Brief Report

Bioinformatic analyses and an expression study of a novel gene associated with metabolic syndrome

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Summary **The investigation of novel genes involved in the derangement of glucose and lipid metabolism is of particular importance in understanding the development of metabolic syndrome (MS). In the present study, bioinformatic analyses were carried out to explore the structures and roles of the proteins encoded by the four cDNA sequences identified in our previous studies as associated with MS. Homology analyses demonstrated that the proteins encoded by Sequence 1, Sequence 2, Sequence 3, and Sequence 4 were homologous with fibrinogen gamma polypeptide, liver fibrinogen-like 1, chromosome 10 open reading frame 104, and an unnamed protein product, respectively. Because the structures were well-known for fibrinogen gamma polypeptide and liver fibrinogen-like 1, further analyses were performed only for Sequence 3 and Sequence 4. Analyses of functional domains showed that the predicted proteins encoded by Sequence 3 and Sequence 4 had multiple phosphorylation and myristoylation sites. These results indicated that the two predicted proteins might be intermediate proteins in some signaling pathways. In order to explore the possible association of Sequence 3 with MS, HepG2 cells, a human hepatoma cell line, were treated with different concentrations of glucose (mannitol as osmotic control) for 48 h. Glucose at concentrations of 22 and 33.3 mM significantly increased the mRNA expression of Sequence 3 compared to glucose at 5.6 mM while mannitol had no significant effect on the mRNA expression of Sequence 3. These results indicated that the mRNA expression of Sequence 3 was positively associated with glucose higher than physiological concentrations.**

Keywords: Metabolic syndrome, bioinformatic analysis, mRNA expression, intermediate proteins

1. Introduction

Metabolic syndrome (MS) is a multigenic disorder that encompasses abnormalities such as visceral (abdominal or central variant) obesity, insulin resistance, glucose intolerance, hypertension, and dyslipidemia characterized by elevated triglyceride and decreased HDL concentrations (*1*). This syndrome has been found to be significantly associated with coronary heart disease, stroke, type 2 diabetes mellitus, and increased risk of various cancers (*2-6*). MS has become increasingly common in many populations. A national cross-sectional survey in China indicated that the age-standardized prevalence of MS was 9.8% in men and 17.8% in women (*7*). In the United States, 24% of US adults were reported to have MS in the Third National Health and Nutrition Examination Survey (*8*).

Investigation of novel genes involved in the derangement of glucose and lipid metabolism is of particular importance in understanding the development of MS. In our previous studies, we introduced a rat model having some features of MS using a high-carbohydrate diet and constructed and screened hepatic subtraction cDNA libraries of the model rats (*9*). Four full-length cDNAs were identified by screening a human hepatic cDNA library with a mixture of

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probes of the differentially expressed fragments from the rat hepatic subtraction cDNA libraries (*10*). Sequencing and homology analyses of the four full-length cDNAs demonstrated that Sequence 1, Sequence 2, and Sequence 3 were highly homologous with fibrinogen gamma mRNA, liver fibrinogen-related gene-1 (LFIRE1) mRNA, and chromosome 10 open reading frame 104 mRNA, respectively. Sequence 4 had homology with carbamoyl phosphate synthetase 1 mRNA.

In the present study, bioinformatic analyses of functional domains were carried out for the proteins encoded by Sequence 3 and Sequence 4 because homology analyses of the proteins encoded by the four cDNA sequences showed that the two were novel proteins. Expression studies were also performed using real-time quantitative PCR (RTQ-PCR) to validate the existence of the gene of Sequence 3 in hepatic cells and its possible association with MS.

2. Materials and Methods

2.1. *Reagents*

Glucose-free Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Low-glucose DMEM was from Gibco, Grand Island, NY, USA. Penicillin, L-glutamine, D-glucose, and mannitol were all from Amresco, Solon, OH, USA. Newborn calf serum was from Hangzhou Sijiqing Co., Hangzhou, Zhejiang, China. Bovine serum albumin (BSA) was from Roche Applied Science, Mannheim, Germany. RNA extraction kit and DNase I (RNase-free) were from TaKaRa, Dalian, China. PCR primers and Taqman probes were synthesized by TaKaRa Biotech, Dalian, China. ReverTra Ace reverse transcriptase and Real-time PCR Master Mix were both from Toyobo, Osaka, Japan.

2.2. *Bioinformatic analyses*

Homology analyses of the proteins encoded by the four cDNA sequences were conducted using the Blastp program (*http://www.ncbi.nlm.nih.gov/BLAST*) (*11*). Bioinformatic analyses were performed by Compute pI/Mw, ScanProsite, SignalP, Psort, Motif, and InterPro Scan software in the ExPASy Server (*http://www.expasy.org*) to predict physical and chemical properties, signal peptides, subcellular localization, and functional domains of the novel proteins encoded by Sequence 3 and Sequence 4 (*12*).

2.3. *Cell culture and treatment*

HepG2 cells, a human hepatoma cell line, were maintained in DMEM (5.6 mM glucose) supplemented with 10% heat-inactivated newborn calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM L-glutamine at 37°C and 5% CO_2. After grown to 90% confluence, cells were starved for 24 h in serum-free DMEM, and then incubated with glucose- and serum-free medium with 0.5% BSA at different concentrations of glucose (5.6, 22.0, and 33.3 mM) for 48 h. The cells in the control group were treated with serum-free medium (5.6 mM glucose) with 0.5% BSA at different concentrations of mannitol (0, 16.4, and 27.7 mM) which was used as the osmotic pressure control.

2.4. *Extraction and reverse transcription of total cellular RNA*

Total RNA was isolated from cultured HepG2 cells using the RNA extraction kit and possible remaining DNA was digested using RNase free DNase I. ReverTra Ace reverse transcriptase was used to perform reverse transcription of total RNA.

2.5. *Real-time quantitative PCR*

RTQ-PCR was performed using an ABI 7300 Real-Time PCR System and Sequence Detection Software (version 1.3.1) using the following cycle parameters: 1 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. All data were normalized to *β-actin* expression. For each sample, RTQ-PCR was conducted in triplicate with a reaction volume of 25 µL. The following primers and probes were used for the PCR of Sequence 3: Forward primer, 5'-GGGTCAGTGGAAGTTCTGTCAC-3'; Reverse primer, 5'-TCTCTCAGAGCCATCTTCTAACATC-3'; Oligonucleotide probe, 5'-FAM-CTGGTTTCAGTGTCTCAGACCTTGCCC-TAMRA-3'. The primers and probes used for the PCR of *β-actin* were as follows: Forward primer, 5'-ACCCTGAAGTACCCCATCGAG-3'; Reverse primer, 5'-ACATGATCTGGGTCATCTTCTCG-3'; Oligonucleotide probe, 5'-FAM-TCACCAACTGGGACGACATGGAGAAA-TAMRA-3'.

2.6. *Statistical analysis*

All quantitative values were expressed as mean ± S.D. The significant differences of the quantitative values among the different concentrations of glucose and mannitol were analyzed by one-factor analysis of variance or rank sum test. Two-sided p values below 0.05 were considered to be statistically significant.

3. Results and Discussion

Homology analyses of the proteins showed that the proteins encoded by Sequence 1 and Sequence 2 were respectively highly homologous with fibrinogen gamma polypeptide and liver fibrinogen-like 1 (Table 1). Disturbances in the thrombotic and fibrinolytic

Table 1. Homology analyses of the proteins encoded by MS-associated cDNAs

Sequences	Length	GHH	Length	SH	Gaps (%)	Identities (%)	Scores	E	Description
1	361	> gi|30583001	437	1	0/0 (0)	309/310 (99)	636	0.0	Fibrinogen, gamma polypeptide [Homo sapiens]
2	81	> gb|AAP35281.1	312	1	0/0 (0)	81/81 (100)	191	5e-48	Fibrinogen-like 1 [Homo sapiens]
3	110	> gb|AAH09530.1	110	1	0/0 (0)	110/110 (100)	216	1e-55	Chromosome 10 open reading frame 104 [Homo sapiens]
4	67	> gi|34533908	130	1	2/38 (5)	26/38 (68)	50.8	1e-05	Unnamed protein product [Homo sapiens]

GHH: Known gene of highest homology in GenBank DNA database; SH: Stretch of homology; E: Expect.

systems are features of MS (*13*). The associations have been reported between fibrinogen and other cardiovascular risk factors of MS (*14,15*). Bonora *et al.* (*16*) demonstrated that subjects with MS had disturbances in coagulation (thrombophilia) and showed higher levels of fibrinogen. It was also shown in our preliminary study that alteration in the expression of the fibrinogen gene was associated with MS (*9*). Fibrinogen-like 1 is encoded by LFIRE1 and specifically expressed in the liver (*17*). It is a member of the fibrinogen family. The specific relationship remains unclear between LFIRE1 and MS. The protein encoded by Sequence 3 was highly homologous with chromosome 10 open reading frame 104 and the protein encoded by Sequence 4 had homology with an unnamed protein product (Table 1). These results strongly suggest that for the previously un-annotated chromosome 10 open reading frame 104 and the unnamed protein product, they would likely be functionally related to MS.

Since homology analyses showed that the proteins encoded by Sequence 3 and Sequence 4 were novel proteins, further bioinformatic studies were performed to analyze the functional domains of these two proteins. The results demonstrated that the predicted protein encoded by Sequence 3 contained 110 amino acids with a putative isoelectric point (pI) of 4.91 and molecular weight (MW) of 11,667.04. This protein had no signal peptide and was predicted to localize in cytoplasm. It had four *N*-myristoylation sites at amino acids 11-16 (GGvsGS), 12-17 (GVsgSS), 15-20 (GSsvTG), and 20-25 (GSgfSV), one casein kinase II phosphorylation site at amino acids 24-27 (SvsD), and two protein kinase C phosphorylation sites at amino acids 50-52 (SeR) and 66-68 (TlK). The predicted protein encoded by Sequence 4 contained 67 amino acids with a putative pI of 10.30 and MW of 8,277.72. This protein had signal peptides and was predicted to localize in cytoplasm. It had two protein kinase C phosphorylation sites at amino acids 23-38 (SpK) and 50-52 (SlR), one cAMP and cGMP dependent protein kinase phosphorylation site at amino acids 40-43 (KRfS), and one *N*-myristoylation site at amino acids 55-60 (GLqfCF).

Phosphorylation and acylation of proteins are of great significance in biology. Moreover, phosphorylation and dephosphorylation of proteins are ubiquitous regulation methods of signal transduction *in vivo* and are involved in almost all of the physiological and pathological processes, such as, cell growth and development, gene expression, and regulation of hepatic glucose and lipid metabolism (*18-21*). Proteins encoded by Sequence 3 and Sequence 4 might be intermediate proteins in some signaling pathway. Both proteins might be phosphorylated by protein kinase C, casein kinase II, or cAMP and cGMP dependent protein kinase, and transmit signals to downstream intermediates.

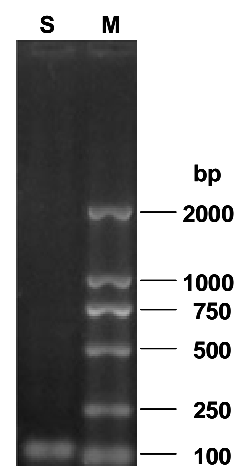

Figure 1. Gel electrophoresis of reverse transcription-PCR products of Sequence 3. S, Sequence 3; M, Marker DL2000.

Total RNA was isolated from cultured HepG2 cells and reverse transcription-PCR for the mRNA of Sequence 3, which we were interested in, was performed to validate the expression of Sequence 3 in hepatic cells. As shown in Figure 1, length of the PCR product of Sequence 3 was 122 bp. This result suggested that Sequence 3, identified by screening a human hepatic cDNA library, was expressed in HepG2 cells.

One of the featured pathophysiologies of MS is its derangement of glucose metabolism. Sequence 3 was screened from a rat model having some characteristics of MS induced by a high-carbohydrate diet. Therefore, we investigated the effect of glucose on the expression of Sequence 3 in HepG2 cells to explore the possible

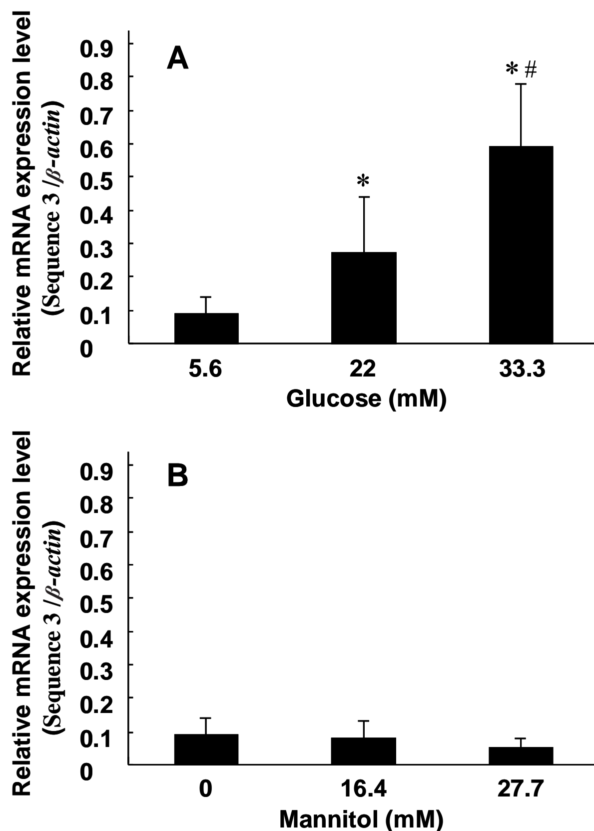

Figure 2. Effects of glucose and mannitol on the mRNA expression of Sequence 3 in HepG2 cells. Values are expressed as mean ± S.D. of four independent experiments. $^{*}p < 0.05$ compared with the concentration of 5.6 mM; $^{\#}p < 0.05$ compared with the concentration of 22 mM.

association of Sequence 3 with MS. HepG2 cells possess similar morphology and function compared to freshly isolated hepatic cells (*22*). HepG2 cells were treated with glucose at different concentrations for 48 h. The mRNA expression of Sequence 3 was normalized to *β-actin* expression. There was a significant difference among the cells incubated with glucose at 5.6, 22.0, and 33.3 mM in terms of the mRNA expression of Sequence 3 (Figure 2). mRNA expression of Sequence 3 was increased by glucose above physiological concentrations. Mannitol, an osmotic control, had no effect on mRNA expression of Sequence 3 in HepG2 cells. This result revealed that mRNA expression of Sequence 3 was positively associated with glucose higher than physiological concentrations.

In conclusion, the characteristics of the proteins encoded by cDNA sequences associated with MS were preliminarily obtained by bioinformatic analyses. The proteins encoded by Sequence 3 and Sequence 4 had multiple phosphorylation and myristoylation sites and might play roles in some signaling pathways. mRNA expression of Sequence 3 was positively associated with glucose higher than physiological concentrations. Therefore, Sequence 3 might play roles in some signaling pathways regulating hepatic glucose and lipid metabolism. More studies are needed to validate the existence of Sequence 4 and its possible association with MS.

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