Short Communication

Variation in the *FABP2* promoter affects gene expression: implications for prior association studies

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Abstract

Aims/hypothesis. An Ala54Thr polymorphism in the *FABP2* gene has previously been associated with insulin resistance and lipid oxidation rates in Pima Indians. Ala54Thr functionally alters the protein's ability to bind and transport dietary fatty acids. In the current report, we sought additional functional variation in *FABP2* by sequencing putative regulatory regions.

Methods. More than 1.2 Kb of the putative promoter of *FABP2* was sequenced in 20 Pima subjects. Variations were genotyped in 84 additional Pima Indian subjects to assess haplotype combinations. Functional activities of variant and nonvariant promoters were compared in Caco-2 cells transfected with luciferase reporter constructs.

Results. Seven variations were identified in the *FABP2* promoter in Pima Indians. Genotypes of these variants were in complete concordance with each other, and were in complete concordance with Ala54Thr. Therefore, only two promoter alleles were observed in Pima

Indians, an Ala54-associated promoter and a Thr54-associated promoter. In contrast, genotyping of these variants in Caucasian DNA showed multiple genotypic combinations. In vitro reporter assays indicated that the Thr54-associated promoter in Pima Indians resulted in a threefold reduction in promoter activity as compared to Ala54-associated promoter.

Conclusion/interpretation. Two functional variations exist in *FABP2*—the coding Ala54Thr and the variant promoter. In the Pima Indian population, but not the Caucasian population, these two functional variants are always carried on the same allele. Therefore, some of the in vivo phenotypic associations previously attributed to the Ala54Thr substitution, which alters binding characteristics of the protein, could instead be due to promoter variation, which alters expression levels. [Diabetologia (2004) 47:349–351]

Keywords Intestinal fatty acid binding protein · IFABP · *FABP2* · Insulin resistance · Diabetes candidate gene · Pima Indians

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Abbreviations: A, Adenosine · Ala, alanine · CEPH, genomic DNA set, originating from Caucasians from Utah, USA · *FABP2*, intestinal fatty acid binding protein gene · G, guanosine · IFABP, intestinal fatty acid binding protein · PCR, polymerase chain reaction · Thr, threonine

The human *FABP2* gene encodes the intestinal fatty acid binding protein (IFABP) that is expressed in epithelial cells of the proximal small intestine. We have previously reported that a single nucleotide polymorphism (G to A) within the second exon (codon54) of *FABP2* predicts an alanine to threonine (Ala54Thr) substitution in the protein. The threonine-encoding allele is associated with higher fasting plasma insulin concentrations, lower insulin-stimulated glucose uptake rates, and higher rates of fat oxidation in Pima Indians [1, 2]. The Ala54Thr substitution has subsequently been genotyped in several population-based studies [reviewed in 3, 4] and has been shown to be associated with a variety of phenotypes including vis-

ceral fat, triglyceride concentrations, BMI and % fat, whereas other studies have shown no association.

We have further reported that the Ala54Thr substitution affects the function of IFABP. The substitution is positioned at a turn that forms a portal between two β -sheets. Structural changes at this portal can affect the passage of a ligand through the entry portal [5], and presumably due to this difference in passage, the Thr54 protein has a twofold higher binding affinity for long-chain fatty acids as compared to the Ala54 protein [1]. Transfection studies in Caco-2 cells have further shown that Thr54 IFABP transports long-chain fatty acids and secretes triglycerides to a greater degree than the Ala54 IFABP [2]. In human intestinal explants, Thr54 homozygote subjects have increased secretion of newly esterified triglycerides, augmented de novo synthesis, and increased chylomicron output as compared to Ala54 homozygous subjects [6].

In the current study, we report additional sequence variability in the *FABP2* promoter that alters transcriptional activity.

Subjects and methods

Identification and genotyping of variants. Genomic DNA samples, from Pima Indians who were known to be homozygous for either Ala54 ($n=10$) or Thr54 ($n=10$) [1], and were not first-degree relatives among themselves, were selected for sequence variant detection. A 1225 base pair region of the *FABP2* promoter (nucleotides -1052 to +173, accession number M18079) was PCR-amplified with primers: forward = 5'-GTAATATCTTGGGCAAGCCCTAGAG-3' and reverse = 5'-CACTCCGGTCTACCTTCCAAGTGCTGTC-3'. Sixty ng of genomic DNA was amplified using an X-long PCR Kit (Applied Biosystems, Foster City Calif., USA). PCR conditions were 94°C for 10 min, followed by 32 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, ending with a final extension at 72°C for 10 min on a 9600 thermal cycler (Applied Biosystems). The 1225 base pair fragment was gel-purified and re-amplified with three pairs of nested primers in buffer containing 1.5 mmol/l $MgCl_2$, 0.25 mmol/l dNTPs and 0.15 μ l of AmpliTaq Gold (Applied Biosystems) DNA polymerase. Sequences for the nested primers are available upon request. DNA cycle sequencing was carried out using the Big Dye Terminator on an automated DNA sequencer (Model 377, Applied Biosystems). All variants detected in the 20 samples were genotyped in 85 additional Pima Indian DNA samples by the method of DNA sequencing described above. All studies were approved by the Tribal Council of the Gila River Indian Council and the Institutional Review Board of the National Institutes of Diabetes and Digestive and Kidney Diseases.

The *FABP2* promoter region of 48 Caucasian (CEPH) DNA (Coriell Institute for Medical Research, Camden N.J., USA) was also sequenced as described above.

Luciferase expression from the *FABP2* promoters. DNA fragments (1225 bp), encompassing all seven of the promoter variants, were amplified from genomic DNA of subjects homozygous for the Ala54 or the Thr54 promoters. PCR amplification was done as above, except that the primers contained additional sequences for KpnI (forward primer) or SacI (reverse primer) restriction sites. Amplified products were ligated into Luci-

Fig. 1. Variations identified in the *FABP2* promoter in Pima Indians. The more common allele for each variant is listed first. The relative positions of each variant is based on the *FABP2* sequence (accession number M18079), where the transcription start site is +1. Also shown, the concordance between each promoter variation and Ala54Thr allele in the given population

ferase reporter plasmids, pGL3 basic (Promega, Madison Wis., USA), at the KpnI and SacI sites.

The human colonic carcinoma cell line Caco-2 (ATCC), was maintained in DMEM (Life Technologies, Rockville Md., USA), supplemented with 0.1 mmol/l nonessential amino acids (Gibco/Invitrogen), 100 μ g/ml penicillin or streptomycin (Life Technologies), and 15% fetal bovine serum (Life Technologies). Cells were seeded into Falcon six-well tissue culture plates at a density of 1×10^6 cells/ml, and incubated at 37°C overnight. Prior to transfection, the media was replaced with media lacking serum and penicillin and streptomycin. Cells were transiently transfected with 1900 ng DNA, according to the Guide to Eukaryotic Transfections with cationic lipid reagents (LipofectAMINE and PLUS, Invitrogen, Carlsbad Calif., USA). All transfections were done in triplicate. Cells were harvested on day 3, post-transfection, using Passive Lysis Buffer (Promega technical manual No. 040 for dual-luciferase reporter assay system, Promega). Cell lysates were assayed for firefly luciferase and Renilla luciferase activities (Promega dual-luciferase reporter assay system) in a luminometer (Model B-LC-1000, Bioscan, Washington D.C., USA). Three replicate assays were done for each of the triplicate transfections. Luciferase activities were averaged and expressed as fold increase over the control (no insert vector). A p value of less than 0.05 was considered statistically significant.

Results

Sequencing of a 1225-base pair region of the *FABP2* promoter in DNA from 20 Pima Indians identified seven polymorphic variations (Fig. 1). Genotypes of these seven polymorphisms showed complete concordance in 85 full-blooded Pima Indians (Fig. 1). Among the Pima subjects, these genotypes were also completely concordant with the previously genotyped Ala54Thr in exon 2. Therefore, in Pima Indians, only two distinct *FABP2* alleles were observed, an Ala54-associated promoter and a Thr54-associated promoter (Fig. 1). The 7 promoter variants and the Ala54Thr were also genotyped in 48 Caucasian (CEPH) DNA samples. The promoter variants were not in complete concordance with the Ala54Thr, predicting multiple allelic combinations (Fig. 1).

To determine whether the Pima Indian Ala54-associated promoter and Thr54-associated promoter differed in their ability to drive transcription, 1.2 Kb

Fig. 2. Luciferase reporter expression from the *FABP2* promoters. The Ala54 promoter construct and the Thr54 promoter construct were each transfected in triplicate into Caco-2 cells. For each transfected cell lysate, three replicate luciferase assays were done. The luciferase activities were averaged and expressed as fold-increase over the control (no insert vector). Luciferase activity from the Ala54 promoter was threefold greater than luciferase activity from the Thr54 promoter ($p < 0.02$)

fragments of each promoter were PCR amplified and ligated into the Luciferase reporter plasmid, pGL3 basic. Luciferase reporter activity from constructs controlled by the Ala54-associated *FABP2* promoter was threefold greater than reporter activity from constructs regulated by the Thr54-associated *FABP2* promoter (Fig. 2).

Discussion

Seven variants were identified in *FABP2* promoter of Pima Indians. All of these variants were also identified in Caucasian (CEPH) DNA, and some of the variants have been described in other Caucasian populations [8, 9]. However, in contrast to the Caucasian samples, all seven promoter variants and the Ala54Thr were in complete genotypic concordance in Pima Indians. Thus, in Pima Indians, but not in Caucasian subjects, the Thr54 variant is always carried on the same allele as all the promoter variations.

Our in vitro studies suggest that the promoter carried on the Ala54 allele results in threefold higher expression as compared to the promoter carried on the Thr54 allele. Consistent with these findings, the functional analysis of two common promoter haplotypes identified in non-Hispanic white subjects from the San Luis Valley Diabetes study showed a twofold difference in expression [9]. Therefore, it appears that in Pima Indians, as well as other populations, there are two

common functional variations in *FABP2*—one affects gene expression and one affects protein binding and transport of long-chain fatty acids. In Pima Indians, the relative contribution of each variant to the observed in vivo phenotypes of insulin resistance and lipid oxidation cannot be delineated since both variations are always carried on the same allele. However, in other populations, the two functional variants may not be in complete concordance, allowing association studies to delineate whether these functional variants interact with an additive effect or function independently. Further studies in other populations will be very useful in establishing the relative contribution of the variant promoters, compared to the Ala54Thr, in influencing metabolic parameters associated with Type 2 diabetes and obesity.

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