Gene structure of human cholecystokinin (CCK) type-A receptor: body fat content is related to CCK type-A receptor gene promoter polymorphism

Akihiro Funakoshi a,*, Kyoko Miyasaka b, Hideo Matsumoto c, Shunji Yamamori c, Sohichi Takiguchi d, Kazuhiro Kataoka d, Yutaka Takada d, Kimihiko Matsusue d, Akira Kono d, Hiroshi Shimokata e

a Department of Gastroenterology, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 811-1395, Japan
b Department of Clinical Physiology, Tokyo Metropolitan Institute of Gerontology, Tokyo 173-0015, Japan
c Department of Cellular Genetics, Mitsubishi Kagaku Bio- Clinical Laboratories, INC, Tokyo 174-8355, Japan
d Department of Chemotherapy, National Kyushu Cancer Center, Fukuoka 811-1395, Japan
e Department of Epidemiology, National Institute for Longevity Sciences, Ohsli 474-8522, Japan

Received 22 November 1999; received in revised form 24 December 1999

Edited by Masayuki Miyasaka

Abstract The transcriptional start site of the human cholecystokinin (CCK)-A receptor gene was determined by the Capsite Hunting method. Two sequence changes were detected, a G to T change in nucleotide –128, and an A to G change in nucleotide –81. The homozygote (T/T, G/G) was detected in 25 of 1296 individuals (1.9%) in the cohort study. This polymorphism showed a significantly higher percent body fat and higher levels of serum insulin and leptin, compared with wild type and heterozygotes. Our study provided the possibility that polymorphism in the promoter region of the CCK-A receptor gene may be one of genetic factors affecting fat deposition.

© 2000 Federation of European Biochemical Societies.

Key words: Fat deposition; CCK-A receptor promoter; Capsite Hunting; Polymorphism; Leptin

1. Introduction

Cholecystokinin (CCK) is known as an essential gastrointestinal hormone, as well as an important neurotransmitter. Two types of cholecystokinin (CCK) receptors (type A and type B) have been identified on the basis of their affinities for a structurally and functionally related family of peptides with identical COOH-terminal pentapeptide sequences [1]. These receptors were cloned recently, and the CCK-B receptor (CCK-BR) gene was found to be identical to the gastrin receptor gene [2,3]. CCK-A receptor (CCK-AR) has been demonstrated to be present in the vagus nerve and localized areas of the central nervous system, and mediates the satiety effect [1,4]. In addition, it is present in the gastrointestinal system, mediating pancreatic growth and enzyme secretion, as well as smooth muscle contraction of the gallbladder and stomach. Abnormalities in gallbladder contraction in response to exogenously administered CCK have been observed in patients with irritable bowel syndrome, suggesting a generalized abnormality of smooth muscle function and possibly altered CCK-AR function and structure.

Many genetic and environmental factors are considered to be responsible for obesity. Recently, we demonstrated absence of the CCK-AR gene expression due to a genetic abnormality in OLETF rats, which show obesity and non-insulin dependent diabetes mellitus (NIDDM), although the expression of CCK-BR gene was intact [5,6]. Administration of intracerebroventricular CCK failed to inhibit food intake in this model [4]. Thus, dysfunctional CCK-AR may cause human disorders such as hyperphagia, obesity, hyperglycemia and gallstone disease. However, mutations of human CCK-AR gene appear to be rare [7,8].

The transcriptional start site of the human CCK-AR gene has not been determined. In the present study, we cloned and sequenced the 5′ upstream region of human CCK-AR gene using a method to replace the cap structure of a mRNA with an oligoribonucleotide (rOligo) to label the 5′ end of eukaryotic mRNAs [9]. Identification of the 5′ end of mRNA is an indispensable step for establishing the promoter region of a gene. Two sequence changes were detected in this promoter region. We also examined the polymorphism of the CCK-AR gene promoter, this is associated with obesity (body fat content), in 1296 individuals between 40–79 years of age.

2. Materials and methods

2.1. RNA extraction

Samples of healthy gallbladder were obtained at surgery from patients with adenocarcinoma of the pancreas. Informed consent was obtained for all subjects prior to the operation. Immediately after removal of the healthy gallbladder, one part was used for histological examination, whereas the other part was rapidly frozen at –80°C. All samples were evaluated histopathologically, and no signs of abnormalities or inflammations were detected. Total RNA was extracted according to the acid-guanidium thiocyanate-phenol-chloroform method. The quantity and purity of RNA were determined from the absorbances at 260 and 280 nm. Ribosomal RNA bands were visualized under ultraviolet light after ethidium bromide staining. Samples showing no signs of degradation were employed for Northern transfer analysis using cDNA probes of the coding regions of the human CCK-AR [10] and β-actin cDNA (Wako Chemical Industries, Osaka, Japan); the former probe was kindly provided by Dr. S.A. Wank, NIH, Bethesda, MD, USA.

2.2. Determination of the transcription start site

Human genomic DNA library (Stratagene, USA) was screened us-
ing the cDNA probe of the coding regions of the human CCK-AR. Approximately 1000000 phage clones were screened under standard plaque hybridization conditions. Fourteen positive phage clones were isolated, and further characterized by Southern blot analysis and restriction mapping. DNA fragments, which hybridized with the cDNA probe of CCK-AR, were isolated from lambda H24 and H14 phage clones and subcloned into M13 mp19 for further shotgun sequencing method. Sequence data were linked using the inherit sequence analysis system (Perkin-Elmer, USA).

To determine the transcription start site of human CCK-AR mRNA, a Capsite Hunting method was used in accordance with manufacturer's protocol (Nippon Gene, Tokyo, Japan). Briefly, the 5'-terminal m'GpppN cap structure was removed by pyrophosphorylase reaction of tobacco nucleotide pyrophosphatase and recapped with the 3' end of a 35 mer-specific oligonucleotide (rOligo) (5'-GAUGCUAGCUGCGAGUCAAGUGCAGGAGCAG-3'). A single-strand cDNA library was prepared from the recapped mRNA by the synthesis of first-strand cDNA with Moloney murine leukemia virus reverse transcriptase and random primer. To amplify CCK-AR-specific transcripts from single-strand cDNA library, nested PCR was performed. The first round of PCR was performed using a sense DNA primer complementary to rOligo (RC1, 5'-GATGCTAGCTGGAGTAGTCGACGAAGUGCAGGGAG-3') paired with target-gene specific primer 1 (TGS-1, 5'-GTGATGAACTCCAGCCGAGG-3'). The second PCR was performed using a sense DNA primer complementary to rOligo (RC2, 5'-CGAAGCTTGAAGAGGGAGG-3'), paired with target-gene specific primer 2 (TGS-2, 5'-GACAGCTGAAGCCCGG-3'). A single band of second round PCR product was isolated and subcloned into pGEM-T vector, and two independent clones were sequenced. The transcription start site was determined by identification of the boundary sequence between rOligo and CCK-AR mRNA sequences.

2.3. Subjects
The subjects consisted of 646 males and 650 females between 40 and 79 years of age. This cohort study was approved by the National Institute for Longevity Sciences-Longitudinal Study of Aging. Informed consent was obtained for all subjects prior to testing. Body mass index (BMI) was calculated by dividing the weight by height squared. Body fat percent was measured both by dual energy X-ray absorptiometry (DXA). Fasting serum leptin and insulin were measured by RIAs using the LINCO kit (St Louis, MO, USA) and Dina-bot kit (Tokyo, Japan), respectively. Genomic DNA was prepared from leukocytes according to established procedures.

3. Results and discussion
Lambda H24 and H14 cover the 21.8 kb genomic sequence that contains the entire region of CCK-AR gene according to previously reported cDNA sequences [7,10]. These sequences were shown in the GenBank database under accession number D85606. The sequence of CCK-AR gene digested by PvuII, including exon 1 as well as the approximately 900 bp 5'-flanking sequences of CCK-AR gene are partially shown in Fig. 1. There were no differences between the Japanese and Caucasian individuals. The Capsite Hunting method showed that the transcription start site is 205 bp upstream of the initiating ATG (Fig. 1). The first base in the mRNA is labelled +1, and the first base downstream of the promoter is labelled −1. This is the first report identifying the transcription start site of human CCK-AR. The first 400 bp are rich in GC content and lack the typical TATA-box motif.

The polymorphisms of promoter region were examined by the PCR-cloning method using sense primer (nucleotides −351 to −332, 5'-TTCTAAGCTTTAAGGGAGG-3') and antisense primer (nucleotides +197 to +176, 5'-TTCTGAATTCTGCTTTCCACCAAGTGC-3').

Fig. 1. Exon mapping and nucleotide sequence of the 5'-flanking region of the human CCK-A receptor gene. Close boxes in the genomic DNA show exons which are numbered below. The transcriptional initiation site determined by the Capsite Hunting method was assigned as +1. The positions of sense and antisense primers used for the polymorphism determination are underlined. Asterisks indicate the nucleotide change positions (G to T change in nucleotide −128, and an A to G change in nucleotide −81). The coding region in exon 1 was boxed. These genomic sequences have been deposited in the GenBank database under accession number D85606.
Table 1
CCK-A receptor genotypes and various body fat content parameters

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild (G/G, A/A) and heterozygote types (n = 1271)</th>
<th>Homozygote (T/T, G/G) type (n = 25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.8 (0.09)</td>
<td>23.9 (0.78)</td>
<td>0.076</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>5.7 (0.14)</td>
<td>8.8 (2.18)</td>
<td>0.003</td>
</tr>
<tr>
<td>Serum insulin (µU/ml)</td>
<td>7.9 (0.13)</td>
<td>9.8 (1.32)</td>
<td>0.038</td>
</tr>
<tr>
<td>DXA fat (%)</td>
<td>26.3 (0.20)</td>
<td>29.3 (1.67)</td>
<td>0.041</td>
</tr>
</tbody>
</table>

Mean (S.E.M.). Statistical differences in phenotypic parameters between the two genotypic groups were analyzed by Student’s t-test. Statistical significance was assumed with a P value < 0.05.

Increased body fat content. BMI showed higher levels, but these were not significant. These results suggest that polymorphism of the CCK-AR gene is related to the increase in body fat content associated with weight control difficulties in obese subjects, and is affected by lifestyle as well as eating habits. Therefore, polymorphism in the promoter region of the CCK-AR gene may be one of the genetic factors that affect obesity (fat deposition).

The real mechanism of relationship of CCK-AR polymorphism and obesity has not been clarified, yet. Many CG-rich segments are observed in the CCK-AR promoter region and nucleotide −128 position was found to be methylated. Thus, a G to T change in nucleotide −129 may affect the gene expression of CCK-AR [12]. The xenobiotic responsive element (XRE) motif (CAGCGW) [13] is also observed in the CCK-AR promoter region around the −128 position (CAGCT-CA). Further functional studies to assess the potential biological consequences of this CCK-AR gene promoter polymorphism are in progress in our laboratory.

Acknowledgements: This study was supported in part by a Research Grant for Longevity Sciences (9C-03) and Comprehensive Research on Aging and Health from the Ministry of Health and Welfare, and the Naito Foundation in Japan.

References