Original Article

A Functional Variant of the Adipocyte Glycerol Channel Aquaporin 7 Gene Is Associated With Obesity and Related Metabolic Abnormalities

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Aquaporin 7 (AQP7), the gateway protein controlling glycerol release, has recently emerged as a modulator of adipocyte metabolism. AQP7 knockout mice develop obesity and hyperglycemia. The contribution of AQP7 to these abnormalities in humans is unknown. We examined whether common single nucleotide polymorphisms (SNPs) in the AQP7 gene modulate the risk of obesity and related abnormalities. Among several SNPs we identified, A-953G in the AQP7 promoter was associated with type 2 diabetes in 977 (530 female/447 male) Caucasians: odds ratio for XG (i.e., AG+GG) versus AA individuals was 1.36 (95% CI 1.01–1.84), $P = 0.04$. This finding was entirely due to the association among females (1.8 [1.2–2.6], $P = 0.004$), which was no longer significant when adjusted for BMI. In fact, BMI was higher in XG than in AA females (30.8 ± 6.6 vs. 28.9 ± 5.2, $P = 0.002$). This association was confirmed in independent case-control study ($n = 299$ female subjects) for morbid obesity (1.66 [1.01–2.74], $P = 0.04$). Luciferase and mobility shift assays showed that, compared with −953A, the −953G promoter had reduced transcriptional activity ($P = 0.001$) and impaired ability to bind CCAAT/enhancer binding protein (C/EBP)β transcription factor ($P = 0.01$). Finally, AQP7 expression in adipose tissue decreased from AA to AG to GG individuals ($P = 0.036$). These data strongly suggest that AQP7 downregulation is pathogenic for obesity and/or type 2 diabetes. *Diabetes* 56:1468–1474, 2007

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AQP7, aquaporin 7; C/EBP, CCAAT/enhancer binding protein; FFA, free fatty acid; HWE, Hardy-Weinberg equilibrium; LD, linkage disequilibrium; MAF, minor allele frequency; PPAR, peroxisome proliferator–activated receptor; RXR, retinoid X receptor; SNP, single nucleotide polymorphism; UTR, untranslated region.

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Obesity and related abnormalities, including type 2 diabetes and dyslipidemia, are becoming epidemic, thus representing major health care problems (1). Although these abnormalities have a clear genetic component, the involved genes are mostly unknown (2,3).

Adipose tissue is an endocrine organ adapting metabolic fluxes to the amount of stored energy (4). When energy is required, adipose tissue triglycerides are hydrolyzed into free fatty acid (FFA) and glycerol (5). Glycerol serves as a substrate of gluconeogenesis into the liver and, under certain circumstances, also as a source of glycerol-3-phosphate for triglyceride resynthesis in adipocytes (6,7). Thus, glycerol production and its efflux from adipocytes modulates lipid and glucose homeostasis and, eventually, body weight control (6,8).

Aquaporin 7 (AQP7/AQPap) is a unique adipocyte-specific glycerol channel (9) whose expression is tightly regulated by nutritional status and insulin levels being increased during fasting and insulin deficiency (10) and downregulated after refeeding and exposure to insulin (11). Both up- and downregulation of AQP7 expression play a role in the control of glucose and lipid homeostasis in animal models. Altered insulin signaling–mediated AQP7 overexpression and subsequent increased glycerol efflux from adipocyte have been proposed as molecular mechanisms linking obesity and insulin resistance to hyperglycemia in obese diabetic *db/db* mice (10). Conversely, intra-adipocyte glycerol accumulation and triglycerides resynthesis are responsible for increased fat mass (6,12) and subsequent hyperglycemia (6) in AQP7 knockout (KO) mice. By contrast, so far the role of AQP7 expression changes has been poorly investigated in humans where AQP7 downregulation was reported in a small sample of obese subjects (13). In addition, human AQP7 gene maps to a chromosomal region, 9p13.3-p21.1, reported to be linked to type 2 diabetes (14,15), increased BMI (14), and related abnormalities (16), thus pinpointing the gene as a candidate for these metabolic alterations. The aim of the present study was to investigate whether common variants of the AQP7 gene play a role in the risk modulation of type 2 diabetes and/or obesity.

TABLE 1
Clinical features of type 2 diabetic patients

	Diabetic subjects
n	685
Age (years)	60.2 ± 9
Sex (male/female)	340/345
BMI (kg/m^2)	31.0 ± 6
Age at diagnosis (years)	50.0 ± 10
Duration of disease (years)	10.1 ± 9
A1C (%)	8.7 ± 2.0
Treatment	
Diet alone	82 (12)
OHA	322 (47)
Insulin ± OHA	281 (41)
Dislipidemia	594 (87)
Hypertension	567 (83)

Data are means ± SE or n (%). OHA, oral hypoglycemic agent.

RESEARCH DESIGN AND METHODS

Recruitment of a case-control study for the association with type 2 diabetes. Both case and control subjects were of Caucasian origin and resident in central Italy. Case subjects comprised 685 (340 male/345 female) patients with type 2 diabetes, consecutively recruited at the Endocrine Unit, Scientific Institute CSS in San Giovanni Rotondo, who met the following criteria: *1*) diabetes diagnosed after age 30 years, *2*) insulin treatment not required for at least 2 years after diabetes diagnosis, and *3*) absence of clinically evident autoimmune disease. Clinical features of patients studied are shown in Table 1. The control group consisted in 292 (107 male/185 female, BMI 27.0 ± 4.5 kg/m^2) unrelated subjects with the same age range (35–76 years, mean age 45.5 ± 8.2 years) as case subjects, being part of a larger sample of 661 healthy nondiabetic individuals thus far recruited for an ongoing study on the genetics of insulin resistance. In 541 individuals (203 male/338 female, age 36.6 ± 12 years, BMI 25.5 ± 4.6 kg/m^2) from this sample, we had the opportunity to measure serum FFA levels (17), which were then compared between the different AQP7 genotypes at the A-953G single nucleotide polymorphism (SNP). Selection criteria of this control group were as follows: fasting plasma glucose <6.1 mmol/l, no medications known to affect glucose and lipid metabolism, and absence of systemic diseases.

Recruitment of a case-control study for association with morbid obesity. Both case (n = 211, age 38.8 ± 13 years, 67 male/144 female, BMI >40 kg/m^2) and control (n = 270, age 37.3 ± 12 years, 120 male/155 female, BMI <30 kg/m^2) subjects were of Caucasian origin and resident in central Italy. Selection criteria were as follows: fasting plasma glucose <6.1 mmol/l, no medications known to affect glucose and lipid metabolism, and absence of systemic diseases.

All studies were performed according to the Declaration of Helsinki, and the protocol was approved by the local ethical committee. All subjects recruited provided written informed consent.

Resequencing and genotyping. The coding region of AQP7 was amplified and resequenced as previously reported (18) (Fig. 1). Specific primers were designed to amplify and resequence untranslated regions (UTRs) and promoter regions of the gene. Due to the existence of three AQP7 pseudogenes (18), the 2.5 kb of promoter sequence was first amplified with a single set of primers ensuring the selection of genuine AQP7 and then analyzed by sequencing of several nested PCR fragments covering the entire promoter length (sequences of primers used and reaction conditions are available from the authors upon request). Genotyping at A-953G and C-1123T SNPs was performed by PCR and direct sequencing.

All PCRs were performed in 25-μl reaction volume containing 50 ng genomic DNA, 25 pmol of each oligonucleotide primer (forward 5′TGGATC CCAATTCCCTCC3′ and reverse 5′GGTCTGCAGACACTGAGGA3′), and 2 units of *Taq Gold* (Applied Biosystem) in 1.5 mmol/l $MgCl_2$. PCR conditions were as follows: denaturation at 94°C for 12 min; 35 cycles of denaturation (94°C, 30 s), annealing (64°C, 30 s), and extension (72°C, 30 s); and a final extension at 72°C for 7 min. Sequencing reactions were performed with the same primer sets used for amplification and big dyes v.3.1 sequencing reagents (Applied Biosystem) and then run on an automated DNA sequencer ABI Prism 3100 (Applied Biosystem).

Luciferase assay. A 1,720-bp fragment of human AQP7 promoter (+11/ −1,731, according to the previously reported sequence numbering [18]) containing the −953A variant, in haplotypic combination with the major allele of the SNP rs3758269 (i.e., −1123C), was amplified from genomic DNA and inserted in the pGL2 basic luciferase expression vector (Promega) (Fig. 2, *left panel*). The −953G mutant promoter vector was obtained using the Quick-Change Site-Directed Mutagenesis kit (Stratagene). Both, peroxisome proliferator–activated receptor (PPAR)γ2 and retinoid X receptor (RXR)α full lengths were cloned in pcDNA3.1 expression plasmid (Invitrogen). All plasmids were confirmed for validity and directionality by DNA sequencing.

Both −953A wild-type and −953G promoter vectors were transiently cotransfected with the phRL-Renilla plasmid in addition to PPARγ2 and RXRα pcDNA3.1 plasmids (both required for the promoter activity of AQP7 through its PPARγ responsive element) in COS1 cells.

Cells (1.5×10^5/well) were grown on a 12-well plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and rosiglitazone (BRL 49653; Cayman Chemical) was added 4 h after transfection at three different concentrations (50, 500, and 5,000 nmol/l). After 24 h, transfection mixture was removed, cells were harvested with passive lyses buffer (Promega), and luciferase activities were measured by a microplate luminometer (EG&G Berthold) using the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol. Activity detected in cells transfected with empty vector was defined as nonspecific and subtracted from counts obtained in AQP7 promoter transfect cells stimulated with the same rosiglitazone concentration. The phRL-Renilla vector was used as an internal control for transfection efficiency and to normalize the results.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay was performed as previously described, with slight modifications (19). The pcDNA3.1 vector containing the full length of C/EBPβ was used to synthesized in vitro the corresponding protein. Reaction was carried out using the TNT T7 Quick Coupled Transcription/Translation system (Promega). Single-stranded oligonucleotides in the sense and antisense directions were synthesized to study binding to C/EBPβ transcription factor. The sense probe sequences are 5′-CCTTCCCACCC**A**TGGAGCCTGGTGCCGGAAGTTCCCAGGACTT-3′ and 5′-CCTTCCCACCC**G**TGGAGCCTGGTGCCGGAAGTTCCCAGGACTT-3′ (corresponding to the −923/−964 nucleotides of the AQP7 promoter) containing the predicted C/EBPβ binding site (underlined) in addition to either −953A or −953G variant (bold nucleotide). Additional set of oligonucleotides, containing a C/EBPβ consensus binding sequence, was prepared as previously described (20) and used as a positive control of DNA-C/EBPβ binding. Double-strand oligonucleotides were ^{32}P radiolabeled using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP. Ten micrograms of the in vitro C/EBPβ translation product was incubated with or without 25 pmol of unlabeled oligonucleotides (competitors). Subsequently, 5^4 cpm of labeled oligonucleotides were added to both reaction mixes for 30 min at room temperature. The DNA protein complexes were resolved from the free probe by electrophoresis on a 4% poliacrylamide gel in 0.5× Tris/borate/EDTA buffer. Gels were then dried and autoradiographed at −80°C. Bands were quantified by densitometric analysis using the ImageQuant software (Amersham).

AQP7 expression in human subcutaneous adipose tissue. Total RNA samples were obtained from subcutaneous adipose tissue of 13 nondiabetic, untreated, 3-month weight-stable individuals who underwent elective abdominal surgery (cholecystectomy or bariatric [in either nonobese or obese individuals, respectively] intervention). One microgram of each RNA sample was reverse transcribed to cDNA by using the T-primed first-strand kit Ready-To-Go (Amersham) according to the manufacturer's protocol. AQP7 gene expression was then evaluated by real-time PCR using premade Fam-labeled TaqMan assays (Applied Biosystem). Reactions were performed on ABI Prism 7000HT Sequence Detection system (Applied Biosystems). The human housekeeping GAPDH (4333764F) gene was used as reference to normalize AQP7 (Hs00357359_m1) expression levels, thus compensating for any differences in reverse transcriptase efficiency. All samples were analyzed in triplicate. Data were obtained as C_t values according to the manufacturer's guidelines. Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method (21).

Statistical analysis. Values are reported as mean ± SD. Comparisons between groups were tested by unpaired Student's t test. Comparisons among groups were tested by one-way ANOVA. Mean values, after adjusting for covariates, were evaluated by ANCOVA test. In the case-control study, χ^2 test was used to test for association between type 2 diabetes and genotype and to test Hardy-Weinberg equilibrium (HWE). To model the effect of different genotypes on disease risk multivariate logistic regression analysis was used. Interaction between A-953G SNP and sex on the risk of type 2 diabetes and on BMI values was tested by logistic regression analysis and general linear model, respectively. All analysis were performed using the SPSS software program, version 12.0 for Windows (Chicago, IL).

Pairwise disequilibria measures (D′ and r^2) were calculated between polymorphisms by software programs PHASE (22) version 2.0 and Haploview (23) version 3.32. Haploview was also used to identify haploblocks, which were defined as solid spines of linkage disequilibrium (LD) (D′ > 0.9).

PHASE was also used to reconstruct individual haplotypes. Haplotype

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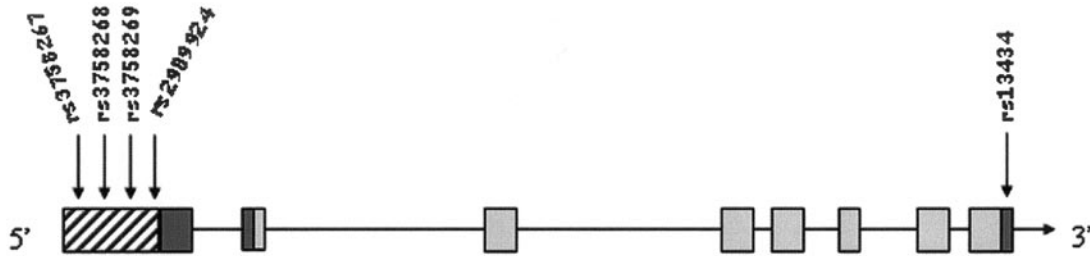

FIG. 1. Schematic diagram of AQP7 gene organization. Solid gray boxes show exons, while thick line shows introns; dark gray boxes corresponds to UTRs, and striped box corresponds to the promoter. The arrows indicate the SNPs identified in the present study.

association analyses were performed using Haplo Stats (24) and considered nonsignificant if the empirical global P value was ≥ 0.05.

RESULTS

Resequencing and LD assessment. Using HapMap data (release 20/Gen06), the haploblock structure of the CEPH sample of Utah residents with European ancestry (CEU group) was evaluated. Defining a solid spine of LD as $D' > 0.90$, six haploblocks across ~50 kb of genomic region encompassing the AQP7 locus (Fig. 1 and online appendix Table 1 [available at http://dx.doi.org/10.2337/db06-1389]) were identified. Pattern of LD observed along this region was rather modest. In detail, a highly preserved LD was observed in the 5′ region of the AQP7 gene, while little significant LD was detected between 5′ and 3′ regions (online appendix Fig. 1).

To verify and/or identify new AQP7 variants in our population, the entire coding region (seven exons) and regulatory sequences, both 5′ and 3′ UTRs and 2.5 kb of the promoter (18), were resequenced in a screening sample consisting of 50 individuals (25 healthy subjects and 25 obese type 2 diabetic patients). No variants, including previously described missense mutations (18), were identified in the coding region, while five SNPs, already reported in public database (National Center for Biotechnology Information dbSNP), were recognized in regulatory regions: rs13434 (minor allele frequency [MAF] = 0.04), rs2989924 (MAF = 0.46), rs3758269 (MAF = 0.11),

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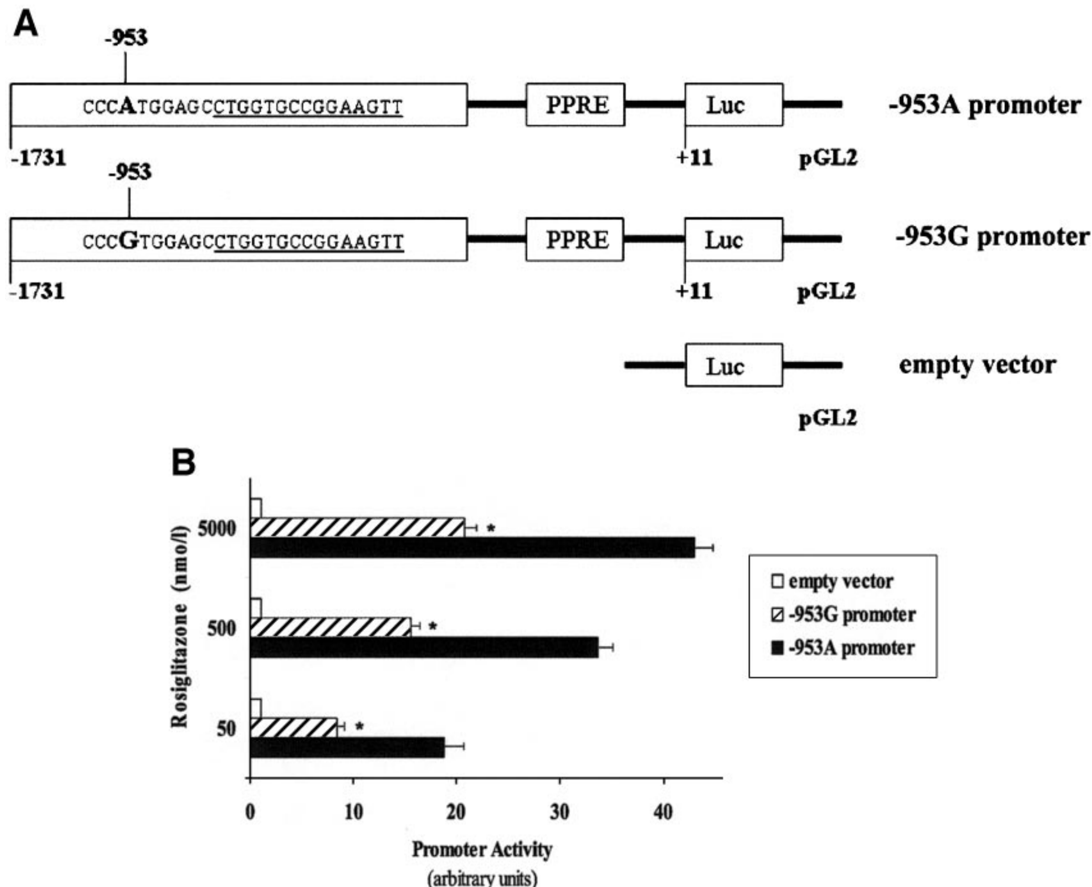

FIG. 2. Luciferase assay. *A*: The 1720-bp fragment of AQP7 promoter (+11/−1731) containing either the −953A or −953G variant (i.e., −953A and −953G promoter, respectively) was inserted in the pGL2 basic luciferase expression vector. Empty vector: pGL2 basic vector. Nucleotide sequence of predicted C/EBPβ binding element is underlined. *B*: Transcriptional activity of both the −953A and the −953G promoter as evaluated by luciferase assay under the stimulation of increasing concentrations of rosiglitazone. Luciferase activity of pGL2 basic in each experiment was arbitrarily set as 1.0. Bars are means ± SE of three different experiments, each performed in duplicate. *P = 0.001 vs. −953A promoter.

TABLE 2
Risk of having type 2 diabetes according to the AQP7 A-953G polymorphism in female and male subjects

	Females			Males	
	Control	Type 2 diabetes		Control	Type 2 diabetes
n	185	345	n	107	340
AA (n = 151)	36.2	24.3	AA (n = 117)	24.3	26.8
XG (n = 379)	63.8	75.7	XG (n = 330)	75.7	73.2
OR (95% CI)	1.8 (1.1–3.2)		OR (95% CI)	0.7 (0.4–1.3)	
P	0.03		P	0.30	

Data are percent unless otherwise indicated. AA, subjects carrying the A-953/A-953 genotype; OR, odds ratio; XG, subjects carrying either theA-953/G-953 or the A-953/A-953 genotype. P values are given after adjusting for age.

rs3758268 (MAF = 0.07), and rs3758267 (MAF = 0.11); rs13434 was located in the 3′ UTR and the other four in the promoter (Fig. 1). LD degree evaluated among the SNPs in our screening sample was very similar to that obtained with the CEU sample in the HapMap, with a highly preserved LD observed only in the 5′ region of the gene (online appendix Fig. 2).

Pairwise disequilibrium measures showed that rs3758269 (which was not genotyped in the HapMap sample) was in perfect LD (r^2 = 1) with rs3758267 but not with rs2989924 (r^2 = 0.1). Noteworthy, both rs3758269 and rs2989924 are close to a predicted binding site for C/EBPβ transcription factor (18). Based on their MAF (i.e., excluding uncommon variants defined as having MAF < 0.1), LD degree, and possible biological significance, SNPs rs2989924 (A-953G) and rs3758269 (C-1123T) were tested in association studies.

Association studies. As a first attempt, a case-control study including 685 patients with type 2 diabetes and 292 nondiabetic individuals was carried out. Clinical features of diabetic individuals are shown in Table 1. The proportion of the three genotypes at the A-953G SNP (AA, AG, and GG) was in HWE and significantly different between control and type 2 diabetic patients: 93 (32%), 129 (44%), and 70 (24%) vs. 175 (26%), 361 (53%), and 149 (22%), respectively, P = 0.041. According to a dominant model, subjects carrying the −953G variant (AG + GG, named as XG) had an increased risk to have diabetes (odds ratio 1.3 [95% CI 1.0–1.8] or 1.2 [0.8–1.7] after adjusting for age). This finding was entirely due to the association among female but not male subjects (Table 2). The female-specific association was confirmed by a significant gene-sex interaction in modulating the risk of type 2 diabetes (age-adjusted P for interaction = 0.024). The association observed in female subjects (Table 2) was no longer significant when BMI was also considered in the model (1.5 [0.8–2.8]). In fact, when all 977 study subjects (i.e., both control and case subjects) were pooled and analyzed together, BMI was significantly higher in female XG subjects than in female AA subjects (28.9 ± 5.2 vs. 30.8 ± 6.6, P = 0.007, age adjusted) but not male subjects (29.7 ± 4.9 vs. 29.0 ± 4.8, P = 0.214, age adjusted). Difference in BMI among female subjects remained significant after adjusting for absence/presence of type 2 diabetes (P = 0.034), thus indicating it was partly independent of diabetes status. Similarly to that observed in the modulation of type 2 diabetes risk, a significant gene-sex interaction in modulating BMI was observed (age-adjusted P for interaction = 0.002).

At variance, the proportion of genotypes at the C-1123T SNP (CC, CT, and TT) (which was in HWE) did not differ between control subjects and type 2 diabetic patients: 232 (79.5%), 58 (19.9%), and 2 (0.7%) vs. 527 (76.9%), 142 (20.7%), and 16 (2.4%), respectively, P = 0.19. Also, no difference in BMI values was observed across the three different genotypes in all study subjects considered together (data not shown).

We then investigated the role of haplotypes given by the combination of both A-953G and C-1123T SNPs (i.e., the first within and the second outside the high LD region, respectively, online appendix Fig. 2) on type 2 diabetes and/or BMI. Computational phase inference indicated three possible haplotypes, namely AC, AT, and GC, whose proportions were not different between control subjects and type 2 diabetic patients (43.4, 10.7, and 45.9% vs. 38.8, 12.6, and 48.6%, respectively, P = 0.133). Similarly, a different BMI was not observed in individuals carrying the three different haplotypes (data not shown). Taken altogether, data from this first association study clearly suggest that, according to a dominant model, the −953G variant is a marker of increased BMI and eventually, through body weight changes, type 2 diabetes. To replicate the association with increased BMI, independently of type 2 diabetes, a second differently designed case-control study was performed by comparing nondiabetic individuals with extreme BMI phenotypes (25), namely nonobese (BMI <30 kg/m^2, 24.8 ± 2; n = 275) versus morbid obese (BMI ≥40 kg/m^2, 48.1 ± 6; n = 211) subjects. As shown in Table 3, the proportion of XG individuals was significantly higher in morbid obese than nonobese female subjects; this was, in contrast, not the case among male subjects.

KO mice for the AQP7 gene show increased serum FFA levels as a consequence of intracellular adipocyte metabolic derangement (6). To investigate whether the A-953G SNP had a role on serum FFA concentrations, 541 nondiabetic, healthy individuals (338 female subjects, aged 36 ± 12 years, BMI 24.9 ± 4.8 kg/m^2 and 203 male subjects, aged 37 ± 12 years, BMI 26.4 ± 3.9 kg/m^2) were studied. Compared with AA individuals, FFA levels were higher in XG carriers among female subjects (0.55 ± 0.2 vs. 0.60 ± 0.2 mmol/l, AA vs. XG, respectively, age-adjusted P = 0.040) but not among male subjects (0.58 ± 0.3 vs. 0.54 ± 0.2 mmol/l, AA vs. XG, respectively, P = 0.363, after adjusting for age). Statistical significance in female subjects did not change much (P = 0.045) after also adjusting for BMI, whose mean values were not different across the different genotype groups (24.9 ± 4.7 and 24.9 ± 4.9 kg/m^2 in AA and XG individuals, respectively, P = 0.918) most likely because, according to selection criteria, these were healthy and mostly young and nonobese individuals (see RESEARCH DESIGN AND METHODS).

Functional and gene expression studies. To investigate whether the A-953G SNP affects promoter transcriptional activity, luciferase reporter gene assay was carried

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TABLE 3
Risk of having morbid obesity (BMI >40 kg/m^2) according to the AQP7 A-953G polymorphism in female and male subjects

	Females			Males	
	Control	Obese		Control	Obese
n	155	144	n	120	67
AA (n = 93)	36.1	25.7	AA (n = 53)	27.5	29.9
XG (n = 206)	63.9	74.3	XG (n = 134)	72.5	70.1
OR (95% CI)	1.6 (1.0–2.7)		OR (95% CI)	0.8 (0.4–1.7)	
P	0.04		P	0.7	

Data are percent unless otherwise indicated. Control subjects are subjects with BMI <30 kg/m^2. AA, subjects carrying the A-953/A-953 genotype; OR, odds ratio; XG, subjects carrying the A-953/G-953 or the A-953/A-953 genotype. P values are given after adjusting for age.

out in COS1 cells transfected with either the A- or G-953 variant (Fig. 2*A*). In the same cells, cDNAs of both the PPARγ2 and the RXRα, which are essential transcription factors for the AQP7 promoter activity (26), were cotransfected. Under stimulation with different rosiglitazone concentrations, the −953G promoter showed ~50% reduction of transcriptional activity compared with the −953A promoter (n = 3, P = 0.001) (Fig. 2*B*). Of note, the −953G variant is 7 bp close to a predicted C/EBPβ transcription factor binding element (18), which is known to be a major player in adipocyte differentiation (27). A gel shift assay was then performed to investigate whether the A-953G SNP affects C/EBPβ binding. Compared with the radiolabeled oligonucleotide carrying the −953A, the one carrying the −953G variant showed a 30 ± 7% reduced binding to C/EBPβ (n = 3, P = 0.01) (Fig. 3). This suggests that a reduced C/EBPβ-DNA binding is responsible, at least partly, for the impaired AQP7 transcriptional activity showed by the −953G promoter.

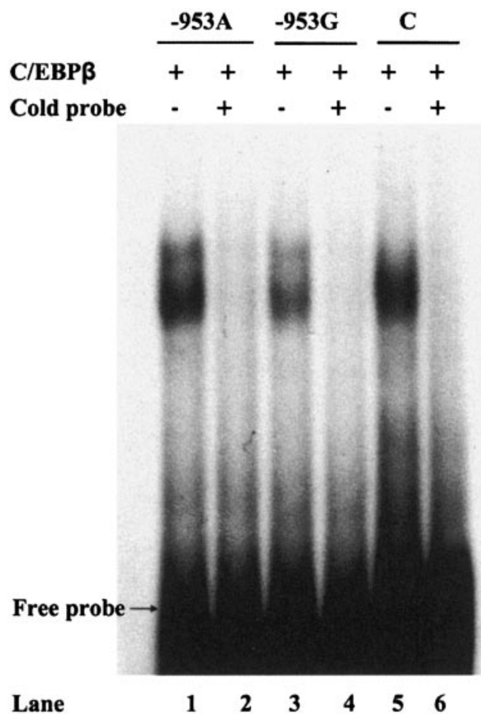

FIG. 3. Electrophoretic mobility shift assay. DNA-C/EBPβ transcription factor binding as evaluated by electrophoretic mobility shift assay. A representative experiment, out of the three performed, is shown. *Lanes 1* and *3* show binding of the two different (either the −953A or −953G) AQP7 promoters. *Lane 5* shows binding of the C/EBPβ consensus binding element of the α-SMA promoter, used as positive control (C). Binding specificity is indicated by inhibition given by the excess of unlabeled probe (*lanes 2*, *4*, and *6*).

We also had the opportunity to measure AQP7 expression levels in subcutaneous adipose tissue of 13 nondiabetic untreated individuals: 8 (3 male/5 female, aged 40.4 ± 11.9 years) were nonobese (BMI <30 kg/m^2, 23.7 ± 3.0) and 5 (2 male/3 female, aged 40.2 ± 16.4 years) were obese (BMI ≥30 kg/m^2, 43.0 ± 12.2). Obese individuals showed a marked (P = 0.001) downregulation of AQP7 expression in respect to nonobese individuals (Fig. 4*A*). In addition, although data are obtained in a small number of individuals and have to be therefore interpreted with great caution, AQP7 mRNA levels seemed to progressively decrease according to the number of −953G alleles (from zero to two alleles in AA, AG, and GG individuals, respectively, P = 0.036 by linear regression analysis) with the

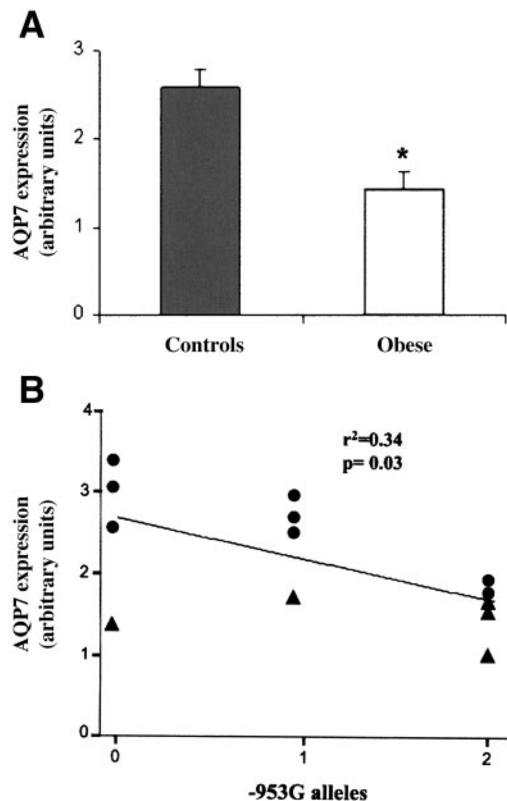

FIG. 4. AQP7 mRNA expression in human subcutaneous adipose tissue according to the A-953G genotype. *A*: AQP7 expression quantified by real-time PCR in RNAs obtained from subcutaneous adipose tissue of 13 nondiabetic untreated individuals who underwent elective abdominal surgery. Data are means ± SE of individual data of either control (BMI <30 kg/m^2, n = 8) or obese (BMI ≥30 kg/m^2, n = 5) individuals. *B*: Individuals with 0, 1, and 2 −953G alleles are carriers of the AA, AG, and GG genotype, respectively. All individual data are the mean of two different experiments, each run in triplicates. ●, control individuals; ▲, obese individuals.

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A-953G SNP being able to predict 34% of AQP7 mRNA level variability (Fig. 4*B*). Although this association was no longer significant when adjusted for BMI ($P = 0.085$), it was still observable when only nonobese individuals were analyzed ($r^2 = 0.68$, $P = 0.012$), thus leaving open the possibility of a direct effect of the −953G variant on in vivo AQP7 expression independent of BMI changes.

DISCUSSION

To the best of our knowledge, this is the first data to describe the association between a common polymorphism in the promoter region of the human AQP7 gene (i.e., the A-953G SNP) and several metabolic alterations including obesity, type 2 diabetes, and high circulating FFA levels. In addition, measurement of mRNA content in adipose tissue and functional studies in transfected cells clearly support a biological role of the −953G variant in negatively modulating AQP7 expression by reducing C/EBPβ-DNA binding, which in turn impairs AQP7 promoter transcriptional activity. Of note, as shown in KO mice, the lack of AQP7 gene is responsible for reduced glycerol efflux from adipocyte, which has profound deleterious effects on lipid accumulation, body weight, and glucose homeostasis, resulting in a phenotype very similar to that observed here in individuals carrying the −953G variant. Based on our present data, it is therefore possible to speculate that also in humans AQP7 plays a role in metabolic pathophysiology. In detail, the entire set of our data suggests that subjects carrying the −953G variant have a reduced AQP7 expression in adipose tissue, which in turn increases the risk of obesity and eventually type 2 diabetes. A role of AQP7 in human metabolic derangements is also suggested by preliminary data showing a reduced AQP7 expression in obese individuals (13) (a finding we were able to replicate in this study) and by the observation that the only subject thus far described being homozygous for an AQP7 missense functional mutation showed a marked reduction in exercise-induced changes in serum glycerol concentration (18).

Despite higher FFA levels, no evidence of insulin resistance (i.e., as indicated by surrogates of this condition including serum insulin levels and/or homeostasis model assessment index) was observed in nondiabetic untreated −953G carriers. This finding is likely to be due to the low sensitivity of surrogates in detecting subtle changes of insulin-resistance as those presumably given by the small increase in circulating FFA concentrations observed in −953G carriers.

All the genotype-phenotype associations we observed seem to be sex-specific, being observable among female but not male subjects. As a matter of fact, a sex-specific genetic effect on BMI modulation has been previously reported in several animal models (28,29) as well as in humans (30). In addition, sexual dimorphism has been also reported in the modulation of circulating FFA concentration (31), a finding that might be due to a sex-specific genetic effect, like the one we here describe. Whether the sex specificity we observed is due to interaction of AQP7 gene with either sex-linked genes and/or sexual hormone effects is not known and deserves further, specifically designed, studies.

It should be pointed out that we have performed a complete resequencing of coding and regulatory but not of intronic regions, which, according to the latest HapMap release (21 July 2006), contains only two intragenic common variants, one of which is located within a recombinant hot spot. Thus, due to these intrinsic limitations, the study we were able to design does not represent a comprehensive investigation of the entire AQP7 gene and is in fact mainly restricted to the promoter region. This leaves open the possibility that other yet unidentified SNPs may turn out to be associated with obesity and related abnormalities.

We acknowledge that in complex disorders, genotype-phenotype associations may represent false-positive results due to population stratification and/or chance. This does not seem to be the case of the present study for the following reasons. First, the population studied is relatively homogeneous, all individuals being Caucasians from central Italy, making the possibility of population stratification remote. Second, the association with increased BMI was replicated in a second independent sample. Finally, expression data in adipose tissue and functional studies in transfected cells clearly support a biological role of the −953G variant in negatively modulating AQP7 expression. These data reinforce the plausibility of the observed genotype-phenotype association. Therefore, although our data cannot presently be claimed as definitive, they are unlikely to represent false-positive findings and might serve the function of new hypothesis generating to be unequivocally confirmed in further larger studies.

In conclusion, present data obtained in −953G carriers highly resemble those obtained in AQP7 KO mice (6), thus suggesting that also in humans AQP7 downregulation is pathogenic for obesity and related abnormalities, which makes AQP7 a new attractive drug target. Of note, thiazolidinediones, the recently developed insulin sensitizer drugs currently used for the treatment of type 2 diabetes, upregulates AQP7 expression (26,32). If data on the −953G variant are confirmed in additional studies, they may serve an important function to help identify at-risk individuals whom targeting specific and early preventive strategies should be aimed.

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