Research Article

Poly r(C) binding protein (PCBP) 1 expression is regulated by the E3 ligase UBE4A in thyroid carcinoma

Ming-Peng Zhang, Wei-San Zhang, Jin Tan, Ming-Hui Zhao, Lin-Juan Lian and Jie Cai

Department of Geriatrics, Tianjin Medical University General Hospital, Tianjin Geriatrics Institute, Tianjin 300052, China

Correspondence: Ming-Peng Zhang (mpzhang_med@126.com)

Thyroid cancer patients with high miR-490-3p inhibit translation of PCBP1 mRNA, whereas in patients with low miR-490-3p PCBP1 mRNA expression is high; however, the resultant protein is targeted for degradation through the proteasome. The objective of the present study was to evaluate the molecular mechanism that regulates post-translation degradation of poly r(C) binding protein (PCBP) 1 expression in thyroid cancer cells. Mass spectrometric analysis of PCBP1 immunoprecipitates from MG-132 treated TPC1 cells revealed a list of ubiquitin ligases associated with PCBP1. RNAi-mediated silencing of the candidate ubiquitin ligases revealed that knockdown of the ubiquitin ligase UBE4A stabilized PCBP1 in TPC1 cells. Concurrent overexpression of the candidate ubiquitin ligases in the normal thyroid epithelial cell line Nthy-ori 3-1 confirmed that ubiquitin conjugation factor E4 A (UBE4A) is the ubiquitin ligase that is degrading PCBP1. Coimmunoprecipitation of HA-tagged PCBP1 in TPC1 cells cotransfected with FLAG–UBE4A revealed robust polyubiquitinated smear of PCBP1, thus confirming UBE4A as the ubiquitin ligase of PCBP1. UBE4A expression mimicked PCBP1 mRNA expression in thyroid cancer patients and was inversely correlated to PCBP1 protein expression. Low UBE4A expression level was associated with a better prognosis in thyroid cancer patients. Our data reveal a post-translational regulatory mechanism of regulating PCBP1 expression in thyroid cancer cells.

Introduction

Thyroid cancer is the major form of all endocrine tumors [1,2]. Thyroid cancer has shown steady increase in incidence especially within male patients [1]. Almost 30% of cases initially diagnosed as thyroid disorders ultimately turn out to be malignant progression [3]. Papillary thyroid cancer (PTC) is the major form of thyroid cancer, the other forms being follicular, anaplastic, or medullary [4-6]. Mortality associated with thyroid cancer is largely associated with metastatic dissemination to lymph nodes within the neck, mechanisms of which are slowly evolving [7-9]. Hence, finding biomarkers that would aid in early diagnosis or help better in indicating prognosis are actively sought. Our previous work has revealed that the RNA-binding protein, heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1), or poly r(C) binding protein (PCBP)1 function as tumor suppressor in thyroid cancer [10,11].

We have shown that expression of PCBP1 mRNA can be suppressed at the translation stage by the microRNA, miR-490-3p [10] or in cases of thyroid cancer where miR-490-3p are expressed in low levels, PCBP1 protein can be degraded through the proteasome [11]. Hence, redundant mechanisms act in sync to regulate PCBP1 protein expression or stability during thyroid cancer. This is not entirely without precedence.

PCBP1 functions as a tumor suppressor by inhibiting translation at the elongation phase of a cohort of genes required for metastatic progression in breast, ovarian, lung, and pancreatic cancer, as well as in...
Burkitt lymphoma [12-18]. The regulatory function of PCBP1 in cancers can be determined through loss expression of PCBP1 or phosphorylation-mediated inhibition of binding to its cognate targets [10,12-18].

The objective of the present study was to define how PCBP1 expression is suppressed at the post-translational level in thyroid cancer. Our results show that the E3 ligase, ubiquitin conjugation factor E4 A (UBE4A) targets PCBP1 for proteasomal degradation and that UBE4A expression can serve as a novel biomarker in thyroid cancer patients.

**Materials and methods**

**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. Inclusion criteria were patients that did not undergo preoperative local or systemic treatment and for whom follow-up data were available. Of the 47 patients, PCBP1 mRNA expression was determined as described below and ten patients each with low and high PCBP1 expression were included for the final analysis.

**Cell culture and treatment**

TPC cells were obtained from the cell and tissue bank at our center, whereas the normal thyroid epithelial cell line Nthy-ori 3-1 was obtained from Sigma-Aldrich, Shanghai, China. Both cells were cultured in RPMI1640 medium, containing glutamine, 5% FBS (Lonza, Germany), and penicillin/streptomycin. Cells were maintained at 37°C under a humidified atmosphere of 5% carbon dioxide, where indicated cells were treated with 10 μM MG-132 (Sigma-Aldrich, Shanghai, China) for 6 h before being harvested.

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

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) to detect PCBP1 expression were done as described previously [11]. Data were represented as mean ± standard deviation of three independent experiments, each done in triplicates.

**Gene construction and transfection**

Scrambled siRNA (control) or siRNAs targeting indicated genes were obtained from Life Technologies (Silencer Select; Shanghai, China). Indicated overexpression constructs were generated by amplifying the coding region from cDNA obtained from TPC1 cells and sub-cloned into pEF-HA vector (Addgene). TPC1 cells (4 × 10⁶) were transiently transfected with indicated plasmids or siRNAs using Lipofectamine 3000 (Life Technologies, Beijing, China). Cells were harvested 72 h after transfection and analyzed as indicated.

**Mass spectrometry**

In-gel trypsin digestion of silver-stained gel was performed with 5–10 ng/l of trypsin (Mass Spectrometric grade) and 50 mM ammonium bicarbonate and incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted three times with 10–20 μl of 5% trifluoroacetic acid in 50% acetoniitrile and dried using a vacuum centrifuge for 30 min. The dried samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager-DE PRO, ThermoFisher Scientific) for peptide mass fingerprinting at a local core facility. Peptides were analyzed using the MASCOT algorithm. Prioritization of putative E3 ligases to be subsequently tested was done based on peptide coverage threshold of 40%.

**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 as described previously [11].

**Immunohistochemistry**

Tissue specimens from 20 patients with thyroid cancer were stained for PCBP1 expression (#ab-133421, Abcam). The stained slides were scored by a pathologist as percent staining (0–100%) blinded to the identity of the tissue cores.
**Figure 1. PCBP1 is post-translationally degraded in thyroid cancer cells**

(A) Western blot analysis of PCBP1 protein expression in normal thyroid epithelial cells, Nthy-ori 3-1, and the thyroid cancer cell line, TPC1. Blots were re-evaluated with anti-GAPDH antibody to confirm equal loading. (B) Quantitative RT-PCR analysis of PCBP1 mRNA expression in Nthy-ori 3-1 and TPC1. Data were normalized to GAPDH expression. (C) Silver stain of PCBP1 immunoprecipitates from untreated and MG-132 treated TPC1 cells. Bands were cut and analyzed by mass spectrometry. Indicated is PCBP1 that could be detected only in the MG-132 treated cells.

**Survival analysis**

Overall survival analysis of the patients in our data set was performed using the Kaplan–Meier method and assessed via log-rank test.

**In vitro ubiquitination reaction**

To detect ubiquitinated-PCBP1, TPC1 cells were transiently transfected with HA–PCBP1 or FLAG–UBE4A as indicated. For immunoprecipitation (IP), whole cell lysates were prepared as above. Cell lysates (2000 µg) were incubated for overnight at 4°C with 50 µg of either mouse anti-HA antibody or mouse 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 centrifugation, washed five times in IP lysis buffer, and eluted with glycine. The eluant were resolved by SDS/PAGE and probed by anti-Ubiquitin antibody (#ab-7780, Abcam).

**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 *PCBP1* expression can be inhibited at the translation level by miR-490-3p (10) and in cells where miR-490-3p expression is low, PCBP1 is degraded by proteasomal degradation. Hence, our goal was to find the E3 ligase that mediates degradation of PCBP1.

We initially confirmed PCBP1 protein expression in the normal thyroid epithelial cell line, Nthy-ori 3-1, and the thyroid cancer cell line TPC1. Whereas robust PCBP1 protein expression was detected in the Nthy-ori 3-1 cells, it was hardly detected in the TPC1 cells (Figure 1A). Differential protein expression was not due to difference in *PCBP1* mRNA expression (Figure 1B) (*P*>0.05). To identify the cognate E3 ligase, we performed mass spectrometry analysis using a local core facility. PCBP1 and its interacting partners were immunoprecipitated from untreated and MG-132 treated TPC1 cells under the rationale that MG-132 stabilizes the protein and thus comparison of the two cases will indicate differential association with putative E3 ligases (Figure 1C). Our mass spectrometry analysis revealed 43
Figure 2. UBE4A is a putative E3 ligase that causes PCBP1 protein degradation

(A) TPC1 cells were transiently transfected with siRNAs targeting indicated E3 ligases. Western blot analysis to detect PCBP1 expression in mock transfected (control) or transfected with indicated siRNAs was performed. The blot was stripped and probed with GAPDH to serve as a loading control. (B) Nthy-ori 3-1 cells were transiently transfected with ectopic overexpression construct of indicated E3 ligases and lysates were probed for PCBP1 expression. The blot was stripped and probed with GAPDH to serve as a loading control.

Figure 3. UBE4A interacts with and ubiquitylates PCBP1 in thyroid cancer cells

(A) Western blot analysis of UBE4A protein expression in normal thyroid epithelial cells, Nthy-ori 3-1, and the thyroid cancer cell line, TPC1. Blots were re-evaluated with anti-GAPDH antibody to confirm equal loading. (B) TPC1 cells were transiently transfected with HA–PCBP1 and FLAG–UBE4A as indicated. Seventy-two hours post-transfection lysates were harvested and immunoprecipitated (IP) as indicated. Immunoprecipitates were resolved by SDS/PAGE and probed with anti-HA (to detect PCBP1) and anti-FLAG (to detect UBE4A). (C) TPC1 cells were transiently transfected with HA–PCBP1 and FLAG–UBE4A as indicated. Seventy-two hours post-transfection cells were treated +− MG-132 for 6 h. Lysates obtained from these cells were immunoprecipitated with HA antibody and resolved by SDS/PAGE. The membrane was probed with anti-Ubiquitin antibody. Shown are poly-ubiquitinated bands of PCBP1 in MG-132 treated cells that were transfected with FLAG–UBE4A, but were absent where FLAG–UBE4A was not cotransfected, or cells were not treated with MG-132.

putative E3 ligases that were interacting with PCBP1. This list was prioritized to ten putative E3 ligases based on a cut-off of at least 40% peptide coverage in the mass spectrometry analysis.

For each of the ten E3 ligases, TPC1 cells were transiently transfected with siRNA targeting those E3 ligase and then PCBP1 protein expression was determined. Transfection of siRNA targeting UBE4A, encoding ubiquitin conjugation factor E4 A E3 ligase, induced accumulation of PCBP1 protein expression (Figure 2A), indicating this might be the ligase that targets PCBP1 for proteasomal degradation. To confirm the same, we overexpressed each of the ten E3 ligases in the normal thyroid cell line, Nthy-ori 3-1, and found that only overexpression of UBE4A resulted in loss of detection of PCBP1 (Figure 2B), confirming that UBE4A has a role to play in the stability of PCBP1 protein.

We next determined endogenous expression level of UBE4A in the TPC1 and Nthy-ori 3-1 cell lines. UBE4A was robustly expressed in TPC1 cell line and hardly detectable in the Nthy-ori 3-1 cell line (Figure 3A), which was inverse to PCBP1 protein expression observed in these cell lines (Figure 1A). Immunoprecipitation analysis revealed
UBE4A expression was a novel prognostic factor for thyroid cancer patients. Heat map depicting log2-fold change in mRNA expression of indicated genes between tumor-normal matched pairs for 153 TCGA patients. UBE4A mRNA levels were determined in tissue specimens and tumor adjacent normal tissue obtained from 20 patients. PCBP1 expression was determined by immunohistochemistry and scored as percent staining. UBE4A expression and PCBP1 histology scores were plotted against each other and found to be inversely correlated in these 20 patients with thyroid carcinoma. Pearson correlation demonstrating the inverse relation between UBE4A and PCBP1 in paired samples (P<0.05; Pearson correlation, r = −0.9113). Kaplan–Meier survival analysis was done in thyroid cancer patients with high and low UBE4A expression levels and assessed via log-rank test. Higher UBE4A expression was associated with a significantly less favorable outcome (log-rank P-value = 0.01963).

Discussion

In the present study, our experimental results show that PCBP1 protein is targeted for degradation by the E3 ligase UBE4A in thyroid cancer patients that have high PCBP1 mRNA expression and basal miR-490-3p expression. We also showed that UBE4A is the E3 ligase that degrades PCBP1 protein in thyroid cancer cells but not in normal thyroid epithelial cells. Our findings highlight a novel post-translational mechanism to regulate PCBP1 expression.
In fact, data mining showed that UBE4A and PCBP1 protein expression follow an inverse correlation in thyroid cancer patients and that thyroid cancer patients can be divided into two distinct cohorts based on PCBP1 and UBE4A mRNA expression. UBE4A expression could serve as a putative prognostic marker in these patients. The profound induction in relative expression of PCBP1 in Nthy-ori 3-1 cells and its active degradation in the TPC1 cells suggests that UBE4A functions as a putative tumor promoter in thyroid cancer.

UBE4A and UBE4B are U-box-containing ubiquitination enzymes and are the two human homologues of yeast UFD2 ubiquitination factor. They have been shown to be mutated in different cancer, including neuroblastoma and colorectal cancer [19,20]. The yeast homologue Ufd2 has been shown to ubiquitylate and degrades the cell cycle kinase Mps1 that is a core component of the anaphase promoting complex E3, and that this function is conserved in humans [21]. UBE4A expression has also been shown to be dependent on cell cycle progression [22], substantiating its role on cell growth and proliferation. However, role of UBE4A has not been previously elucidated in the context of thyroid cancer.

Our previous work has shown that in thyroid cancer patients with high miR-490-3p expression, PCBP1 mRNA is translationally inhibited, whereas in patients with low or basal miR-490-3p expression, PCBP1 protein is targeted for degradation [10,11]. We now show that it is UBE4A that functions as the E3 ligase to target PCBP1 to the degradation machinery. It remains to be determined what regulates UBE4A and miR-490-3p in thyroid cancer patients such that they are expressed in a mutually exclusive fashion in these patients to ensure that PCBP1 expression is down-regulated. Given that PCBP1 is a well-established tumor suppressor [10-18] and UBE4A is an enzyme, determination of this cross-talk in normal physiological conditions and in pathological context will be vital for rational design of therapeutic strategy to target UBE4A expression.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Funding
This study was supported by the project Mechanism and Effect of Hypothyroidism Induced by Chronic Intermittent Hypoxia (grant number 2013KZ138) from the Science and Technology Foundation of Tianjin Municipal Health Bureau.

Author Contribution
M.-P.Z. and W.-S.Z. designed the experiment; J.T. and M.-H.Z. prepared the manuscript and carried out the analysis; L.-J.L. and J.C. collected samples. All authors read and approved the final manuscript.

Abbreviations
GAPDH, Glycerinaldehyde-3-phosphate dehydrogenase; IP, immunoprecipitation; PCBP, poly r(C) binding protein; PTC, papillary thyroid cancer; UBE4A, ubiquitin conjugation factor E4 A.

References
1 Hodgson, N.C., Button, J. and Solorzano, C.C. (2004) Thyroid cancer: is the incidence still increasing? Ann. Surg. Oncol. 11, 1093–1097
2 Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J. and Thun, M.J. (2009) Cancer statistics, 2009. CA Cancer J. Clin. 59, 225–249
3 Mackenzie, E.J. and Mortimer, R.H. (2004) Thyroid nodules and thyroid cancer. Med. J. Aust. 180, 242–247
4 Kondo, T., Ezzat, S. and Asa, S.L. (2006) Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nat. Rev. Cancer 6, 292–306
5 Fitzgibbons, S.C., Brams, D.M. and Wei, J.P. (2008) The treatment of thyroid cancer. Am. Surg. 74, 389–399
6 Kita, Y., Shimizu, K., Ito, K., Tanaka, S. and Emi, M. (2001) Allelotype of follicular thyroid carcinoma: frequent allelic losses in chromosome arms 7q,11p, and 22q. J. Clin. Endocrinol. Metab. 86, 4268–4272
7 Nikiforov, Y.E. (2008) Thyroid carcinoma: molecular pathways and therapeutic targets. Mod. Pathol. 21, S37–S43
8 Suster, S. (2006) Thyroid tumors with a follicular growth pattern: problems in differential diagnosis. Arch. Pathol. Lab. Med. 130, 984–988
9 Vasko, V.V. and Saji, M. (2007) Molecular mechanisms involved in differentiated thyroid cancer invasion and metastasis. Curr. Opin. Oncol. 19, 11–17
10 Zhang, M., Wang, X., Tan, J., Zhao, M., Lian, L. and Zhang, W. (2016) Poly r(C) binding protein (PCBP1) is a negative regulator of thyroid carcinoma. Am. J. Transl. Res. 8, 3567–3573
11 Zhang, M.P., Zhang, W.S., Tan, J., Zhao, M.H., Lian, L.J. and Cai, J. (2017) Poly r(C) binding protein (PCBP1) 1 expression is regulated at the post-translation level in thyroid carcinoma. Am. J. Transl. Res.
12 Cho, S.J., Jung, Y.S. and Chen, X. (2013) Poly (C)-binding protein 1 regulates p63 expression through mRNA stability. PLoS One 8, e71724
13 Chaudhury, A., Hussey, G.S., Ray, P.S., Jin, G., Fox, P.L. and Howe, P.H. (2010) TGF-beta-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. Nat. Cell Biol. 12, 286–293
14 Hussey, G.S., Chaudhury, A., Dawson, A.E., Lindner, D.J., Knudsen, C.R., Wilce, M.C. et al. (2011) Identification of an mRNP complex regulating tumorigenesis at the translational elongation step. Mol. Cell 41, 419–431

© 2017 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
15 Wang, H., Vardy, L.A., Tan, C.P., Loo, J.M., Guo, K., Li, J. et al. (2010) PCBP1 suppresses the translation of metastasis-associated PRL-3 phosphatase. Cancer Cell 18, 52–62
16 Liu, Y., Gai, L., Liu, J., Cui, Y., Zhang, Y. and Feng, J. (2015) Expression of poly(C)-binding protein 1 (PCBP1) in NSCLC as a negative regulator of EMT and its clinical value. Int. J. Clin. Exp. Pathol. 8, 7165–7172
17 Zhang, Z.Z., Shen, Z.Y., Shen, Y.Y., Zhao, E.H., Wang, M., Wang, C.J. et al. (2015) HOTAIR long noncoding RNA promotes gastric cancer metastasis through suppression of poly r(C)-binding protein (PCBP) 1. Mol. Cancer Ther. 14, 1162–1170
18 Wagener, R., Aukema, S.M., Schlesner, M., Haake, A., Burkhardt, B., Claviez, A. et al. (2015) IGCG MMML-Seq-Project: “Molecular Mechanisms in Malignant Lymphomas” Network Project of the Deutsche Krebsforschung. The PCBP1 gene encoding poly(rC) binding protein 1 is recurrently mutated in Burkitt lymphoma. Genes, Chromosomes Cancer 54, 555–564
19 Carén, H., Holmstrand, A., Sjöberg, R.M. and Martinsson, T. (2006) The two human homologues of yeast UFD2 ubiquitination factor, UBE4A and UBE4B, are located in common neuroblastoma deletion regions and are subject to mutations in tumours. Eur. J. Cancer 42, 381–387
20 Naudin, C., Sirvent, A., Leroy, C., Larive, R., Simon, V., Pannequin, J. et al. (2014) SLAP displays tumour suppressor functions in colorectal cancer via destabilization of the SRC substrate EPHA2. Nat. Commun. 5, 3159
21 Liu, C., van Dyk, D., Choe, V., Yan, J., Majumder, S., Costanzo, M. et al. (2011) Ubiquitin ligase Ufd2 is required for efficient degradation of Mps1 kinase. J. Biol. Chem. 286, 43660–43667
22 Contino, G., Amati, F., Pucci, S., Pontieri, E., Pichiorri, F., Novelli, A. et al. (2004) Expression analysis of the gene encoding for the U-box-type ubiquitin ligase UBE4A in human tissues. Gene 328, 69–74