Biochemical and Biophysical Research Communications 448 (2014) 437–442

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The RNA-binding protein PCBP2 facilitates gastric carcinoma growth by targeting miR-34a

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ARTICLE INFO

Article history:
Received 17 April 2014
Available online 2 May 2014

Keywords:
PCBP2
Gastric cancer
miR-34a
Apoptosis

ABSTRACT

Gastric carcinoma is the fourth most common cancer worldwide, with a high rate of death and low 5-year survival rate. However, the mechanism underling gastric cancer is still not fully understood. Here in the present study, we identify the RNA-binding protein PCBP2 as an oncogenic protein in human gastric carcinoma. Our results show that PCBP2 is up-regulated in human gastric cancer tissues compared to adjacent normal tissues, and that high level of PCBP2 predicts poor overall and disease-free survival. Knockdown of PCBP2 in gastric cancer cells inhibits cell proliferation and colony formation *in vitro,* whereas opposing results are obtained when PCBP2 is overexpressed. Our *in vivo* subcutaneous xenograft results also show that PCBP2 can critically regulate gastric cancer cell growth. In addition, we find that PCBP2-depletion induces apoptosis in gastric cancer cells *via* up-regulating expression of pro-apoptotic proteins and down-regulating anti-apoptotic proteins. Mechanically, we identify that miR-34a as a target of PCBP2, and that miR-34a is critically essential for the function of PCBP2. In summary, PCBP2 promotes gastric carcinoma development by regulating the level of miR-34a.

1. Introduction

Gastric cancer is the fourth most common cancer worldwide, with a high rate of death and low 5-year survival rate [1]. Despite extensive research to identify novel diagnostic and therapeutic agents, patients with advanced gastric cancer suffer from a poor quality of life and poor prognosis, and treatment is dependent mainly on conventional cytotoxic chemotherapy [2]. To improve the quality of life and survival of gastric cancer patients, a better understanding of the underlying molecular mechanisms, and their application towards the development of novel targeted therapies, is urgently needed.

Poly(C)-binding proteins (PCBPs) are generally known as RNA-binding proteins that interact in a sequence-specific fashion with single-stranded poly(C). This family can be divided into two groups: hnRNP K and PCBP1-4. PCBPs are expressed broadly in human and mouse tissues and all members of the PCBP family are related evolutionarily. These proteins are involved mainly in various posttranscriptional regulations (e.g., mRNA stabilization or translational activation/silencing) [3]. It has been suggested that PCBPs play a critical role in carcinogenesis. hnRNP K induces transcriptional activation of the oncogenes c-SRC and c-Myc [4,5], suggesting that hnRNPK may cooperate with additional oncoproteins to overexpress genes that promote tumor growth. PCBP1 is downregulated in metastatic cervical cancer cells [6] and metastatic breast cancer cells [7]. Knockdown of PCBP1 in the prostate cancer cell line LNCaP promotes androgen receptor (AR) protein targeting to the 3′ untranslated region of AR transcripts [8]. The recent finding that transforming growth factor-β-mediated phosphorylation of PCBP1 induced epithelial–mesenchymal transdifferentiation (EMT) [9], and the evidence that PCBP1 can down-regulate metastasis-associated PRL-3 phosphatase translation [10], indicate that PCBP1 could be a tumor suppressor. It has been suggested that PCBP2 also plays an important role in cancers such as leukemogenesis [11] and oral cancer [12]. Recently, two related reports demonstrated that PCBP2 is overexpressed in human glioma and facilitates glioma growth by targeting four-and-a-half LIM domain 3 (FHL3), which was recently shown to act as tumor suppressor [13,14]. PCBP2 overexpression is due to the down-regulation of SIRT6, which targets to the promoter of PCBP2 and deacetylates H3K9ac [14].

However, the role of PCBPs in other cancer types, gastric carcinoma for example, remains unknown. Recently, Ghanem et al. [15] showed a specific enrichment of the RNA-binding proteins PCBP1 and PCBP2 in chief cells of the murine gastric mucosa. PCBP1/2 expression correlates with gastric gland elongation in the

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http://dx.doi.org/10.1016/j.bbrc.2014.04.124
0006-291X/

post-natal period. This finding indicates that PCBPs may act in gastric development and diseases. Here in this study, we show that PCBP2 is overexpressed in human gastric carcinoma and predicts poor survival. We provide evidence that PCBP2 facilitates gastric cancer growth and inhibits apoptosis by targeting miR-34a.

2. Material and method

2.1. Patients

One hundred and fifty-four cases of gastric cancer with full case history and paraffin-embedded tissue between January 1997 and December 2006 were collected at Huashan Hospital, Fudan University (Shanghai). The patients included 87 (56.5%) males and 67 (43.5%) females with a mean age of 59 years. Tumors were histologically classified into 73 (47.7%) intestinal gastric cancer and 81 (52.3%) diffuse gastric cancer. The diagnosis of gastric cancer was established using World Health Organization (WHO) morphological criteria [16]. Clinicopathological parameters of the patients were listed in Supplementary Tables 1 and 2. For the non-cancer normal gastric mucosa (NGM), 18 biopsy-tissue specimens were obtained from the antrum and the body of the normal stomach separated by a distance of 5 cm. Those normal samples were collected at Huashan Hospital, Fudan University (Shanghai). A written form of informed consent was obtained from all patients and donors. The study was approved by the Clinical Research Ethics Committee of Huashan Hospital, Fudan University (Shanghai).

2.2. Cell culture and infection

Gastric epithelial cell lines (GES-1 and HFE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28) were purchased from the American Type Culture Collection. All the cells were cultured in RPMI1640 (Gibco; #11875-093) medium supplemented with penicillin–streptomycin (Gibco; #15140-122), GlutaMAX-1 (Gibco; #06690302A) and 10% fetal bovine serum (Gibco; #10100-147) at 37 °C with 5% CO_2.

Control shRNA and three non-overlapping specific sh-RNAs targeting PCBP2 were purchased from Invitrogen, and the corresponding sequences (Supplementary Table 3) were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. miR-34a was knocked down with a specific miR-34a sponge. pHIV-D2EGFP was generated by replacing EGFP in pHIV-EGFP with a destabilized variant, D2EGFP (Clontech). To construct the miR-34a sponge, 13 repeats of modified miR-34a binding sites with a bulge at position 9–12 were cloned into pHIV-D2GFP using BamHI and KpnI [17]. For transduction, cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for 72 h with puromycin (2 mg/ml) or hygromycin (200 mg/ml). Adenovirus carrying control GFP (#SL100708) or human PCBP2 (#SL174118) were purchased from SignaGen Laboratories.

2.3. Tumor xenograft experiments

Equal numbers of gastric cancer cells stably with either control or PCBP2 knockdown or overexpression (5×10^6) in 100 μl of a 1:1 mixture of culture medium and growth factor–reduced Matrigel were implanted subcutaneously into the forelegs of 4-week-old male BALB/c athymic nu/nu mice (Vital River). When the tumors reached approximately 7–10 mm in diameter, they were prepared to form a brei and then injected subcutaneously into nude mice. Tumor weight was evaluated at the terminal of the experiment.

2.4. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (Sigma; #TOX1-1KT) in according to the guidelines.

2.5. Soft sugar colony formation assay

Gastric cancer cells were suspended in 1.5 ml complete medium supplemented with 0.45% low melting point agarose (Gibco; #18300-012). The cells were placed in 35 mm tissue culture plates containing 1.5 ml complete medium and agarose (0.75%) on the bottom layer. The plates were incubated at 37 °C with 5% CO_2 for 2 weeks. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope.

2.6. Statistics

Statistical differences between two groups were determined using Student's *t* test. The Kaplan–Meier method was used to estimate overall and disease-free survival. Survival differences according to PCBP2 expression were analyzed by the log-rank test. Linear regression analysis was performed to analyze the relation between PCBP2 and miR-34a expression in human gastric cancers. The statistical analysis was performed with GraphPad Prism 6 software. *p* Values of less than 0.05 were considered statistically significant.

3. Results

3.1. PCBP2 overexpresses in human gastric cancer

To study the potential participation of the PCBP family in human gastric cancer, we firstly evaluated the mRNA levels of the members of PCBP family in human normal gastric mucosa (NGM) and gastric carcinoma tissues. The results showed that the PCBP2 was significantly increased in gastric carcinoma tissues compared to NGM, whereas the mRNA levels of other members did not change (Fig. 1A). To investigate whether this significance change in PCBP2 expression also exists in a larger cohort, we tested the mRNA level of PCBP2 in 18 NGM and 154 gastric carcinomas with different stages. Markedly, the expression of PCBP2 was up-regulated in gastric carcinoma tissues and high PCBP2 level was correlated with high disease stage (Fig. 1B). In addition, the western blot results revealed that PCBP2 protein level also increased in human gastric carcinoma tissues (Fig. 1C). Finally, we investigated the levels of PCBP2 in normal gastric epithelial cell lines (GES-1 and HFE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28). In consistence with the condition in human gastric cancer patients, the mRNA level of PCBP2 was significantly increased in gastric cancer cell lines compared to normal gastric epithelial cell lines (Fig. 1D). Taken together, we show that PCBP2 is overexpressed in human gastric carcinoma and gastric cancer cell lines.

3.2. PCBP2 predicts prognosis of human gastric cancer

Since PCBP2 is correlated with disease stage (Fig. 1B), it may serve as a factor predicting patients' prognosis. We found that high PCBP2 mRNA level was correlated with high liver and lymph node metastasis stage, large tumor size, total extent of gastrostomy, and high disease stage (Supplementary Table 1). We performed Kaplan–Meier and log-rank test to analyze the difference in survival durations between PCBP2 low and high expression groups. The results indicated that high expression of PCBP2 predicted poor overall survival (Fig. 2A; $p = 0.008$). Furthermore, PCBP2 expression

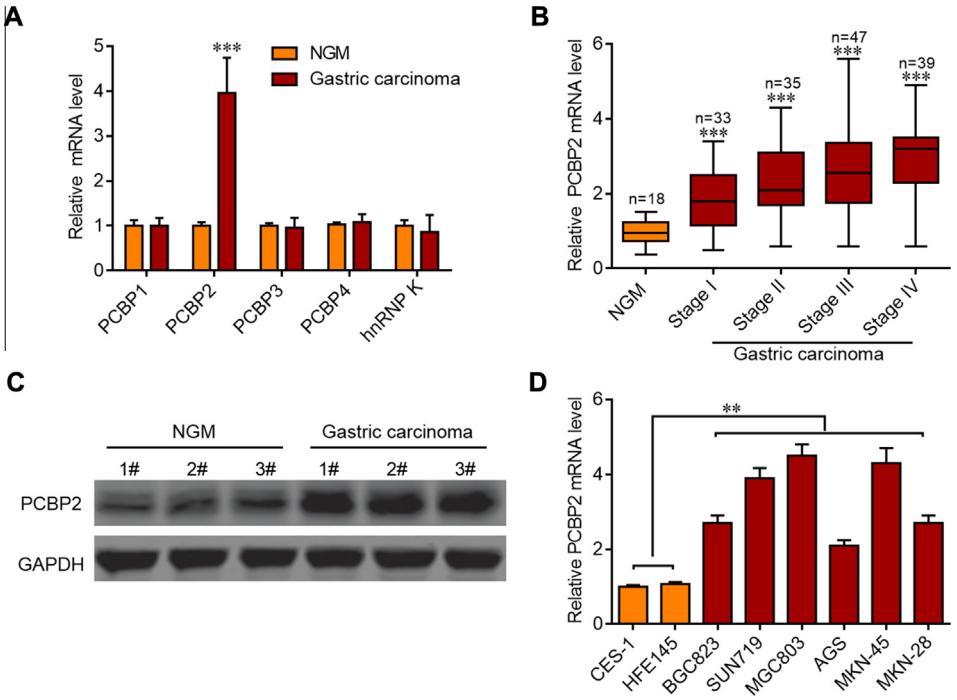

Fig. 1. PCBP2 is overexpressed in human gastric cancer tissues and cell lines. (A, B) Human gastric cancer and normal gastric mucosa (NGM) tissues were extracted for RNA, which was then subjected to cDNA synthesis and q-PCR analysis with indicated primers. (A) The mRNA levels of PCBP family members in normal gastric mucosa and cancer tissues. $N = 5$ in each group. $^{***}p < 0.001$ *vs.* NGM. (B) The mRNA levels of PCBP2 in NGM and gastric cancer tissues. $^{***}p < 0.001$ *vs.* NGM. (C) Representative western blot showing PCBP2 protein level upregulates in human gastric cancer tissues. Total protein was extracted from human gastric cancer and NGM tissues and were subjected to western blot analysis with indicated antibodies. (D) PCBP2 mRNA in normal gastric epithelial cell lines (GES-1 and HFE145) and gastric cancer cell lines (BGC823, SNU-719, MGC803, AGS, MKN-45 and MKN-28).

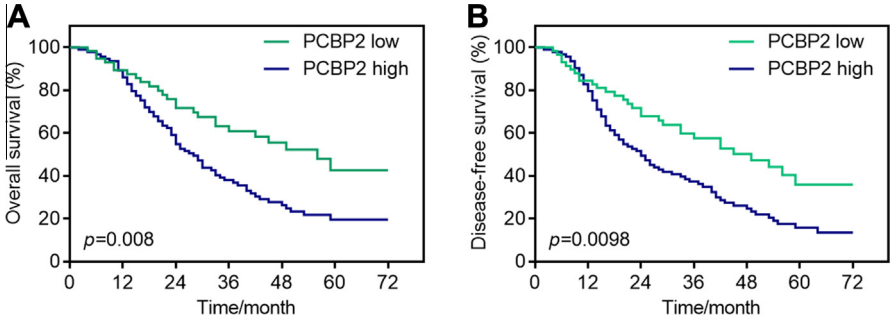

Fig. 2. PCBP2 predicts poor overall and disease-free survival. Kaplan–Meier plot of survival durations in gastric cancer patients with different PCBP2 expression are shown. Survival differences according to PCBP2 expression were analyzed by the log-rank test. (A) High PCBP2 expression predicts poor overall survival. (B) High PCBP2 expression predicts poor disease-free survival. $N = 61$ in PCBP2 low group and $n = 93$ in PCBP2 high group.

was correlated with disease recurrence, because high PCBP2 expression also predicted poor disease-free survival (Fig. 2B; $p = 0.0098$). Importantly, the univariate and multivariate survival analyses indicated that PCBP2 could serve as an independent prognostic factor for outcome of gastric cancer patients. In summary, PCBP2 is a significant prognostic factor for predicting poor survival. Those results implicate that PCBP2 may participate in human gastric carcinoma development and prognosis.

3.3. PCBP2 regulates gastric cancer cell growth in vitro and in vivo

We next investigated whether PCBP2 can regulate gastric cancer growth. We knocked down PCBP2 in gastric cancer cell line MGC803, SUN791 and MKN-45 cells (Suppl. Fig. 1). We showed that PCBP2 knockdown reduced the proliferation rate of MGC803, SUN791 and MKN-45 cells (Fig. 3A–C and Suppl. Fig. 2A), whereas PCBP2 overexpression facilitated MGC803 cell proliferation (Suppl. Fig. 3A, B). We next probed the contribution of PCBP2 in the transformative properties of gastric cancer cells. PCBP2-depleted cells possessed reduced colony-forming activity in MGC803, SUN791 and MKC-45 cells (Fig. 3D–F and Suppl. Fig. 2B), while PCBP2 overexpression promoted colony-forming activity in MGC803 cells (Suppl. Fig. 3C). These findings indicated the PCBP2 regulated gastric cancer growth *in vitro* but, we are still interested whether this regulation exists *in vivo*. Therefore, we also performed tumor xenograft experiments and evaluated the effects of PCBP2 on tumor weight at the terminal of the experiment. Significantly, PCBP2 knockdown inhibited gastric cancer growth *in vivo* (Fig. 3G–I), whereas PCBP2 overexpression promoted gastric cancer cell growth (Suppl. Fig. 3D). In summary, our evidence strongly demonstrates that PCBP2 promotes gastric cancer growth *in vitro* and *in vivo*.

3.4. PCBP2 regulates miR-34a and cell apoptosis in gastric cancer

At last, we wanted to make it clear how PCBP2 affects gastric cancer growth. We knocked down PCBP2 in gastric cancer cells

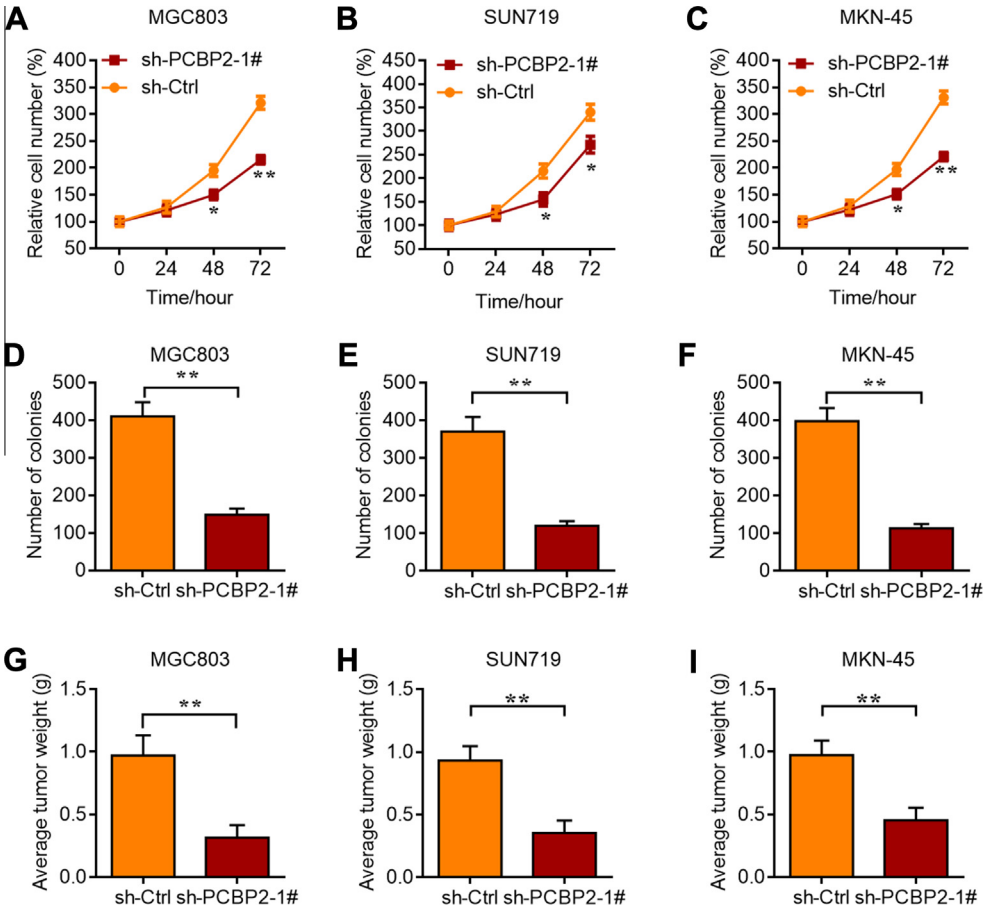

Fig. 3. PCBP2 regulates gastric cancer growth *in vitro* and *in vivo*. (A–C) PCBP2 facilitates gastric cancer cell proliferation. PCBP2 was knocked down in MGC803, SUN791 or MKN-45 cells and cell number was evaluated at the indicated time points with MTT method. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* the control group. (D–F) PCBP2 promotes colony formation of gastric cancer cells. MGC803, SUN791 or MKN-45 cells with PCBP2 knockdown were subjected to soft sugar colony formation assay. At the terminal of the experiments, the colony numbers were evaluated. $^{**}p < 0.01$ and $^{***}p < 0.001$. (F–I) PCBP2 facilitates gastric cancer growth *in vivo*. MGC803, SUN791 or MKN-45 cells with PCBP2 knockdown were subjected to xenograft mice experiments. The tumor weight was evaluated at the terminal of the experiments. $^{**}p < 0.01$. $n = 10$ in each group.

and analyzed cell apoptosis by fluorescence-activated cell sorting (FACS). Interestingly, PCBP2 knockdown significantly increased the percentage of apoptotic cells in MGC803, SUN791 and MKC-45 cells (Fig. 4A and Suppl. Fig. 4). In addition, PCBP2 knockdown increased the levels of pro-apoptotic proteins (Bax and cleaved Caspase 3) and decreased the level of the anti-apoptotic protein Bcl-2 in MGC803 cells (Fig. 4B). These results indicate that PCBP2 is critically essential for gastric cancer cell survival and depletion of PCBP2 promotes apoptosis.

To further study the underling mechanism by which PCBP2 regulates gastric cancer cell apoptosis, we evaluated the level of miR-34a, which was reported to act as a pro-apoptotic microRNA and be downregulated in human gastric carcinoma [18–20]. We found that miR-34a was indeed down-regulated in human gastric carcinoma (Fig. 4C). Linear regression analysis was performed to test whether PCBP2 is correlated with the level of miR-34a in human gastric carcinoma. Interestingly, our data showed that miR-34a level was significantly but negatively correlated with PCBP2 mRNA level (Fig. 4D), indicating PCBP2 may regulate miR-34a level or miR-34a may regulate PCBP2. To test this hypothesis, we knocked down PCBP2 or miR-34a in MGC803 cells. Significantly, we showed that PCBP2 knockdown restored the expression of miR-34a in MGC803 cells (Fig. 4E), whereas miR-34a knockdown did not change the expression of PCBP2 (data not shown). To further determine whether miR-34a is critically essential for PCBP2 function in gastric cancer cells, we performed rescue experiments by knockdown miR-34a with a miR-34a sponge (Suppl. Fig. 5). Significantly, miR-34a sponge treatment abolished PCBP2 knockdown-induced apoptosis, reduction of gastric cancer cell proliferation and colony formation (Fig. 4F–H; Suppl. Fig. 6). In summary, PCBP2 inhibits miR-34a expression and cell apoptosis in gastric cancer cells.

4. Discussion

In the present study, we evaluated the changes in expression of PBCPs in human gastric carcinoma compared to normal gastric mucosa, and found that PCBP2 was overexpressed in human gastric carcinoma. The high expression of PCBP2 predicted poor overall and disease-free survival. We further demonstrated that PCBP2 facilitated gastric cancer cell growth and transformation using *in vitro* and *in vivo* evidence. Mechanically, we showed that PCBP2 maintained the survival of gastric cancer cells and miR-34a is involved in this process.

PCBPs regulate gene expression at various levels, including transcription, mRNA processing, mRNA stabilization, and translation. It has been suggested that PCBPs play a critical role in various carcinomas. PCBP2 is one of the least studied proteins in human cancers among PCBPs. Most of the reports on PCBP2 have focused on its posttranscriptional and translational controls in RNA viruses. For instance, PCBP2 has been reported to participate in the replication

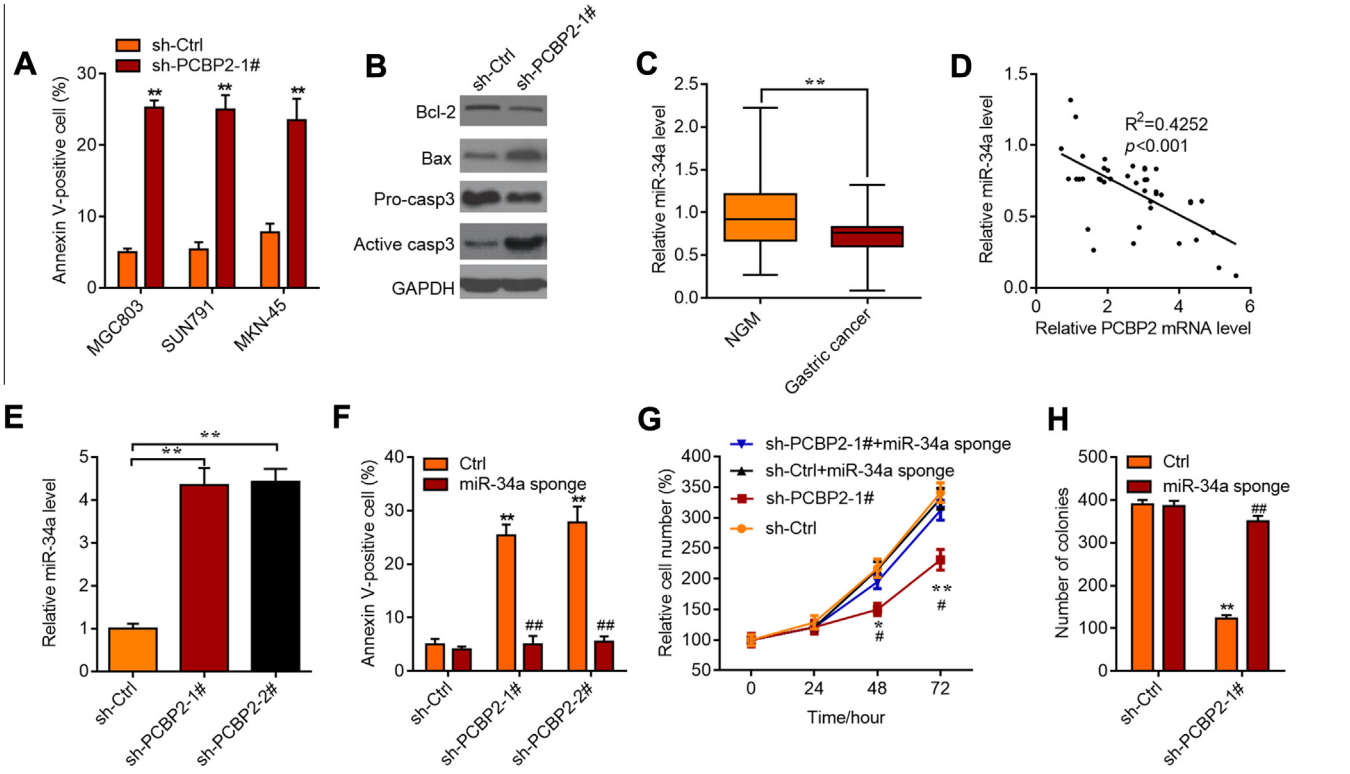

Fig. 4. PCBP2 knockdown induces apoptosis of gastric cancer cells *via* targeting miR-34a. (A, B) PCBP2 knockdown induces apoptosis of gastric cancer cells. Gastric cancer cells MGC803, SUN791 and MKN-45 cells were infected with retrovirus expressing sh-Ctrl or sh-PCBP2 for 48 h. Then the cells were subjected to fluorescence-activated cell sorting (FACS) analysis with indicated markers or were extracted for protein for western blot analysis with indicated antibodies. (A) Apoptotic cells were detected by FACS using Annexin V and propidium iodide (PI) in control MGC803 cells, or cells expressing sh-PCBP2. Quantitative analysis of the results is shown. The values are the means of three independent experiments. $^{**}p < 0.01$. (B) PCBP2 knockdown induces the expression of pro-apoptotic proteins (Bax, active caspase 3) and reduces the level of anti-apoptotic proteins (Bcl-2) in MGC803 cells. (C) miR-34a level decreases in gastric cancer tissues compared to normal gastric mucosa tissues. $N = 18$ in normal NGM group and $n = 44$ in gastric cancer group. (D) Linear regression analysis showing miR-34a level negatively correlates with PCBP2 level in human gastric cancer tissues. (E) PCBP2 knockdown restores the expression of miR-34a in MGC803 cells. (F) miR-34a silence abolishes PCBP2 knockdown induced apoptosis in MGC803 cells. $^{**}p < 0.01$ *vs.* sh-Ctrl. $^{\#\#}p < 0.01$ *vs.* sh-PCBP2. (G) miR-34a silence abolishes PCBP2 knockdown induced reduction of proliferation rate in MGC803 cells. $^{*}p < 0.05$; $^{**}p < 0.01$ *vs.* sh-Ctrl. $^{\#}p < 0.05$ *vs.* sh-PCBP2-1# + miR-34a sponge. (H) miR-34a silence abolishes PCBP2 knockdown induced reduction of colony formation in MGC803 cells. $^{*}p < 0.05$; $^{**}p < 0.01$ *vs.* sh-Ctrl. $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ *vs.* sh-PCBP2-1#.

and translation of many RNA viruses, including poliovirus [21], coxsackievirus [22], and rhinovirus [23]. PCBP2 can also bind to the 5′ UTR [24] and 3′ UTR [25] of the HCV gene. PCBP2 was induced after viral infection and interacted with MAVS, which showed that PCBP2 was a negative regulator of MAVS-mediated antiviral signaling [26].

Recent discoveries have revealed that PCBP2 is a regulator of tumor development. Molinaro et al. [27] found that 2′,5′-oligoadenylate synthetase (OAS) activation may occur in prostate cancer cells *in vivo* when stimulated by Raf kinase inhibitor protein (RKIP) and PCBP2. In leukemic blasts, PCBP2 expression was induced by BCR/ABL through constitutive activation of MAPK-ERK1/2 [28] and its regulation of CEBPA mRNA was influenced by the decoy activity of miR-328 [29]. Recently, Han et al. [13] showed that PCBP2 modulated glioma growth by targeting FHL3. As PCBP2 is a RNA-binding protein, Han et al. used RIP-ChIP analysis to fish PCBP2-binding RNAs and they identified preferentially 35 mRNAs that are associated with PCBP2. However, no non-coding RNAs, neither miRNA nor lncRNA, were present in their list. Here we identified PCBP2 as an oncogenic protein in gastric carcinoma and miR-34a as a novel target of PCBP2 in gastric cancer. When this work is under preparation, Chen et al. [14] uncovered the mechanism underling PCBP2 overexpression in tumor tissues. They showed that SIRT6 bound to the promoter of PCBP2 and deacetylates H3K9ac, leading to the transcription regression of PCBP2. However, in glioma tissues, SIRT6 is significantly down-regulated and subsequently the H3K9 on PCBP2 promoter is hyperacetylated, which results in high expression of PCBP2. Interestingly, it was shown that mice without SIRT6 have a higher risk of gastrointestinal cancers [30], implicating that SIRT6 may b1 account for the change in PCBP2 expression in gastric carcinoma. Nevertheless, further work may be critically essential to prove the hypothesis.

MiR-34a belongs to the miR-34 family and acts as a tumor suppressor in several cancer types. Ectopic miR-34 expression induces apoptosis, cell-cycle arrest or senescence [31]. Recently, miR-34a was reported to be down-regulated in human gastric cancer and miR-34a can serve as an independent prognostic factor [18–20]. However, it remains unknown how miR-34a is down-regulated in gastric cancer, although some studies in other cancers indicate hypermethylation of promoter DNA silent this miRNA. In the present work, we showed that the level of miR-34a is critically controlled by PCBP2. However, further work may be still essential to identify the detailed mechanism underling PCBP2 inhibits miR-34.

In summary, we identify PCBP2 as an oncogenic protein in human gastric cancer. PCBP2 overexpresses in human gastric carcinoma and predicts overall and disease-free survival. PCBP2 promotes gastric cancer cell growth *in vitro* and *in vivo*. Mechanically, PCBP2 maintains gastric cancer cell survival through inhibiting miR-34a. Therefore, PCBP2 may serve as a prognostic marker and a candidate drug target for intervention of gastric carcinoma.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.124.

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