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A novel link between *Slc22a18* and fat accumulation revealed by a mutation in the spontaneously hypertensive rat

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ABSTRACT

Two different strains of the spontaneously hypertensive rat (SHR) exist, either with or without a *Cd36* mutation. In the F2 population derived from a cross between these two SHR strains, the mutant *Cd36* allele was tightly linked to differences in metabolic phenotypes but not to those in fat pad weight. This suggested the existence of another crucial mutation related to adiposity. Linkage analysis of this F2 population showed a significant linkage between the rat chromosome 1 region (*D1Rat240–D1Wox28*) and fat pad weight. By integrating both positional and expression information, we identified a donor splice site mutation in the gene for solute carrier family 22 member 18 (Slc22a18) in SHR with reduced fat pad weight. This mutation was located at the linkage peak with a maximum logarithm of odds score of 7.7 and caused skipping of the whole exon 9 that results in a complete loss of a whole membrane-spanning region of the rat Slc22a18 protein. *Slc22a18* mRNA was abundantly expressed in isolated adipocytes and in a differentiation-dependent manner in 3T3-L1 cells. Knockdown of the *Slc22a18* mRNA via infection of adenoviral vectors markedly inhibited both triglyceride accumulation and adipocyte differentiation in 3T3-L1 cells. By contrast, overexpression of the *Slc22a18* mRNA had the opposite effects. These results reveal a novel link between *Slc22a18* and fat accumulation and suggest that this gene could be a new therapeutic target in obesity.

1. Introduction

The spontaneously hypertensive rat (SHR) is a widely-used animal model of essential hypertension and demonstrates a series of manifestations of insulin resistance syndrome [1]. We previously reported that there were two different SHR strains, depending on the presence or absence of a *de novo* mutation in the gene for Cd36, and that they manifested significant differences in several important phenotypes [2]. The SHR strain with a *Cd36* null mutation (hereafter referred to as SHR/NCrj) had significantly reduced blood glucose and triglyceride levels and increased blood free fatty acid levels as compared with the SHR strain without this mutation (referred to as SHR/Izm). Furthermore, the SHR/NCrj strain also showed markedly decreased epididymal fat pad weight as compared with the SHR/Izm strain, although both SHR strains had comparable body weights [2].

Cd36 is a multi-functional transporter that is involved in glucose and lipid metabolism and is known to facilitate the uptake of long-chain fatty acids in adipocytes [3]. Taking into consideration the known function of Cd36 and the extreme genetic similarity between these two SHR strains, we hypothesised that the phenotypic differences observed between these two strains could be attributable to a *Cd36* mutation.

In the present study, we initially examined for possible linkages between the mutant *Cd36* allele and the respective phenotypes in an F2 population derived from a cross between these SHR strains. Comparing these relatively identical strains provides a unique opportunity for linkage analysis with minimal effects of genetic noise. In this F2 cross, as expected, the mutant *Cd36* allele was significantly linked to altered metabolic phenotypes, but unexpectedly, not to the differences in fat pad weight, which strongly suggested another crucial mutation underling the differences in adiposity between these two rat strains. Based on the results of linkage and mRNA expression analyses, we report a novel link between solute carrier family 22 member 18 (Slc22a18) and fat accumulation.

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2. Materials and methods

2.1. Animals

NCrj rat strains were purchased from Charles River (Japan) and Izm strains were from Funabashi Farm (Chiba, Japan). All rats were maintained under stable conditions on a 12-h light–dark cycle and fed standard laboratory chow (MF purchased from Oriental Yeast, Ltd., Japan) and water ad libitum. At 12 weeks of age, after measuring its body weight, a male rat was sacrificed after an overnight fast. Arterial blood and tissue samples were collected rapidly, frosen in liquid nitrogen, and kept at −80 °C prior to analyses. Whole blood glucose levels were measured using a glucometer (Sankyo, Japan), and serum cholesterol and free fatty acid levels were determined by enzymatic assays (Kyowa, Japan). All animal procedures were performed in accordance with the guidelines for the care and use of laboratory animals approved by University of Tokyo Graduate School of Medicine.

2.2. Isolation of genomic DNA, genotyping, and DNA sequencing

DNA was isolated from a whole blood sample of each rat with a DNA extractor WB kit (WAKO, Japan). DNA (100 ng) was used for polymerase chain reaction (PCR) as previously described [4]. The resulting PCR products were analysed with 1–4% NuSieve 3:1 agarose gels (Takara Bio, Japan) or with a GenePhor DNA Separation System (GE Healthcare) for single strand conformation polymorphism (SSCP) analysis. A polymorphic (CA)*n*-repeat site was found 13-bases downstream of the *Slc22a18* mutation site, and tight linkage was observed between the wild-type G allele and $(CA)_{18}$ and between the mutant A allele and $(CA)_{28}$ (Supplementary Table 1). PCR was run with a pair of primers (upstream: 5′-CTGCTGAGATCCAGTGTACT-3′, downstream: 5′-TGGAGGATGGCTTGAGACCT-3′) that spanned both the mutation and (CA)*n*-repeat sites, which generated PCR-products of 142 base-pairs (bp) for the wild-type allele and of 162 bp for the mutant allele. Genotyping of the *Cd36* mutation was performed as previously described [2]. Genotyping for 11 other polymorphic microsatellite markers (*D1Wox32*, *D1Rat240*, *D1Wox28*, *D1Got209*, *D1Wox10*, *D1Rat169*, *D1Rat75*, *D1Rat77*, *D1Mit7*, *D1Rat119*, *D1Wox25*) was performed according to recommended conditions available in public rat databases. Direct DNA sequencing of the PCR-amplified products was performed as described [4].

2.3. Isolation of RNA, northern blot and microarray analyses, and real-time reverse-transcription PCR (RT-PCR)

Total RNA was extracted from tissues or cells using 1 ml of TRIzol reagent (Invitrogen). The purity and concentration of each RNA sample were determined by measuring the absorbance at 260/280 nm. To further determine the quality of RNA, 1 μg of total RNA was run on a 1% agarose gel to inspect the quality of the 28 S and 18 S ribosomal bands. cDNA was synthesised using a high capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. A full-length cDNA probe for rat *Slc22a18* mRNA was prepared by cloning its RT-PCR product from rat liver into pGEM-T easy vectors (Promega) and its authenticity was confirmed by DNA sequencing. Total RNA (10 μg) was electrophoresed on a 1% agarose gel containing formaldehyde, and then transferred to a nylon membrane, Hybond N (Amersham Biosciences). Membranes were hybridised with [^{32}P] dCTP-labeled *Slc22a18* cDNA probes using a Megaprime DNA labeling kit in rapid-hyb buffer (Amersham Biosciences). After washing with 0.1× SSC, 0.1% SDS at 65 °C, the membranes were exposed to Kodak XAR-5 films (Kodak). Total RNA from rat epididymal adipose tissue was also subjected to an Oligotex mRNA purification kit (Takara Bio, Japan) and then analysed with a Rat Genome 230 2.0 Array (Affymetrix), which detected 20,861 rat genes expressed in adipose tissues. Gene expression was quantitatively analysed using real-time RT-PCR with LightCycler 480 SYBR Green I Master or LightCycler 480 Probes Master on a LightCycler 480 System II (Roche) as described previously [5]. DNA sequences of primers and probes that were used are listed in Supplementary Table 2. Messenger RNA of *36B4* was used as an RNA loading control for northern blot experiments and as an internal control in real-time RT-PCR.

2.4. Western blot analysis

A rabbit polyclonal antibody against rat Slc22a18 was generated by immunizing a rabbit with a synthetic peptide (C + KPLSQKGDKAR) for the C-terminal region of rat Slc22a18. Anti-mouse Slc22a18 antibody was also generated against a synthetic peptide (C + KPLSQKGEKAR) for its C-terminus. Anti V5-horseradish peroxidase (HRP)-conjugated antibody was obtained from Invitrogen, and antibodies against PPARγ and α-tubulin from Santa Cruz Biotechnology. Western blot analysis was performed as previously described [5] with some modifications. Whole cell lysates were prepared using lysis buffer (25 mM HEPES (pH7.9), 50 mM KCl, 6% glycerol, 5 mM EDTA, 5 mM $MgCl_2$, 1% TritonX100, 1 mM DTT, protease inhibitor cocktail (Roche)). Protein samples (50 μg) were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) or native–PAGE without SDS. Proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare), immunoblotted with appropriate antibodies, and visualised with an ImageQuant LAS 4000 mini (GE Healthcare).

2.5. Isolation of rat primary adipocytes and 3T3-L1 cell culture

Male rat epididymal fat pads were excised and minced in PBS with 0.5% BSA. Collagenase (Sigma–Aldrich) was added at 1 mg/ml before incubation at 37 °C for 2 h with shaking. Suspensions were centrifuged at 200*g* for 1 min to remove cellular debris and oil. Precipitated materials were resuspended as a vascular stromal fraction (VSF). Total RNA of floating adipocytes and the VSF were extracted separately with TRIzol reagent and subjected to RT-PCR. Mouse 3T3-L1 cells were maintained and differentiated in DMEM (WAKO, Japan) with 10% FBS as previously described [5]. On Day 0, 3T3-L1 preadipocytes were stimulated with a mixture comprising insulin, dexamethasone (DEXA) and 3-isobutyl-1-methylxanthine (IBMX) (all from Sigma), which induced the differentiation of preadipocytes into mature adipocytes. Oil Red O staining of 3T3-L1 cells was performed as previously described [5].

2.6. Adenoviral expression

Two independent constructs for short hairpin RNA (shRNA) targeting of *Slc22a18* were subcloned into the U6 entry vector using primer sequences specific for mouse *Slc22a18* cDNA (#1: 5′-gtgtaaccgttgacctgaaacgtgtgctgtccgtttcgggtcaatggttgcac-3′, #2: 5′-gggtcattatactcatctaacgtgtgctgtccgttaggtgagtatgatgatcc-3′) to generate adenoviral vectors by homologous recombination with the pAd promoterless vector (Invitrogen). Recombinant adenovirus was produced in 293A cells and purified as previously described [6]. The titer of adenovirus was determined using Adeno-X Rapid Titer kit (BD Biosciences). Four days before differentiation, 3T3-L1 cells were infected with adenoviral vectors for expressing shRNA (Ad-shSlc#1, Ad-shSlc#2, or Ad-shLacZ(control)) at the multiplicity of infection (MOI) of 30. Adenoviruses expressing GFP (Ad-GFP) or V5-tagged mouse Slc22a18 (Ad-Slc22a18V5) were also generated using the pAd/CMV/V5-DEST