Comparison of Proteome between Hepatitis B Virus- and Hepatitis C Virus-Associated Hepatocellular Carcinoma

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ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) is one of the most common malignancies associated with chronic infection by the hepatitis B virus (HBV) or the hepatitis C virus (HCV) throughout the world. Differential expression of the proteome in HBV- and HCV-associated HCC was investigated to identify any useful biomarkers indicating virus-specific hepatocarcinogenesis.

Experimental Design: Twenty-one pairs of specimens (tumorous and surrounding nontumorous liver tissues) were obtained from 21 HCC patients. They were divided into three HCC types by viral markers: 7 hepatitis B surface antigen-positive (B-type HCC), 7 anti-HCV-positive (C-type HCC), and 7 hepatitis B surface antigen-negative and anti-HCV-negative. Total proteins were analyzed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and alterations in the proteome were examined.

Results: Sixty proteins were identified that show significant changes in the expression level between nontumorous and tumorous tissues. Among these, 14 proteins were commonly changed in all three of the HCC types, but 46 proteins showed a tendency of viral marker specificity.

Conclusions: The identified proteins were classified according to the viral factor as being involved in B-type and C-type HCC. These results suggest strongly that the expression pattern of proteome in HCC tissues is closely associated with etiologic factors. The different protein profiles between B-type and C-type HCC indicate that the pathogenetic mechanisms of hepatocarcinogenesis may be different according to the viral factor, HBV and HCV.

INTRODUCTION

Worldwide, HCC5 is a common malignant tumor that takes the lives of ~1 million people annually. It is known that HCC develops from a chronic inflammatory liver disease due to the HBV infection, the HCV infection, and the exposure to carcinogens such as aflatoxin B1 (1). In fact, most chronic inflammatory liver diseases result from infection by the two hepatitis viruses, HBV and HCV (2, 3). Although the virologic features of the two viruses are entirely different, both viruses infect human liver and initiate a series of processes leading to chronic hepatitis, cirrhosis, and HCC (4, 5). Some epidemiological findings suggest different modes of disease progression and HCC promotion between HBV and HCV infection (6, 7). The mechanisms underlying such differences are unknown.

There have been several reports comparing the expression profiles of genes or proteins in HCC tissues with those in nontumor liver tissues by using microarray analysis or 2-DE analysis (8–17). Among these, two studies suggested that gene expression profiles in HCC tissues are different according to the infected hepatitis viruses, HBV or HCV (9, 17). Our previous study and others suggested that 2-DE method for proteome analysis in HCC tissues may be helpful, as HCC may have some characteristic features in the protein expression pattern (15, 16). However, in most of the studies, the sample sizes were too small to identify the relationship between etiologic factors and protein expression. In addition, there have been no reports on comparing the protein expression pattern between B-type HCC and C-type HCC using proteomics tools.

As a microarray method, 2-DE method together with MALDI-TOF MS has greatly promoted systematic analysis of global protein expression, which provides new insights into pathogenetic mechanisms of various human diseases. Such improved capability to identify disease-specific profiles of proteins leads to improved prediction of disease class and new development of clinical methods. The studies of cancer proteome have

5 The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; 2-DE, two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; IEF, isoelectric focusing; B-type HCC, hepatocellular carcinoma associated with hepatitis B virus; C-type HCC, hepatocellular carcinoma associated with hepatitis C virus; NBNC, negative for both hepatitis B surface antigen and antihepatitis C virus; HBsAg, hepatitis B surface antigen; mAb, monoclonal antibody; CPSASE I, carbamoyl-phosphate synthase; SOD, superoxide dismutase; GABA-AT, 4-aminobutyrate aminotransferase.
definitely shown such capability of 2-DE and MALDI-TOF MS. This study using the same method was designed to identify proteins that show hepatitis virus specificity. As in the cases of microarray study, the expression profiles of proteins in HCC tissues are different according to the viral factor, HBV and HCV. We found 46 proteins that showed a tendency of hepatitis virus specificity. These data will be discussed.

MATERIALS AND METHODS

Tissue Specimens. Twenty-one pairs of HCC tissues and surrounding nontumorous liver tissues were included in this study. They were collected from surgically resected liver specimens and carefully snap-frozen in a liquid nitrogen tank. The average age of the patients was 60 (range, approximately 40–76), and all but one were male. Samples were divided into three HCC types by serological markers of hepatitis virus, and there were 7 pairs in each group: a HBsAg-positive (B-type HCC), an anti-HCV-positive (C-type HCC), and a HBsAg-negative and anti-HCV-negative (NBNC-type HCC). All of the cases were differentiation grade I or II as determined by the Edmondson-Steiner grading system; 3 cases were grade I, 3 cases were grade I > II, 4 cases were grade I < II, and 11 cases were grade II. Clinical stages were determined according to the Classification of the Korean Liver Cancer Study Group and the method described by Desmet et al. (18).

Sample Lysis Before 2-DE. Human liver tissues were dissolved at a concentration of −0.2 g/ml in lysis buffer (Ready-Prep Sequential extraction kit, Reagent 3; Bio-Rad, Hercules, CA) consisting of 5 M urea, 2 M thiourea, 2% (w/v) 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 2% (w/v) SB 3–10, 40 mM Tris, 0.2% (w/v) Bio-Lyte 3/10, and 2 mM tributyl phosphine. Samples were sonicated on ice for 2 min using an ultrasonic processor (Sonics & Materials Inc., Newtown, CT) and centrifuged for 1 h at 100,000 × g to remove DNA, RNA, and any particulate materials. The protein concentration of sample was measured on an Emax precision microplate reader (Molecular Devices Co., Sunnyvale, CA) using a Bio-Rad Protein Assay kit (Bio-Rad). All of the samples were stored at −70°C until use.

2-DE. 2-DE was performed as described previously (15, 19). The first dimensional IEF was performed on precast 17 cm immobilized nonlinear pH 3–10 gradient (IPG) strips (Amersham Pharmacia Biotechnology, Uppsala, Sweden) at 20°C using a Protean IEF Cell (Bio-Rad). Five hundred μg of total proteins were mixed with a rehydration buffer containing 9 M urea (Bio-Rad), 4% (w/v) 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (Bio-Rad), 100 mM DTT (Sigma, St. Louis, MO), 0.2% (w/v) Bio-Lyte (Bio-Rad), and a trace of bromphenol blue (Amresco, Solon, OH), to a total volume of 500 μl. The mixtures were loaded onto the IPG strip. After rehydration for 12 h, IEF was carried out under the following conditions: (a) 250V, 125Vh; (b) 500V, 500Vh; (c) 1,000V, 1,000Vh; (d) 2500V, 2,500 Vh; and (e) 5000V, 90,000Vh. After IEF, the gel strip was first equilibrated for 15 min in the equilibration buffer containing 130 mM DTT, 6 M urea, 2% SDS (Bio-Rad), 0.375 M Tris-HCl, pH 8.8 (Bio-Rad), and 20% glycerol (Amresco). Then the gel strip was equilibrated for another 15 min in the same equilibration buffer, except that DTT was replaced with 135 mM iodoacetamide (Sigma). The second dimensional SDS-PAGE was performed in 11% acrylamide gels using the Protein XL system (Bio-Rad). Proteins were visualized by SYPRO orange (Bio-Rad).

Image Acquisition and Analysis. The stained two-dimensional gels were scanned on an FLA-2000 (Fuji Photo Film Co., LTD, Tokyo, Japan). After scanning the two-dimensional gels were stained with CBB R-250 (Amresco) and stored at 4°C. The images were processed using Photoshop 6 (Adobe) software. The image analysis and two-dimensional gel proteome database management were done using the ImageMaster 2D Elite software 4.01 (Amersham Pharmacia Biotechnology). The ImageMaster software calculated the two-dimensional spot intensity by integrating the absorbance over the spot area. The integrated spot intensity is called spot volume for convenience.

Tryptic In-Gel Digestion of 2-DE Resolved Proteins. Protein spots were excised and transferred to a siliconized 1.5 ml Eppendorf tube (Sigma). Enough 25 mM ammonium bicarbonate/50% acetonitrile (1:1; Sigma) was added to the tube to cover the gel piece, and the tube was incubated at room temperature for 10 min. The liquid was discarded, and washing was repeated. After the gel pieces were shrunk by dehydration in 100% acetonitrile, which was then removed, they were dried in a vacuum centrifuge. The gel pieces were swollen in 10 μl of 25 mM ammonium bicarbonate buffer containing 5 μl/g tryspin (Roche Applied Science, Mannheim, Germany) and incubated at 37°C for 20 h.

MALDI-TOF MS and Protein Identification. Mass spectra were obtained using Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Cambridge, MA) with delayed extraction and reflectron. The 337-nm nitrogen laser had a pulse width of 3 ns. The accelerating voltage was 25 kV, and the grid voltage was set at 70% of the accelerating voltage. After a delay of 350 ns, the accelerating voltage was used to extract the ions. The matrix solution was prepared by dissolving 10 mg α-cyano-4-hydroxycinnamic acid (Sigma) in 1 ml of 50% acetonitrile and 0.1% trifluoroacetic acid in deionized water. The peptide digest (1 μl) was mixed with 1 μl of matrix solution, and 1 μl of this mixture was applied to the stainless steel plate. A matrix peak (m/z = 379.0930 Da) and a trypsin fragment peak (m/z = 2163.0616 Da) served as internal standards for mass calibration. The database searches were carried out with the MS-Fit, which is accessible on the internet. MS-Fit performs fast database searches by comparing experimentally determined masses from the proteolytic digestion of protein with peptide database masses calculated from the SWISS-PROT protein database. The following search parameters were applied: all of the mass searches included human sequences; alkylation of cysteine by carbamidomethylation and oxidation of methionine were considered as possible modifications; peptide masses were monoisotopic; at least four matching peptide masses; a mass tolerance of 50 ppm; and one incomplete cleavage were allowed. The criteria used to accept identifications included

* Internet address: http://prospector.ucsf.edu/.
## RESULTS

### Proteomic Analysis of Nontumorous and Tumorous Tissues

The two-dimensional gel in Fig. 1 shows a typical separation of total proteins from tumorous tissues in NBNC-,
B-, and C-type HCC into 1000–1200 spots. The 2-DE experiment was repeated at least 7 times on both nontumorous and tumorous tissue samples from 21 HCC patients. Approximately 300 two-dimensional gel images were analyzed, and 3 of the most reproducible images from each tissue sample were selected for statistical analysis. Statistical analysis was performed using the 126 images (21 patients × 2 type tissues × 3 images). For each patient, the percentage of volume of the protein spots was compared between nontumorous and tumorous tissue samples. Wilcoxon signed-rank test was performed separately on NBNC-, B-, and C-type HCC groups, and protein spots satisfying ρ < 0.05 were selected. After the statistical analysis, MALDI-TOF mass spectrometric analysis was performed for all of the selected protein spots. This analysis was performed at least three times for each protein spot, and 60 proteins were clearly identified. The identified proteins are listed in Tables 1 and 2, and their positions on the two-dimensional map are annotated in Fig. 1.

### Proteins Differentially Expressed in All of the HCCs

The extent of sequence coverage, the number of peptides matched, molecular weight search score (the probabilistic score), and also molecular weight and isoelectric point of identified proteins should match estimated values obtained from image analysis.

#### Table 1

<table>
<thead>
<tr>
<th>ANa</th>
<th>NBNCb (n = 7)</th>
<th>HBVc (n = 7)</th>
<th>HCVb (n = 7)</th>
<th>Total (n = 21)</th>
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<tr>
<td>Up-regulated proteins (T&gt;N)</td>
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<tr>
<td>P14625</td>
<td>94 kDa glucose regulated protein (GRP94)</td>
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<tr>
<td>P11021</td>
<td>78 kDa glucose regulated protein (GRP78)</td>
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<td>7</td>
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<tr>
<td>P38646</td>
<td>75 kDa glucose regulated protein (GRP75)</td>
<td>4</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>P11142</td>
<td>Heat shock cognate 71 kDa protein (HSC71)</td>
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<td>5</td>
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<tr>
<td>P07900</td>
<td>Heat shock 90 kDa protein (HSP90)</td>
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<td>7</td>
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<td>P08107</td>
<td>Heat shock 70 kDa protein (HSP70)</td>
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<td>P10809</td>
<td>Heat shock 60 kDa protein (HSP60)</td>
<td>2</td>
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<td>Q99798</td>
<td>Aconitase hydratase (Aconitase)</td>
<td>4</td>
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<td>5</td>
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<tr>
<td>P13639</td>
<td>Elongation Factor 2 (EF-2)</td>
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<tr>
<td>P06748</td>
<td>Nucleophosmin</td>
<td>7</td>
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<tr>
<td>P08670</td>
<td>Vimentin</td>
<td>4</td>
<td>7</td>
<td>2</td>
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<tr>
<td>P15531</td>
<td>Nucleoside diphosphate kinase A (NDK A)</td>
<td>6</td>
<td>3</td>
<td>6</td>
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</table>

| Down-regulated proteins (N>T) | | | | |
| P00966 | Argininosuccinate synthase | 6 | 5 | 5 | 16 (76%) / 11.3 ± 32.9 |
| P31327 | Carbamoyl-phosphate synthase (CPSASE I) | 7 | 7 | 6 | 20 (95%) / 12.3 ± 8.7 |

- a N, nontumorous tissue; T, tumorous tissue; AN, SWISS-PROT accession number.
- b HBsAg-negative and anti-HCV-negative cases.
- c Number of cases with >1.5-fold increase or decrease in tumorous tissue.
- d HBsAg-positive cases.
- e Anti-HCV-positive cases.
Proteome in HBV- and HCV-Associated HCC

The frequency is the number of cases with >1.5-fold increase or decrease in tumorous tissue relative to nontumorous tissue. The differential expression of the two proteins in nontumorous and tumorous tissues is evident in the spot images on the two-dimensional gel in Fig. 2. The 2-DE result was confirmed by determining the expression level of nucleophosmin by immunoblot analysis (Fig. 3A).

of 21, 12.3 ± 8.7) were observed in >95% of HCCs. The frequency is the number of cases with >1.5-fold increase or decrease in tumorous tissue relative to nontumorous tissue. The differential expression of the two proteins in nontumorous and tumorous tissues is evident in the spot images on the two-dimensional gel in Fig. 2. The 2-DE result was confirmed by determining the expression level of nucleophosmin by immunoblot analysis (Fig. 3A).
**Hepatitis Virus-Associated Differential Protein Expression.** Analysis of proteins differentially expressed as a result of hepatitis virus infection led to identification of proteins associated with HBV or HCV, as well as NBNC proteins. Expression of SOD (Cu-Zn) showed a tendency to decrease in B-type HCC (5 of 7, 2.5 ± 1.1). GABA-AT generally increased in C-type HCC (6 of 7, 3.9 ± 2.6), and dihydrolipoamide dehydrogenase increased in NBNC-type HCC (7 of 7, 2.4 ± 0.5; Table 2). The differential expressions of SOD (Cu-Zn) and GABA-AT are evident in the spot images on the two-dimensional gel in Fig. 2. Changes in the expression level of SOD (Cu-Zn) were also measured by immunoblot analysis, and results similar to the 2-DE results were obtained (Fig. 3B). Interestingly, the expression levels of HSP27 and enoyl-CoA hydratase were decreased in B-type HCC (6 of 7, 19.4 ± 28.2; 6 of 7, 2.7 ± 1.1, respectively), whereas they were increased in C-type HCC (7 of 7, 4.4 ± 4.2; 6 of 7, 3.9 ± 2.6, respectively; Table 2).

Ribosomal binding protein showed little change in NBNC-type HCC, whereas it was clearly decreased in B- and C-type HCC (6 of 7, 3.6 ± 2.8; 6 of 7, 9.0 ± 10.1, respectively; Table 2). The differential expression of this protein in nontumorous and tumorous tissues is evident in the spot images on the two-dimensional gel in Fig. 2. Thiosulfate sulfurtransferase was increased in both NBNC- and B-type HCC (6 of 7, 16.3 ± 24.7; 6 of 7, 6.7 ± 7.6, respectively) but not in C-type HCC (Table 2). On the other hand, aflatoxin B1 aldehyde reductase 2 was increased in NBNC- and C-type HCC (6 of 7, 16.3 ± 24.7; 6 of 7, 6.7 ± 7.6, respectively) but not in B-type HCC (Table 2).

**DISCUSSION**

It has been demonstrated in previous reports that protein expression profiles of cancers (e.g., breast cancer, colon cancer, lung cancer, and so forth) obtained by 2-DE and MALDI-TOF MS could provide information useful for cancer classification, establishment of diagnostic markers, and selection of therapeutic target candidates (20–22). Microarray technique is rapid and efficient for comparing mRNA expression pattern in cancer research. However, the mRNA and protein expressions do not necessarily match. Sev-
eral earlier works report results obtained by both microarray and 2-DE analyses for the same type of cancer (9–16). The gene profiles observed by transcriptome analysis using microarray, and proteome analysis using 2-DE and MALDI-TOF MS were quite different. Clearly, both analyses provide valuable information.

Both HBV and HCV infection cause chronic hepatitis and cirrhosis, and increase the probability of leading to HCC. However, HBV and HCV appear to play different roles in some clinical manifestations and modes of disease progression (6, 7). The biological and pathogenic activities of the two viruses are different, and, thus, it is suspected that the processes involved in the development of hepatitis and HCC might be distinct for both viruses (8). In the present work, 2-DE and MALDI-TOF MS

![Fig. 3 Immunoblot analysis of expression of (A) nucleophosmin and (B) SOD (Cu-Zn) in nontumorous and tumorous tissues of four representative NBNC-, B-, and C-type HCC patients, respectively. Immunoblotting with a nucleophosmin and SOD (Cu-Zn) mAb after SDS-PAGE was performed as described in “Materials and Methods.” β-Actin was used as a reference. N, nontumorous tissue; T, tumorous tissue; P, patient]
were used as a proteomics tool, and protein expression profiles were compared between B-type and C-type HCC. It was hoped that the results could provide key information in understanding hepatocarcinogenesis caused by HBV or HCV infection as well as in identifying biomarkers useful for diagnosis and treatment of each type of HCC. Several previous reports compared mRNA expression pattern between B-type and C-type HCC using microarray (9, 17). However, our work is the first attempt to compare protein expression patterns in B-type and C-type HCC tissue samples using 2-DE and MALDI-TOF MS.

We identified 14 proteins of which the expression levels were significantly different between nontumorous and tumorous tissue irrespective of virus infection. Stress-induced proteins and proteins involved in cell proliferation were enhanced with a high frequency, and several enzymes involved in cellular metabolism and/or catabolism were suppressed. Nucleophosmin was increased and CPSASE I decreased with a particularly high frequency (frequency = 20 of 21 in both).

Kinoshita and Miyata (23) reported that the expression of CPSASE I was decreased in 75% of the tissue samples from 20 HCC patients, which is in agreement with our result. Nucleophosmin is a major nucleolar phosphoprotein that displays a number of activities including a potential role as a positive regulator of cell proliferation. It was found to be significantly more abundant in tumorous and proliferating cells than in normal resting cells (24, 25). According to Imai et al. (26), autoantibodies against nuclear and nucleolar antigens are produced in HCC patients. Changes in autoantibodies against nuclear and nucleolar antigens (from negative to positive) against three autoantigens (NOR-90, fibrillarin, and nucleophosmin/protein B23) were observed at a transition stage from chronic hepatitis to HCC (26). In a previous paper, we reported for the first time a distinct change in nucleophosmin expression in the tissue of HCC patients (15). The result was confirmed by additional 2-DE data in the present work. Immunoblot analysis also showed a clearly different expression level of this protein between nontumorous and tumorous tissues. These two proteins (nucleophosmin and CPSASE I) appear to be useful diagnostic markers of HCC.

On analyzing specific alteration of expression levels associated with hepatitis virus, HBV- and HCV-associated proteins were identified. The expression of SOD (Cu/Zn) showed a HBV- associated decrease. Immunoblot analysis of SOD (Cu/Zn) showed little change in C-type and NBC-type HCC. However, a significant decrease was observed in the tumorous tissue of B-type HCC patients (Fig. 3B). SOD (Cu/Zn) is a metalloenzyme and protects the cell from oxygen-free radicals. A series of reports indicated a significant decrease in SOD (Cu/Zn) in HCC (27, 28). Lin et al. (28) investigated the SOD level in tumorous tissues of 36 HCC patients and observed a positive connection between the SOD level and the survival rate beyond 5 years. They suggested that the SOD level in tumorous tissue could be a useful indicator of the severity of the HCC and a prognostic factor for patients after HCC operation.

Enoyl-CoA hydratase is another protein showing HBV-associated decrease. In the study involving 45 HCC patients by Suto et al. (29), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase and cytosolic carbonyl reductase, which are peroxisomal bifunctional enzymes, were shown to decrease in HCC. Litwin et al. (30) reported a decrease in peroxisomal enzyme as well as peroxisome in HCC. Decrease in catalase, which is a marker for peroxisome, was also reported in HCC.

GABA-AT is an enzyme involved in amino acid metabolism and generally shows HCV-associated increase. Our result is consistent with the increase in GABA-AT in the tumorous tissue of HCV-associated HCC observed by Iizuka et al. (17) using a microarray. The reason why this protein is increased in C-type HCC only is unknown and poses an interesting problem.

Unlike seven stress-associated proteins (GRP94, GRP78, GRP75, HSC71, HSP90, HSP70, and HSP60), which showed an increase in most HCC, the expression of HSP27 was decreased in B-type HCC and increased in C-type HCC. Similarly, enoyl-CoA hydratase was decreased in B-type HCC and increased in C-type HCC. It is not understood why HSP27 and enoyl-CoA hydratase exhibit opposite trends in B-type and C-type HCC.

In conclusion, we carried out an extensive proteomic analysis of liver tissues from 21 HCC patients and identified proteins showing significant differences in the expression level between nontumorous and tumorous tissues. These proteins were classified according to the viral factor as being involved in B-type and C-type HCC. These results suggest strongly that the expression pattern of proteome in HCC tissues is closely associated with etiologic factors. The different protein profiles between B-type and C-type HCC indicate that the pathogenetic mechanisms of hepatocarcinogenesis may be different according to the viral factors, HBV and HCV. It is hoped that our results could help understand the molecular mechanism of different types of HCC and provide clues in the search for treatment of HCC specific for each hepatitis virus infection.

REFERENCES


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