Human fibroblast growth factor-21 serves as a predictor and prognostic factor in patients with hepatitis B cirrhosis combined with adrenal insufficiency

JIAN ZHANG, JUNHONG LI, JUNWEI MA, HONGXIN WANG and YIN YI

Emergency Department, Beijing You'an Hospital, Capital Medical University, Beijing 100069, P.R. China

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Abstract. Hepatitis B cirrhosis is caused by liver cell necrosis, residual liver cell nodular regeneration, connective tissue hyperplasia and fiber formation, which frequently leads to adrenal insufficiency. Previous reports have demonstrated that human fibroblast growth factor (hFGF)-21 is a multifunctional protein that exhibits potential therapeutic value for metabolic diseases. The present study investigated the diagnostic value of hFGF-21 and analyzed the potential molecular mechanism in the progression of hepatitis B cirrhosis combined with adrenal insufficiency. Characteristics of cellular immunity and humoral immunity were analyzed in patients with hepatitis B cirrhosis combined with adrenal insufficiency (PhbA). Results demonstrated that expression levels of hFGF-21 were downregulated in plasma and liver cells isolated from clinical specimens. Plasma concentration levels of hFGF-21 were upregulated in prognostic PhbA. In vitro assays indicated that hFGF-21 treatment decreased the continuous deposition of extracellular matrix and reactive oxygen species in liver cells isolated from clinical specimens. Results also demonstrated that hFGF-21 treatment downregulated inflammatory cytokines. It was observed that hFGF-21 treatment downregulated nuclear factor (NF)-κB and Kruppel-like factor 6. Notably, transforming growth factor (TGF)-β, platelet-derived growth factor and epidermal growth factor levels were improved by hFGF-21 treatment.

In conclusion, these results indicated that hFGF-21 inhibits inflammation by regulation of the NF-κB-mediated TGF-β signaling pathway, which may serve as a predictor and prognostic factor in PhbA.

Introduction

Liver cirrhosis is a kind of metabolic disease that is reversible and may be treated when it is identified at an early stage (1). Liver cirrhosis is divided into hepatitis B cirrhosis and hepatitis C cirrhosis according to pathogenesis in clinical research (2,3). Previous reports have indicated risks for alcoholic liver cirrhosis, and regression of fibrosis/cirrhosis by glycine propionyl-l-carnitine treatment has been also investigated in D-Galactosamine-induced chronic liver damage (4,5). The main pathogenesis of liver cirrhosis is progressive fibrosis (6). A comprehensive review has evaluated the management of patients with autoimmune hepatitis with decompensated cirrhosis (7). Furthermore, a study by Wang et al (7) suggested that chronic hepatitis B and hepatitis B virus-related cirrhosis contributes to other metabolic syndromes, which further influences renal function and increases the risk of renal damage, hypophosphatemia, and adrenal insufficiency (8-10).

Fibroblast growth factor (FGF)-21 is an atypical member of the FGF family, as well as a multifunctional protein predominantly secreted by adipose tissue, the pancreas and liver, which has been regarded as an efficient polypeptide for the treatment of metabolic disorders (11,12). Previous research has reported that metabolic hormone effects of FGF-21 on energy metabolism were essential for human vascular endothelial cells (13,14). A study by Wang et al (15) indicated that FGF-21 is positively associated with atrial fibrosis in patients with atrial fibrillation with rheumatic heart disease. FGF-21 has been reported as a novel liver safeguard (16), as well as being identified as a momentous controller and regulator of glucose and lipid metabolism, and long-term energy balance (17,18). Notably, transplantation of basic FGF-pretreated adipose tissue-derived stromal cells enhances regression of liver fibrosis in mice (19). However, the molecular mechanisms of liver fibrosis associated with FGF-21 are not well understood or clearly elaborated.

Chronic inflammation associated with hepatitis C virus infection contributes to hepatic transforming growth factor (TGF)-β signaling that promotes cirrhosis and hepatocellular carcinoma (20). Research has also indicated protective effects of allopurinol against acute liver damage and cirrhosis induced by carbon tetrachloride through modulation of nuclear factor (NF)-κB, cytokine production and oxidative stress (21). The present study analyzed the potential diagnostic value of human
(h)FGF-21 and investigated the hFGF-21-mediated signaling pathway of hepatitis B cirrhosis combined with adrenal insufficiency in liver cells. The present data indicated that plasma concentration levels of hFGF-21 were downregulated in patients with hepatitis B cirrhosis combined with adrenal insufficiency (PhbA), which may be associated with the NF-κB-mediated TGF-β signaling pathway.

**Patients and methods**

**Patients and healthy volunteers.** A total of 186 PhbA (90 male and 96 female) and 68 healthy volunteers (35 male and 33 female) were recruited in the present clinical investigation following presentation to Beijing You'an Hospital, Capital Medical University (Beijing, China) between May 2014 and October 2015. The mean age was 38.5 (16.4-62.5 years) and 34.2 (22.5-46.2 years) in PhbA and healthy volunteers, respectively. A total of 10 patients [male/female; 5/5; 34.2 years old (22.5-46.2)] who had recovered from hepatitis B cirrhosis combined with adrenal insufficiency (PPhbA) were also recruited to the present study. Patients with diabetes mellitus and digestive tract diseases were excluded from the present study. Patients were diagnosed with PhbA as described previously (22). All participants were required to provide written informed consent prior to initiation of the study. The present study was approved by the Ethics Committee of Beijing You'an Hospital, Capital Medical University (Beijing, China).

**Cell culture.** Liver and renal epithelial cells were obtained from PhbA using a biopsy needle as previously described (23). Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Liver and renal epithelial cells were incubated with hFGF-21 (1.0 mg/ml, Sigma-Aldrich; Merck KGaA) for 24 h to analyze protein expression with non-treated cells used as controls. The cells were cultured in a humidified atmosphere containing 5% of CO₂ at 37°C.

**ELISA.** Serum levels of hFGF-21 (cat. no. DF2100), tumor necrosis factor (TNF)-α (cat. no. DTA00C), interleukin (IL)-1β (cat. no. DLBS50), IL-6 (cat. no. D6050) and IL-8 (cat. no. D8000C) were detected in PhbA and healthy volunteers using ELISA kits (IBL International GmbH, Hamburg, Germany), according to the manufacturer's protocol. The serum levels of hFGF-21 were also analyzed between PhbA and PPhbA on day 30 following treatments. The serum concentration levels of hFGF-21, TNF-α, IL-1β, IL-6 and IL-8 were measured by an enzyme microplate reader at 450 nm.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from liver and renal epithelial cells using an RNeasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA), according to the protocol provided by the manufacturer. RNA was reversed transcribed using a PrimeScript RT Master Mix kit (Takara Bio, Inc., Otsu, Japan). All forward and reverse primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA; Table I). For amplification diluted cDNA was combined with a reaction mixture containing SYBR-Green PCR Core Reagents (cat. no. 4304886; Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative mRNA expression levels were calculated using the 2−ΔΔCq method (24). PCR cycling was performed under the following conditions: 94°C for 30 sec and 45 cycles of 95°C for 5 sec, 57°C for 10 sec and 72°C for 10 sec. The results were expressed as the n-fold of the control.

**Flow cytometry.** The following antibodies were used: FITC-conjugated anti-CD11b (cat. no. 557686; clone M1/70; BD Biosciences, Franklin Lakes, NJ, USA), allophycocyanin-conjugated anti-Ly-6B.2 (cat. no. NBP2-13077APC; clone 7/4; Bio-Rad Laboratories, Inc.), FITC-conjugated anti-CD4 (cat. no. MCA2649; clone RM 4-5; ebioScience; Thermo Fisher Scientific, Inc.), PercP-conjugated anti-CD8α (cat. no. 555369; clone 53-6-7; BD Biosciences), PE-conjugated anti-CD45R/B220 (cat. no. A15835; clone RA3-6B2; ebioScience; Thermo Fisher Scientific, Inc.) for 12 h at 4°C after Western blot analysis. Liver and renal epithelial cells from PhbA were incubated with hFGF-21 (2 mg/ml) for 12 h at 37°C. Cells not treated with hFGF-21 were used as the controls. Cells were homogenized in a lysate buffer containing protease-inhibitor (P3480; Sigma-Aldrich; Merck KGaA) and were centrifuged at 4,000 x g at 4°C for 10 min. Western blot analysis was subsequently performed as previously described (25). Protein concentration was measured by a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein samples (20 μg/lane) were resolved by 15% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Merck KGaA). Monoclonal rabbit anti-human epidermal growth factor (EGF), platelet-derived growth factor (PDGF; ab32570), hFGF-21 (ab64857), TNF-α (ab6671), IL-1β (ab2105), IL-6 (ab6672) and IL-8 (ab7747), TGF-β (ab31013), NF-κB (ab32360) and Kruppel-like factor 6 (KLF6; ab35783), extracellular matrix (ECM; ab28666), reactive oxygen species (ROS; ab5512) and β-actin (ab8227) antibodies (all 1:200; Abcam, Shanghai, China) were incubated with protein samples for 1 h at room temperature. After blocking with 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA), followed by incubation with horseradish peroxidase-conjugated polyclonal anti-rabbit immunoglobulin G antibodies (1:10,000; PV-6001; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. Signals were visualized by chemiluminescence detection (Z370398; Sigma-Aldrich; Merck KGaA). Densitometric quantification of the immunoblot data was performed using Quantity-One software (version 3.24; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Gene knockdown with small interfering RNA (siRNA).** Liver cells (1×10⁴/well) were incubated with hFGF-21 (2 mg/ml) for 5 days at 37°C in a six-well plate. To silence NF-κB gene expression, liver cells were transfected with 100 pmol siRNA-NF-κB sense, 5′-CUUUGGCUAUCUCAAGAUAtt-3′ and antisense, 5′-UAUCUGAGAUGUACCAAGc-3′; with siRNA-vector sense, 5′-CGGACAAAAGCGUCAUUUt-3′ and antisense, 5′-AAAGUGAGCCGUUUUGCcgg-3′ as a control (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a Cell Line Nucleofector kit L (Lonza Group, Ltd., Basel, Switzerland), according to the manufacturer's protocol (26). The cells were analyzed 48 h following transfection.
blocking with 1% bovine serum albumin for 2 h at 37˚C. All antibodies were used at a dilution of 1:100. B-lymphocytes were identified as CD11bhiLy6G-7/4hi/lo and macrophagocytes were identified as CD11chi. Cells were washed three times with 0.1% tris-buffered saline-Tween-20. Cells were analyzed using a flow cytometer (LSR II; BD Biosciences). Data was analyzed using BD FACSDiva™ software version 8.0.1 (BD Biosciences).

Statistical analysis. All data were presented as the mean ± standard deviation of triplicate independent trials in each experiment. All data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA). Statistical differences between groups were assessed using analysis of variance with the post hoc Dunnett’s test. P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of expression levels of hFGF-21 in PhbA. Expression levels of hFGF-21 were analyzed in serum and cellular units in PhbA. Characteristics of patients were summarized in Table II. Plasma concentration levels of hFGF-21 were significantly downregulated in PhbA compared with the those in healthy volunteers (P<0.01) (Fig. 1A). Western blotting demonstrated that hFGF-21 protein expression levels were significantly downregulated in liver cells isolated from PhbA compared to those from healthy volunteers (P<0.01) (Fig. 1B). These outcomes suggested that hFGF-21 is downregulated in PhbA. Healthy.

Analysis of expression levels of hFGF-21 in PhbA and PPhbA. Expression levels of hFGF-21 were detected in PPhbA. As demonstrated in Fig. 2A, plasma concentration levels of hFGF-21 were significantly increased in PPhbA compared with those in PhbA on day 30 (P<0.01). Cellular hFGF-21 mRNA and protein expression levels in liver cells isolated from PPhbA were significantly upregulated compared with those isolated from PhbA (P<0.01) (Fig. 2B). These results indicated that hFGF-21 may be a prognostic indicator in PhbA.

Association of hFGF-21 plasma concentration with cellular immunity and humoral immunity in PhbA. Characteristics of cellular immunity and humoral immunity were investigated in clinical PhbA prior and post treatments. Results demonstrated that the B lymphocyte level increased as the hFGF-21 plasma concentration increased during treatment (Fig. 3A). Macrophagocyte concentration levels were demonstrated to be positively associated with hFGF-21 plasma concentration during treatment (Fig. 3B). Results indicated that the percentage of cluster of differentiation (CD)4+ and CD8+ cells increased in serum as the hFGF-21 plasma concentration increased during treatment (Fig. 3C and D). These results indicated that hFGF-21 plasma concentration may be associated with cellular immunity and humoral immunity in PhbA during treatment.

Effects of hFGF-21 on inflammatory cytokine expression levels in liver cells isolated from clinical patients. Inflammatory cytokine levels were investigated in PhbA. As demonstrated in Fig. 4A-D, plasma concentration levels of TNF-α, IL-6, IL-1β and IL-8 were significantly upregulated in PhbA compared with those in healthy volunteers (P<0.01). Western blot analysis and RT-qPCR indicated that protein and mRNA expression levels of TNF-α, IL-6, IL-1β and IL-8 were significantly downregulated in the PPhbA groups compared with PhbA groups (P<0.01) (Fig. 4E and F). These results indicated that hFGF-21 treatment decreases inflammatory cytokine expression levels in liver cells isolated from clinical patients.

Effects of hFGF-21 on inflammatory cytokine expression levels in renal epithelial cells isolated from clinical patients. Inflammatory cytokines in renal epithelial cells isolated

| Gene name | Reverse | Forward |
|-----------|---------|---------|
| FGF-21    | CTGCTGGGGGTCTACCAAG | CTGCCTACACCAGTTGTTCC |
| TNF-α     | TCCAGACTTCTCTGAGACA | GGCAGTACGAGACAACCT |
| IL-6      | CCACACAGACCCACTCA   | CATCCATCTTTTTTCAGCATCT |
| IL-1β     | GGCTGCTTCACACCTTGA  | GAAGACACGGATTCATGGA |
| IL-8      | TACTCCAAATTTTCCACCCC | AACCTTCTCCACACCCCT |
| β-actin   | CGGAGTCGCCGATTGTC   | AGCCTTCTCCATGGTCGTA |

FGF, fibroblast growth factor; TNF, tumor necrosis factor; IL, interleukin.

Table I. Primer sequences used in the study for polymerase chain reaction.

| Characteristics | Patients | Healthy volunteers |
|-----------------|----------|--------------------|
| Number          | 186      | 68                 |
| Age, years (range) | 16.4-62.5 | 22.5-46.2        |
| Sex, n          |          |                    |
| Male            | 70       | 30                 |
| Female          | 116      | 38                 |

Table II. Characteristics of patients and healthy volunteers.
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from clinical patients were analyzed following treatment with hFGF-21. As demonstrated in Fig. 5A and B, gene and protein expression levels of TNF-α, IL-6, IL-1β and IL-8 were significantly downregulated by hFGF-21 treatment in renal epithelial cells isolated from clinical patients compared to the levels in the control cells (P<0.01). These outcomes indicated that hFGF-21 suppresses inflammatory cytokine expression in renal epithelial cells isolated from clinical patients.

hFGF-21 regulates inflammatory cytokines through downregulation of the NF-κB-mediated TGF-β signaling pathway.

In order to analyze the potential mechanism mediated by hFGF-21, the NF-κB-mediated TGF-β signal pathway was investigated in liver cells isolated from clinical patients. Results demonstrated that hFGF-21 treatment significantly inhibited deposition of ECM and ROS expression levels in liver cells compared with the levels in control cells (P<0.01) (Fig. 6A). Western blotting indicated that expression levels of TGF-β, NF-κB and KLF6 were significantly downregulated and PDGF and EGF expression levels were significantly upregulated by hFGF-21 treatment in liver cells compared with the levels in the control cells (P<0.01) (Fig. 6B and C). Knockdown of NF-κB with siRNA-NF-κB significantly inhibited the
Figure 3. Association of hFGF-21 plasma concentration with cellular immunity and humoral immunity in clinical patients. Relationship between concentration levels of hFGF-21 and (A) B lymphocyte level and (B) macrophagocyte level in patients during treatment. Association of hFGF-21 plasma concentration with percentage of (C) CD4+ and (D) CD8+ cells in serum in patients during treatment. hFGF-21, human fibroblast growth factor-21; CD, cluster of differentiation.

Figure 4. Effects of hFGF-21 on inflammatory cytokine expression levels in liver cells isolated from clinical patients. Plasma concentration levels of (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-8 in PhbA and healthy volunteers. (E) mRNA and (F) protein expression levels of inflammatory cytokines in cells treated with hFGF-21 isolated from patients and the PPhbA control group. **P<0.01. hFGF-21, human fibroblast growth factor-21; PhbA, patients with hepatitis B cirrhosis combined with adrenal insufficiency; PPhbA, patients who recovered from hepatitis B cirrhosis combined with adrenal insufficiency; TNF, tumor necrosis factor; IL, interleukin.
Figure 5. Effects of hFGF-21 on inflammatory cytokine expression levels in renal epithelial cells isolated from clinical patients. (A) Gene expression levels of TNF-α, IL-6, IL-1β and IL-8 in renal epithelial cells isolated from clinical patients treated with hFGF-21 or not treated with hFGF-21. (B) Protein expression levels of TNF-α, IL-6, IL-1β and IL-8 in renal epithelial cells isolated from clinical patients treated with hFGF-21 or not treated with hFGF-21. *P<0.01. hFGF-21, human fibroblast growth factor-21; TNF, tumor necrosis factor; IL, interleukin.

Figure 6. hFGF-21 regulates inflammatory cytokines through downregulation of the NF-κB-mediated TGF-β signaling pathway. (A) Effects of hFGF-21 on deposition of ECM and ROS expression levels in liver cells. (B) Effects of hFGF-21 on expression levels of TGF-β, NF-κB and KLF6 in liver cells. (C) Effects of hFGF-21 on expression levels of PDGF and EGF in liver cells. (D) Knockdown of NF-κB with Si-NF-κB increases TGF-β and KLF6 expression in liver cells. (E) Knockdown of NF-κB with Si-NF-κB suppresses PDGF and EGF expression in liver cells. (F) Effects of Si-NF-κB on protein expression levels of TNF-α, IL-6, IL-1β and IL-8 in liver cells. *P<0.01. hFGF-21, human fibroblast growth factor-21; NF, nuclear factor; TGF, transforming growth factor; ECM, extracellular matrix; ROS, reactive oxygen species; KLF6, Kruppel-like factor 6; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; TNF, tumor necrosis factor; IL, interleukin; Si, small interfering RNA.
hFGF-21-induced suppression of TGF-β and KLF6 expression and hFGF-21-promoted PDGF and EGF expression levels in liver cells compared with the levels in cells transfected with siRNA-vector (P<0.01; Fig. 6D and E). Findings also indicated that knockdown of NF-κB significantly inhibited the suppression of protein expression levels of TNF-α, IL-6, IL-1β and IL-8 in liver cells induced by hFGF-21 compared with the levels in cells transfected with siRNA-vector (P<0.01; Fig. 6F). These results indicated that hFGF-21 regulates inflammatory cytokines through downregulation of the NF-κB-mediated TGF-β signaling pathway.

Discussion

Hepatitis B-induced liver cirrhosis poses a great threat to health and frequently leads to adrenal insufficiency that further affects the endocrine system and disturbs liver metabolism (27,28). Pathophysiologic and clinical evidences have suggested that inflammation is associated with hepatitis B-induced liver cirrhosis and inflammatory cytokines, including TNF and IL-1, which may be potential target agents in decompensated cirrhosis (29). Research has also indicated that FGF is altered and molecular signaling pathways are regulated by attenuating the expression of TGF-β (30). The present study detected hFGF-21 serum concentration and expression levels in PhbA. Outcomes indicated that hFGF-21 suppressed inflammatory cytokine levels in liver cells isolated from clinical specimens through regulation of the NF-κB-mediated TGF-β signaling pathway. These findings suggested that hFGF-21 may serve as a predictor and prognostic factor in PhbA.

Beneficial effects of inhibition of oxidative stress and inflammation have been reported in hepatitis C virus-positive patients with liver cirrhosis and findings indicate that inflammation inhibition influences microinflammation and the metabolism of iron in hepatitis C virus-positive patients with liver cirrhosis, which subsequently appeared to reduce the production of oxidative stress, possibly leading to a decrease in the occurrence of hepatocellular carcinoma (31). A study by Prystupa et al (32) indicated that proinflammatory cytokines (IL-1β and IL-6) and hepatocyte growth factor were upregulated in patients with alcoholic liver cirrhosis. Additionally, the levels of ghrelin, leptin, TNF-α and IL-8 in liver cirrhosis were increased following hepatitis B and hepatitis D virus infection (33,34). The present findings suggested that hFGF-21 treatment inhibits mRNA and protein expression levels of TNF-α, IL-6, IL-1β and IL-8 in liver cells. Inhibitory effects of hFGF-21 were demonstrated in the present study, indicating that hFGF-21 regulates inflammatory cytokines by downregulation of the NF-κB-mediated TGF-β signaling pathway.

Target-specific systemic delivery of siRNA for TGF-β has been proposed for the treatment of liver cirrhosis and has demonstrated feasible therapeutic effects on liver cirrhosis by reduction of nodule formation, collagen content and hepatic stellate cell numbers (35). A study by Chávez et al (36) suggested that Sulfasalazine prevents the increase in TGF-β, cyclooxygenase-2 and NF-κB translocation and fibrosis in carbon tetrachloride-induced liver cirrhosis in rats. A study by Aldaba-Muruato et al (21) indicated that modulation of NF-κB, cytokine production and oxidative stress may protect the liver against allopurinol-induced acute liver damage and cirrhosis induced by carbon tetrachloride. Therefore, we assumed that the regulation of inflammatory cytokines by hFGF-21 may be associated with the NF-κB signaling pathway. The present results supported this hypothesis and the findings suggested that hFGF-21 treatment suppresses ECM and ROS expression levels and downregulates TGF-β, NF-κB and KLF6 expression levels in liver cells.

Previous research has demonstrated that FGF-21 resulted in insulin resistance by inhibiting the activation of NF-κB (37). FGF-21 also served an endocrine hormone role in blocking somatic growth, leading to growth hormone resistance (38). Furthermore, FGF-21 has been reported to be associated with lipid metabolism and the incidence of cardiovascular disease (39), as well as various human diseases and metabolic syndromes, including geriatric obesity, type 2 diabetes mellitus and congenital hypothyroidism (40-42). In the present study, changes of hFGF-21 plasma concentration levels in PhbA were analyzed. Outcomes suggested that hFGF-21 is downregulated in clinical patients suffering with hepatitis B cirrhosis combined with adrenal insufficiency. Therefore, hFGF-21 may serve as a predictor and prognostic factor for hepatitis B cirrhosis combined with adrenal insufficiency.

In conclusion, the present study indicated that hFGF-21 improved inflammatory cytokine expression levels in renal epithelial cells and liver cells isolated from clinical patients. The results demonstrated the potential molecular mechanism mediated by hFGF-21 in liver cells in the progression of hepatitis B cirrhosis combined with adrenal insufficiency. The present study suggested that hFGF-21 administration downregulates inflammatory cytokine levels through the NF-κB-mediated TGF-β signaling pathway. Changes in hFGF-21 plasma concentration prior and post treatment were observed for PhbA, suggesting that hFGF-21 possesses the potential to act as an alternative predictor and prognostic indicator for the evaluation of prognosis of hepatitis B cirrhosis combined with adrenal insufficiency.

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