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ORIGINAL DATA

The rs11705701 G>A Polymorphism of IGF2BP2 is Associated With IGF2BP2 mRNA and Protein Levels in the Visceral Adipose Tissue - A Link to Type 2 Diabetes Susceptibility

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■ Abstract

BACKGROUND: Insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) regulates translation of IGF2, a growth factor that plays a key role in controlling fetal growth and organogenesis including adipogenesis and pancreatic development. In Caucasians, the rs4402960 G>T polymorphism of *IGF2BP2* has been shown to predispose to type 2 diabetes (T2D) in multiple populations. In this study, we tested whether rs4402960 G>T and rs11705701 G>A contribute to the development of T2D in a Russian population. **METHODS**: Both markers were genotyped in Russian diabetic (n = 1,470) and non-diabetic patients (n = 1,447) using a Taqman allele discrimination assay. The odds ratio (OR) for the risk of developing T2D was calculated using logistic regression assuming an additive genetic model adjusted for age, sex, HbA1c, hypertension, obesity, and body mass index (BMI). Multivariate linear regression analyses were used to test genotype-phenotype correlations, and adjusted for age, sex, hypertension, obesity, and BMI. Expression of IGF2BP2 in the visceral adipose tissue was quantified using real-time PCR. The content of IGF2BP2 protein and both its isoforms (p58 and p66) in the adipose tissue was measured using Western blot analysis. **RESULTS**: There was no significant association between rs4402960 and T2D. Whereas, allele A of rs11705701 was associated with higher T2D risk (OR = 1.19, $p < 0.001$). Diabetic and non-diabetic carriers of genotype TT (rs4402960) had significantly increased HOMA-IR ($p = 0.033$ and $p = 0.031$, respectively). Non-diabetic patients homozygous for AA (rs11705701) had higher HOMA-IR ($p = 0.04$), lower HOMA-β ($p = 0.012$), and reduced 2-h insulin levels ($p = 0.016$). Non-obese individuals (diabetic and non-diabetic) homozygous for either AA (rs11705701) or TT (rs4402960) had higher levels of IGF2BP2 mRNA in the adipose tissue than other *IGF2BP2* variants. Also, allele A of rs11705701 was associated with reduced amounts of the short isoform (p58) and increased levels of the long isoform (p66) of the IGF2BP2 protein in adipose tissue of non-obese diabetic and non-diabetic subjects. **CONCLUSIONS**: *IGF2BP2* genetic variants contribute to insulin resistance in Russian T2D patients. The short protein isoform p58 of IGF2BP2 is likely to play an anti-diabetogenic role in non-obese individuals.

Keywords: type 2 diabetes · insulin resistance · adipose tissue · insulin-like growth factor 2 · polymorphism · isoform

Introduction

Insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP2) belongs to a family of mRNA-binding proteins involved in RNA localization, stability, and translation. The protein family comprises of three closely related members, IGFBP1-3, with an overall sequence identity of 59% [1]. All family members have six characteristic RNA-binding modules, namely two N-terminal RNA

recognition motifs (RRM1 and 2) and four C-terminal heterogeneous nuclear ribonucleoprotein (hnRNP) K-homology (KH1 to 4) domains. Among the RNA-binding modules, four KH domains constitute a functional entity in terms of high-affinity RNA-binding, granular RNP assembly, and RNA localization [2].

IGF2BPs are mainly cytoplasmic, but the presence of two nuclear export signals implies that they attach to their target mRNAs in the nucleus, and facilitate nuclear export of the transcripts to enable protein synthesis at specific locations in the cytoplasm [3]. They form large RNP granules dispersed around the nucleus and in cellular protrusions [4]. The RNP granules move into the cytoplasm. They are enriched with IGF2BPs and other proteins, including those responsible for the secretory pathway, endoplasmic reticulum-associated quality control, and ubiquitin-dependent metabolism. The presence of these proteins thus supports the concept of RNP granules as post-transcriptional operons [5].

Except for mRNA trafficking, IGF2BPs also control both the translatability and stability of particular mRNAs. IGF2BPs impair translation of leader 3 IGF2 mRNA that exhibits at least six IGFBP-binding elements in the 5' untranslated region (UTR) and one site within the 3'-UTR [1]. The molecular target of rapamycin (mTOR), a key member of PI3K/Akt/p70-S6 kinase 1 signaling (that stimulates cell growth and protein synthesis), has been shown to phosphorylate IGF2BP2 at two sites, which promote IGF2BP binding to the IGF2 leader 3 mRNA 5' UTR [6].

The physiological role of IGF2BP is still unexplained. In mice, all three forms of IGF2BP are highly expressed in the embryo. Their expression declines with development towards birth, and is low or undetectable in most adult animals [1]. Transgenic overexpression of IGF2BP3 causes subtle morphological alterations in the pancreas [7]. Loss-of-function analysis in *Xenopus* embryos indicated that the *IGF2BP3* orthologue Vg1-RBP is required for establishment of the pancreatic fate within the endoderm [8].

The IGF2BP2 protein is encoded by 16 exons. The *IGF2BP2* gene, located at chromosome 3q27.2, has a very long 125-kb intron, which is by far the largest intron among mammalian species. In genome-wide association studies (GWAS), several single nucleotide polymorphisms (SNPs) situated in this intron showed significant association with type 2 diabetes (T2D) [9-11]. The *IGF2BP2* association has been widely replicated in various Caucasian [12-14] and Asian [15-19] populations. Among *IGF2BP2* intron 2 SNPs, rs4402960 G>T showed the most significant association with the disease. According to the results of two recent meta-analyses, the rs4402960 polymorphism of the *IGF2BP2* gene was related to an increased risk of T2D for T vs. G allele (OR 1.14; 95% CI 1.11-1.16) [20, 21]. In the analysis of different ethnicities, significantly increased risks were found in East Asian, Caucasian, and Indian populations.

Abbreviations:
ACTB - actin beta
Akt - serine/threonine kinase, also known as protein kinase B
BMI - body mass index
BP - blood pressure
cDNA - complementary DNA
CI - confidence interval
ΔΔCT - comparative cycle threshold (method for calculating relative quantitation of gene expression)
DNA - deoxyribonucleic acid
ECL - enhanced chemiluminescence
FPG - fasting plasma glucose
GAPDH - glyceraldehyde 3-phosphate dehydrogenase
GWAS - genome-wide association studies
HbA1c - glycated hemoglobin
HEPES - N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HOMA-β - homeostatic model assessment of β-cell function
HOMA-IR - homeostatic model assessment of insulin resistance
IGF2BP2 - insulin-like growth factor 2 mRNA-binding protein 2
IR - insulin resistance
KH - K-homology
LD - linkage disequilibrium
MAF - minor allele frequency
mRNA - messenger RNA
mTOR - molecular target of rapamycin
OGTT - oral glucose tolerance test
OR - odds ratio
PBS - phosphate-buffered saline
PCR - polymerase chain reaction
PG - plasma glucose
PI3K - phosphoinositol 3-kinase
PVDF - polyvinylidene difluoride
RNA - ribonucleic acid
rpm - rounds per minute
RRM - RNA recognition motif
SD - standard deviation
SDS - sodium dodecyl sulfate
SEM standard error of mean
SNP - single nucleotide polymorphism
T2D - type 2 diabetes
UTR - untranslated region
Vg1 - transforming growth factor-β family member, localized to the vegetal cortex during oogenesis
Vg1-RBP - Vg1 RNA-binding protein
v/v% - volume-volume percent (relation of volume of solute to volume of solution in percent)

Table 1. Clinical and metabolic characteristics of the study participants

Characteristic	Type 2 diabetes (n = 1470)	Control (n = 1447)	p
Age (yr)	59.9 ± 7.9	61.6 ± 9.7	NS*
Male (%)	45%	46%	NS
Diabetes duration (yr)	11.2 ± 7.2	-	-
BMI (kg/m^2)	28.3 ± 5.9	26.9 ± 4.8	NS
Systolic BP(mmHg)	134.7 ± 16.8	128.5 ± 15.2	NS
Diastolic BP(mmHg)	98.3 ± 14.7	87.7 ± 12.4	0.025
HbA1c (%)	7.7 ± 1.2	5.0 ± 0.7	0.031
Fasting PG(mmol/l)	10.1 ± 1.6	5.5 ± 0.6	0.003
2-h PG (mmol/l)	12.5 ± 1.3	6.6 ± 0.7	< 0.001
Fasting serum insulin (mU/l)	14.6 ± 7.4	10.6 ± 6.5	0.001
2-h serum insulin (mU/l)	82.8 ± 31.7	48.0 ± 19.4	< 0.001
HOMA-β	44.2 ± 20.8	106.0 ± 46.3	< 0.001
HOMA-IR	6.6 ± 1.2	2.6 ± 0.5	0.008
Hypertension (%)	35.5%	33.2%	NS*
Obesity (%)	20.7%	15.1%	0.001*

Legend: Data are mean ± SD, or percentages. * Comparisons were made using the chi-squared test; other data were compared using the unpaired Student's *t*-test. *Abbreviations*: BMI - body mass index, BP - blood pressure, HbA1c - glycated hemoglobin, HOMA-β - homeostasis model assessment of β-cells, HOMA-IR - homeostasis model assessment of insulin resistance, PG - plasma glucose, NS - not significant.

The diabetes-associated allele G of rs4402960 has been found to be linked to decreased early-phase insulin release, reduced homeostatic model assessment of β-cell function (HOMA-β), lowered fasting insulin, and other indices of impaired pancreatic β-cell function [22-25]. Also, rs4402960 and rs11705701, located in the promoter of *IGF2BP2*, showed associations with abdominal total fat and visceral total fat in Canadian Caucasians [26] and Mexican Americans [27], suggesting a possible role of *IGF2BP2* in insulin resistance (IR). Conclusive evidence that polymorphisms within the *IGF2BP2* gene affect diabetes susceptibility through changes in the activity of the IGF2BP2 protein *per se* is lacking, but it seems plausible that IGF2BP2 might influence the development and/or function of the pancreas or adipose tissue through effects on the expression of IGF2 and/or other proteins [28].

It has been reported that SNP rs4402960 is in high linkage disequilibrium (LD) with rs11705701 G>A, a polymorphic marker located in the promoter region, ~1.48-kb upstream from exon 1 of *IGF2BP2* [27]. The diabetes-associated allele A of rs11705701 has been shown to be related to the percentage of body fat, IR [27], and the expression of a novel short *IGF2BP2* isoform that lacks the N-terminal RRM1 motif and that is highly expressed in the pancreatic and adipose tissue [29, 30]. In the present study, we investigated whether the rs4402960 and 11705701 polymorphisms are associated with T2D, diabetes-related traits, and IGF2BP2 expression levels in adipose tissue derived from Russian diabetic and non-diabetic patients.

Materials and methods

Patients

A total of 2,917 people including 1,470 diabetic and 1,447 non-diabetic subjects aged 50 years and older were studied. A total of 1,211 (622 affected and 589 non-affected) individuals who live in Moscow and neighborhood were recruited by the Endocrinology Research Center, Moscow. The second cohort (760 diabetic and 742 non-diabetic residents of Tyumen) was recruited by the Tyumen State Medical Academy. The study population was ethnically homogenous. According to the patients' questionnaires, 2,602 participants (89.2% of 2,917) had grandparents of Russian ancestry, whereas the remaining 265 patients had three grandparents of Russian ancestry and one of either Ukrainian or Belarusian descent.

T2D was defined according to the American Diabetes Association [31] and the International Expert Committee diagnostic criteria [32] as follows:

1. Fasting plasma glucose concentration ≥7.8 mmol/l
2. And plasma glucose concentration ≥11.1 mmol/l, 2 hours after a 75-g oral glucose tolerance test (OGTT)
3. And/or glycated hemoglobin (HbA1c) ≥6.5%

Hypertension was defined as systolic pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg in at least two separate measurements, or

Table 2. Association of *IGF2BP2* polymorphisms with type 2 diabetes

SNP	Alleles (major/minor)*	MAF	Genotype frequency (T2D/controls)	OR (95%CI)	p	Genotype frequency (T2D/controls)‡	OR (95%CI)	p
rs4402960	G>**T**	0.311	658/633/161	1.12	0.13	605/574/150	1.05	0.18
			689/579/153	(1.02-1.24)		689/579/153	(0.94-1.19)	
rs11705701	G>**A**	0.38	477/710/270	1.19	< 0.001	436/646/251	1.21	< 0.001
			552/680/202	(1.08-1.33)		552/680/202	(1.1-1.36)	

Legend: OR and CI are calculated using logistic regression adjusted for age, sex, HbA1c, hypertension, obesity, and BMI. * Minor allele is shown in bold. ‡ Diabetic patients diagnosed on the basis of HbA1c alone excluded. *Abbreviations*: MAF - minor allele frequency, OR - odds ratio, CI - confidence interval.

in the case of a history of hypertension. Obesity was defined as BMI ≥ 30 kg/m^2 [33]. The control subjects had no history of glucose intolerance, with HbA1c < 6.4%, normal OGTT, and no family history of diabetes. To avoid interference from biological variables, individuals with a previous diagnosis of type 1 diabetes, gestational diabetes, rare forms of T2D, secondary diabetes (pancreatitis, hemochromatosis), and those with hypercholesterolemia or undergoing treatment with cholesterol-lowering drugs were excluded from the study. The study protocol was approved by the Review Board of the Endocrinology Research Center. All participants provided written informed consent.

Biochemical measurements

Plasma glucose was measured by a standard enzymatic assay. Plasma insulin levels were determined by means of an enzymatic immunoassay. Homeostasis model assessment of β-cell function (HOMA-β) was calculated as 20 x fasting plasma insulin (mU/l)/(fasting glucose (mmol/l) - 3.5). Homeostasis model assessment of insulin resistance (HOMA-IR) was computed as fasting plasma insulin (mU/l) x fasting glucose (mmol/l)/ 22.5 [34]. HOMA-IR ≥ 3.8 was considered as threshold score reflecting the presence of IR. Clinical and metabolic characteristics of diabetic and non-diabetic subjects are summarized in Table 1.

Measuring IGF2BP2 protein levels in adipose tissue

IGF2BP2 protein was quantified in human visceral adipose tissue samples. Visceral adipose tissue biopsies were taken from the greater epiploon region. Human adipocytes were isolated by treating the adipose tissue biopsies with Collagenase NB 6 GMP Grade from *Clostridium histolyticum* (Serva Electrophoresis, Heidelberg, Germany), as described in the manufacturer's protocol [35]. Isolated adipocytes were washed with PBS and then solubilized in the lysis buffer (50 mmol/l HEPES, 150 mmol/l $NaCl_2$, 10% (v/v) glycerol, and 1% (v/v) Triton X-100). Cell lysates were centrifuged at 18,000 rpm for 10 min to remove cell debris and unlysed cells. Before loading on SDS-polyacrylamide gel, extracted proteins were mixed with 4 x SDS loading buffer and heated at 95ºC for 5 min.

After electrophoresis, proteins were transferred to Immunobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, NA, USA) for Western blot analysis. The membranes were incubated with antibodies against human IGF2BP2 (Abgent, San Diego, CA, USA) specific to the C-terminal region (dilution 1/5,000). Horseradish peroxidase-coated polyclonal goat anti-IgG antibodies (Thermo Fisher Scientific, Rockford, IL, USA) diluted at 1/1,000 were used as secondary antibodies. Chemiluminiscence, enhanced with Pierce enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific), was measured on the chemiluminometer Lumipol 3 (Lumipol Techniques, Bratislava, Slovakia). The recombinant human IGF2BP2 (OriGene Technologies, Rockwell, MD, USA) was used as a positive control.

Quantification of IGF2BP2 expression

IGF2BP2 expression was measured in samples of the human visceral adipose tissue. Total RNA was isolated from adipose tissues with the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol, and digested with DNase I (RNase-Free DNase set, Qiagen). RNA quality was evaluated by measuring the 260/280 nm absorbance ratio (≥1.8) and by using

electrophoresis. First-strand cDNA was synthesized using an equal amount of total RNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative polymerase chain reaction (PCR) was performed in a final volume of 20 µl, which contained 10 ng of reverse-transcribed cDNA, 10 µl of 2X Taqman Fast Universal PCR Master Mix (Applied Biosystems), and 1 µl Taqman Assay predesigned by Applied Biosystems for the detection of *IGF2BP2*, *ACTB*, and *GAPDH* (product numbers: Hs01118009_m1, Hs03023880_g1, and Hs02758991_g1, respectively). *ACTB* and *GAPDH* were both used as housekeeping genes. All reactions were performed in triplicate, and carried out in 96-well plates by using the 7500 Real-Time PCR System (Applied Biosystems). Real-time PCRs were performed with transcription efficiency >90%. The analysis was performed using the ΔΔCT method [36].

DNA analysis

Total DNA was isolated from whole-blood samples pretreated with proteinase K using a standard protocol for extraction with phenol-chloroform. Genotyping of rs11705701 and rs4402960 was performed by a Taqman-based allelic discrimination on the 7500 Real-Time PCR System 7500 using the recommended protocol [37]. Overall, genotype calling rates were 98.5% (rs4402960) and 99.1% (rs11705701), respectively.

Statistical analysis

Data were analyzed using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). Results are given as mean ± SD or percentages. Skewed variables for the continuous traits were log-transformed before statistical comparisons were made. Unpaired Student's *t*-test was applied to compare quantitative data in the groups of affected and non-affected subjects. Odds ratios (OR) for the risk of developing T2D were calculated using logistic regression, assuming an additive genetic model adjusted for age, sex, HbA1c, hypertension, obesity, and BMI. Multivariate linear regression analysis, adjusted for age, sex, hypertension, obesity, and BMI, was applied to test genotype-phenotype correlations.

The nucleotide sequence flanking rs11705701 was screened for the presence of putative binding sites of transcription factors with the help of the JASPAR database (http://jaspar.cgb.ki.se/). The Microinspector v1.5 program [38] was implemented to search the sequence flanking rs4402960

Table 3. Association of *IGF2BP2* polymorphisms with blood glucose levels in type 2 diabetic patients and non-diabetic controls

SNP	Genotype	Fasting glucose[§]		2-h glucose[§]	
		T2D	Control	T2D	Control
rs4402960	GG	9.6± 1.9	5.2± 0.5	11.8± 1.3	6.3± 0.8
	GT	9.4± 1.7	5.2± 0.4	11.7± 1.1	6.5± 0.6
	TT	10.2± 1.7	6.3± 0.6	12.9± 1.3	7.3± 0.8
Beta[‡]		0.38	0.13	0.12	0.03
SEM		0.27	0.23	0.15	0.10
p-value		NS	NS	NS	NS
rs11705701	GG	9.5± 2.0	5.3± 0.6	12.5± 1.4	6.5± 0.6
	AG	9.7± 1.3	5.1± 0.4	12.2± 1.3	6.4± 0.6
	AA	10.4± 1.7	5.9± 0.8	13.1± 1.6	6.9± 0.7
Beta[‡]		0.16	0.11	0.20	0.08
SEM		0.17	0.10	0.16	0.25
p-value		NS	NS	NS	NS

Legend: Data are mean ± SD. [§] in mmol/l. [‡] Regression coefficient from multivariate linear regression analysis, corresponding to correlation coefficient. Calculated by adjusting for gender, age, BMI, obesity, HbA1c, and hypertension. *Abbreviations*: NS - not significant, SEM - standard error of mean, T2D - type 2 diabetes.

Table 4. Association of *IGF2BP2* polymorphisms with insulin levels in type 2 diabetic patients and non-diabetic controls

SNP	Genotype	Fasting insulin[§]		2-h insulin[§]	
		T2D	Control	T2D	Control
rs4402960	GG	15.4± 7.3	10.7± 6.5	89.3± 32.7	50.6± 20.5
	GT	14.2± 8.2	9.4± 5.8	80.8± 30.7	47.3± 19.2
	TT	14.0± 8.1	9.1± 5.9	79.7± 31.1	46.5± 19.4
Beta[‡]		-0.26	-0.13	-0.11	-0.01
SEM		0.15	0.13	0.12	0.05
p-value		0.08	NS	NS	NS
rs11705701	GG	14.8± 5.9	11.0± 6.2	88.5± 28.2	53.5± 18.0
	AG	14.0± 7.7	10.4± 6.8	85.5± 36.9	49.0± 17.1
	AA	14.3± 7.8	10.4± 5.2	77.0± 30.8	42.7± 16.7
Beta[‡]		-0.72	-0.60	-0.16	-0.39
SEM		0.53	0.35	0.17	0.16
p-value		NS	NS	NS	0.016

Legend: Data are mean ± SD. [§] in mU/l. [‡] Regression coefficient from multivariate linear regression analysis, corresponding to correlation coefficient. Calculated by adjusting for gender, age, BMI, obesity, HbA1c, and hypertension. *Abbreviations*: NS - not significant, SEM - standard error of mean, T2D - type 2 diabetes.

Table 5. Association of *IGF2BP2* polymorphisms with homeostasis model assessment (HOMA) levels in type 2 diabetic patients and non-diabetic controls

SNP	Genotype	HOMA-β		HOMA-IR	
		T2D	Control	T2D	Control
rs4402960	GG	46.0 ± 22.9	96.4 ± 44.9	6.0 ± 1.5	2.1 ± 0.5
	GT	48.1 ± 21.2	94.0 ± 47.5	5.9 ± 1.5	2.2 ± 0.6
	TT	54.9 ± 21.9	97.5 ± 44.2	7.0 ± 1.7	2.9 ± 0.6
Beta‡		0.11	-0.014	0.43	0.32
SEM		0.21	0.049	0.20	0.15
p-value		NS	NS	0.033	0.031
rs11705701	GG	49.3 ± 19.5	112.4 ± 50.9	6.3 ± 1.7	2.0 ± 0.4
	AG	45.2 ± 22.4	110.0 ± 41.1	6.0 ± 1.4	2.3 ± 0.5
	AA	41.5 ± 20.9	86.7 ± 45.3	6.6 ± 1.6	2.7 ± 0.5
Beta‡		-0.40	-0.51	0.027	0.06
SEM		0.23	0.30	0.022	0.11
p-value		0.079	0.012	NS	0.04

Legend: Data are mean ± SD. ‡ Regression coefficient from multivariate linear regression analysis, corresponding to correlation coefficient. Calculated by adjusting for gender, age, BMI, obesity, HbA1c, and hypertension. *Abbreviations*: HOMA-β - homeostasis model assessment of β-cells, HOMA-IR - homeostasis model assessment of insulin resistance, NS - not significant, SEM - standard error of mean, T2D - type 2 diabetes.

for possible microRNA binding sites. The 2LD software [39] was used to access LD (measured by D') between rs4402960 and rs11705701.

In the quantitative analysis of IGF2BP2 mRNA and protein, statistical significance of differences between the groups was tested using non-parametric tests: Kruskal-Wallis rank sum test for comparison of three genotypes and Mann-Whitney *U*-test for comparisons between two groups. P-values of less than 0.05 were considered as significant.

Results

For both rs4402960 and rs11705701, observed genotype frequencies were in accordance with the Hardy-Weinberg equilibrium (data not shown).

We found no significant association between rs4402960 and T2D (Table 2). SNP rs11705701 of *IGF2BP2* showed an association with T2D, with allele A contributing to higher diabetes risk (OR = 1.19, $p < 0.001$). Since specificity and sensitivity of HbA1c as a screening tool for diabetes diagnosis is limited, we excluded 125 T2D patients whose diagnosis was based on HbA1c alone. However, exclusion of these subjects did not markedly influence the association. For rs4402960, both diabetic and non-diabetic carriers of genotype TT had significantly increased HOMA-IR ($p = 0.033$ and $p = 0.031$, respectively), as compared to homozygotes GG and heterozygotes GT (Tables 3-5). This observation suggests that rs4402960 of *IGF2BP2* is implicated in the development of IR in the population studied.

Compared to the carriers of other rs11705701 genotypes, non-diabetic subjects homozygous for AA showed higher HOMA-IR ($p = 0.04$), lower HOMA-β ($p = 0.012$), and reduced 2-h insulin levels ($p = 0.016$), suggesting an involvement of this *IGF2BP2* variant in both IR and impaired β-cell function (Tables 4 and 5).

The expression of *IGF2BP2* was measured in adipose tissue of obese-and non-obese diabetic patients and control subjects with and without obesity (Table 6). *ACTB* and *GAPDH* were used as internal controls to normalize quantitative gene expression data. The expression of both genes showed a good replication rate, with a variability between the samples of only 1-5%.

To verify whether different IGF2BP2 isoforms with five or six RNA-binding motifs [30] could arise from the alternative splicing, we generated primers and Taqman probes specific to exon 1, which encodes the first N-terminal 59 amino acids lacking in the truncated IGF2BP2 isoform. However, the expression levels of IGF2BP2, measured by means of exon 1-specific primers, did not differ from IGF2BP2 mRNA amounts quantified using the RT-PCR detection assay (cat. number Hs01118009_m1) from Applied Biosystems (data not shown). The primers and the Taqman probe predesigned by Applied Biosystems are specific to the *IGF2BP2* region that differs from exon 1. Indeed, we found that the truncated IGF2BP2 isoform is unlikely to result from the alternatively spliced transcript lacking exon 1.

Overall, IGF2BP2 mRNA levels were significantly elevated in obese diabetic subjects compared to obese controls (7.56 ± 2.75 vs. 3.4 ± 1.56, $p = 0.007$). Similarly, in adipose tissue of non-obese T2D individuals, IGF2BP2 expression was higher than that in non-obese controls (6.1 ± 2.53 vs. 2.17 ± 1.19, $p = 0.011$). Non-obese individuals (diabetic and non-diabetic) homozygous for either AA (rs11705701) or TT (rs4402960) had increased levels of *IGF2BP2* mRNA in adipocytes compared to other *IGF2BP2* variants (Table 6). However, we did not find any significant difference in IGF2BP2 expression in obese patients carrying different rs11705701 and rs4402960 genotypes.

The role of the stimulatory effect of the T allele of rs4402960 on IGF2BP2 expression is unclear because this polymorphism is located in the long, non-coding intron 2, at nucleotide 29,254, distinct from exon 2. We tested whether the nucleotide variation at rs4402960 may alter a putative binding site for microRNA. In the vicinity of rs4402960, we found two potential binding sequences for hsa-miR-4646-3p and hsa-miR-197 (in 6 and 9 nucleotides upstream from the polymorphic site). However, none of these sequences could be influenced by rs4402960.

The rs11705701 is located in the 5' promoter region of *IGF2BP2*, approximately 1.48 kb upstream of exon 1 and 32.62 kb apart from rs4402960. We did not observe any significant pairwise LD between these polymorphic markers (D' = 0.53, p = 0.22). We found that allele G of rs11705701 may disrupt a potential sequence for binding transcription factors NFIC (atggcA, score 0.88) and ETS1 (Aatcct, score 0.90). This could explain the activating effects of minor allele A in *IGF2BP2* transcription. However, it is necessary to test whether these transcription factors stimulate the expression of IGF2BP2.

Using anti-IGF2BP2 antibodies specific to the C-terminal region of this protein, we were able to detect both IGF2BP2 isoforms (p58 and p66) in the adipose tissue (Figure 1). The recombinant IGF2BP2 purchased in OriGene Technologies was used as a positive control, and revealed a single full-length protein isoform p66. We found that the short isoform p58 can constitute a significant percentage (up to 45%) of a total IGF2BP2 protein in the adipose tissue. In total, average IGF2BP2 levels did not significantly differ between non-obese and obese diabetic patients. Similarly, there were no significant differences between adipose IGF2BP2 protein levels between non-obese and obese non-diabetic controls. Compared to control subjects, adipose tissue of T2D patients involved a 2-fold increased IGF2BP2 protein level (32.6 ± 18.5 pg/mg tissue vs. 17.1 ± 7.9 pg/mg tissue, p = 0.0003) as a consequence of enhanced expression of this factor in the diabetic adipose tissue.

We also tested whether polymorphic variants of *IGF2BP2* were associated with IGF2BP2 protein levels in adipocytes, but we found no significant correlation between IGF2BP2 protein amounts

Table 6. Expression of *IGF2BP2* mRNA in adipose tissue of obese and non-obese diabetic and non-diabetic carriers of different genotypes of rs4402960 and rs11705701

SNP	Patients	*IGF2BP2* genotype	Relative *IGF2BP2* mRNA expression	p‡
rs4402960	Non-obese T2D	GG	6.52 ± 2.35	0.0085
		GT	7.73 ± 3.05[1]	
		TT	8.60 ± 2.77[2]	
	Non-obese controls	GG	2.97 ± 1.50	0.017
		GT	3.35 ± 1.71	
		TT	3.56 ± 1.66[3]	
rs11705701	Non-obese T2D	GG	5.17 ± 2.08	0.0014
		AG	7.23 ± 2.55[4]	
		AA	9.60 ± 2.77[5]	
	Non-obese controls	GG	2.67 ± 1.32	0.011
		AG	3.56 ± 1.44	
		AA	4.12 ± 1.91[6]	
rs4402960	Obese T2D	GG	5.77 ± 2.18	NS
		GT	6.24 ± 2.41	
		TT	6.44 ± 3.20	
	Obese controls	GG	1.95 ± 1.07	NS
		GT	2.29 ± 1.32	
		TT	2.38 ± 1.26	
rs11705701	Obese T2D	GG	5.55 ± 2.02	NS
		AG	5.94 ± 2.32	
		AA	6.20 ± 2.50	
	Obese controls	GG	2.07 ± 1.36	NS
		AG	2.44 ± 1.33	
		AA	2.21 ± 1.15	

Legend: Data are mean ± SD. ‡ Kruskal-Wallis test. [1] GT vs. GG (non-obese T2D patiens), p = 0.023 (Mann-Whitney *U*-test). [2] TT vs. GG (non-obese T2D patients), p = 0.014 (Mann-Whitney *U*-test). [3] TT vs. GG (non-obese controls), p = 0.025 (Mann-Whitney *U*-test). [4] AG vs. GG (non-obese T2D patiens), p = 0.019 (Mann-Whitney *U*-test). [5] AA vs. GG (non-obese T2D patients), p = 0.016 (Mann-Whitney *U*-test). [6] AA vs. GG (non-obese controls), p = 0.022 (Mann-Whitney U-test). *Abbreviations*: mRNA - messenger ribonucleic acid. NS - not significant, T2D - type 2 diabetes.

and rs4402960 (Table 7). In contrast, in non-obese individuals (both diabetic and non-diabetic) homozygous for AA of rs11705701, p58 isoform levels were reduced, while p66 was significantly increased in comparison with other rs11705701 variants. In obese subjects, we found no correlation between the p66 levels and rs11705701, while p58 was significantly reduced in both diabetic and non-diabetic carriers of genotype AA compared with AG and GG carriers (Table 7). Since we observed a reverse correlation between isoform p58 levels and the presence of the disease-predisposing allele A, this isoform may play a protective role against T2D.

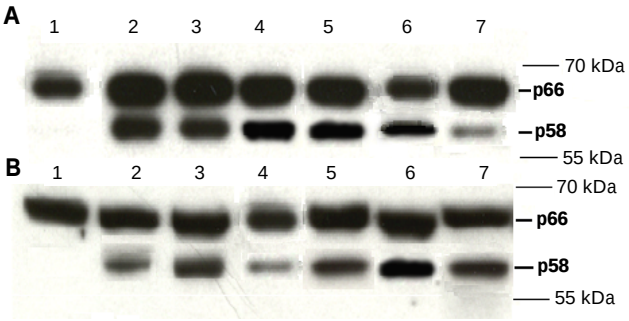

Figure 1. Western blot analysis of two IGFBP2 isoforms (p58 and p66) in the adipose tissue of obese T2D patients. **A**1 - recombinant IGFBP2 (positive control), 2 and 3 - carriers of the genotype GG (rs11705701), 4 and 5 - genotype AG, 6 and 7 - genotype AA. **B**1 - recombinant IGFBP2 (positive control), 2 and 3 - carriers of the genotype GG (rs4402960), 4 and 5- genotype GT, 6 and 7 - genotype TT.

Discussion

Despite repeated demonstration of an association of rs4402960 of *IGF2BP2* with diabetes in many populations, there was no significant correlation in our Russian population. In agreement with our finding, several large-scale population studies reported a lack of association in some Caucasian groups, including Frenchmen [40, 41], Islanders [42], and US Whites [43]. The *IGF2BP2* variant has a very small effect in T2D. Based on all studies, the reported association with the *IGF2BP2* locus (OR 1.14, 95% CI 1.10-1.17) represents a difference in allele frequency of only 3% between case and control groups in over 34,000 subjects [41]. Thus, a significant increase in population size is needed to detect the effect of this *IGF2BP2* genetic variant.

Despite the lack of association between the rs4402960 *IGF2BP2* variant and diabetes, we found a significant association between another *IGF2BP2* marker (rs11705701) and T2D. The minor alleles of both SNPs were associated with increased HOMA-IR, suggesting that *IGF2BP2* is involved in the development of IR in a Russian population. In line with this finding, Li *et al*. reported a relationship between rs11705701 and adiposity detected by an association with abdominal and visceral total fat [27]. It is likely that the association between *IGF2BP2* polymorphic markers and IR could be explained, at least in part, by correlation between the presence of the genotype TT (rs4402960) and/or AA (rs11705701) and increased levels of IGF2BP2 mRNA in adipose tissue. We observed a more than two-fold increase in *IGF2BP2* expression levels in the adipose tissue of diabetic patients compared to controls. Similarly, Parikh *et al*. detected altered expressions of *IGF2BP2* in adipocytes of T2D subjects compared with healthy people [44]. In two other studies, augmented expression of *IGF2BP2* has been shown in the pancreas of T2D patients [45, 46]. Jin *et al*. reported upregulated expression of *IGF2BP2* in peripheral monocytes of monkeys fed with a high-fat diet [47].

IGF2 plays a pivotal role in regulating fetal growth and organogenesis, including adipogenesis [48] and pancreatic development [49]. In Goto-Kakizaki rats, a spontaneous model for T2D, suppression of IGF2 production in embryonic pancreas results in lowered β-cell mass that precedes the onset of hyperglycemia [50]. Since IGF2BPs repress translation at late developmental stages [1], fetal overproduction of IGF2BP2 in pancreatic and adipose tissues could downregulate the translation of IGF2 mRNA at earlier prenatal steps, and thus lead to abnormalities in the development of pancreas and adipocytes. Generally, IGF2 shortage may contribute to reduced birthweight that is considered an independent risk factor for T2D [51].

Van Hoek *et al*. detected a nominally significant role of rs4402960 of *IGF2BP2* and its interaction with intrauterine malnutrition to early induction of glucose intolerance [52]. Impaired islet β-cell development leads to impairment in insulin secretion, thereby promoting the manifestation of hyperglycemia and IR [53]. Association of rs4402960 with T2D may be realized not only through impaired β-cell development and function, but also through alterations in adipose tissue [27].

In our study, we confirmed recent data from Li *et al*. who detected the existence of two isoforms of IGF2BP2 generated by alternative translation ini-

Table 7. Levels of IGF2BP2 protein isoforms in adipose tissue of obese and non-obese diabetic and non-diabetic carriers of different genotypes of rs4402960 and rs11705701

IGF2BP2 isoform	Patients	rs4402960 genotype	IGF2BP2 protein (pg/mg tissue)	p‡	rs11705701 genotype	IGF2BP2 protein (pg/mg tissue)	p‡
p66	Non-obese T2D	GG	17.8 ± 14.5	0.0054	GG	23.5 ± 11.7	NS
		AG	26.6 ± 10.2[1]		GT	27.1 ± 13.6	
		AA	30.3 ± 13.0[2]		TT	26.8 ± 12.2	
	Non-obese controls	GG	11.9 ± 6.2	0.012	GG	13.9 ± 5.5	NS
		AG	13.2 ± 4.8		GT	14.4 ± 4.8	
		AA	17.6 ± 6.5[3]		TT	16.1 ± 5.2	
p58	Non-obese T2D	GG	13.2 ± 6.7	0.005	GG	10.9 ± 5.6	NS
		AG	7.9 ± 6.1[4]		GT	9.2 ± 4.3	
		AA	5.5 ± 4.3[5]		TT	8.6 ± 4.7	
	Non-obese controls	GG	4.8 ± 3.5	0.037	GG	3.8 ± 2.1	NS
		AG	4.2 ± 2.7		GT	3.8 ± 2.3	
		AA	2.7 ± 1.5[6]		TT	3.3 ± 1.7	
p66	Obese T2D	GG	18.6 ± 13.3	NS	GG	14.5 ± 10.6	NS
		AG	16.2 ± 11.7		GT	16.5 ± 9.9	
		AA	19.3 ± 12.5		TT	18.0 ± 12.5	
	Obese controls	GG	10.4 ± 7.2	NS	GG	10.5 ± 7.2	NS
		AG	11.9 ± 6.5		GT	11.4 ± 5.2	
		AA	11.4 ± 5.9		TT	12.7 ± 6.6	
p58	Obese T2D	GG	12.8 ± 9.2	0.027	GG	11.7 ± 7.3	NS
		AG	11.5 ± 6.7		GT	12.5 ± 5.8	
		AA	9.6 ± 5.4		TT	10.9 ± 6.1	
	Obese controls	GG	3.9 ± 1.9	0.044	GG	3.3 ± 1.5	NS
		AG	3.4 ± 1.6		GT	2.5 ± 1.3	
		AA	2.6 ± 1.7[8]		TT	2.9 ± 1.9	

Legend: Data are mean ± SD. ‡Kruskal-Wallis test. [1] AG vs. GG (non-obese T2D patiens), p = 0.015 (Mann-Whitney *U*-test). [2] AA vs. GG (non-obese T2D patients), p = 0.009 (Mann-Whitney *U*-test). [3] AA vs. GG (non-obese controls), p = 0.014 (Mann-Whitney *U*-test). [4] AG vs. GG (non-obese T2D patiens), p = 0.017 (Mann-Whitney *U*-test). [5] AA vs. GG (non-obese T2D patients), p = 0.081 (Mann-Whitney *U*-test). [6] AA vs. GG (non-obese controls), p = 0.03 (Mann-Whitney U-test). [7] AA vs. GG (non-obese T2D patients), p = 0.081 (Mann-Whitney *U*-test). [8] AA vs. GG (non-obese controls), p = 0.03 (Mann-Whitney *U*-test). *Abbreviations*: NS - not significant, T2D - type 2 diabetes.

tiation [27]. The isoforms are abundantly distributed in different tissues, preferentially in adipose and pancreatic tissues. We found that adipose levels of the short IGF2BP2 isoform p58, having a molecular weight of 58 kDa and lacking the first 58 amino acids, can be modulated by the rs11705701 polymorphism of *IGF2BP2*.

The first RRM domain of IGF2BP2, which is absent in the small isoform, contains highly conserved RNP1 and RNP2 motifs important for RNA recognition, in contrast to the second RRM domain, which has poor RNA-binding signatures [54]. In addition to binding to RNA, RRM domains can participate in protein-protein interaction and facilitate inter- or intra-molecular dimerization of RRM domain-containing proteins [54]. Thus, loss of the RRM1 domain in the small isoform might affect its binding affinity and specificity to both RNA and protein partners. It may also affect its own structure, dimer formation, and stability.

Compared to the canonic full-length isoform p66 that has six RNA-binding motifs, the truncated isoform p58 may have decreased RNA-binding capacity and reduced affinity to IGF-2 mRNA. Due to the less efficient capacity of the short isoform to downregulate translation of IGF-2 mRNA, this mRNA would be better translated to produce more IGF-2, and in turn, to improve insulin action in peripheral tissues. In the context of diabetes, reduced levels of p58 accompanied with increased production of IGF2BP2 isoform p66 could predispose to the development of IR by im-

pairing insulin action in the adipose tissue of carriers of the genotype AA of 11705701.

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