Original Article

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.[3-5] 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 neuroendocine 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

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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 immunosorbent 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 supplmented with 10% fetal bovine serum, 80 U/mL penicillin, and 80 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 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′-CGTGGAGCTTAGCTGTGAGA-3′, and reverse 5′-CTCCTCGGTGTATCTGCTCA-3′; β-actin: forward 5′-ATCATCCCTGCCTCTACTGG-3′, and reverse 5′-GTCAGGTCCACCACTGACAC-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 $2^{-\Delta\Delta T}$ 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, and 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 peroxide 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 χ^2 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 differently 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. Frist 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 ($r^2 = 0.713$, $P = 0.003$).

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].

Table 1: Comparison of serum lipid and bile composition in CGS and non-CGS patients

	Non-CGS	CGS
Case (male/female)	5/5	10/10
Age (years)	49.63±3.64	50.17±2.67
BMI (kg/m^2)	23.34±0.90	22.70±1.12
Triglyceride (mM)	1.20±0.22	1.33±0.23
Serum total cholesterol (mM)	3.22±0.60	3.90±1.32
Biliary cholesterol (mol%)	5.72±1.11	8.05±0.37*
Bile acid (mol%)	74.12±2.75	72.26±1.39
Bile phospholipids (mol%)	20.16±1.73	19.68±1.29
Bile total fat (g/dL)	15.48±1.46	11.01±1.40*
CSI	0.78±0.12	1.24±0.06*

*P<0.05, compared with non-CGS group

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,

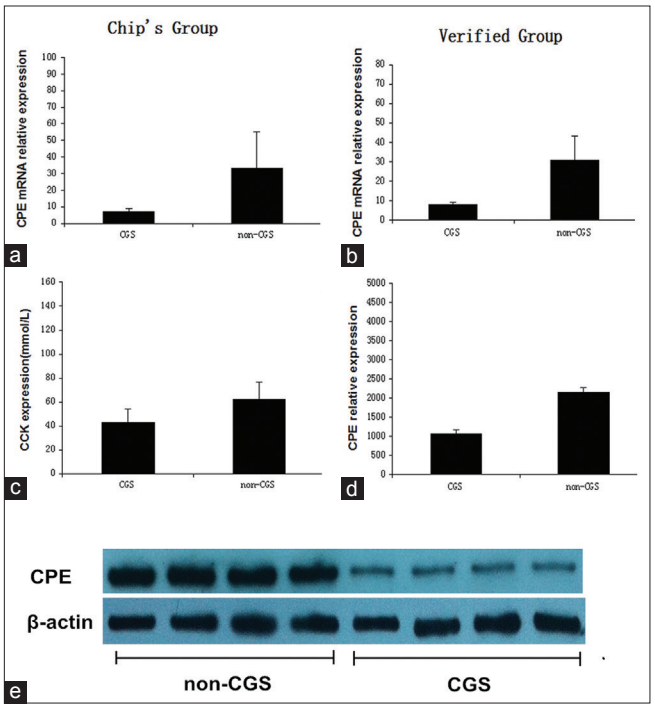

Figure 1: Decreased CCK and CPE expression in patients with cholesterol gallstone. (a) CPE relative mRNA level in Chip's CGS group, β-actin served as the internal control. *P < 0.05. (b) CPE relative mRNA level in Verified's CGS group, β-actin served as the internal control. *P < 0.05. (c) CCK level measured by ELISA. *P < 0.05. (d) CPE protein level was determined by Western blot analysis, β-actin served as loading control. *P < 0.05. (e) The blots showing hepatic CPE protein level

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)	CHI3L1	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
Fibrinogen, B beta polypeptide	FGB	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	HSXIAPAF	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, (*Drosophila*)	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
FBJ 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...

Table 2: Contd...

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)	HMMR	−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

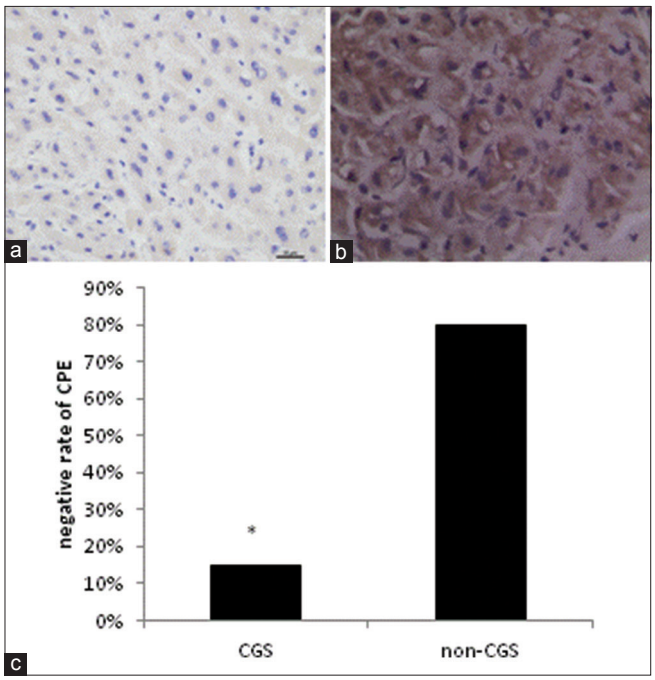

Figure 2: Immunohistochemical staining of CPE in human liver tissues. (a) CPE staining was weak in the liver tissues of CGS patients (×400). (b) CPE staining was strong in the nuclei and cytoplasm in the liver tissues of non-CGS group (×400). (c) Quantitative analysis of CPE staining in the liver tissues of CGS and non-CGS groups. *$P < 0.05$

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

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.[16] After treatment with CPE, the level of CCK in brains of CPE (fat) mice was elevated about 51-fold higher compared with control group.[17] 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.

REFERENCES

1. Stokes CS, Krawczyk M, Lammert F. Gallstones: Environment, lifestyle and genes. Dig Dis 2011;29:191-201.
2. Everhart JE, Yeh F, Lee ET, Hill MC, Fabsitz R, Howard BV, *et al*. Prevalence of gallbladder disease in American Indian populations: Findings from the Strong Heart Study. Hepatology 2002;35:1507-12.
3. Stinton LM, Shaffer EA. Epidemiology of gallbladder disease: Cholelithiasis and cancer. Gut Liver 2012;6:172-87.
4. Attili AF, De Santis A, Attili F, Roda E, Festi D, Carulli N. Prevalence of gallstone disease in first-degree relatives of patients with cholelithiasis. World J Gastroenterol 2005;11:6508-11.
5. Gilat T, Feldman C, Halpern Z, Dan M, Bar-Meir S. An increased familial frequency of gallstones. Gastroenterology 1983;84:242-6.
6. Katsika D, Grjibovski A, Einarsson C, Lammert F, Lichtenstein P, Marschall HU. Genetic and environmental influences on symptomatic gallstone disease: A Swedish study of 43,141 twin pairs. Hepatology 2005;41:1138-43.
7. Bouchard G, Johnson D, Carver T, Paigen B, Carey MC. Cholesterol gallstone formation in overweight mice establishes that obesity *per se* is not linked directly to cholelithiasis risk. J Lipid Res 2002;43:1105-13.
8. Carey MC. Critical tables for calculating the cholesterol saturation of native bile. J Lipid Res 1978;19:945-55.
9. Lammert F, Carey MC, Paigen B. Chromosomal organization of candidate genes involved in cholesterol gallstone formation: A murine gallstone map. Gastroenterology 2001;120:221-38.
10. Zhang X, Che FY, Berezniuk I, Sonmez K, Toll L, Fricker LD. Peptidomics of Cpe (fat/fat) mouse brain regions: Implications for neuropeptide processing. J Neurochem 2008;107:1596-613.
11. Rodriguiz RM, Wilkins JJ, Creson TK, Biswas R, Berezniuk I, Fricker AD, *et al*. Emergence of anxiety-like behaviours in depressive-like Cpe (fat/fat) mice. Int J Neuropsychopharmacol 2013;16:1623-34.
12. Utsunomiya N, Ohagi S, Sanke T, Tatsuta H, Hanabusa T, Nanjo K. Organization of the human carboxypeptidase E gene and molecular scanning for mutations in Japanese subjects with NIDDM or obesity. Diabetologia 1998;41:701-5.
13. Harrap SB, Wong ZY, Scurrah KJ, Lamantia A. Genome-wide linkage analysis of population variation in high-density lipoprotein cholesterol. Hum Genet 2006;119:541-6.
14. Behar J, Mawe GM, Carey MC. Roles of cholesterol and bile salts in the pathogenesis of gallbladder hypomotility and inflammation: Cholecystitis is not caused by cystic duct obstruction. Neurogastroenterol Motil 2013;25:283-90.
15. Glasbrenner B, Dominguez-Munoz JE, Nelson DK, Pieramico O, Holzwarth C, Riepl RL, *et al*. Postprandial release of cholecystokinin and pancreatic polypeptide in health and in gallstone disease: Relationships with gallbladder contraction. Am J Gastroenterol 1994;89:404-10.
16. Cain BM, Wang W, Beinfeld MC. Cholecystokinin (CCK) levels are greatly reduced in the brains but not the duodenums of Cpe (fat)/Cpe (fat) mice: A regional difference in the involvement of carboxypeptidase E (Cpe) in pro-CCK processing. Endocrinology 1997;138:4034-7.
17. Wang W, Cain BM, Beinfeld MC. Adult carboxypeptidase E-deficient fat/fat mice have a near-total depletion of brain CCK 8 accompanied by a massive accumulation of glycine and arginine extended CCK: Identification of CCK 8 Gly as the immediate precursor of CCK 8 in rodent brain. Endocrine 1998;9:329-32.

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