ACTA ABP BIOCHIMICA POLONICA

Vol. 62, No 1/2015
77–82
http://dx.doi.org/10.18388/abp.2014_779

Regular paper

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

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Dysregulation of miRNA is widely involved in human cancers, including hepatocellular carcinoma (HCC). Array data for miRNAs indicated that miR-331-3p might be one of the disorderly expressed miRNAs in HCC cell lines, but the function of miR-331-3p in HCC remains unclear. In this study, quantitative real time polymerase chain reaction (qRT-PCR) results indicated that miR-331-3p was up-regulated in HepG2.2.15 cells, Ad-HBV-HepG2 cells and pCH9/3091transfected SMMC7721 cells compared with their control group, respectively. miRNA target prediction software was used, and VHL was found to be one of the target genes of miR-331-3p. qRT-PCR and western blot analysis indicated VHL expression was decreased when miR-331-3p was over-expressed and increased when miR-331-3p was inhibited in SMMC7721 cells. The luciferase reporter activity was inhibited in SMMC7721 cells when co-transfected with miR-331-3p expression vector and VHL 3′-UTR wild type vector and increased in HepG2.2.15 transfected with miR-331-3p inhibitor compared to its control group respectively. When co-transfected with miR-331-3p expression vector and VHL 3′-UTR mutated type vector in SMMC7721 cells the luciferase reporter activity was recovered. All of these results show that HBV up-regulated miR-331-3p expression in HCC cell lines and miR-331-3p could inhibit VHL expression by directly targeting its 3′-UTR. This provided useful information in exploring the mechanism of HCC induced by HBV infection.

Key words: HCC, HBV, miRNA, Hsa-miR-331-3p and VHL

Received: 23 April, 2014; revised: 05 December, 2014; accepted: 09 January, 2015; available on-line: 26 February, 2015

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 miRNA expression (Lin & Flemington *et al.*, 2011). Lots of reports indicate that HBV can induce carcinogenesis in HCC in a miRNAs involved pathway (Wang *et al.*, 2011; Xu *et al.*, 2013; Zou *et al.*, 2014). Different miRNAs expression patterns between HepG2 cells and HepG2.2.15 (a HBV stable expression HCC cell line based on HepG2) cells have been shown by a miRNA microarray analysis in a previous study (Zhang *et al.*, 2011). There, we found that miR-331-3p was up-regulated in HepG2.2.15 cells compared to its control HepG2 cells. It has also been reported that miR-331-3p plays an important role in tumors, like prostate cancer, gastric cancer and glioblastoma multiforme (Guo *et al.*, 2010; Epis *et al.*, 2011; Epis *et al.*, 2012; Epis *et al.*, 2014), but whether miR-331-3p is involved in HCC development and progression remains unknown.

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

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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 37ºC with 5% CO_2. 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 Lipofectamine^{TM}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'-CGCCGCTCGAGATAATATCCTAAACAAAGCA-3', reverse primer: 5'-ACGCGTCGACTTTTAGGGCTAAGTTGCTTC-3' (underlined parts are restriction enzyme cut sites). The fragment (694 bp) containing miR-331-3p was amplified with polymerase chain reaction (PCR) by using HepG2.2.15 cell genomic DNA as a template, and cloned into the XhoI/SalI sites of pTARGET vector. For generating pGL3-Control-VHL-WT, the following primers were used. Forward primer: 5'-AGCTCTAGAATGCCTGCCCATTAGAGAAG-3', reverse primer: 5'-AGCTCTAGAACAAGAGTGGACCGAGAAAG-3' (underlined parts are restriction enzyme cut sites). The VHL 3'-UTRs fragment containing miR-331-3p binding site (CCAGGGG) was amplified by PCR from genomic DNA of HepG2.2.15 cells, and cloned into the XbaI site of pGL3-Control vector. pGL3-Control-VHL-MUT plasmid was constructed based on the pGL3-Control-VHL-WT, and contains a 3'-UTRs mutated sequence (AATCCCC) instead of the wild type by using the following primers, forward primer: 5'-AGAGGAACAAAAATCCCCACACTTTGTTAG-3', reverse primer: 5'-CTAACAAAGTGTGGGGATTTTTGTTCCTCT-3'. All plasmids constructed were verified by DNA sequencing (Invitrogen). Ad-HBV adenovirus and its control Ad-GFP adenovirus were constructed by our laboratory as following: HBV 1.3 fold genome was ligated into shuttle vector pAdTrack-TO4, then, pAdTrack-TO4-HBV1.3 was linearized by PmeI and transfected into BJ5183 cells containing pAd-Easy1 to form a recombinant plasmid. After that, the confirmed recombinant plasmid was linearized by PacI restriction endonuclease and transfected into HEK-293 cells to generate recombinant adenoviruses (Luo *et al.,* 2007).

RNA interference. MiR-331-3p inhibitor and negative control (NC) was designed and synthesized by Invitrogen. Sequences were as follows, miR-331-3p inhibitor: 5'-UUCUAGGAUAGGCCCAGGGGC-3', random microRNA inhibitor NC: 5'-CAGUACUUUUGUGUAGUACAA-3'.

Total RNA extraction, reverse transcription and qRT-PCR. The total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase I (Promega, USA). Total RNAs were reversely transcribed using miRNA cDNA Kit (Cwbio, China) or the Reverse Transcription System (Promega, USA). qRT-PCR was performed using miRNA Real-Time PCR Assay Kit and UltraSYBR mixture (Cwbio) to confirm the expression levels of mRNAs and miRNAs. The miR-331-3p specific forward primer was 5'-GCCCCTGGGCCTATCCTAGAA-3' and a universal reverse primer was provided by miRNA Real-Time PCR Assay kit. U6 was used as a miRNA internal control, and the primers for U6 were as follows, forward primer: 5'-AGAGCCTGTGGTGTCCG-3' and reverse primer: 5'-CATCTTCAAAGCACTTCCCT-3'. The primers for VHL were forward primer: 5'-ACATCGTCAGGTCGCTCTAC-3' and reverse primer: 5'-ATCTCCCATCCGTTGATGTG-3'. β-actin was used as an endogenous control, forward primer: 5'-GTGGATCAGCAAGCAGGAGT-3' and reverse primer: 5'-TGTGTGGACTTGGGAGAGGA-3'. The qRT-PCR reactions were performed in triplicate and included no-template controls. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ 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 (166 pmol) or miR-331-3pinhibitor (166 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 24well 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

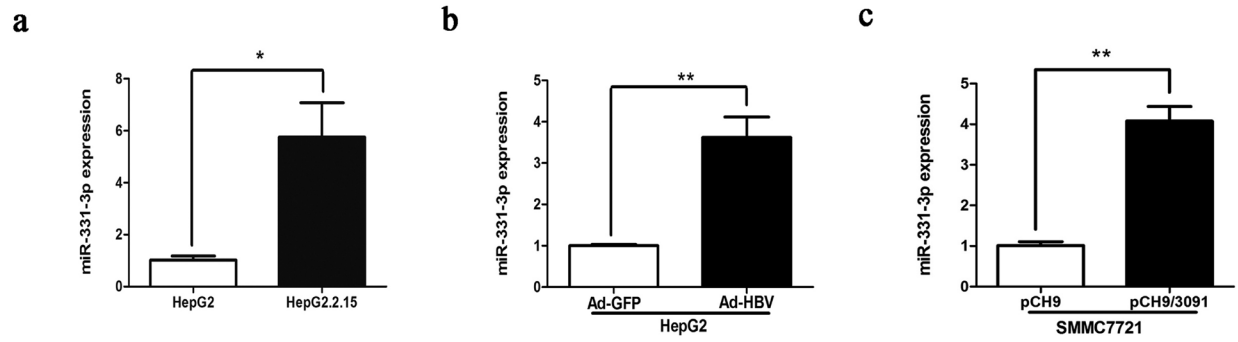

Figure 1. MiR-331-3p was up-regulated in HBV expressing HCC cell lines.
(**a**) Relative miR-331-3p expression in HepG2 and HepG2.2.15cells. (**b**) Relative miR-331-3p expressions in HepG2 cells infected with Ad-GFP or Ad-HBV. (**c**) Relative miR-331-3p expressions in SMMC7721 cells transfected with pCH9 or pCH9/3091 plasmids. miRNA abundance was normalized to U6 RNA. **P*<0.05, ***P*<0.01.

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-

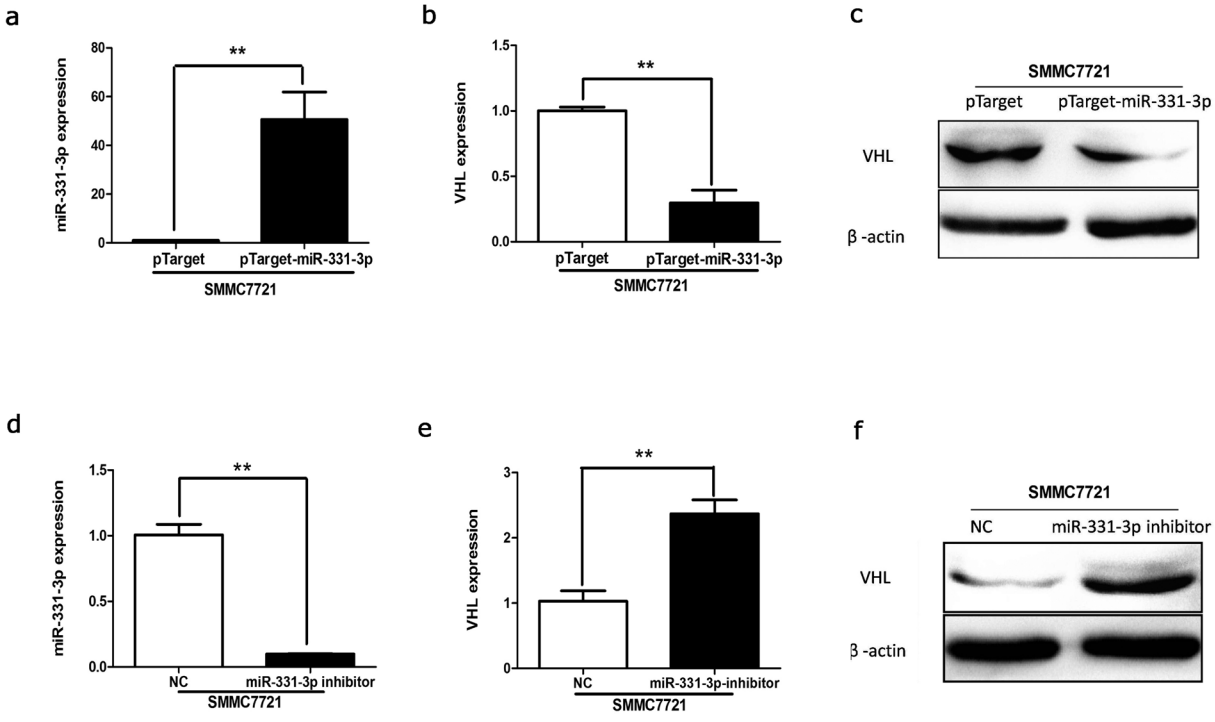

Figure 2. MiR-331-3p inhibited VHL expression.
(**a**) Relative expression of miR-331-3p in SMMC7721 cells transfected with pTARGET or pTARGET-miR-331-3p. MiRNA abundance was normalized to U6. (**b, c**) VHL mRNA and protein expression levels in SMMC7721 cells transfected with pTARGET or pTARGET-miR-331-3p. (**d**) Relative expression of miR-331-3p in SMMC7721 cells transfected with miR-331-3p inhibitor or inhibitor NC. (**e, f**) VHL mRNA and protein expression levels in SMMC-7721 cells transfected with miR-331-3p inhibitor or inhibitor NC. β-actin was used as an internal quantitative control. **P*<0.05, ***P*<0.01.

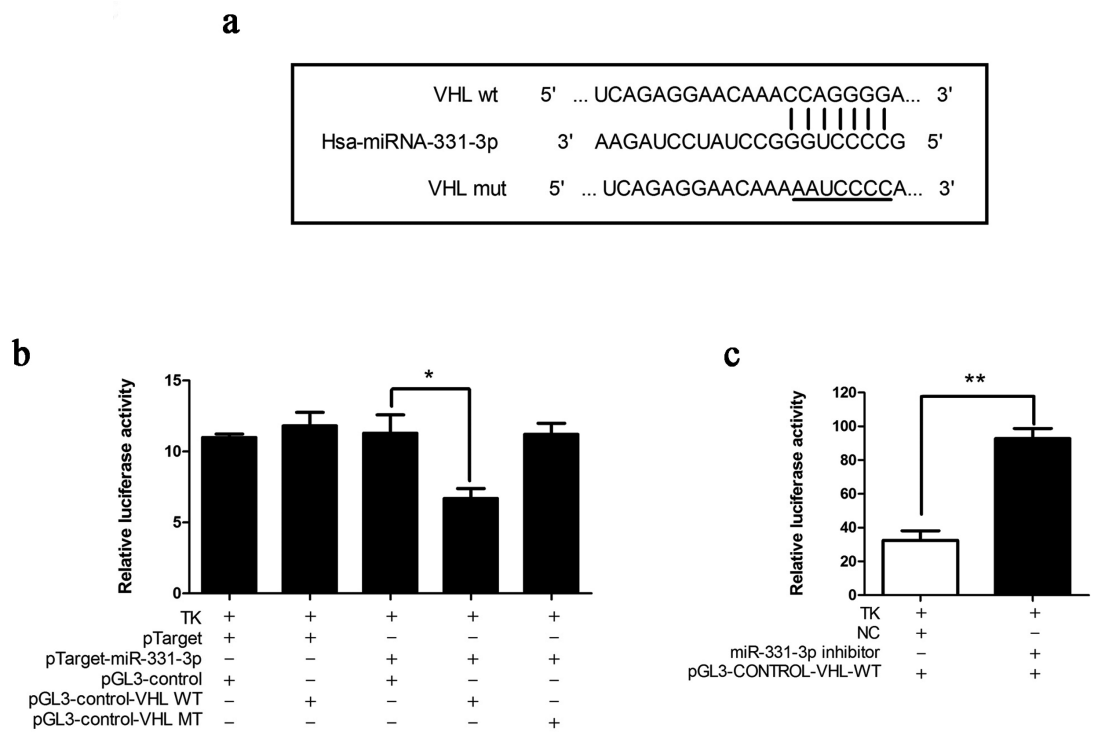

Figure 3. MiR-331-3p down-regulated the expression of VHL through its 3′-UTR binding sites.
(**a**) Schematic diagram of wild-type 3′-UTR binding site or mutated type of miR-331-3p. (**b**) Luciferase reporter assays for SMMC7721 cells which were co-transfected with different plasmid DNAs as indicated. (**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.

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

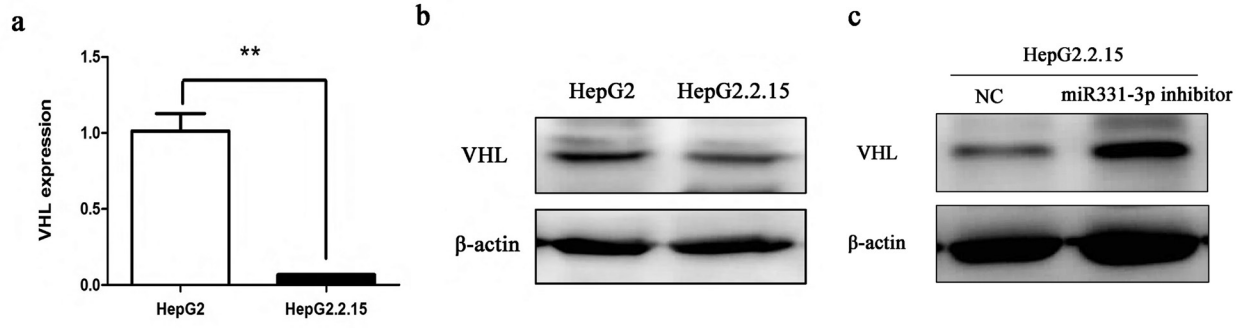

Figure 4. VHL expression was decreased in HepG2.2.15 cells compared to HepG2 cells.
(a) Relative expression of VHL in HepG2 and HepG2.2.15 cells was analyzed by qRT-PCR. β-actin was used as an internal quantitative control. *P<0.05, **P<0.01. **(b)** VHL protein expression levels in HepG2 and HepG2.2.15 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±9.77) 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 pGL3-Control-VHL-WT and has little influence on pGL3- 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 (cstc2013jcyjC10002, ALH), and Natural Science Foundation Project of CQ CSTC (2010BB5359).

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