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ORIGINAL ARTICLE

Ribotrap Analysis of Proteins Associated with FHL3 3'Untranslated Region in Glioma Cells[△]

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Objective To screen the proteins associated with four-and-a-half LIM domains 3 (FHL3) 3' untranslated region (3'UTR) in glioma cells.

Methods Western blot was adopted to detect the regulatory effect of poly(C)-binding protein 2 (PCBP2) on FHL3. Biotin pull-down and sliver staining were employed to screen and verify the candidate binding proteins of FHL3 3'UTR. Then liquid chromatography-tandem mass spectrometry (LC-MS/MS) and molecule annotation system were used to identify and analyze the candidate binding proteins. Immunoprecipitation was conducted to study the interaction between PCBP2 and polypyrimidine tract-binding protein 1 (PTBP1), a binding protein identified by LC-MS/MS.

Results PCBP2 could bind to FHL3 mRNA 3'UTR-A and inhibited the expression of FHL3 in T98G glioma cells. 22 candidate binding proteins were identified. Among them, there were 11 RNA binding proteins, including PCBP2. PTBP1 associated with FHL3 mRNA 3'UTR and interacted with PCBP2 protein.

Conclusion PCBP2 and PTBP1 can both associate with FHL3 mRNA 3'UTR through forming a protein complex.

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RIBOTRAP is an immunoaffinity tool to identify RNA-binding proteins (RBPs) that specifically bind to mRNAs of interest.¹ Multiple RBPs may associate with a given RNA and vice versa, each RBP may associate with a number of RNAs, and the RNA-RBP interactions are responsive to regulatory signals.² Although the characterization of mRNAs targeted by a specific RBP has been accelerated by the application of RBP immunoprecipitation-Chip (RIP-Chip),³ reliable methods to describe the endogenous assembly of ribonucleoproteins (RNPs) are needed.

We previously reported that poly(C)-binding protein 2 (PCBP2), an important member in heterogeneous nuclear ribonucleoprotein (hnRNP) family, plays an oncoprotein-like role in human glioma tissues and cell lines. Using RIP-Chip analysis, we identified 35 mRNAs that preferentially bind PCBP2. Four-and-a-half LIM domain 3 (FHL3) has been confirmed as a novel binding target of PCBP2 and can inhibit glioma cells proliferation and anti-apoptosis ability. We further found that PCBP2 binds to the FHL3 mRNA 3' untranslated region (UTR), and negatively regulates FHL3 expression through affecting its mRNA stability.⁴

In addition, FHL3 is a negative regulator of proliferation in breast cancer and hepatocellular carcinoma (HCC).^{5,6} FHL3 protein was also reported to inhibit hypoxia-inducible factor 1 transcriptional activity in HCC cells and identified as a novel Angiogenin binding partner,^{7,8} which suggested that FHL3 will be a key gene involved in cancer progression. Therefore it is meaningful to explore the regulatory mechanism of FHL3 itself, such as in the posttranscriptional level.

In this study, we applied a Ribotrap methodology in FHL3 mRNA 3'UTR specific purification of a target RNP by biotin pull-down assay and identified the proteins associated with FHL3 3'UTR in glioma cells, including PCBP2, by mass spectrometry, so as to lay the foundation for exploring the upstream molecular mechanism of FHL3 in glioma.

MATERIALS AND METHODS

Glioma cell culture and plasmids transfection

Human glioma cell line T98G was purchased from American Type Culture Collection and cultured according to the guidelines recommended by the center. Biotin acceptor peptide (BAP)-PCBP2 was constructed as previously described.⁴ The plasmid was purified using the EndoFree Plasmid Maxi Kit (QIAGEN, Germantown, MD, USA) and transfected into T98G cells using VigoFect (Vigorous Biotechnology, Beijing).

Western blot

Whole-cell extracts were obtained by lysing cells in TNTE buffer (50 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 0.5% Triton X-100, 1 mmol/L sodium vanadate, and 25 mmol/L sodium fluoride) containing protease inhibitors (5 µg/ml phenylmethanesulfonyl fluoride, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, and 0.5 µg/ml aprotinin). The protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Amersham, USA) and detected using polyclonal rabbit anti-PCBP2 (1:2000, Beijing Aviva Systems Biology or MBL International Corporation), rabbit anti-FHL3 (1:500, Proteintech Group, Chicago, IL, USA), anti-PTBP1 (1:3000, generated by our laboratory) or mouse anti-β-actin (1:5000, Sigma, St. Louis, MO, USA) antibodies.

Biotin pull-down

Fragments located in the 3'UTR-A or -B of FHL3 mRNA were constructed into the pGEM-3zf vector containing a T7 promoter. The primers sequences were shown as follows: 3'UTR-A, (forward) 5'-CGGGATCCCAGGACTGTGGCTCCTTTTC-3', (reverse) 5'-CCCAAGCTTGCACCCATAGGAGACCTGAA-3'; 3'UTR-B, (forward) 5'-CGGGATCCGGACTGTTCTCAGGCTTGAC-3', (reverse) 5'-CCCAAGCTTCACACCGCTTTATTGCAGAATC-3'. The biotin-labeled sense RNA probes were synthesized *in vitro* using T7 RNA polymerase (Takara, Dalian, China). Cytoplasmic cell extracts were isolated from T98G cells using nuclear protein extraction reagents (NE-PER) (Thermo Scientific, Waltham, MA, USA). RNA affinity capture was subsequently conducted with streptavidinsepharose beads as previously described.⁹

Immunoprecipitation

For the immunoprecipitation assay, equal amounts (1200-1500 µg) of the cell lysates were incubated with 1/100 (wt/wt) of rabbit IgG or the anti-PTBP1 antibody overnight at 4°C. Subsequently, the lysates were incubated with Protein A sepharose (Roche Co., Mannheim, Germany) while rotating for 3 hours at 4°C. Immunoprecipitates were washed four times with lysis buffer without the protease inhibitor, eluted in 20 µl of 6×loading buffer at 98°C and analyzed by immunoblot analysis.

Silver staining

To identify all the FHL3 mRNA 3'UTR specific binding proteins, the transcribed *in vitro* biotin-labeled RNA probes, FHL3 3'UTR-A and 3'UTR-B, were used as the templates for assembling bound proteins from T98G cells in biotin

pull-down assay. Silver staining presents the proteins associated with FHL3 mRNA 3'UTR-A or 3'UTR-B (the experimental group) compared with negative control. The protein samples from biotin pull-down were separated using 10% SDS-PAGE, and the gel was then incubated with the stationary liquid (50% methanol, 5% acetic acid) for 20 minutes. After washed with washing buffer for 10 minutes, the gel was further incubated with sensitization buffer (0.05 g Na₂S₂O₃, 250 ml H₂O) for 1 minute. It was later incubated with silver staining buffer for 20 minutes at 4°C and then with color reagent (4 g Na₂CO₃, 80 µl formaldehyde, 200 ml H₂O) for 6 minutes.

Liquid chromatography-tandem mass spectrometry

The stained protein bands after silver staining on the gel were carved, digested, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the National Center of Biomedical Analysis. Sequence analysis was performed with MASCOT (version 2.2, Matrix Sciences, Boston, MA, USA) using the non-redundant protein database from Mascot's web site (http://www.matrixscience.com/help/data_file_help.html). The bound proteins were screened according to ions scores, in which a number greater than 38 indicates identity or extensive homology ($P < 0.05$).

Bioinformatical analysis

We utilized gene ontology (GO) to investigate the nature of the FHL3 3'UTR associated proteins identified by LC-MS/MS. The GO terms were analyzed using the web-based tool CapitalBio molecule annotation system (MAS V3.0, CapitalBio Corporation, Beijing, China). Molecular function, biological process and cellular component of the candidate proteins were analyzed.

RESULTS

Effect of PCBP2 on target FHL3 expression

The expression of FHL3 was decreased after overexpressing BAP-PCBP2 fusion construct in T98G glioma cell line (Fig. 1A). The fragments A and B located in the 3'UTR of FHL3 mRNA were predicted to contain potential binding sites by bioinformatical analysis (Fig. 1B). PCBP2 was not detected in the FHL3 3'UTR-B pull-down complex, while FHL3 3'UTR-A showed a strong binding of PCBP2 (Fig. 1C).

Candidate binding proteins to FHL3 3'UTR identified by LC-MS/MS

18 bands were selected in the experimental group (Fig. 2). Based on the ions scores and MASCOT database, 22 candidate proteins were identified after eliminating the same proteins (Table 1). Among the molecular functions, approximately 86.4% (19/22) and 50.0% (11/22) of the candidate proteins were related to the functions of protein binding and RNA binding, respectively. More than 50% of the proteins were localized in the nucleus (16/22, 72.7%) or cytoplasm (12/22, 54.5%) according to the result of cellular component analysis. The biological processes showed enrichment in RNA processing, export and stability (40.9% gene frequency), regulation of transcription (27.3% gene frequency), and cell grow and apoptosis (27.3% gene frequency) (Fig 3).

Possible interaction between PTBP1 and PCBP2

Among the 22 proteins identified by LC-MS/MS, we discovered another member of hnRNP family, PTBP1. It was also reported up-regulated in gliomas. With biotin pull-down assay, we confirmed that PTBP1 could combine with FHL3 3'UTR (Fig 4A). PTBP1 also interacted with PCBP2 as shown by immunoprecipitation (Fig 4B).

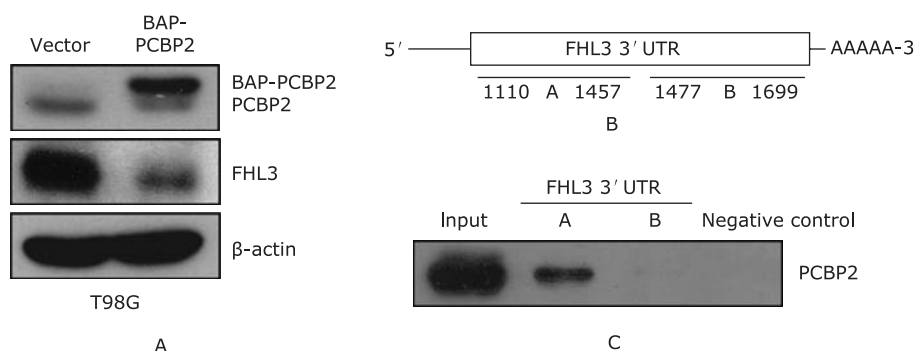


Figure 1. PCBP2 inhibits expression of FHL3 and binds to FHL3 mRNA 3' untranslated region (UTR).

A. Representative Western blot shows the decreased FHL3 protein level in biotin acceptor peptide (BAP)-PCBP2 overexpressing T98G cells. β-actin was used as a loading control.

B. Schematic of FHL3 mRNA 3'UTR demonstrates the specific fragments A and B used as templates for the synthesis of biotin-labeled RNAs and the nucleotide positions amplified by PCR.

C. Biotin pull-down analysis of complexes formed *in vitro* using biotin-labeled FHL3 3'UTR-A or 3'UTR-B and T98G whole-cell lysates and detected using anti-PCBP2 antibody. A nonsense sequence from pGEM-3zf vector was included as negative control.

Table 1. The result of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for FHL3 3'UTR associated proteins

Number	Protein	GI	Score	Description
1	SFPQ	4826998	295	splicing factor proline/glutamine-rich (polypyrimidine tract binding protein associated)
2	ILF3	119604539	458	interleukin enhancer binding factor 3, 90kDa
3	IGF2BP1	56237027	510	insulin-like growth factor 2 mRNA binding protein 1
	KHDRBS1	5730027	257	KH domain containing, RNA binding, signal transduction associated 1
	IGF2BP3	30795212	246	insulin-like growth factor 2 mRNA binding protein 3
	SYNCRIP	119569012	149	synaptotagmin binding, cytoplasmic RNA interacting protein
	IGF2BP2	4191610	130	insulin-like growth factor 2 mRNA binding protein 2
4	PTBP1	4506243	360	polypyrimidine tract binding protein 1
5	SERBP1	12803339	425	SERPINE1 mRNA binding protein 1
6	HNRNPH1	5031753	104	heterogeneous nuclear ribonucleoprotein H1 (H)
7	PCBP2	14141166	270	poly(rC) binding protein 2
8	HNRNPA2/B1	14043072	115	heterogeneous nuclear ribonucleoprotein A2/B1
9	unnamed protein	194387672	128	unknown
10	GNB2L1	5174447	274	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1
11	PHB	4505773	172	prohibitin
12	NDUFS3	4758788	101	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)
13	EEF2	4503483	235	eukaryotic translation elongation factor 2
	CSE1L	951338	207	CSE1 chromosome segregation 1-like (yeast)
	TRIM28	1699027	155	tripartite motif-containing 28
	EIF4G2	110611823	121	eukaryotic translation initiation factor 4 gamma, 2
14	ILF3	119604539	547	interleukin enhancer binding factor 3, 90kDa
15	IGF2BP1	56237027	418	insulin-like growth factor 2 mRNA binding protein 1
16	RUVBL1	4506753	303	RuvB-like 1 (E. coli)
17	PHB	4505773	303	prohibitin
18	EEF1A1	31092	64	eukaryotic translation elongation factor 1 alpha 1

GI: MASCOT serial number, which could link to the mascot search result and NCBI BLAST search; score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event; an individual ion score > 38 indicates identity or extensive homology ($P < 0.05$).

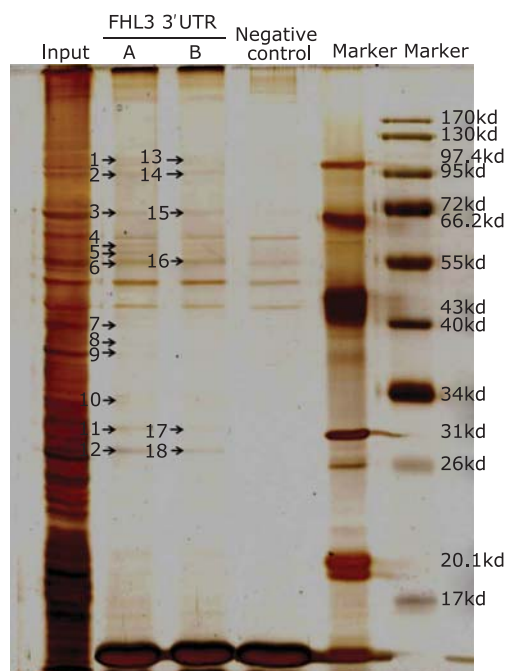


Figure 2. Silver staining of proteins which combined with FHL3 3'UTR-A, FHL3 3'UTR-B, or negative control by biotin pull-down. Input from T98G glioma cell lysate was used as a positive control. The arrows show the carved bands to be identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The numbers of carved bands (1-18) correspond with the results of LC-MS/MS in Table 1.

DISCUSSION

The 3'UTR of eukaryotic transcripts plays an important role in regulating gene expression in posttranscriptional level. In this region exist the 3' end processing signals which instruct the 3' end processing. 3'UTR not only controls mRNA stability and the rate of mRNA degradation, but also helps to determine mRNA localization, regulate translation initiation and translation efficiency as well as translation time. The interactions between 3'UTR and trans-acting factors can influence the expression of one or more genes, thus possibly leading to the occurrence of diseases. Regulatory sequence of mRNA 3'UTR and the specific bound proteins will become new molecular targets in drug design.¹⁰

In our previous study, we have confirmed that knockdown of PCBP2 can increase FHL3 protein level through stabilizing FHL3 mRNA.⁴ In this study, 22 proteins associated with FHL3 3'UTR were identified by LC-MS/MS. Among them, the seventh band represented the PCBP2 protein, which was consistent with the results of our biotin pull-down assay. Eleven RBPs that we are most interested in in Table 1 are SFPQ, IGF2BP1, KHDRBS1, IGF2BP3, SYNCRIP, IGF2BP2, PTBP1, SERBP1, HNRNPH1, PCBP2, and HNRNPA2/B1. These proteins regulate RNA splicing,

transport, stability and translation shuttling between the nucleus and cytoplasm. Other proteins also have important functions in cellular biological process. According to GO analysis, ILF3, TRIM28, RUVBL1, and PHB are involved in the regulation of transcription; CSE1L, NDUFS3 and EIF4G2 will modulate cell proliferation or apoptosis; while EEF1A1 and GNB2L1 are related to GTPase activity.

It is notable that 4 hnRNP proteins, PTBP1, hnRNP H1, hnRNP A2/B1, and PCBP2 were detected by LS-MS/MS in this study. Similar to PCBP2, the other 3 proteins are also overexpressed in glioblastomas and are correlated with poor prognosis.¹¹⁻¹³

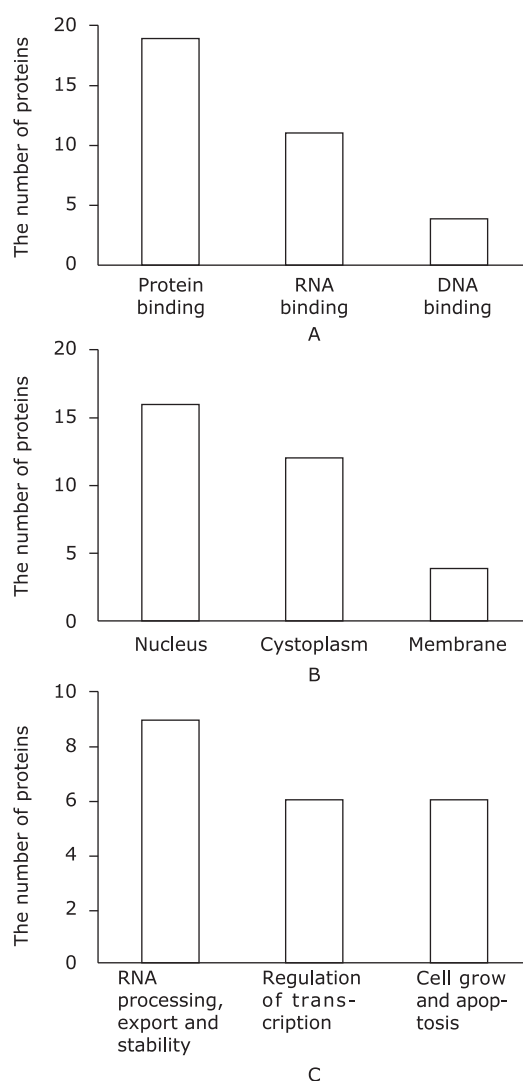


Figure 3. The candidate proteins are analyzed in terms of their gene ontology (GO) using molecule annotation system. FHL3 3'UTR-associated 22 proteins were annotated to the GO term "molecular function" (A), "cellular component" (B) and "biological process" (C) using the web-based tool MAS V3.0. The first three terms related to the largest gene number in each ontology were presented in the histogram.

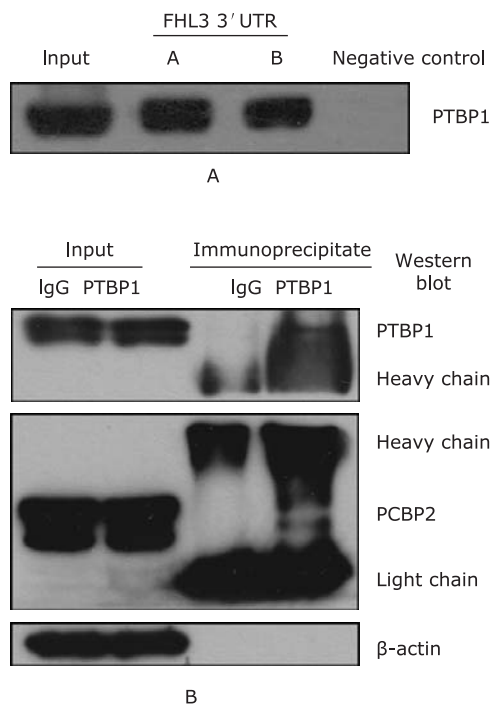


Figure 4. PTBP1 can bind to FHL3 mRNA 3'UTR and interact with PCBP2.

A. Biotin pull-down analysis of complexes formed *in vitro* using biotin-labeled FHL3 3'UTR-A or FHL3 3'UTR-B and T98G whole-cell lysates and detected using anti-PTBP1 antibody.

B. T98G cell lysates were immunoprecipitated with IgG (control) and anti-PTBP1 antibody (experimental), then the precipitates were analyzed by Western blot with anti-PTBP1 and anti-PCBP2 antibodies. Immunoglobulin heavy and light chains are also shown.

We verified by using biotin pull-down that PTBP1 associated with FHL3 mRNA 3'UTR. Not only 3'UTR-A, but also 3'UTR-B could bind to PTBP1. A specific differential band at the FHL3 3'UTR-B paralleling to the fourth band compared to negative control was found in the silver staining PAGE gel. Immunoprecipitation experiments also revealed that PTBP1 can interact with PCBP2. We thus hypothesized that PTBP1 and PCBP2 may work together to involve in posttranscriptional regulation of FHL3 by forming a protein complex in glioma cells. In fact, PTBP1 has been reported to be associated with many hnRNP members. The complexes with PTBP1 and PCBP2 are required for the hepatitis C virus internal ribosome entry site-dependent initiation in mammalian cells.^{14,15} The specific cooperative interactions among PTBP1/2, hnRNP H/F and KH-type splicing-regulatory protein regulate the splicing of c-src N1 exon in neuronal cells by binding to an intronic cluster of RNA regulatory elements.¹⁶ In glioma cells, PTBP1 and the alternative splicing repressors hnRNP A1/A2 influence pyruvate kinase isoform expression and the switch to aerobic glycolysis.¹⁷

In conclusion, PCBP2, PTBP1 and other RBPs can bind to FHL3 3'UTR, forming a protein complex, and co-regulate FHL3 expression, thus affecting the proliferation and apoptosis of gliomas.

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