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Original Article

MicroRNA-214 targets PCBP2 to suppress the proliferation and growth of glioma cells

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Abstract: PCBP2, a member of the poly(C)-binding protein (PCBP) family, is involved in posttranscriptional and translational regulation by interacting with single-stranded poly(C) motifs in target mRNAs. Recent studies have shown that PCBP2 is overexpressed and plays an important role in human cancers, including glioma. However, the molecular basis for its up-regulation remains poorly understood. Here, we show that microRNA-214 (miR-214) interacts with the 3'-untranslated region of PCBP2 mRNA and induces its degradation, leading to reductions in its protein expression. As a result, overexpression of miR-214 mimics significantly inhibited, while its antisense oligos proliferation and growth of glioma cells. Restoration of PCBP2 remarkably reversed the tumor-suppressive effects of miR-214 on cell proliferation and growth. In summary, our data indicate that miR-214 may function as tumor suppressor in glioma by targeting PCBP2.

Keywords: PCBP2, miR-214, glioma, tumor suppressor

Introduction

Glioma, derived from glial cells, has become one of the most common primary malignant brain tumor in China [1]. Despite multimodal therapies, such as surgery, radiotherapy, and chemotherapy, the average life expectancy remains limited [2, 3]. Therefore, a better understanding of the molecular mechanism of glioma may provide novel therapeutic strategies for its treatment.

Poly (C)-binding protein 2 (PCBP2), characterized by its high-affinity and sequence-specific interactions with polycytosine (poly (C), regulates gene expression at various levels, including transcription, mRNA processing, mRNA stabilization, and translation [4, 5]. It has been reported that PCBP2 participated in the replication and translation of many RNA viruses, including poliovirus, coxsackievirus, and rhinovirus [6-8]. Recent studies demonstrate that PCBP2 may play a crucial role in the cell proliferation and tumorigenesis. For instance, depletion of PCBP2 led to an induction of cyclin-dependent kinase inhibitor (CDKN1A) and G1 arrest in the human hematopoietic cells [9]. Besides, PCBP2 promoted gastric cancer cell proliferation and colony formation [10]. More importantly, Han et al. found that PCBP2 was up-regulated in human glioma tissues and cell lines. Depletion of PCBP2 inhibited glioma growth in vitro and in vivo through suppression of cell-cycle progression and induction of caspase-3-mediated apoptosis [11]. However, the mechanism by which PCBP2 is regulated in glioma remains poorly understood.

Increasing studies have highlighted the importance of microRNAs, a class of small and non-coding RNAs, in the regulation of gene expression [12, 13]. Given that multiple miRNAs are deregulated and involved in the glioma progression [14-16], whether PCBP2 is regulated by miRNAs has not been previously studied.

Materials and methods

Human samples collection

Twenty-five pairs of glioma tissues and adjacent normal tissues were collected from routine therapeutic surgery at our department. All samples were obtained with informed consent and approved by the hospital institutional review board.

Cell culture

Colon cancer cells (U251 and SHG-44 cells) were purchased from American Type Culture Collection (Rockville, MD, USA). Cells were culture in RPMI 1640 medium (GIBCO, Shanghai, China) supplemented with 10% fetal bovine serum (GIBCO). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

RNA isolation and quantitative real-time PCR

Total RNA from tissue samples and cell lines was harvested using the RNA Isolation Kit (Ambion, USA). Expression of mature miRNAs was assayed using Taqman MicroRNA Assay (Applied Biosystems) specific for hsa-miR-214. Quantitative real-time PCR was performed by using an Applied Biosystems 7900 Real-time PCR System and a TaqMan Universal PCR Master Mix. All the primers were obtained from the TaqMan miRNA Assays. Small nuclear U6 snRNA (Applied Biosystems) was used as an internal control. Differences in gene expression, expressed as fold-changes, were calculated using the $2^{-\Delta\Delta Ct}$ method.

miR-214 mimics, antisense and transfection

Human miR-214 mimics, antisense or negative controls were obtained from Genepharm Company (Shanghai). For transfection, cells were cultured in a 6-well plate and transiently transfected at 70-80% confluence using the LipofectamineTM 2000 reagent (Invitrogen, CA, USA) as per the manufacturer's instructions.

BrdU and cell invasion assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA). For cell invasion assays, cells were analyzed using extracellular matrix-coated invasion chambers (Millipore, CA, USA), and quantitated with a colorimetric microplate reader at 570 nm, according to the manufacturer's instructions.

Western blot

Cells or tissues were harvested and lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, pH 7.5). Proteins were quantified and separated by 8% SDS-agarose gel, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-PCBP2 antibody was purchased from Abcam Company (USA). Protein levels were normalized to total GAPDH, using a mouse anti-GAPDH antibody (Santa Cruz, USA).

Luciferase reporter assay

3'-UTR of human PCBP2 gene that were predicted to interact with miR-214 were synthesized and inserted into pMir-Report (Ambion, USA), yielding pMir-Report-PCBP2. Mutations within potential miR-214 binding sites were generated by nucleotide replacement of wild type sequence to inhibit miR-214 binding. Cells were transfected with the pMir-Report vectors containing the 3'-UTR variants, and miR-214 mimics for 36 hours. The pRL-SV40 vector (Promega, USA) carrying the Renilla luciferase gene was used as an internal control to normalize the transfection efficiency. Luciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega).

Statistics

Data are expressed as the mean ± SEM from at least four separate experiments. Differences between groups were analyzed using Student's t-test. A value of $P < 0.05$ was considered statistically significant.

Results

miR-214 down-regulates PCBP2 expression by targeting its 3'-untranslated region

Using the three bioinformatics software (miRWalk, miRanda and Targetscan) based on seed recognition, we identified several miRNAs that could potentially interact with the PCBP2 transcript. To determine whether these miRNAs could regulate PCBP2, the 3'-untranslated region (UTR) of PCBP2 gene was cloned and inserted into a luciferase reporter construct. As shown in the **Figure 1A**, overexpression of miR-214 mimics, but not others, significantly inhib-

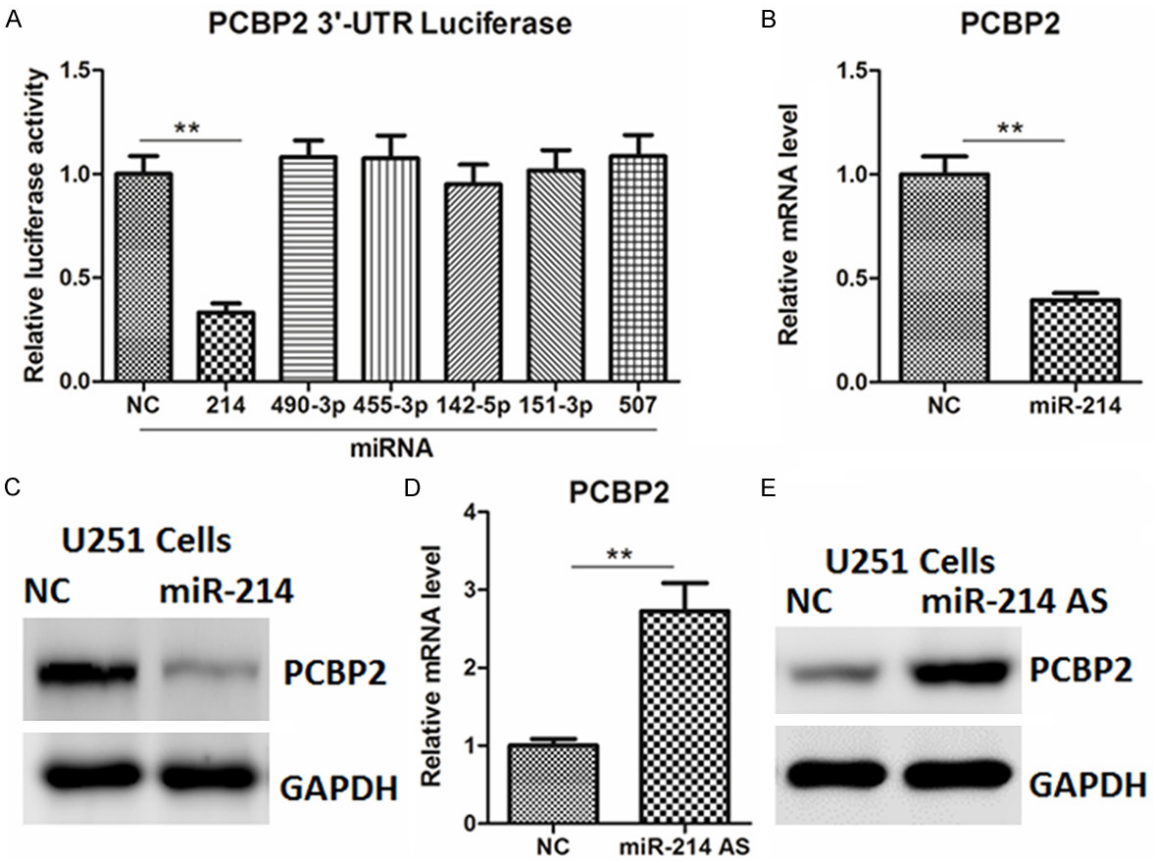

Figure 1. Effects of miR-214 on the regulation of PCBP2 in glioma cells. (A) List of miRNAs that could potentially interact with the PCBP2 transcript. (B, C) mRNA (B) and protein (C) levels of PCBP2 in U251 cells transfected with miR-214 mimics or negative control (NC) for 24 or 48 hr, respectively. (D, E) mRNA (D) and protein (E) levels of PCBP2 in U251 cells transfected with miR-214 antisense oligos or negative control (NC) for 24 or 48 hr, respectively.

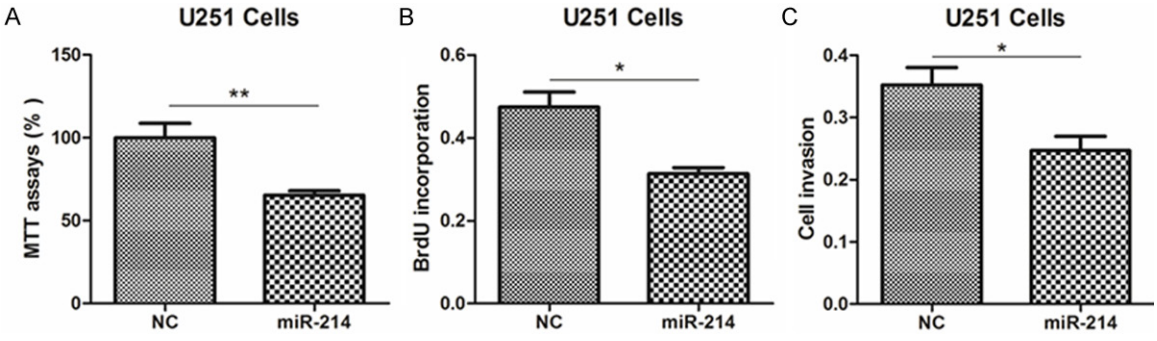

Figure 2. miR-214 mimics inhibits glioma cell proliferation. (A-C) The cell viability (A), proliferative potential (B) and invasion abilities were determined in U251 cells transfected with miR-214 mimics or negative control (NC).

ited the reporter activity of PCBP2 3'-UTR in U251 cells. Next, the endogenous expression of PCBP2 was examined by quantitative real-time PCR and western blot analysis. As expected, forced expression of miR-214 mimics decreased PCBP2 mRNA and protein levels (**Figure 1B**, **1C**). In agreement, inhibition of miR-214 by its antisense oligos increased PCBP2 expression (**Figure 1D**, **1E**). Similar results were also observed in SHG-44 cells (Supplementary Figure 1A-D).

miR-214 regulates glioma cell growth in vitro

Next, to clarify the roles of miR-214 in glioma, we introduced miR-214 mimics into U251 and

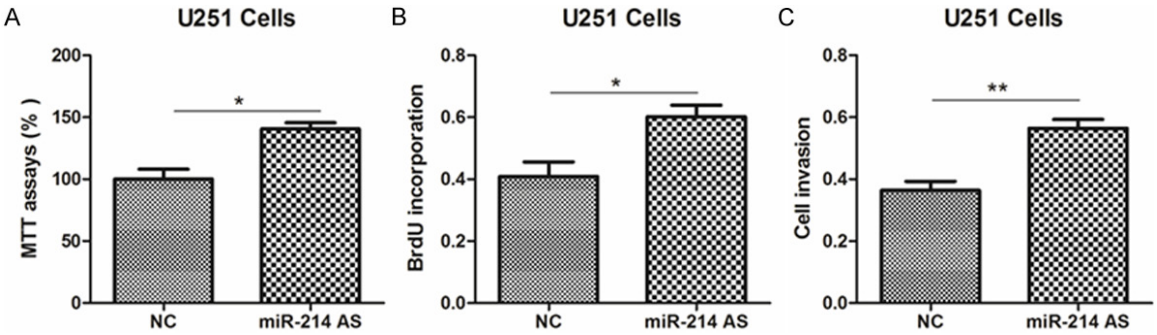

Figure 3. Inhibition of miR-214 promotes glioma cell proliferation. (A-C) The cell viability (A), proliferative potential (B) and invasion abilities were determined in U251 cells transfected with miR-214 antisense or negative control (NC).

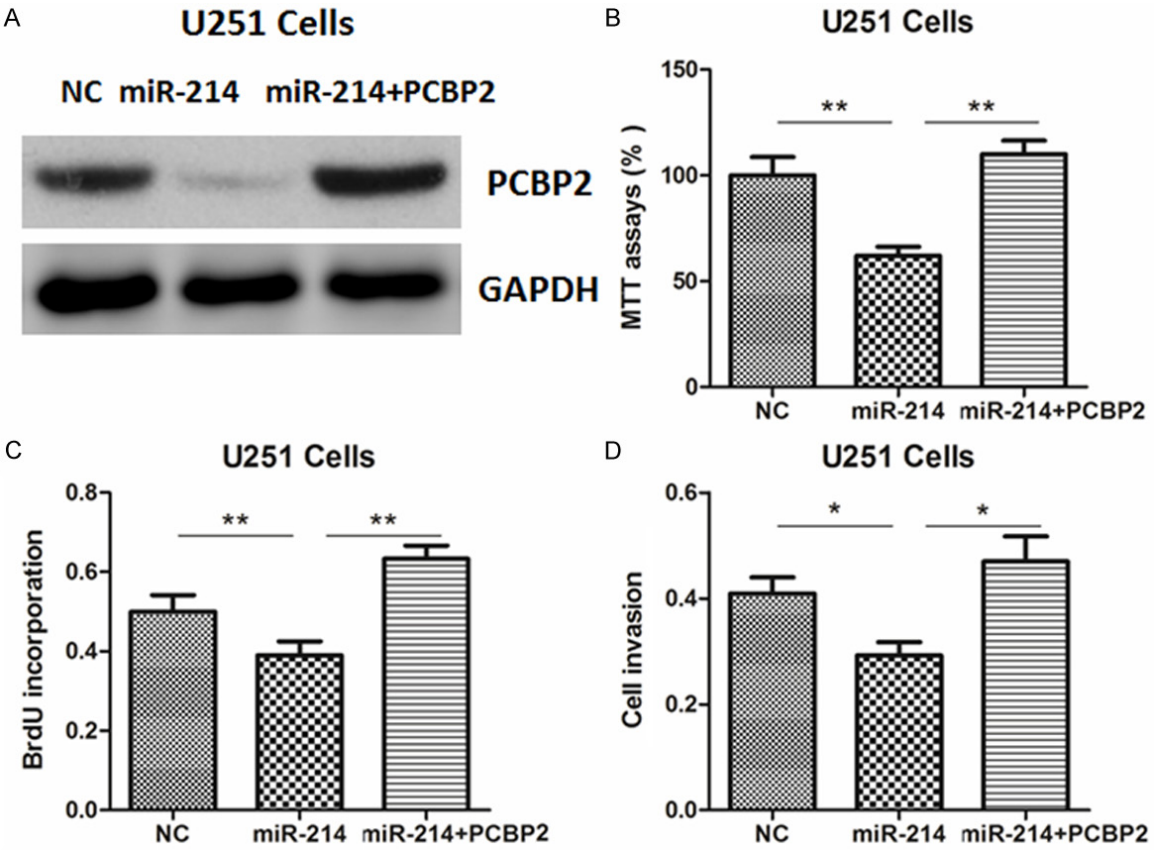

Figure 4. PCBP2 re-introduction reverses the anti-proliferative roles of miR-214. (A) PCBP2 protein expression was determined by western blot in U251 cells. Cells were pre-transfected with miR-214 mimics or negative control (NC) for 24 hr, and then transfected with expression plasmids for PCBP2 or empty vector (EV) for another 24 hr. (B-D) The cell viability (B), proliferation (C) and invasion abilities (D) were determined in U251 cells.

SHG-44 cells. As a result, miR-214 mimics significantly inhibited the abilities of cell viability, proliferation and invasion, compared with negative control (NC) (**Figure 2A-C**, Supplementary Figure 2A-C). Moreover, anti-miR-214 promoted the growth and invasion of glioma cells, compared to NC-transfected cells (**Figure 3A-C**, Supplementary Figure 3A-C). These results provide strong evidence that miR-213 could regulate glioma cell growth *in vitro*.

PCBP2 re-introduction reversed the anti-proliferative role of miR-214

To further verify the functional connection between miR-214 and PCBP2, U251 cells were

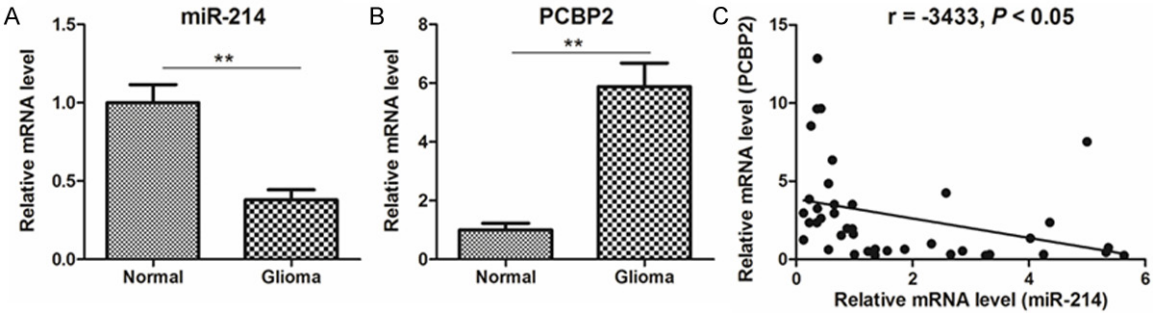

Figure 5. Expression of miR-214 and PCBP2 in glioma tissue specimens. A, B. Relative expression levels of miR-214 and PCBP2 in glioma and adjacent normal tissues. C. An inverse correlation between the expression of miR-214 and the level of PCBP2 mRNA in two groups of tissues.

transfected with PCBP2 plasmids or empty vector (EV) (**Figure 4A**). As shown in **Figure 4B-D**, PCBP2 overexpression reversed the tumor suppressive roles of miR-214, underlining the specific importance of the PCBP2 for miR-214 action in the cell proliferation and invasion.

The expression level of miR-214 is down-regulated and inversely correlated with PCBP2 mRNA in glioma tissues

Given that miR-214 could regulate PCBP2 expression and cell growth in vitro, its expression level was determined in glioma tissues. As shown in the **Figure 5A** and **5B**, the expression levels of miR-214 were significantly higher, while PCBP2 was up-regulated in glioma tissues, compared with adjacent normal tissues. Furthermore, across all specimens tested, we found an inverse correlation between the expression of miR-214 and the level of PCBP2 mRNA (**Figure 5C**), further suggesting that miR-214 may be involved in the regulation of PCBP2 in glioma.

Discussion

The results from our study indicate that mRNA and protein levels of PCBP2 are negatively regulated by miR-214. Although the functional significance of PCBP2 in glioma has been studied *in vitro* and *in vivo* [11], the molecular mechanisms for its up-regulation remain poorly understood. Therefore, our data highlight an important role of miR-214 in the regulation of PCBP2. However, whether this regulatory pathway occurs in other human cancers remain to be determined.

Moreover, we found that miR-214 was down-regulated in glioma tissues and could inhibit cell proliferation and growth. It has been shown that miR-214 is down-regulated in human cervical cancer tissue compared with normal tissue [17]. miR-214 represses HeLa cell proliferation by targeting the noncoding regions of MEK3 and JNK1 mRNAs [17]. Besides, miR-214 reduces cell survival and enhances cisplatin-induced cytotoxicity via down-regulation of Bcl2l2 in cervical cancer cells [18]. On the other hand, miR-214 was up-regulated in pancreatic cancer tissues and the elevation of miR-214 expression in pancreatic cancer specimens might be related to the poor response of pancreatic cancer cells to chemotherapy [19]. Moreover, miR-214 was noted to be highly overexpressed in gastric cancer tissues and cell lines [20]. As a result, knockdown of miR-214 could significantly inhibit proliferation, migration and invasion of gastric cancer cells [20]. Therefore, miR-214 could act as an oncogenic miRNA or a tumor suppressor. Although the inconsistence for these observations remains largely unknown, we speculate that the precise role of miR-214 might be tissue or cell-specific.

Increasing reports have demonstrated that some miRNAs are dys-regulated and play a key role in the development of human glioma [14-16]. Together with these studies, our results suggest that identification of specific miRNAs and their targets may provide novel insight for the diagnosis and treatment of glioma.

Disclosure of conflict of interest

None.

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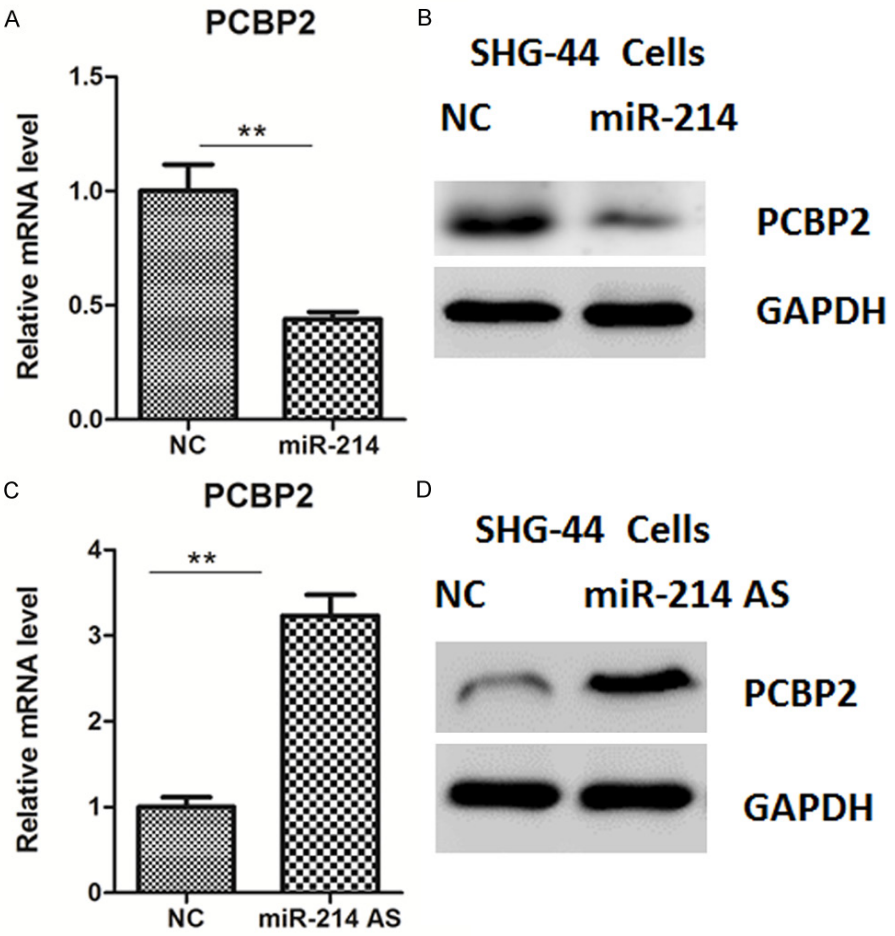
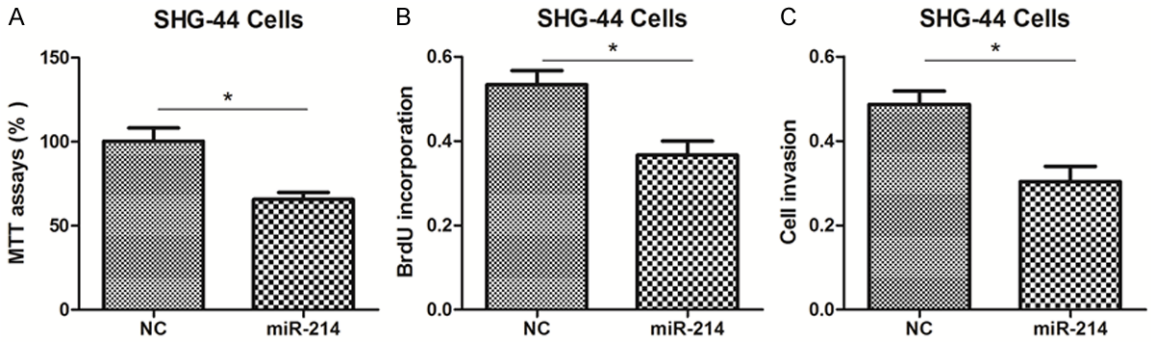
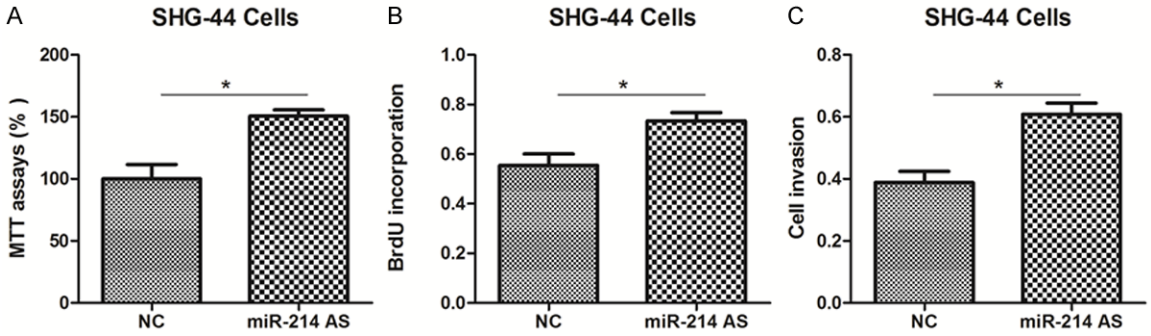


Supplementary Figure 1. (A, B) mRNA (A) and protein (B) levels of PCBP2 in SHG-44 cells transfected with miR-214 mimics or negative control (NC) for 24 or 48 hr, respectively. (C, D) mRNA (C) and protein (D) levels of PCBP2 in SHG-44 cells transfected with miR-214 antisense oligos or negative control (NC) for 24 or 48 hr, respectively.



Supplementary Figure 2. (A-C) The cell viability (A), proliferative potential (B) and invasion abilities were determined in SHG-44 cells transfected with miR-214 mimics or negative control (NC).



Supplementary Figure 3. (A-C) The cell viability (A), proliferative potential (B) and invasion abilities were determined in SHG-44 cells transfected with miR-214 antisense or negative control (NC).