The Expression of Hepatic Carboxypeptidase E is Decreased in Patients with Cholesterol Gallstone
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ABSTRACT

Background/Aims: Decreased carboxypeptidase E (CPE) expression is associated with numerous pathophysiological conditions. This study aimed to investigate the potential function of hepatic CPE in cholesterol gallstone formation. Patients and Methods: Patients with cholesterol gallstone (CGS group) and patients without cholesterol gallstones (non‑CGS group) were enrolled. The serum total cholesterol, triglyceride, and biliary composition were analyzed. Eight liver samples from two patients without CGS and six patients with CGS were subjected to cDNA microarray analysis. Hepatic CPE expression was detected by quantitative real‑time polymerase chain reaction (qRT‑PCR), Western blot, and immunohistochemical analysis. Plasma CCK level was measured by ELISA. Results: cDNA microarray identified CPE as a gene downregulated in the CGS group. RT‑PCR showed that CPE mRNA level was lower in CGS group than in control (P < 0.05, t‑test). Moreover, Western blot and immunohistochemistry analysis showed that CPE protein level was significantly lower in CGS group than in the control group. In addition, plasma CCK level was lower in CGS group than in the control group. A positive correlation was found between serum CCK level and hepatic CPE mRNA level (r² = 0.713, P = 0.003). Conclusions: Down‑expression of liver CPE may reduce the secretion of serum CCK and contribute to the formation of cholesterol gallstone.

Key Words: Carboxypeptidase E, cholesterol gallstone, cholecystokinin, liver

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Gallstone disease has become one of the most common digestive disorders in the world. With the development of economic conditions in recent years, cholesterol gallstone (CGS) has become the main type of gallstones.[1] Gallbladder stone may induce pancreatitis, severe biliary infection, and malignant biliary tumor. Therefore, it is of great significance to understand the pathogenesis of cholesterol gallstone and develop effective strategy for the treatment of cholesterol gallstone.

The supersaturation of biliary cholesterol is very important for the formation of cholesterol gallstones. The main components of bile are the products of hepatic synthesis, intake, and secretion. Thus, hepatic metabolism is related to the supersaturation of biliary cholesterol. On the other hand, a large‑scale epidemiological study showed that the formation of gallstones is associated with genetic factors.[2] Family history can increase the risk of gallstone disease.[1‑5] Twin studies suggest that up to 25% of gallstone disease is due to genetic factors.[6] To identify the genes associated with CGS, in the present study we performed cDNA microarray analysis to compare hepatic gene expression in CGS patients with patients without cholesterol gallstone (non‑CGS patients).

CPE is found primarily in endocrine and neuroendocrine cells, and functions to generate biologically active peptide hormones. In human beings, deregulated CPE has been associated with numerous pathophysiological conditions, such as obesity, diabetes, and cancer. CPE mutation enhanced the prevalence of cholelithiasis.[7] This study aimed to investigate the potential function of hepatic CPE in cholesterol gallstone formation, and we detected the expression of hepatic CPE in both CGS group and non‑CGS group.

PATIENTS AND METHODS

Patients and tissue samples
This study was performed at Nanjing First Hospital, Nanjing
Medical University (Nanjing, China). Liver tissues and blood samples were obtained from CGS (n = 26) and non-CGS (n = 12) patients who visited the hospital between June 2012 and October 2013. The basic demographic data of all patients were obtained from medical records. The liver tissues were snap frozen in liquid nitrogen and stored at −80°C. The blood samples were centrifuged at 3000 rpm for 10 min and the separated serum was stored at 4°C. The study was approved by the Ethics Committee of Nanjing Medical University Affiliated Nanjing Hospital, and informed consent was obtained from all the participants.

**Analysis of the serum lipid and bile**
The serum lipid and bile were analyzed at the clinical laboratory of Nanjing Hospital. The levels of triglyceride, serum total cholesterol, biliary cholesterol, bile acid, bile phospholipids, and bile total fat were determined with an autoanalyzer. Biliary cholesterol saturation index (CSI) was measured according to Carey method.[8]

**Serum CCK**
Serum CCK was measured by enzyme-linked immunosorbotent assay (ELISA kit by KeyGEN BioTECH -Nanjing, China).

**Cholesterol of the gallstone**
Gallstone was washed with saline and kept overnight in dry atmosphere at 37°C. Total cholesterol level of the gallstone was measured according to the Oxidase method.

**Cell culture**
The normal liver HL-7702 cell line was purchased from Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM medium supplemented with 10% fetal bovine serum, 80 U/mL penicillin, and 80 μg/mL streptomycin in a humidified atmosphere with 5% CO₂ at 37°C.

**cDNA microarray**
Total RNA was extracted from liver HL-7702 cells and liver tissues by RNAeasy kit (Qiagen, Inc, Valencia, CA, USA) and used for cDNA synthesis and labeling, microarray, hybridization, followed by flour-labeled cDNA hybridization on the chip. The data were analyzed by Tigr Lowess. Relative gene expression levels in liver tissues were compared between patients with cholesterol gallstone and control group, and gene expression levels in HL-7702 cells served as the internal control.

**Quantitative real-time polymerase chain reaction**
Total RNA was extracted from liver samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green kit (Takapa, Tokyo, Japan) on a PRISM 7500 real-time PCR detection system (Labnet, USA). Sequences of the primers were as follows: CPE: forward 5′-GGGCGGCGCTGCTGAGA-3′, and reverse 5′-AGGCCTGGTATGCTGCA-3′; β-actin: forward 5′-ATCATCCCTGCTCTACTTG-3′, and reverse 5′-GTCAGGTCCACACTGACAC-3′. Reaction conditions were predenaturation at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 40 s. CPE mRNA level was normalized to β-actin mRNA level and calculated by comparative 2^{-ΔΔCT} method.

**Western blot analysis**
Total proteins were extracted from the liver tissues, seperated by SDS-PAGE (5% stacking gel and 10% separating gel), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were incubated in 5% skimmed milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C (CPE, 1:1000, Abcam; β-actin, 1:4000, Immunoway). Next, the membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Jackson) for 1 h at room temperature. Finally, immunoreactive bands were detected by enhanced chemiluminescence kit (Amersham, UK) according to the manufacturer’s instructions.

**Immunohistochemistry**
The liver tissues were cut into 5-μm sections, deparaffinized in xylene, and dehydrated through a graded series of ethanol solutions. The antigen retrieval was performed by heating the sections for 20 min in a microwave oven with a citrate buffer. The sections were washed in phosphate-buffered saline (PBS) and treated with 3% hydrogen peroxidase for 15 min to block endogenous peroxidase activity. The tissue sections were then incubated with CPE antibody (1:100, Abcam) for 2 h at 37°C and secondary antibodies for 30 min at 37°C. The results of immunohistochemical staining were evaluated by two pathologists independently. The results were judged as positive if the percentage of positively stained cells was >10%.

**Statistical analysis**
Statistical analysis was performed using SPSS19.0 statistical software. Numerical data were expressed as mean ± SD. The categorical variables were assessed by χ² or Fisher’s exact test. Comparisons of the quantitative variable were analyzed by Student’s t-test or one-way analysis of variance. The Spearman’s rank correlation test was used to determine the correlation. P < 0.05 was considered to indicate a statistically significant difference.
RESULTS

Serum lipid and bile composition in CGS patients
As shown in Table 1, we found no significant differences in triglyceride and serum total cholesterol levels in the CGS group and control group, but biliary cholesterol level was significantly lower in CGS group (P < 0.05). In addition, CSI was higher but bile total fat was lower in CGS group than in non-CGS group.

Differential gene expression in liver tissues of CGS patients
Microarray analysis showed that total 6068 genes were expressed in human liver cell line and liver samples. Seventy-three genes from the patients with CGS were expressed differentially from those in controls, including 26 genes upregulated over twofold and 47 genes downregulated over twofold [Table 2]. Among those upregulated genes, nine were associated with defense response, such as C9 (complement 9), CRP (C-reactive protein), Orosomucoid 1, whereas cholesterol ester transfer protein (CETP) was associated with lipid metabolism. Among those downregulated genes, 17 were associated with physiological regulation, such as early growth response (EGR2), nuclear receptor 2 (NR4A), whereas four were associated with lipid metabolism, including CPE, apolipoprotein E (APOE), lipoprotein lipase (LPL), and 7-dehydrogenation cholesterol reductase.

CCK expression is regulated by CPE
We selected CPE for further analysis. Frst we performed qRT-PCR to confirm the downregulation of CPE in the CGS group. We found that liver CPE mRNA level was lower in CGS group than in the control group (P < 0.05, Figure 1a and 1b). Moreover, Western blot analysis showed that the expression of CPE protein in the CGS group was significantly lower than in the control group (P < 0.05, Figure 1d and 1e). In addition, we found that the level of plasma CCK was higher in non-CGS group than in CGS group (P < 0.05, Figure 1c). The Spearman’s rank correlation test showed that CCK level was positively correlated with CPE mRNA level (r² = 0.713, P = 0.003).

Immunohistochemistry analysis showed that CPE staining was weak in the liver tissues of CGS patients [Figure 2a], but was strong in the nuclei and cytoplasm in the liver tissues of non-CGS group [Figure 2b]. Quantitative analysis showed that CPE positive staining was significantly lower in the liver tissues of CGS group than in those of non-CGS groups [Figure 2c].

DISCUSSION
Biliary cholesterol supersaturation is a prerequisite in the formation of cholesterol gallstone. Epidemiologic studies indicate that cholesterol gallstone formation is genetically determined. It has been reported that 45 candidate genes are associated with cholesterol gallstone formation.[9] In this study, we performed cDNA microarray analysis on normal liver cell line HL-7702 and human liver samples. We found that 26 genes were upregulated over twofold and 47 genes were downregulated over twofold. Some of these genes were associated with cholesterol gallstone formation, such as CPE.
### Table 2: Genes upregulated or downregulated over twofold in liver tissues of CGS patients

| Gene name                                                                 | Symbol | Fold change |
|---------------------------------------------------------------------------|--------|-------------|
| Leukocyte immunoglobulin-like receptor, subfamily B, with TM and ITIM domains, member 6 | LILRB3 | 3.34        |
| Chromatin assembly factor 1, subunit B (p60)                              | CHAF1B | 3.13        |
| C-reactive protein, pentraxin-related                                    | CRP    | 2.95        |
| Protein phosphatase 1, regulatory (inhibitor) subunit 3C                 | PPP1R3C | 2.62       |
| Haptoglobin                                                               | HP     | 2.49        |
| Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) | MX1    | 2.44        |
| Phosphoinositide-3-kinase, class 2, gamma polypeptide                    | PIK3C2G | 2.43        |
| Chitinase 3-like 1 (cartilage glycoprotein-39)                           | CH3L1  | 2.41        |
| 2',5'-oligoadenylate synthetase 1, 40/46kDa                            | OAS1   | 2.36        |
| Dehydrogenase/reductase (SDR family) member 2                            | DHRS2  | 2.31        |
| Transmembrane protein 48                                                 | FLJ10407 | 2.29     |
| Cholesteryl ester transfer protein, plasma                               | CETP   | 2.23        |
| Complement component 9                                                   | C9     | 2.21        |
| Arginine vasopressin receptor 1A                                         | AVPR1A | 2.20        |
| Guanylate cyclase 1, soluble, alpha 2                                    | GUCY1A2 | 2.18       |
| Orosomucoid 1                                                            | ORM1   | 2.17        |
| Lectin, galactoside-binding, soluble, 4 (galectin 4)                     | LGALS4 | 2.17        |
| Chemokine (C-X-C motif) ligand 10                                       | CXCL10 | 2.15        |
| XIAP associated factor-1                                                 | HSXIAF | 2.10        |
| Prolactin receptor                                                       | PRLR   | 2.07        |
| Kinesin family member 1C                                                 | KIF1C  | 2.06        |
| Vanin 1                                                                  | VNN1   | 2.06        |
| CD5 antigen-like (scavenger receptor cysteine rich family)              | CD5L   | 2.04        |
| Interferon, alpha-inducible protein 27                                   | IFI27  | 2.02        |
| Transmembrane 4 L six family member 5                                    | TM4SF5 | 2.01        |
| Transmembrane protease, serine 11D                                      | HAT    | −2.04       |
| Kruppel-like factor 6                                                    | KLF6   | −2.06       |
| ELK4, ETS-domain protein (SRF accessory protein 1)                       | ELK4   | −2.06       |
| Erythrocyte membrane protein band 4.1-like 1                             | EPB41L1| −2.07       |
| Follistatin                                                              | FST    | −2.07       |
| Lipoprotein lipase                                                       | LPL    | −2.09       |
| Argininosuccinate synthetase                                             | ASS    | −2.10       |
| Apolipoprotein E                                                         | APOE   | −2.11       |
| Microphthalmia-associated transcription factor                           | MITF   | −2.11       |
| BCL2-like 11 (apoptosis facilitator)                                     | BCL2L11| −2.11       |
| Fatty acid-binding protein 4, adipocyte                                  | FABP4  | −2.11       |
| Fibulin 2                                                                | FBLN2  | −2.12       |
| 7-dehydrocholesterol reductase                                           | DHCR7  | −2.13       |
| Peroxisomal biogenesis factor 6                                          | PEX6   | −2.14       |
| Chromosome 1 open reading frame 21                                       | C1orf21| −2.15       |
| Hairy and enhancer of split 1, (Orosophila)                              | HES1   | −2.18       |
| Amyloid beta (A4) precursor protein-binding, family A, member 2 binding protein | APBA2BP | −2.18   |
| Cystathionase (cystathionine gamma-lyase)                                | CTH    | −2.19       |
| Transcription factor binding to IGHM enhancer 3                         | TFE3   | −2.22       |
| Tumor-associated calcium signal transducer 1                             | TACSTD1| −2.22       |
| Aryl hydrocarbon receptor nuclear translocator                           | ARNT   | −2.22       |
| FBV murine osteosarcoma viral oncogene homolog B                         | FOSB   | −2.25       |
| KIAA1609 protein                                                         | KIAA1609| −2.34     |
| Aminolevulinate, delta-, synthase 1                                      | ALAS1  | −2.36       |
| BRCA1 associated protein                                                 | BRAP   | −2.36       |
| Mannosidase, alpha, class 1A, member 2                                   | MAN1A2 | −2.36       |

Contd...
CETP, APOE, and APL. We paid special attention to CPE. cDNA microarray analysis suggested that the expression of hepatic CPE decreased about fivefolds. Thus we enlarged sample size and determined CPE expression by qRT-PCR, Western blot, and immunohistochemistry analysis.

CPE gene is located in 4q32.3 of human chromosome. CPE contributes, directly or indirectly, to the production of the majority of neuropeptides.\(^{[10]}\) The absence of CPE activity led to abnormal processing of many peptides and obesity in mice.\(^{[11]}\) However, polymorphism analysis of the promoter and entire coding region of CPE gene in 269 Japanese individuals demonstrated that genetic variation in CPE gene did not play a major role in the pathogenesis of obesity in the Japanese population.\(^{[12]}\) Furthermore, chromosome 4q32.3 is linked with normal population variation in HDL-C and encompasses the gene encoding CPE.\(^{[13]}\) In this study, cDNA microarray showed that hepatic CPE expression decreased in patients with cholesterol gallstone. Furthermore, qRT-PCR, Western blot, and immunohistochemistry analysis showed lower hepatic CPE mRNA and protein expression in CGS group compared with the control group. These data suggest that decreased hepatic CPE expression is associated with the formation of cholesterol gallstone.

CCK is a gastrointestinal peptide hormone that causes gallbladder contraction. Notably, plasma CCK level was significantly decreased in patients with gallstones.\(^{[14]}\) A reduced and delayed postprandial gallbladder contractility and impaired CCK release in the early postprandial phase have been shown to be significantly associated with gallstone disease.\(^{[15]}\) In this study we found decreased plasma CCK level and increased biliary cholesterol and CSI in CGS

| Gene name                                                                 | Symbol | Fold change |
|---------------------------------------------------------------------------|--------|-------------|
| Gelsolin (amyloidosis, Finnish type)                                       | GSN    | −2.39       |
| Fragile X mental retardation 1                                             | FMR1   | −2.39       |
| Early growth response 2 (Krox-20 homolog, Drosophila)                     | EGR2   | −2.43       |
| Serine dehydratase                                                         | SDS    | −2.49       |
| Preferentially expressed antigen in melanoma                               | PRAME  | −2.52       |
| Carboxypeptidase E                                                         | CPE    | −2.53       |
| Vesicle-associated membrane protein 5 (myobrevin)                         | VAMP5  | −2.53       |
| Mesothelin                                                                | MSLN   | −2.59       |
| BUB3 budding uninhibited by benzimidazoles 3 homolog (yeast)              | BUB3   | −2.64       |
| Hyaluronan-mediated motility receptor (RHAMM)                              | HMNR   | −2.65       |
| Activating transcription factor 3                                          | ATF3   | −2.69       |
| Insulin-like growth factor binding protein 1                              | iGFBP1 | −2.71       |
| Guanine nucleotide binding protein (G protein), gamma 7                    | GNG7   | −2.83       |
| Connective tissue growth factor                                            | CTGF   | −2.84       |
| Dual specificity phosphatase 4                                             | DUSP4  | −2.99       |
| Liver cancer associated non-coding mRNA, partial sequence                 | −2.99  |             |
| SNF1-like kinase 2                                                         | SNF1LK2| −3.24       |
| Adrenergic, alpha-2C-, receptor                                            | ADRA2C | −3.38       |
| Glutathione S-transferase theta 1                                          | GSTT1  | −3.46       |
| Hypothetical protein LOC283143                                             | LOC283143 | −3.83 |
| Nuclear receptor subfamily 4, group A, member 1                            | NR4A1  | −4.04       |

**Figure 2:** Immunohistochemical staining of CPE in human liver tissues. (a) CPE staining was weak in the liver tissues of CGS patients (×400). (b) CPE staining was strong in the nuclei and cytoplasm in the liver tissues of non-CGS group (×400). (c) Quantitative analysis of CPE staining in the liver tissues of CGS and non-CGS groups. *P < 0.05
patients compared with non-CGS patients. Interestingly, it was reported that amidated CCK level was decreased by about 74% in whole brain of CPE (fat) mice compared with control group. After treatment with CPE, the level of CCK in brains of CPE (fat) mice was elevated about 51-fold higher compared with control group. Consistent with these reports, in this study we found that plasma CCK level was correlated with CPE mRNA level. Thus we speculate that CPE may convert pro-CCK to CCK, and decreased hepatic CPE expression contributes to lower plasma CCK, which eventually induces the formation of cholesterol gallstone.

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