

Original Article

Poly r(C) binding protein (PCBP) 1 expression is regulated at the post-translation level in thyroid carcinoma

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Abstract: Poly r(C) binding protein (PCBP) 1 or heterogeneous ribonucleoprotein (hnRNP) E1 is a RNA binding protein that plays a vital role in a wide variety of biological processes. PCBP1 has been shown to function as a tumor suppressor by negatively regulating translation of pro-metastatic proteins in different cancers. Loss of *PCBP1* expression or its Akt2-mediated phosphorylation at serine 43 residue has both been indicated to de-repress its regulation of EMT inducer proteins. Our previous work has established that *PCBP1* functions as a tumor suppressor in thyroid cancer, where its translation is inhibited by microRNA-490-3p. Here we show that thyroid cancer patients can be divided into 2 cohorts based on miR-490-3p expression and *PCBP1* mRNA expression-one cohort with high *PCBP1* mRNA expression and basal miR-490-3p expression and a second cohort with low *PCBP1* mRNA expression and high miR-490-3p expression. However, PCBP1 protein expression is also downregulated in the cohort with high *PCBP1* mRNA expression, with expression levels similar to what is observed in patients with the low *PCBP1* mRNA expression. Our analysis shows that *PCBP1* mRNA is actively translated in patients with high *PCBP1* mRNA expression, but that the protein is post translationally degraded by the proteasome machinery. Our results thus elucidate a novel mechanism responsible for down regulation of PCBP1 expression in thyroid cancer. It will be important in future to identify the mechanism that causes degradation of PCBP1 protein and to identify if similar mechanisms are active in other tumors characterized by low PCBP1 protein expression.

Keywords: Poly r(C) binding protein, thyroid carcinoma, miR-490-3p

Introduction

Thyroid cancer accounts for 90-95% of all endocrine tumors [1, 2] and has exhibited a steady increase in incidence, especially in men [1]. Approximately 30% of palpable nodules associated with thyroid disorders turn out to be malignant [3]. Papillary, follicular, medullary, or anaplastic are the different forms of thyroid cancer [4], with papillary thyroid cancer (PTC) accounting for 70% of all thyroid tumors [5, 6]. Death due to PTC mostly result from secondary progression to lymph nodes in necks [7, 8]. However, the underlying mechanisms for metastatic dissemination of PTC is not well understood [9].

We have recently shown that the RNA binding protein, poly r(c) binding protein (PCBP) 1 or heterogeneous nuclear ribonucleoprotein

(hnRNP) E1 functions as a putative tumor suppressor in thyroid cancer [10]. PCBP1 has also been previously shown to function as a tumor suppressor by inhibiting translation of genes required for metastatic progression in pancreatic cancer, ovarian cancer, breast cancer, lung cancer, gastric cancer, and Burkitt lymphoma [11-17]. Loss of *PCBP1* expression in prostate cancer is also associated with enrichment of CD44+CD133+CD24- stem cells [18].

Our previous experiments showed that in thyroid cancer, translation of *PCBP1* mRNA is suppressed by the microRNA miR-490-3p [10]. MiR-490-3p expression similarly functions as an oncomirin hepatocellular carcinoma [19]. However, in Ewing's sarcoma and colon cancer, miR-490-3p is downregulated [20, 21], which would suggest that there might be additional mechanisms to downregulate or inactivate

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PCBP1 expression and function during tumor progression.

The objective of the current study was to determine if additional mechanism(s) regulate PCBP1 protein expression in thyroid cancer. Our results cumulatively reveal that PCBP1 is post-translationally degraded in a cohort of thyroid cancer patients who have low miR-490-3p expression.

Materials

Patient samples

The study protocol was approved by the Institutional Review Board of Tianjin Medical University General Hospital, China. Papillary thyroid carcinoma tissue specimens and corresponding adjacent non-tumorous thyroid tissue samples were obtained from 47 Chinese patients at Tianjin Medical University General Hospital between 2012 and 2015. Patients that did not undergo pre-operative local or systemic treatment were included in this study. Of the 47 patients, *PCBP1* mRNA expression was determined as described below and 10 patients each with low and high *PCBP1* expression were included for the final analysis.

Cell culture and treatment

BCAP, WRO, FTC133, TPC1, NPA, FRO, and ARO cells were obtained from the cell and tissue bank at our center and cultured in RPMI1640 medium, containing glutamine, 5% FBS (Lonza, Germany), and penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% carbon dioxide. Where indicated cells were treated with 10 µM of MG-132 (Sigma-Aldrich, Shanghai, China) for 6 hours before being harvested.

Polyribosomal profiling

BCAP, WRO, FTC133, TPC1, NPA, FRO, and ARO cells were treated with 100 µg/mL cycloheximide (Sigma-Aldrich, Shanghai, China) for one hour at 37°C before being washed with cold PBS containing 100 µg/mL Cycloheximide. Cell lysis and polyribosomal profiling was done by lysing cells in polysome lysis buffer (10 mM Tris-Cl, pH 7.4, 5 mM MgCl₂, 100 mM KCl, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 1000 U/ml RNasin, 2 mM DTT and 100 µg/ml

Cycloheximide in DEPC treated water), incubated on ice for 10 minutes and centrifuged at 16,000× g at 4°C to obtain the clarified post-nuclear lysate. One-tenth (v/v) of the lysate was set aside for total RNA isolation. Post-nuclear clarified lysates (50 OD units) were layered on top of 10-50% sucrose gradients and centrifuged at 28,000 rpm (100,000× g) for 4 hours at 4°C using an SW41 rotor (Beckman, Fullerton, CA, USA).

RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

Trizol was used to isolate total RNA and miRNA from tissue specimens and cultured cells. The expression level of *PCBP1* mRNA, *GAPDH* mRNA, miR-490-3p, and *RNU6B* were detected by TaqMan miRNA assays (Life technology, Shanghai, China). The $-\Delta\Delta C_t$ method was used to analyze the data in each case and normalization was done to *GAPDH* and *RNU6B* expression for mRNA and miRNA, respectively, unless otherwise mentioned.

The cancer genome atlas analysis

The TCGA data portal (tcga-data.nci.nih.gov/tcga/) was used to download thyroid carcinoma RNASeqV2 normalized gene expression data on 153 tumor-normal matched pairs. Statistical analysis was performed using the R statistical software. Tumor-normal log₂-fold changes were calculated as $FC_{ij} = \log_2 [(T_{ij} + 1)/(N_{ij} + 1)]$, where FC_{ij} represents the fold change for patient i in gene j . T_{ij} and N_{ij} represent the normalized read counts for gene j in tumor and matched normal samples of patient i , respectively.

Cell and tissue lysis and western blot

Cell lysis and western blot was done as described previously [10]. Blots were probed with anti-PCBP1 antibody (#ab-133421, Abcam) and anti-GAPDH antibody (#ab-9485, Abcam) (to confirm equal loading per sample).

Immunoprecipitation to detect poly-ubiquitinated PCBP1

To detect ubiquitinated-PCBP1, whole cell lysates from TPC1 cells ± MG-132 were prepared as above. Cell lysates (2 mg) were incubated for overnight at 4°C with 50 µg of either mouse anti-PCBP1 antibody or mouse

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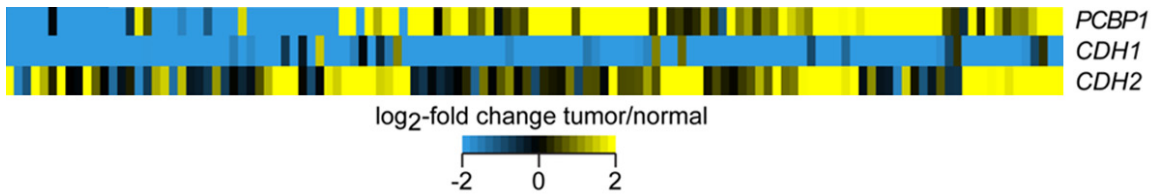


Figure 1. Thyroid cancer patients can be divided into two groups based on *PCBP1* mRNA expression levels. Metagenomic analysis of expression of *CDH1*, *CDH2*, and *PCBP1* in thyroid cancer patients. Heat map depicting log₂-fold change in mRNA expression of indicated genes between tumor-normal matched pairs for 153 TCGA patients.

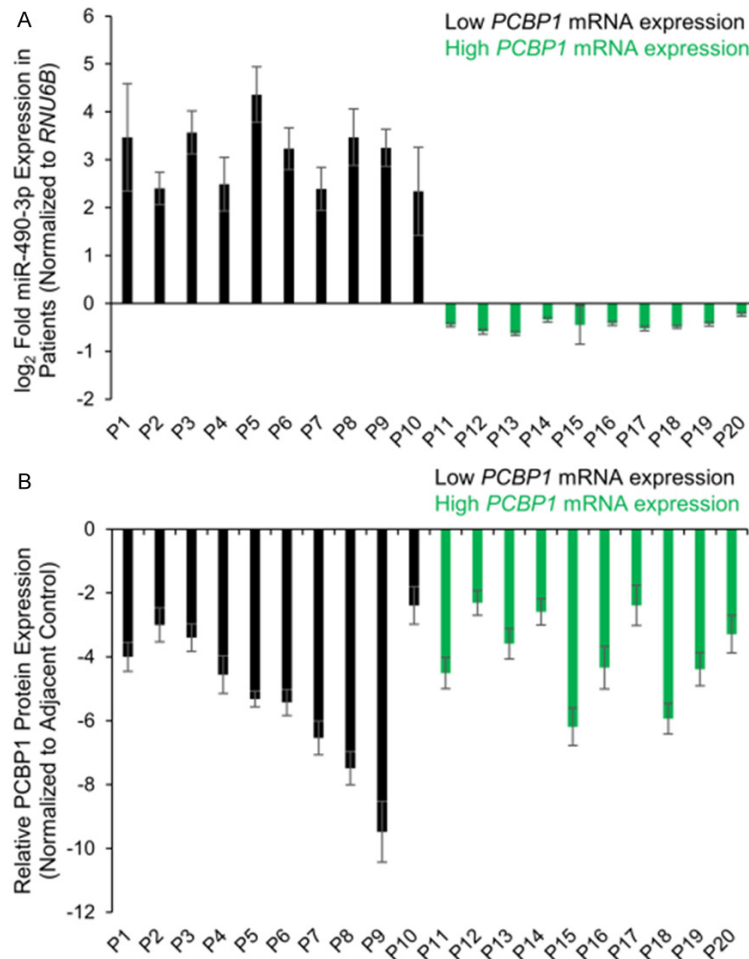


Figure 2. *PCBP1* protein expression is universally suppressed in thyroid cancer patients, irrespective of miR-490-3p expression. A: Steady state expression of miR-490-3p in thyroid cancer and adjacent normal tissues from twenty different patients, ten with high and ten with low *PCBP1* mRNA expression. Data is represented as mean \pm standard deviation. B: Relative *PCBP1* protein expression, normalized to adjacent control tissue, in 20 thyroid cancer tissue specimens. Data is represented as mean \pm standard deviation.

IgG (Sigma-Aldrich, Shanghai, China) cross-linked to protein A/G beads (Pierce Crosslink IP Kit, Life Technologies, Shanghai, China). The immune complexes were collected by centrifu-

gation and eluted with glycine. The eluant were resolved by SDS-PAGE and probed by anti-Ubiquitin antibody (#ab-7780, Abcam) to detect poly-ubiquitinated *PCBP1*.

Statistical analyses

All statistical analyses were performed using the SPSS version 20.0 (IBM Corporation, NY). A *P*-value <0.05 was considered statistically significant.

Results

We have previously shown that miR-490-3p inhibits *PCBP1* translation in thyroid cancer [10]. To determine, if *PCBP1* mRNA was differentially expressed among thyroid cancer patients, we performed metagenomic analysis of The Cancer Genome Atlas (TCGA) data. Along expected lines, *CDH1*, encoding the epithelial cell marker E-cadherin, and *CDH2*, encoding the mesenchymal cell marker N-cadherin, were respectively, down- and up-regulated uniformly among the entire cohort of thyroid cancer patients (Figure 1). However, *PCBP1* mRNA was either up or down regulated

among the cancer patients. Based on *PCBP1* mRNA expression the entire cohort could be sub-divided into 2-one with low *PCBP1* expression and one with high *PCBP1* expression

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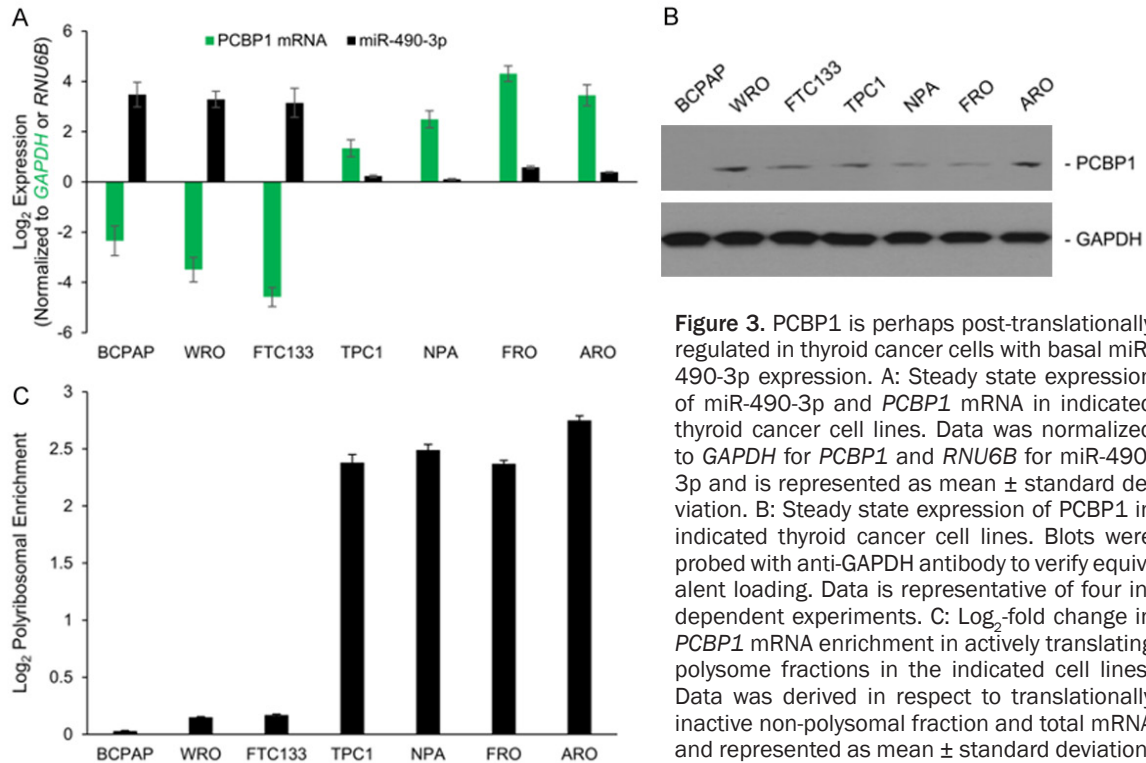


Figure 3. PCBP1 is perhaps post-translationally regulated in thyroid cancer cells with basal miR-490-3p expression. **A:** Steady state expression of miR-490-3p and *PCBP1* mRNA in indicated thyroid cancer cell lines. Data was normalized to *GAPDH* for *PCBP1* and *RNU6B* for miR-490-3p and is represented as mean \pm standard deviation. **B:** Steady state expression of PCBP1 in indicated thyroid cancer cell lines. Blots were probed with anti-GAPDH antibody to verify equivalent loading. Data is representative of four independent experiments. **C:** Log₂-fold change in *PCBP1* mRNA enrichment in actively translating polysome fractions in the indicated cell lines. Data was derived in respect to translationally inactive non-polysomal fraction and total mRNA and represented as mean \pm standard deviation.

(Figure 1), suggesting there are other mechanisms beyond miR-490 that might regulate *PCBP1* expression in thyroid cancer patients.

From our cohort of 47 patients with thyroid cancer, we chose 10 each with low and high *PCBP1* mRNA expression and determined miR-490-3p expression level in these 20 patients. In each of the patients with low *PCBP1* mRNA expression, miR-490-3p expression was significantly high ($P < 0.05$) (Figure 2A). In comparison, in patients with high *PCBP1* mRNA expression, miR-490-3p expression was detected only at the basal level. Our results indicated that *PCBP1* mRNA expression showed an inverse relation to miR-490-3p expression, and the observed cases of low *PCBP1* mRNA expression can be due to the high miR-490-3p expression. Whether transcriptional regulation is also a contributing factor in the later cohort remains to be determined.

We next decide to focus on the cohort of patients with basal miR-490-3p expression and high *PCBP1* mRNA expression. Interestingly, PCBP1 protein expression was significantly suppressed ($P < 0.001$) in all thyroid cancer patients, irrespective of the *PCBP1* mRNA expression level (Figure 2B). This indicated that

in the cohort of patients with high *PCBP1* mRNA and low miR-490-3p expression, there are additional post-transcriptional mechanism (miRNA or RNA-binding protein) or post-translational mechanism that is causing the observed repression of PCBP1 protein.

To find the appropriate *in vitro* model system to further test this observation, we determined miR-490-3p and *PCBP1* mRNA and protein expression in 7 different thyroid cancer cell lines (Figure 3A, 3B). Out of the 7 cell lines tested, miR-490-3p expression was significantly high ($P < 0.05$) in BCPAP, WRO, and FTC133 cells; these were also the cell lines where *PCBP1* mRNA expression was significantly down-regulated ($P < 0.01$) (Figure 3A). In the remaining 4 cell lines, NPA, FRO, ARO, and TPC1, *PCBP1* mRNA expression was high and miR-490-3p was expressed at basal level (Figure 3A). However, PCBP1 protein expression was hardly different in these later 4 cell lines or detectable in any of the 7 cell lines tested (Figure 3B).

To determine if additional post-transcriptional mechanism(s) are operant, we determined polysomal enrichment of *PCBP1* mRNA in each of the aforementioned 7 cell lines (Figure 3C).

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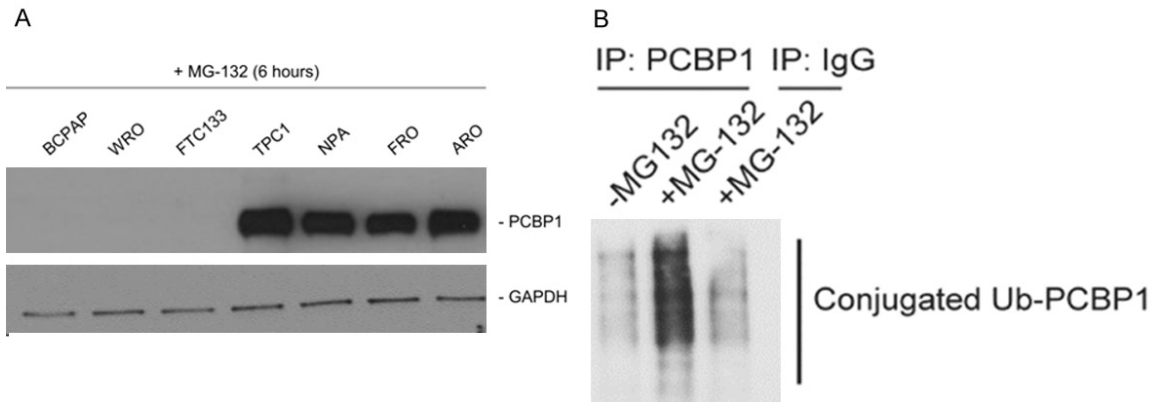


Figure 4. PCBP1 is post translationally modified in thyroid cancer cells. A: Western blot analysis of PCBP1 protein expression in indicated cell lines following treatment with the proteasome inhibitor, MG-132 for six hours. B: PCBP1 immunoprecipitates from untreated and MG-132 treated TPC1 cells were probed with anti-Ubiquitin antibody to determine if PCBP1 protein was being ubiquitinated in these cells. Smear indicates ubiquitinated PCBP1 in the MG-132 treated TPC1 cells.

As has been shown previously [10], and expected based on miR-490-3p expression level, *PCBP1* was excluded from the actively translating polysome fractions in the BCPAP, WRO, and FTC133 cells. However, *PCBP1* was detected at significant levels in the actively translating polysome fractions in each of the 4 cell lines with high *PCBP1* mRNA expression, indicating that protein synthesis was not being inhibited and that post-translational mechanism(s) might be involved.

Hence, we next determined if the difference in the PCBP1 protein expression level was due to altered degradation kinetics in the NPA, FRO, ARO, and TPC1 cells. All the 7 aforementioned cell lines were treated with MG-132, which inhibits proteasomal degradation. Inhibition of proteasomal degradation induced PCBP1 protein expression only in the NPA, FRO, ARO, and TPC1 cells, but not in the BCPAP, WRO, and FTC133 cells (**Figure 4A**), suggesting that differential targeting by the protein degradation machinery is responsible for lack of PCBP1 protein expression in the NPA, FRO, ARO, and TPC1 cell lines.

We next determined if PCBP1 was actually getting ubiquitinated in the TPC1 cells. PCBP1 was immunoprecipitated from MG-132 treated or untreated TPC1 cells and resolved by SDS-PAGE and probed with anti-Ubiquitin antibody. MG-132 treatment lead to detection of a robust poly-ubiquitinated PCBP1 smear in the TPC1 cells (**Figure 4B**).

Discussion

Our results cumulatively show that PCBP1 is indeed a tumor suppressor in the context of thyroid cancer and that these cells have evolved redundant mechanisms to suppress its expression during tumorigenesis and progression. In patients with high miR-490-3p, *PCBP1* mRNA's translation is inhibited; however, in patients with basal miR-490-3p expression, the *PCBP1* mRNA is translated but the protein is targeted for proteasome-mediated ubiquitination and degradation. The degradation machinery remains to be determined in future endeavors.

PCBP1 can regulate the stability of the pro-oncogenic p63 transcript in pancreatic, ovarian and breast cancer cell line [11]. Furthermore, downregulation or loss of *PCBP1* expression has been shown to upregulate translation of genes and long non-coding RNA required for epithelial to mesenchymal transition and metastatic progression in different cancers, and Burkitt lymphoma [12-17]. However, that PCBP1 itself can be regulated at the level of protein stability has not been previously indicated in any of the aforementioned cases.

It will be important to understand the degradation machinery and particularly what regulates the machinery. It can be hypothesized that the same regulatory mechanism will have a way to sense miR-490-3p expression levels to regulate its own activation. Similarly, it will be important to determine if similar redundant mecha-

nisms regulate PCBP1 expression in other cancers.

Of note, in certain cases like colon cancer and Ewing's sarcoma, miR-490-3p expression has been shown to be suppressed [20, 21]. To better understand the regulation of PCBP1 expression, it will be vital to use colon cancer and Ewing's sarcoma as a model to determine if PCBP1 expression is downregulated and if so whether it is by proteasomal degradation, as can be extrapolated from our own findings in thyroid cancer.

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Disclosure of conflict of interest

None.

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