Calpain 10 as a Predictive Gene for Type 2 Diabetes: Evidence from a Novel Screening System Using White Blood Cells of Otsuka Long-Evans Tokushima Fatty (OLETF) Rats

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The mRNA expression of type 2 diabetes-related genes in white blood cells (WBC) was examined before and after onset in Otsuka Long-Evans Tokushima Fatty (OLETF) rat. The level of the calpain 10 (CAPN10) transcript was significantly decreased compared to control animals in WBC before and after onset. Significant decreases in this gene expression were also found in the major insulin-target tissues as well as WBC before onset. These results suggest that gene expression in WBC could be a useful screening system for predicting the incidence of type 2 diabetes before onset in OLETF rats, and that CAPN10 represents a potential candidate gene for predicting type 2 diabetes in human.

Keywords type 2 diabetes; genetic diagnosis; calpain 10; white blood cell; Otsuka Long-Evans Tokushima Fatty rat

More than 60 candidate genes have been identified as type 2 diabetes-related genes, which are related to insulin action, insulin secretion and lipid metabolism. Among these genes, some may be down (or up)-regulated after the onset of type 2 diabetes, the others may be down (or up)-regulated prior to onset. If an altered expression of type 2 diabetes-related genes could be detected before onset, it would be possible to predict the incidence of type 2 diabetes.

Our long-term goal is to predict the incidence of type 2 diabetes by human genetic analysis. Although white blood cells (WBC) are not major insulin-target tissues compared to liver, skeletal muscle, and adipose tissue, an estimation of the level of expression of type 2 diabetes-related genes in insulin-target tissues via measurement of their expression in WBC would be useful. We therefore analyzed some candidate genes which may be down (or up)-regulated before the onset of type 2 diabetes using blood cells, and then examined relationships between the extent of gene expression for WBC and insulin-target tissues.

The use of animal models is desirable in examining the extent of gene expression before and after onset of the type 2 diabetes, since it is difficult to design this protocol in clinical situation. In this study, Otsuka Long-Evans Tokushima Fatty (OLETF) rats were selected as a model animal. Since OLETF rats are known to initiate type 2 diabetes at 18 weeks with hyperglycemia, the extent of gene expression at 6 and 24 weeks in WBC was measured, as well as insulin-target tissues such as liver, muscle, and adipose tissue.

It has been proposed that the pathophysiology of insulin resistance involves an abnormality in insulin signaling in type 2 diabetes. Therefore, insulin receptor (IR) was chosen as an initiator of signaling. Src homology 2-containing inositol 5′-phosphatase (SHIP2) as a negative regulator, and peroxisome proliferator-activated receptor gamma (PPARγ) at the downstream of insulin signaling. In addition, calpain 10 (CAPN10) which has been reported to be linked with type 2 diabetes with the G→A polymorphism (UCSNP-43) in certain human population groups was analyzed.

MATERIALS AND METHODS

Animals Male OLETF and Long-Evans Tokushima Otsuka (LETO) rats at 4 weeks of age were generously donated by the Tokushima Research Institute, Otsuka Pharmaceutical. The rats were housed at 22 °C with light from 7:00 to 21:00 and had free access to water and food. All in vivo experiments were approved by the Institutional Animal Care and Use Committee.

Glucose Tolerance Test (GTT) Rats were fasted overnight and subjected to an intraperitoneal GTT at 5 and 23 weeks of age. Glucose (1 g/kg) was injected intraperitoneally and blood was obtained from a tail vein after 1 h. Serum glucose concentration was determined using a Glucose-B test kit (Wako, Osaka, Japan). Concerning the 23 weeks old OLETF rats, only rats showing a serum glucose concentration above 11.1 mmol/L were classified as diabetic types and were used in the following experiments.

RNA Extraction and First-Strand cDNA At 6 and 24 weeks of age, blood was sampled under ether anesthesia after an overnight fast. Heparin (1.6 U/g) was administered intravenously before sampling. Blood was sampled from the aorta and was perfused from the portal with a cold 0.9% NaCl solution. Liver, right hindlimb skeletal muscle, and adipose tissue were harvested and stored at −80 °C until used for assay. Using a QIAamp RNA blood Mini Kit (QIAGEN, Hilden, Germany), erythrocytes were selectively lysed and total RNA was extracted from WBC. Total RNA was also extracted from liver and skeletal muscle using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), and using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) in the case of adipose tissue. All RNA was purified using RNase-Free DNase Set and RNeasy Mini Spin Columns (QIAGEN, Hilden, Germany). First-strand cDNA synthesis was performed on 1 μg of total RNA using an oligo dT primer and Super Script II RNase H− Reverse Transcriptase as described by the manufacture’s instructions (Invitrogen, Carlsbad, CA, U.S.A.).

Real-Time Quantitative PCR Polymerase chain reaction (PCR) primers were designed based on the nucleotide
sequence cited in GenBank and are shown in Table 1. Each of the mRNA transcripts were quantitated by the real-time SYBR green PCR method using the SYBR green PCR master mix (Applied, Inc., Norwalk, CT, U.S.A.). Samples were prepared in a final volume of 50 μl including 1 μl of each primer (10 μM), 25 μl of the supplied enzyme mix, 19 μl of H2O and finally 4 μl of the template. PCR was performed with a 10 min pre-incubation at 95 °C followed by 40 cycles of 15 s at 95 °C (denaturation), 1 min at 60 °C (annealing and amplification) in an ABI PRISM 7700 Sequence Detection System (Applied, Inc., Norwalk, CT, U.S.A.), according to the manufacturer’s recommendations. All samples were run in duplicate, and data were calculated using the standard curve method and expressed as a ratio to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reference. The PCR products were analyzed by agarose gel electrophoresis and confirmed as objectives by restriction enzyme cleavage and direct sequencing.

Statistical Analysis All values are expressed as the means±S.D. Statistical significance in values for body weights and serum glucose concentration were examined by the Student’s t-test. The experimental data on gene expression in WBC were analyzed by the Student’s t-test. Data relative to a correlation analysis on CAPN10 expression were examined by two-way analysis of variance (ANOVA) followed by the Turkey–Kramer method. Levels of p<0.05 were considered to be significant.

RESULTS

Basic Profiles The body weight and the results of the GTT are shown in Table 2. The body weights of OLETF rats were statistically higher than those of LETO rats after 5 weeks old. No differences in serum glucose levels were observed at 5 weeks, and both strains demonstrated a normal glucose tolerance. However, this value was markedly higher in OLETF rats at 6 weeks old, but no statistical significance was observed (Figs. 1A—C). No significant difference between these three genes at 24 weeks of age was observed (Figs. 1A—C). In contrast, a significant difference in CAPN10 expressions between OLETF and LETO rats was found. Its expression in OLETF rats was decreased by 50% of that in LETO rats at both 6 and 24 weeks of age (Fig. 1D). Thus, the CAPN10 gene has the potential for use in predicting the incidence of type 2 diabetes by genetic diagnosis.

Correlation of CAPN10 Gene Expression among Four Tissues The extent of CAPN10 gene expressions in each tissue was quantified and normalized to that of LETO rats at 6 weeks old (Fig. 2). Data were analyzed by two-way ANOVA against strain and age. A significant difference in strain and age in WBC, liver, and adipose tissue was found. In skeletal muscle, a significant difference was observed only on strain. No interaction between strain and age was found (data not shown). The Turkey–Kramer test was applied to compare OLETF and LETO rats in each age group. The ex-
were examined before and after onset using WBC as a source of mRNA, since such cells represent a suitable sampling tissue for genetic diagnosis in clinics for screening a candidate gene which is down (or up)-regulated prior to onset. IR, SHIP2, PPARγ are expressed in WBC. Although it has been reported that CAPN10 is ubiquitously expressed in all tissues, this is the first report that documents its expression in WBC.

There was no significant difference in the mRNA levels of IR, SHIP2 and PPARγ at 6 weeks of age (Figs. 1A—C). However, a remarkable and significant decrease in the mRNA level of CAPN10 was found, and this decrease was maintained after onset (24 weeks of age) (Fig. 1D). Thus, the decreased CAPN10 gene expression in WBC of OLETF rats occurred prior to impaired glucose tolerance.

Although WBC is one of the most convenient sampling tissues in humans, it is not a major type 2 diabetes-related tissue or major insulin-target tissue. Therefore, relationship of CAPN10 gene expression between WBC and insulin-target tissues was examined. A general tendency of a decrease in CAPN10 both before and after onset was found in major insulin-target tissues examined (liver, muscle and adipose tissue) (Fig. 2). Moreover, significant differences were observed in liver (Fig. 2B) and adipose tissue (Fig. 2D) before onset and in liver (Fig. 2B) after onset, as evidenced by the Turkey–Kramer test. These results suggest that CAPN10 gene expression tend to decrease in major insulin-target tissues in OLETF rat, and this decrease is detectable in blood sample (WBC).

The CAPN10 gene was positionally cloned within the non-insulin-dependent diabetes mellitus (NIDDM) region and UCSNP-43 was associated with the evidence for linkage in the NIDDM1 region in Mexican–American sib pairs concordant for the at-risk genotype (G/G). Although the precise role of CAPN10 in type 2 diabetes remains unclear, some studies on its role in type 2 diabetes have been reported. The presence of CAPN10 mRNA in pancreatic islets, muscle, and adipose tissue, the three most important tissues that control blood glucose levels, suggest that CAPN10 may regulate pathways that affect insulin secretion, insulin action on pancreatic islets, muscle, and adipose tissue.

For UCSNP-43, the G/G genotype showed two-fold higher basal and insulin stimulated rates of lipogenesis, compared with AA/AG genotypes in human subcutaneous adipocytes. These results suggest that the CAPN10 gene predisposes an individual to diabetes by influencing glucose metabolism. Baier et al. reported that G/G homozygotes had a reduced CAPN10 mRNA expression in their skeletal muscle, which correlated well with 24-h carbohydrate oxidation rates. Although there could be species difference between these reports and this study, CAPN10 can be considered as a potent candidate gene responsible for type 2 diabetes, since OLETF rat spontaneously develops disease that resemble those of human type 2 diabetes. Therefore, further clinical studies should be required to verify the role of this gene expression in type 2 diabetes.

In conclusion, CAPN10 represents a candidate gene for predicting the incidence of type 2 diabetes. The gene can be evaluated by measuring mRNA from WBC, based on the screening system introduced here using OLETF rat as a
model for type 2 diabetes. Genome wide screening should also be applied to find other genes for the clinical genetic diagnosis of type 2 diabetes.

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REFERENCES