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# C-Terminus of E1A Binding Protein 1 Stimulates Malignant Phenotype in Human Hepatocellular Carcinoma

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**Background:** The C-terminus of E1A binding proteins (CTBPs) has recently been shown to stimulate tumorigenesis in several human tissues by participating in cell signal transduction. However, to date, the expression profile of CTBP isoforms in hepatocellular carcinoma (HCC) and the impact of CTBPs on HCC cell phenotype have not been fully explored.




**Material/Methods:** The expression level of CTBP1 was investigated in various HCC cell lines and HCC tissues by RT-qPCR, Western blotting, and immunohistochemistry assays. The phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 was utilized to treat hepatic astrocyte cells, and the impact of CTBP1 on proliferation and metastasis of hepatic astrocytes and HCC cells was accessed by CCK-8, clone-forming, Transwell chamber, and cell scratch assays.

**Results:** Increased expression of CTBP1 was observed in HCC tissues and was a predictor of poor prognosis in HCC patients. CTBP1 modified proliferation and migratory activity of HCC cells via the PI3K/protein kinase B (Akt) signaling pathway in hepatic astrocytes. Moreover, genetic loss of CTBP1 significantly reduced the metastatic activity of HCC cells *in vitro*.

**Conclusions:** Our data suggest that the loss of CTBP1 suppresses cell proliferative and invasive activity of HCC cells via the PI3K/Akt pathway.

**MeSH Keywords:** **Carcinoma, Hepatocellular • Genes, Tumor Suppressor • Intracellular Signaling Peptides and Proteins**

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## Background

The C-terminus of the E1A binding proteins (CTBPs) was originally identified as a binding partner of specific transcription factors, such as the adenovirus E1A oncoprotein. The binding was achieved via the PXDLS peptide motif and allowed CTBPs to perform diverse roles in developmental and oncogenic processes [1,2]. CTBPs are classified into 2 diverse protein isoforms named CTBP1 and CTBP2. These isoforms are highly expressed during development and participate in the differentiation of numerous human organs, such as the brain, eyes, heart, placenta, and muscles [3–7]. CTBPs regulate the function of sequence-specific DNA-binding transcription factors and thereby control proliferation, apoptosis, and migration [8–10]. The CTBP corepressor complex exerts a vital effect in various developmental processes, indicating that the upregulation of CTBP proteins in certain human tissues may be involved in carcinogenesis [11–13]. Various transcription factors regulated by CTBPs have been shown to bind to these proteins, as demonstrated by the analysis of certain CTBP-binding transcription factors and their gene promoters [14–16]. Previous studies have also shown that overexpression of CTBP1 is hyperactivated in tumor cells [17–19]. Tumor cells frequently exhibit high levels of NADH, caused by NADH overproduction under hypoxic and pseudo-hypoxic conditions [20–22]. Recent studies have shown that NADH can combine with CTBPs to promote interaction with transcriptional repressors [19,23,24]. It was shown that high NADH levels suppress E-cadherin transcription in tumor cells under hypoxic conditions, suggesting an oncogenic role of CTBPs [19,25,26]. These properties suggest unique applications of CTBPs for tumor-specific treatment that could theoretically spare normal tissues.

To date, the inhibition of metastasis is insufficient in the treatment of HCC, and recurrence of this disease occurs frequently. Following HCC progression to distant metastatic sites, current therapeutic treatments are ineffective [27]. Previously, our group demonstrated that CTBP1 was overexpressed in HCC, but no studies have reported on the influence of CTBPs in the carcinogenesis of human HCC. Consequently, the present study investigated the mechanisms underlying the effects of CTBPs on HCC malignancy.

## Material and Methods

### RT-qPCR

TRIzol reagent (Thermo Fisher Scientific, Inc.) was used to isolate total RNA from HCC cells and tissues. The First-Strand cDNA Synthesis kit and SYBR Green PCR mix (Thermo Fisher Scientific, Inc.) was used to perform this experiment. The relative expression levels of CTBP1/CTBP2 mRNA were evaluated

via the  $2^{-\Delta\Delta q}$  method, and the GAPDH gene was used as an internal control.

### Protein extraction in tissue section

Total protein extraction from HCC and noncancerous hepatic tissues was performed using commercial kits (cat. no. BB-3161-50T, Best Bio). Briefly, 2  $\mu$ l protease inhibitor and 2  $\mu$ l protein stabilizer were added into 400  $\mu$ l cold hepatic tissue protein extraction solution. The reagents were mixed and put on ice for subsequent use.

Fresh tissue samples were immersed in physiological saline (pH 7.4) at 4°C. The tissues were cut into small sections, weighed, and put into a mortar containing liquid nitrogen. Subsequently, they were ground into a fine powder to retain the nitrogen in its liquid form. The tissue powder was added to the centrifuge tube, and 400  $\mu$ l protein extract was added for every 200 mg of hepatic tissue. The samples were homogenized by oscillation at 4°C for 30 min. Homogenization in an ice bath with ultrasound at 80 w at an interval of 10 s was performed 10 times (10 s each time). Subsequently, the protein extract was transferred into another pre-cooled centrifuge tube and centrifuged at 12 000 rpm at 4°C for 15 min. Then, the supernatant was rapidly transferred to another pre-cooled clean centrifuge tube to obtain the total protein.

### Western blotting

The Western blotting process was performed as previously described [28] and the PVDF membranes were incubated with the following primary antibodies at 4°C overnight: rabbit anti-human CTBP1 (cat. no. 8684, Cell Signaling Technology), anti-human CTBP2 (cat. no. 13256, Cell Signaling Technology), anti-human phospho-Akt at Thr308 site (cat. no. 13038, Cell Signaling Technology), anti-human Akt (cat. no. 2920, Cell Signaling Technology), anti-human phospho-phosphatidylinositol-3-kinase p110 $\alpha$  (cat. no. 4255, Cell Signaling Technology), and mouse anti-human  $\beta$ -actin (cat. no. ab 8227, Abcam). The density of each protein band was quantified using Image J software.

### Patients and tissue specimens

Tissues were obtained from 97 patients (age range 52–75 years, average age 64 years) who underwent surgery at the First Hospital of Jilin University. The clinicopathological parameters of HCC patients – age, sex, HBsAg status, serum  $\alpha$ -fetoprotein, distant metastasis and Tumor-Node-Metastasis stage – are summarized in Table 1. Sections of the noncancerous hepatic and cirrhotic tissues were obtained from 97 patients with hepatitis after these tissues were determined to be non-neoplastic. The viability status of each patient was confirmed by an outpatient or telephone interview.

**Table 1.** Expression levels of CTBP1 and the clinicopathological characteristics of patients with HCC.

Characteristic	Cases, n	CTBP1 (+)	CTBP1 (-)	P-value
HCC tissues	97	69	28	<0.01*
Hepatic tissues	97	24	73	
Age (years)				0.118
≤60	34	21	13	
>60	63	48	15	
HBsAg				0.417
+	81	58	23	
-	16	10	6	
Distant metastasis				<0.01*
+	57	45	12	
-	40	24	16	
Serum AFP (ng/ml)				0.819*
≤400	76	55	21	
>400	21	15	6	
TNM stage (AJCC)				0.349
I-II	50	37	13	
III-IV	47	32	15	

\* Statistical significance was detected with the  $\chi^2$  test/e2 Goodness-of-Fit Test. HCC – hepatocellular carcinoma; CTBP1 – C-terminus of E1A binding protein 1; AFP –  $\alpha$ -fetoprotein; TNM – Tumor-Node-Metastasis; HBsAg – hepatitis B virus surface antigen; AJCC – American Joint Committee on Cancer.

### Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded clinical tissues were cut into 4- $\mu$ m sections and underwent deparaffination, rehydration, and antigen retrieval. The tissues were blocked with 10% FBS and incubated with anti-human CTBP1 and p-Akt antibodies at 4°C overnight. Then, the sections were probed with secondary antibody (Abcam) for 60 min and the DAB solution was used for staining. The slides were stained by hematoxylin, dehydrated, and mounted. The IHC score for each slide was calculated by 2 independent pathologists, as previously described [29].

### Cell culture and transfection

The hepatic astrocyte line LX-2 and the 3 HCC cell lines Hep3B, Huh1 and MHCC97H were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). STR profiling was used to determine the identity of all cell lines. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with fetal bovine serum (10% FBS; Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained in a humidified cell incubator at 37°C

supplemented with 5% CO<sub>2</sub>. The pNSE-IRES2-EGFP-C1/CTBP1 plasmid was transfected into hepatic astrocytes cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. A monoclonal population was expanded via G418 (Sigma-Aldrich; Merck KGaA) screening culture.

### PI3K inhibitor LY294002 treatment

The tyrosine phosphorylation inhibitor LY294002 was dissolved in DMSO at a concentration of 50 mM and stored at -20°C. LY294002 was diluted to a final concentration of 40  $\mu$ M. The control cells were treated with DMSO for the same time period.

### CCK-8 assay

The experimental process for the CCK-8 assay was as specified by the manufacturer's instructions (Dojindo Molecular Technologies, Inc.).

### Clone-forming experiment

The experimental process for the clone-forming experiment was conducted as described previously [28].

## Transwell chamber assay

The Transwell chamber assay was performed according to the manufacturer's instructions (3422, Corning, Costar, USA) to investigate the invasive activity. Briefly, the Transwell inserts were coated with 80  $\mu$ l Matrigel (diluted with DMEM at a ratio of 1: 7). Following 24 h of incubation, the cells that invaded through the membranes were stained with crystal violet. The number of invaded cells was calculated by light microscopy.

## Cell scratch assays

The experimental process for the cell scratch assays assay was as described previously [29]. Following removal of the supernatant, 5 horizontal lines were drawn on the bottom of the 6-hole plate with a marking pen. Three scratches were created by a 100- $\mu$ l pipette tip to make sure that were distributed evenly. The scratch points were perpendicular to the marking line and the cell monolayer was washed with 0.01 M phosphate-buffered saline 3 times. A total of 10 images were obtained at the same position using an inverted microscope at 0, 12, and 24 h (magnification,  $\times$ 40). Image J software was used to measure the area at 0, 12, and 24 h.

## shRNA transfection

The plasmids comprising the packaging construct (pHelper 1.0), VSVG-expressing construct (pHelper 2.0), pGCSIL- EGFP, and pGCSIL-scramble construct or pGCSIL CTBP1-shRNA construct were procured from Genechem Biotech Co. Short hairpin RNA transfection was performed as described previously [29]. Briefly, HEK 293T cells were maintained in 6-well dishes at 70–80% confluence. The plasmids comprising the packaging construct (pHelper 1.0), VSVG expressing construct (pHelper 2.0), pGCSIL- EGFP and pGCSIL-scramble construct, or pGCSIL CTBP1-shRNA construct were transfected into the cells using Lipofectamine following the manufacturer's instructions (cat. no. 11668027, Thermo Fisher Scientific, Inc.). The viral stocks were concentrated by ultracentrifugation and dissolved in Hanks' balanced salt solution. The HCC cells were transfected with the viral stocks at a multiplicity of infection (MOI) of 200.

## Statistical analysis

The experiments in the present study were conducted at least 3 times. Each time a duplicate set of samples was employed. The t test or ANOVA with Dunnett's multiple comparison test were used to determine the statistical significance of the differences among groups. In addition, the association between survival time and CtBP1 expression in HCC patients was investigated by Kaplan-Meier survival curves and the log-rank test.

## Results

### CTBP1 is overexpressed in HCC cell

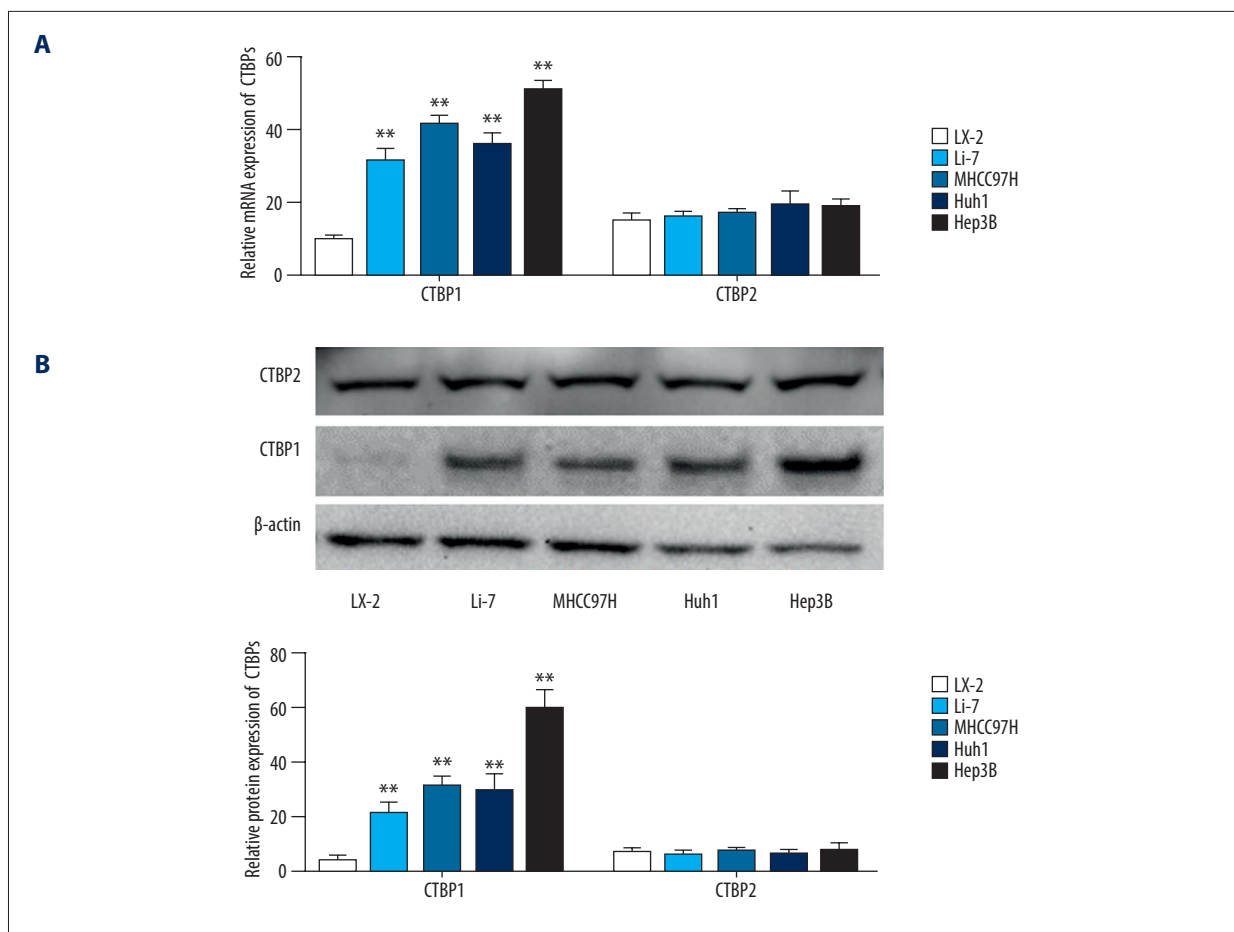
The expression pattern of CTBPs in human HCC cell lines and a hepatic astrocyte line was explored. The data indicated that the mRNA (Figure 1A) and protein (Figure 1B) expression levels of CTBP1 in the HCC cell lines (Hep3B, Huh1 and MHCC97H) were notably higher than those of the hepatic astrocyte line (LX-2). CTBP2 mRNA and protein expression did not reveal a significant difference between the HCC cell lines (Hep3B, Huh1, and MHCC97H) and the hepatic astrocyte line (LX-2).

### CTBP1 was overexpressed in HCC patients and was associated with distant metastases

Western blot and IHC assays were used to explore the expression profile of CTBP1 in 97 HCC and 97 noncancerous hepatic tissues. CTBP1 expression was mainly located in the cell nuclei of HCC tissues (Figure 2A). Expression of CTBP1 was detected in 24.7% (24/97) of noncancerous hepatic tissues and in 71.1% (69/97) of HCC tissues (Table 1). Moreover, CTBP1 expression was significantly associated with distant tumor metastasis ( $P=0.001$ ; Table 1). Western blot analysis was performed to determine the CTBP1 expression in 97 hepatic sections and 97 HCC sections (Figure 2B). The data showed that CTBP1 was markedly upregulated in HCC tissues versus the corresponding expression in non-neoplastic hepatic tissues. The data (Figure 2C) indicated that the HCC patients who had positive CTBP1 protein expression in tumors tissues (overall survival, 33.62 months) exhibited significantly shorter survival time than in patients with negative CTBP1 protein expression (overall survival, 45.24 months) (Kaplan-Meier survival curves and log-rank test,  $P=0.004$ ).

### CTBP1 overexpression promoted the malignant phenotype of hepatic astrocytes

A pNSE-IRES2-EGFP-C1/CTBP1 plasmid was transfected into LX-2 cells and was named the CTBP1 group. Our data revealed that the ratios of the phosphorylated to total Akt ( $P=0.0013$ ) and the expression of PI3K catalytic subunit p110 $\alpha$  ( $P=0.0023$ ) were upregulated in the LX-2 cells following CTBP1 overexpression (Figure 3A). In addition, the growth of LX-2 cells was assessed by the CCK-8 assay (Figure 3B), showing that the proliferation rate of LX-2 cells was significantly enhanced following the CTBP1 overexpression ( $P=0.0013$ ). Moreover, the ability of the cells that overexpressed CTBP1 to form colonies (Figure 3C) was significantly enhanced following the CTBP1 overexpression ( $P=0.0002$ ). The data further suggested that cell migratory activity was accelerated in LX-2 cells that overexpressed CTBP1, as demonstrated by Transwell (Figure 3D) and cell scratch assays (Figure 3E).



**Figure 1.** CTBPs expression levels in hepatic astrocyte and HCC cells. **(A)** Quantification of mRNA expression of CTBPs in hepatic astrocyte and various HCC cells. **(B)** Quantification of protein expression of CTBPs in hepatic astrocyte and HCC cell lines. \*\*  $P < 0.01$  vs. the hepatic astrocytes. HCC – hepatocellular carcinoma; CTBP – C-terminus of E1A binding proteins.

### CTBP1 modified cell migratory capacity via PI3K/Akt signaling in hepatic astrocytes

Modifications in the PI3K/Akt pathway were determined in hepatic astrocytes via Western blotting following treatment with the PI3K tyrosinase inhibitor LY294002 (10 nM) for 24 h. The ratios of the expression of PI3K catalytic subunit p110 $\alpha$  ( $P=0.0021$ ) and phosphorylated to total Akt ( $P=0.0011$ ) were significantly reduced in LX-2 hepatic astrocytes that over-expressed CTBP1 following LY294002 treatment (Figure 4A).

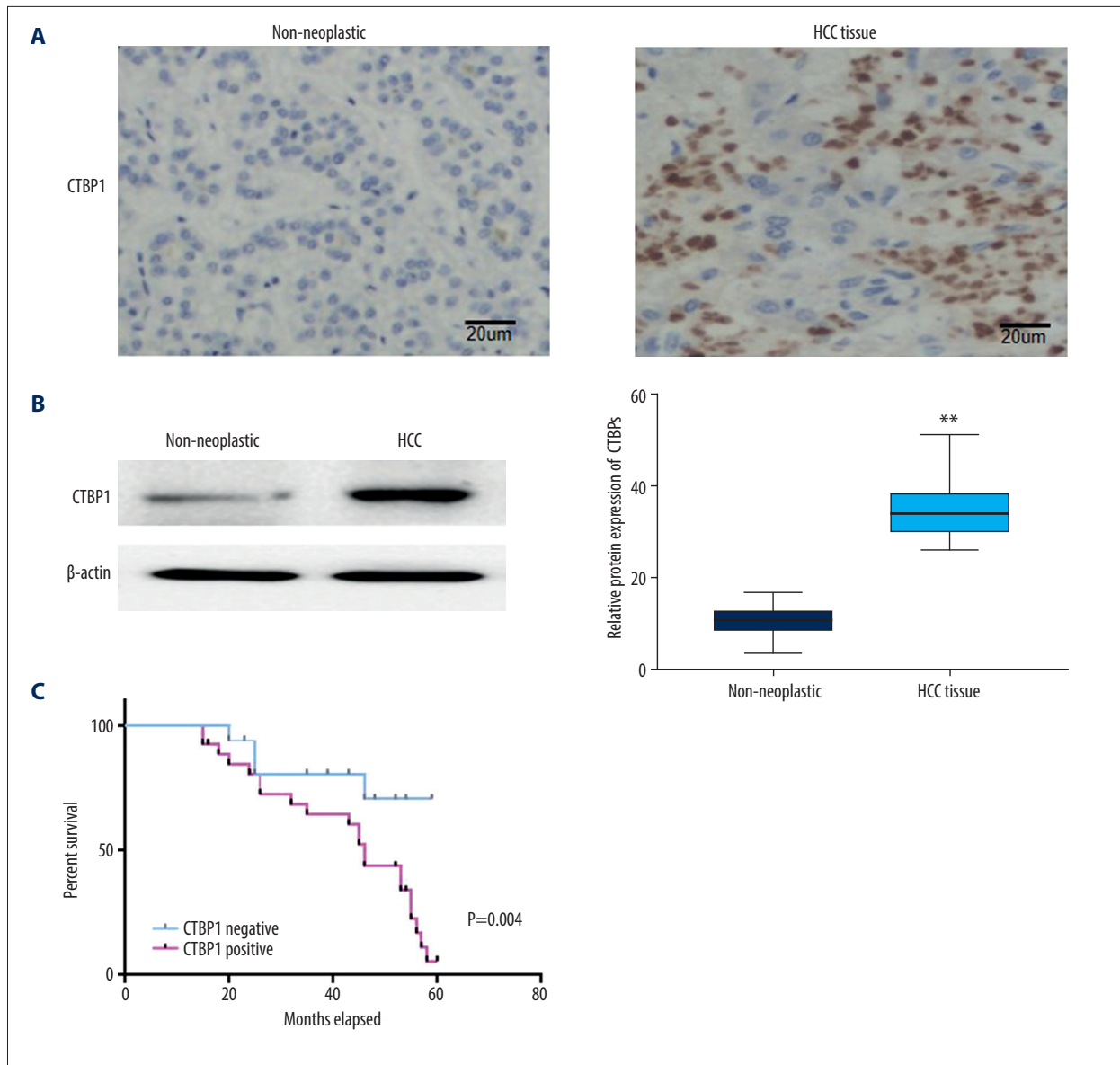
The observations attained from the CCK-8 (Figure 4B) and colony formation (Figure 4C) assays suggested that the proliferation ability ( $P=0.0016$ ) and the number of colonies formed in LY294002-treated cells ( $P=0.0004$ ) were significantly decreased compared to those of the DMSO group. Moreover, there were far fewer invasive LX-2 cells ( $P=0.0012$ ) after LY294002 treatment (Figure 4D). A similar result was noted with regard to the migratory activity of LY294002-treated cells, which was significantly reduced compared to that of the DMSO-treated group

following 12 and 24 h of cell culture ( $P=0.0003$  and  $P=0.0012$ , respectively; Figure 4E).

### CTBP1 silencing reduced the migratory activity of HCC cells

Because overexpression of CTBP1 was detected in HCC cells, further experiments were performed to discover whether CTBP1 influenced HCC carcinogenesis. sh-CTBP1 transfection markedly inhibited CTBP1 protein expression in Hep3B cells (Figure 5A, 5B). The modification of the activation state of the PI3K/Akt pathway in HCC cells was also examined, showing that the ratios of the expression of PI3K catalytic subunit p110 $\alpha$  ( $P=0.0011$ ) and phosphorylated to total Akt ( $P=0.0012$ ) were significantly reduced in CTBP1-silenced Hep3B cells (Figure 5A).

The proliferation rate of Hep3B cells was significantly inhibited after CTBP1 silencing (Figure 5B,  $P=0.0016$ ). A similar effect was noted with regard to the colony formation activity of CTBP1-silenced cells in 2D monolayer cultures (Figure 5C,  $P=0.0001$ ).



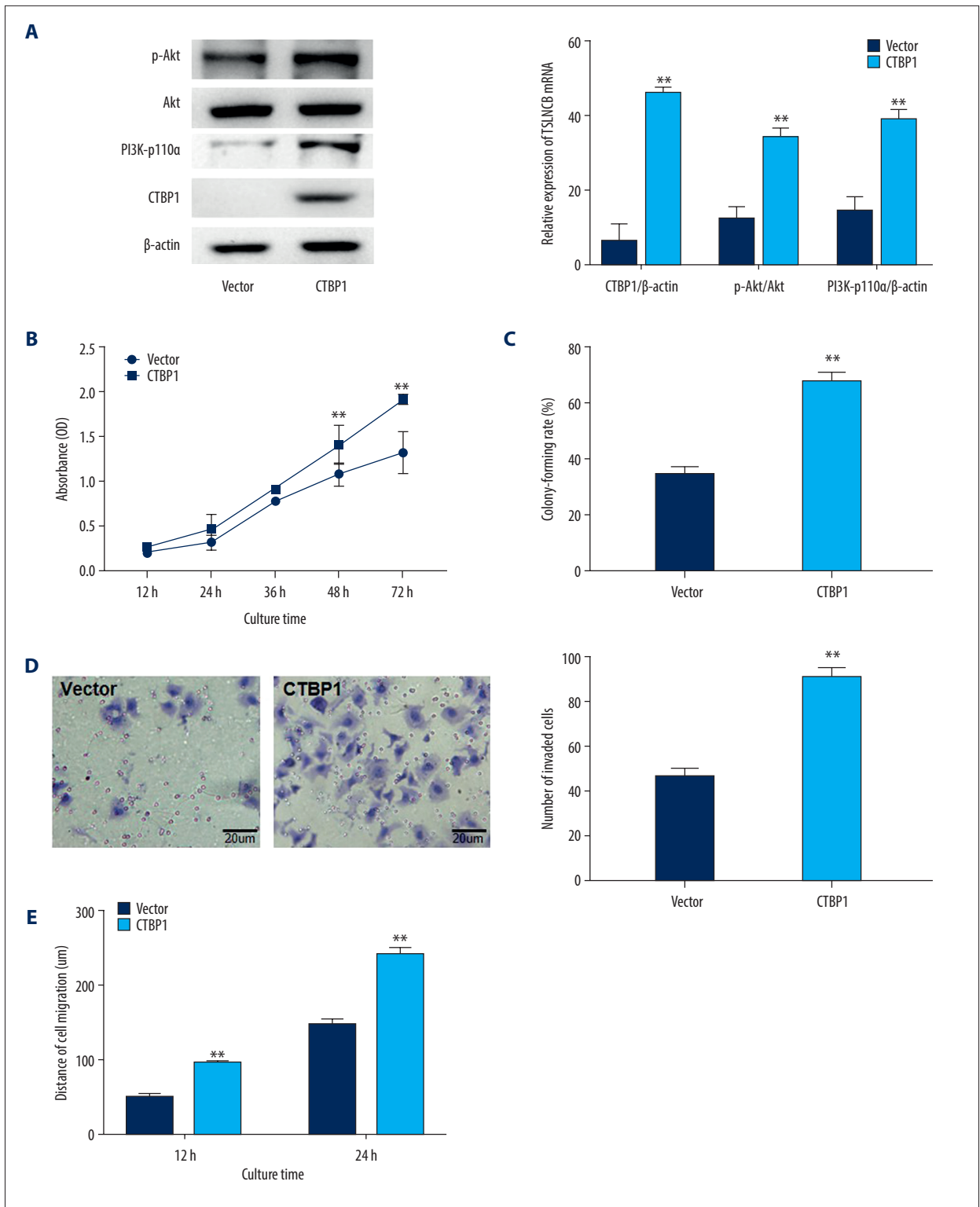
**Figure 2.** CTBP1 was highly expressed in HCC tissues. **(A)** High CTBP1 expression in HCC tissues was observed via IHC. **(B)** The protein of CTBP1 was highly expressed in HCC tissues. \*\*  $P < 0.01$  vs. hepatic tissues. **(C)** Positive CTBP1 expression predicted a shorter survival time. HCC – hepatocellular carcinoma; CTBP – C-terminus of the E1A binding proteins.

Transwell (Figure 5D) and cell scratch assay (Figure 5E) indicated that cell migratory activity was decreased after induction of CTBP1 expression in Hep3B cells. Overall, loss of CTBP1 affected cell migratory activity in Hep3B cells.

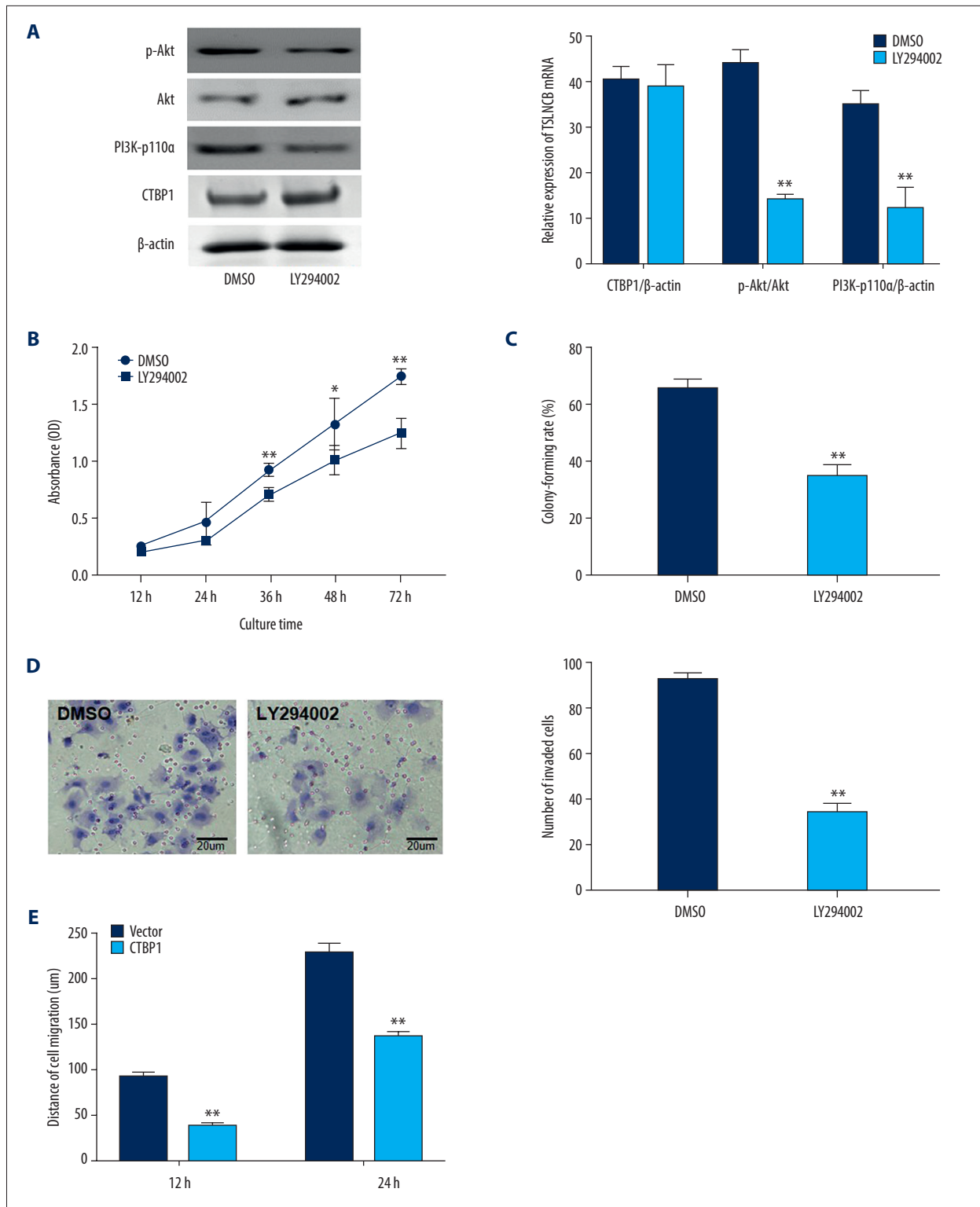
## Discussion

The dysregulation of the CTBP profusion has been considered an initiating step in the formation of colorectal cancer (CRC) tumors [30,31]. CTBP was found to suppress the expression levels of several epithelial and pro-apoptotic genes.

CTBP overexpression in certain human tumor cells accelerates epithelial-mesenchymal transition (EMT) and tumor cell survival [25,32]. In contrast to these observations, decreased CTBP1 expression enhanced the tumor progression of melanoma cells due to the inhibition of gene expression associated with melanoma progression [33]. Therefore, suppression of CTBP1 function is an important anti-tumor therapeutic strategy, although it has never been clinically targeted in HCC [13]. Hence, the expression pattern of CTBPs in HCC patients requires further studies. At present, it appears that the expression levels of CTBP1 in the HCC cell lines were notably higher than those of the hepatic astrocyte line. Similarly, recent research

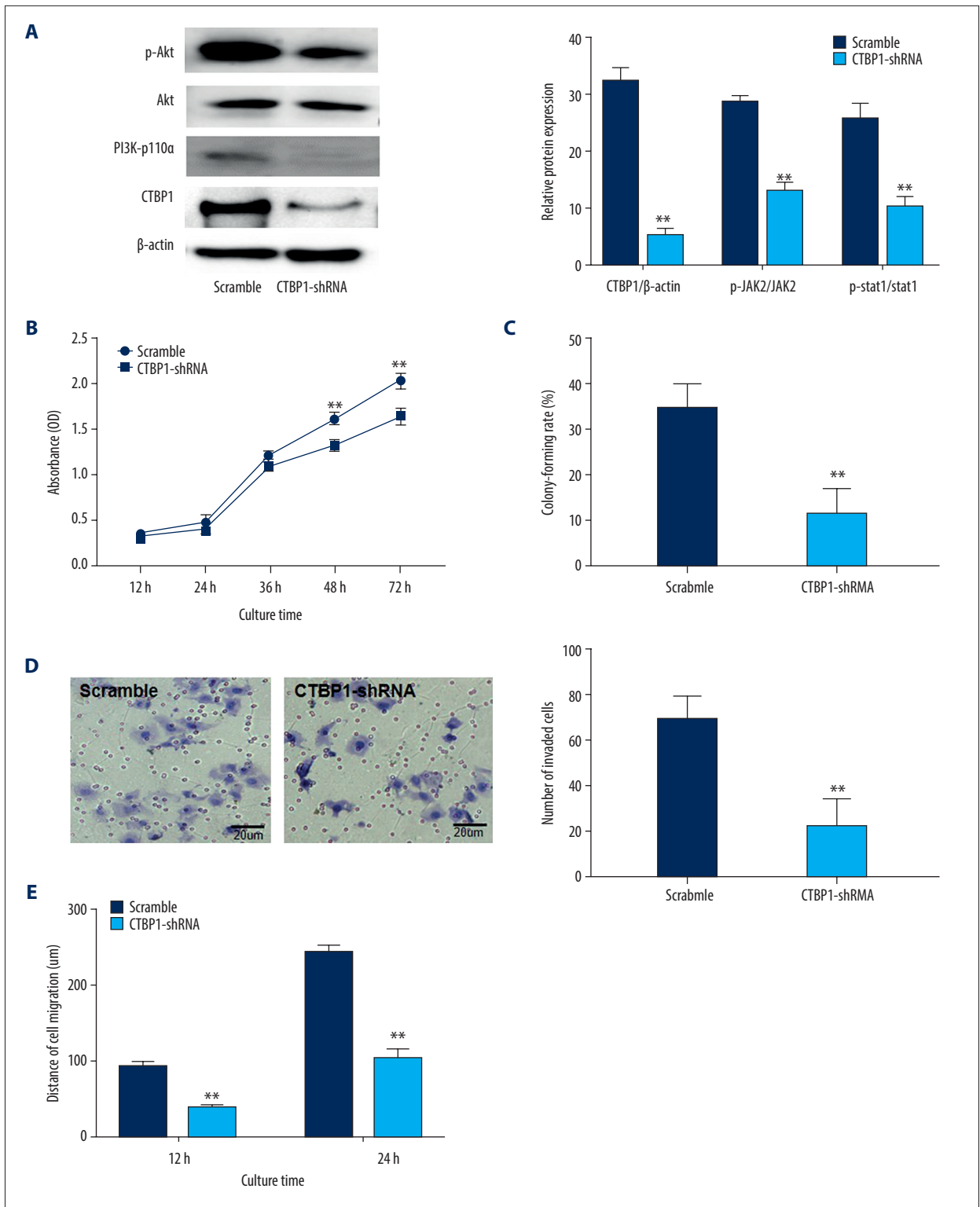


**Figure 3.** CTBP1 promoted the malignancy of hepatic astrocytes. **(A)** Modifications in activation of the PI3K/Akt pathway. **(B)** Growth curve of the LX-2 cells as determined via the CCK-8. **(C)** Colony formation activity in 2D monolayer cultures. **(D)** The *in vitro* invasive activity of LX-2 cells was detected by the Transwell chamber method. **(E)** The *in vitro* migratory activity of LX-2 cells was investigated using the cell scratch assay. \*\*  $P < 0.01$  vs. the vector group. CTBP – C-terminus of the E1A binding proteins.



**Figure 4.** LY294002 suppressed PI3K activity and cell malignancy in LX-2 cells. **(A)** Modifications in the activation of the PI3K/Akt pathway. **(B)** Growth curvature was determined via CCK-8. **(C)** The colony formation activity of LX-2 cells was determined via the clone-forming assay. **(D)** The number of invaded LX-2 cells was explored by the Transwell chamber method. **(E)** The cell scratch assay was used to explore the migratory capacity of LX-2 cells *in vitro*. \*\*  $P < 0.01$  vs. the DMSO group. CCK-8 – Cell Counting Kit-8.





**Figure 5.** CTBP1 silencing reduced the migratory activity of HCC cells. **(A)** The consequences of CTBP1 loss on the PI3K/Akt signaling in Hep3B cells. **(B)** The growth rate of Hep3B cells was assessed via CCK-8 assay. **(C)** The colony formation activity of Hep3B cells was evaluated using 2D culture. **(D)** The *in vitro* invasive activity of Hep3B cells. **(E)** The *in vitro* migratory activity of Hep3B cells. \*\* P<0.01, vs. the scramble group.

revealed that CtBP1, but not CtBP2, is highly expressed in the human invasive osteosarcoma. The reason for the upregulation of CTBP1 in osteosarcoma is that the expression levels of CtBP1, but not CtBP2, are increased by the DNA hypermethylation in the promoter region of miR-485-3p [34]. These results provide a reasonable explanation of why CtBP1 is specifically upregulated in human tumors. The present study also suggests that the high expression of CTBP1 promoted HCC malignancy. Moreover, the impact of CTBP1 on PI3K/Akt signaling was explored in hepatic astrocytes. Our observations demonstrated that the inhibition of the PI3K/Akt signaling generated a reduction in the metastatic activity of hepatic astrocytes that expressed CTBP1. Furthermore, CTBP1 silencing of the HCC cell line Hep3B led to a suppression of the PI3K/Akt signaling pathway and reduced the malignancy of Hep3B cells. Nevertheless, the detailed molecular mechanism of signal transduction involving the nuclear expression of the CTBP1 protein and the activation of the PI3K enzyme require further investigation.

To date, several studies have implicated CTBP as a transcriptional corepressor [14]. Accumulating evidence shows the context-specific influence of CTBP on transcriptional stimulation. For example, the CTBP2 protein was shown to increase T-lymphoma invasion and metastasis-inducing protein 1 (Tiam1) expression via an NADH-dependent method [20,35], and this process activated the transcription factor 4 (TCF-4) signaling pathway [36]. Moreover, a previous study demonstrated that CTBP1 upregulated the expression levels of the multidrug-resistant (MDR) protein 1, thereby promoting drug resistance of MDR cancer cell lines [37]. These observations revealed that the CTBP transcriptional corepressors exerted specific context-dependent effects with regard to oncogenic progression. Consequently, more detailed studies are needed to define the function of CTBP corepressors in HCC carcinogenesis. This can also influence the future application of novel treatment methods.

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## Conclusions

The exact function of CTBP1 and its underlying molecular mechanism in HCC remains obscure. The results of our study suggest that CTBP1 enhanced the malignancy of HCC cells by activating the PI3K/Akt signaling pathway. Considering the inadequate application of HCC therapeutics, the examination of CTBP1 as a molecular therapeutic protein target is of great research significance.

## Ethics approval and consent to participate

Ethics approval (approval no. JLU13657) was obtained from the Ethics Committee of Jilin University. Informed consent for participation was obtained from all patients and their parents.

## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

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## Conflict of interests

None.

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