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A Polymorphic Microdeletion in the *RGS9* Gene Suppresses PTB Binding and Associates with Obesity

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Abstract

Objective: RGS9 is a member of the family of Regulators of G-Protein Signaling (RGS) proteins defined by the presence of an RGS domain which can accelerate the GTPase-activity of G protein Gα subunits. An insertion/deletion (I/D) polymorphism of the nucleotide sequence TTTCT (rs3215227) has been identified in the human RGS9 gene, which matches the consensus high affinity binding motif for the ubiquitously expressed RNA binding Polypyrimidine Tract Binding Protein (PTB). In this study, we evaluate the genetic association and functional relevance of this polymorphism in type 2 diabetes and obesity.

Subjects and methods: We genotyped a larger population of 9272 Chinese and Malaysian individuals for the RGS9 I/D polymorphism using TaqMan allelic discrimination protocols. We found that the D allele of the RGS9 polymorphism was associated with a decreased prevalence of obesity in women (P=0.003, OR=0.753 95%CI 0.625-0.906) and girls (P=0.002, OR=0.604 95%CI 0.437-0.835). The association was moderate in boys (P=0.038, OR=0.724 95%CI 0.533-0.983) and not significant in men. Furthermore, we found that the transcript deletion variant exhibited a 10-fold reduction in PTB binding in vitro and that the splicing of the deletion variant was less affected by PTB co-expression.

Conclusions: We provide genetic and biochemical data to support a genetic role of RGS9 in obesity but unlikely in T2D. The RGS9 I/D polymorphism influence the post-transcriptional processing of the gene through an altered affinity for the splicing factor PTB and are associated with obesity.

Keywords: Association; Obesity; Polypyrimidine tract binding protein; Regulators of G-protein signaling protein; Type 2 diabetes

Abbreviations: BMI: Body Mass Index; PTB: Polypyrimidine Tract Binding Protein; RGS9: Regulators of G-Protein Signaling Protein; T2D: Type 2 Diabetes

Introduction

In the past decade, the prevalence of obesity and Type 2 Diabetes Mellitus (T2D) is increasing worldwide particularly in the developing countries undergoing economic transition to a market economy such as China. A 1% increase in the prevalence of obesity in the country leads to 20 million additional obese cases. The increase in obesity has an important impact on the global incidence of T2D, cardiovascular disease, cancer and other diseases [1-4]. Therefore, identification of the genes and gene variations that regulate Body Mass Index (BMI) will help us to understand the underlying mechanisms of the diseases and possibly aid in the development of novel prevention and therapeutic approaches.

RGS9 protein is a member of the family of Regulators of G-Protein Signaling (RGS) proteins and expression of RGS9 is highly enriched in the striatum region of the brain [5-9]. Dopaminergic circuits in the striatum are activated by stimuli associated with reward and are also densely interconnected with the hypothalamus, a brain region that is critical for regulating energy expenditure [10]. These dopamine-sensitive striatal reward centers have been shown to be involved in obesity which could be produced either by heightening reward responses to food or by suppressing energy expenditure [10-12].

An Insertion/Deletion (I/D) polymorphism of the nucleotide sequence TTTCT (rs3215227 in dbSNP) [13] has been identified in intron 13 of the human RGS9 gene, which precisely matches the

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consensus high affinity binding motif for the ubiquitously expressed RNA binding Polypyrimidine Tract Binding Protein (PTB) [14]. PTB and its neuronally expressed paralog nPTB [15] are the dominant splicing repressor in humans and have been shown to regulate many alternative splicing events. PTB and nPTB have indistinguishable binding specificities and a near complete overlap of functional targets [15]. PTB has also been implicated in polyadenylation, message stabilization, translation from internal ribosome entry sites and as a RNA chaperone [16]. The location of the RGS9 I/D polymorphism [8] and coincidence with a PTB binding motif [14] suggested that the presence of the deletion could alter processing of the RGS9 transcripts and have important functional consequences: exons 13 and 14, that flank the TTTCT I/D polymorphism, code for the conserved RGS domain that mediates GTPase accelerating protein (GAP) function [8,17]. Hence, incorrect splicing of these exons will result in protein products that lack a functional RGS domain.

In a previous study, we demonstrated that rgs9 knockout mice became obese and had increased adiposity compared to their wild-type littermates [18]. Conversely, the virally transduced overexpression, specifically of rgs9 proteins (and not other R7 RGS proteins) in the nucleus accumbens (NAc) of rats produced a reduction in body weight [18]. In that study, we also investigated if there was an association between the RGS9 I/D polymorphism and BMI [18]. Since the micro-deletion allele is rare in European Caucasians [13], we had genotyped for the RGS9 I/D polymorphism in a limited number samples from 491 Asian individuals that was available to us [18] and found that the mean BMI of individuals carrying with the D allele was significantly higher than those that tested negative for the deletion. However, the generalizability of those results was limited by 1) the limited sample size, 2) the heterogeneous ethnic composition of the cohort which included individuals who identified themselves as either Chinese, Japanese, Korean, Pacific Islander, or Southeast Asian and 3) the significant variation in the frequency of the D and I alleles between the different ethnic groups.

To address these concerns, in this study, we asked if the I/D polymorphism was associated with obesity in a larger sample of 2737 Chinese adults and 816 children. We also investigated whether the polymorphism was associated with T2D in a sample of 6535 Chinese and Malaysian. Furthermore, we have expanded on those results by demonstrating allelic differences in the binding of PTB to the RGS9 transcript and present data from minigene experiments that demonstrated allelic, PTB-dependent, effects on the splicing of RGS9 transcripts in vivo.

Materials and Methods

Subjects

In the present study, a total of 9272 subjects in Chinese and Malaysian populations were selected for genetic association study. Clinical characteristics of the subjects are represented in Table 1. Collection of the subjects in each cohort is described briefly as below.

First cohort: Lean and obese Chinese adults: A total of 4138 subjects were recruited in Jiangsu province, China, from a population of 16,034 by epidemiological stratification sampling with ages ranging from 35 to 85 years. According to the criteria of overweight/obesity for Chinese from working group on obesity in China (WGOC) [19], 820 (females 541/ males 279) subjects with BMI>28 kg/m² were defined as having an obese phenotype and 1,100 (females 687/males 413) subjects with BMI ranging from 18.5-23.9 kg/m² were defined as having a lean phenotype.

Second cohort: Chinese children with and without over-weight: Chinese children aged 5-15 years were selected from primary and middle schools located in Jiangsu province, China. The children and their parents provided informed consent and completed a questionnaire to provide information covering age, gender and education. Within this population (n=2,373), 361 (157/204) children were overweight, i.e. had a BMI that exceeded the 80th percentile for their age (P80) and 451 (215/236) children had BMIs that ranged from 20th to 50th percentile (P20-50) for their age.

Third and fourth cohorts: Chinese adults with and without type 2 diabetes (T2D): Chinese adults with and without T2D were selected respectively from the regions of Jiangsu province and Beijing city in China. All patients with T2D were diagnosed according to the World Health Organization (WHO) criteria, 1998 [20]. 1279 patients with T2D and 1990 subjects with normal fasting plasma glucose levels were included in the present study from Jiangsu province. 1049 (496/553) patients with T2D and 1004 (520/484) non-diabetic subjects were enrolled as previously described from regions around Beijing. Subjects with BMI>28.0 kg/m² were excluded from the study [21].

Fifth cohort: Adults with and without T2D from Malaysia: Malaysians subjects including 575 (268/307) non-diabetic controls and 643 (311/332) T2D patients were collected from the university hospitals in the states of Kelantan, Putrajaya and Penang, Malaysia. The ethnic break-up of the Malaysian population was as follows: 64.6% Malay, 17.5% Chinese, 15.5% Indians and 2.4% indigenous. Subjects with BMI>28.0 kg/m² were excluded from the study.

Sample	Origin	Region/Country	Group	N (Female%)	Age (years)	BMI (kg/m²)	FPG (mmol/l)
Sample 1	Chinese	Jiangsu, China	Adults with lean	1100 (62.5)	59.4 ± 10.7	21.5 ± 1.5	5.1 ± 0.8
			Adults with obese	820 (66.0)	59.9 ± 10.4	29.1 ± 2.0*	6.0 ± 2.2*
Sample 2	Chinese	Jiangsu, China	Children without overweight	451 (43.6)	10.1 ± 2.9	16.4 ± 1.5	4.2 ± 1.0
			Children with overweight	361 (47.3)	10.1 ± 3.0	21.3 ± 3.8*	4.3 ± 1.0
Sample 3	Chinese	Jiangsu, China	Non-diabetic controls	1990 (63.5)	60.1 ± 10.3	22.5 ± 2.9	4.4 ± 0.4
			Type 2 diabetes	1279 (65.3)	60.2 ± 9.5	25.4 ± 3.7*	8.7 ± 3.3*
Sample 4	Chinese	Beijing, China	Non-diabetic controls	1004 (51.7)	55.6 ± 9.6	25.3 ± 3.6	5.0 ± 0.3
			Type 2 diabetes	1049 (47.3)	55.8 ± 10.1	25.3 ± 3.4	9.0 ± 3.1*
Sample 5	Malaysian	Penisular, Malaysia	Non-diabetic controls	575 (49.6)	45.8 ± 15.1	26.0 ± 5.1	5.6 ± 0.7
			Type 2 diabetes	643 (51.7)	56.3 ± 10.4	27.8 ± 5.2	8.6 ± 3.1*
Total				9272 (57.1)			

Data are means ± SD. BMI: body mass index. FPG: fasting plasma glucose. *P<0.05 for comparing with controls.

Table 1: Clinical characteristics of subjects.

Written informed consent was obtained for each subject. Written informed consent was obtained from the subject or from the parents or guardians if the subjects were children or minors. The sample and data collection procedures were approved by the local ethics committees, respectively. The full names of the respective ethics committee/ institutional review boards are as follows: the Research Ethics Committee of Nanjing Medical University, Nanjing, China (IRB NMU-1-1-2011), the Ethics Committee of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, Beijing, China (PUMC-019-2011), and the Medical and Research Ethics Committee, Ministry of Health Malaysia, Kuala Lumpur, Malaysia (IMR-3-3-2007). All these studies were carried out in accordance with the declaration of Helsinki II.

Genotyping

The ID number of the RGS9 I/D polymorphism in dbSNP is rs3215227. The position of this RGS9 I/D polymorphism is represented in Figure 1. To genotype the RGS9 I/D polymorphism with TaqMan allelic discrimination, a SNP genotyping assay (ID: AH0I2R) was designed and then synthesized by Life Technologies (Grand Island, USA). In addition, a specific PCR protocol was also designed for genotyping the polymorphism. The primers included RGS9delF1 (5'-aaagaggaaataaatgagtcaa-3'), RGS9delF2 (5'-agttggaagaagaaaagaggaaataaaagaaa-3') and RGS9delR (5'-agttggaagaagaaaagaggaaattaa-3'). The PCR program was 95°C, 5min; (95°C, 30sec; 54°C, 30se; 72°C,30 sec) x 31; 72°C, 8 min. PCR fragments of sizes 104 bp (deletion allele) and 122 bp (insertion allele) were detected with 3% w/v agarose gels and gel electrophoresis.

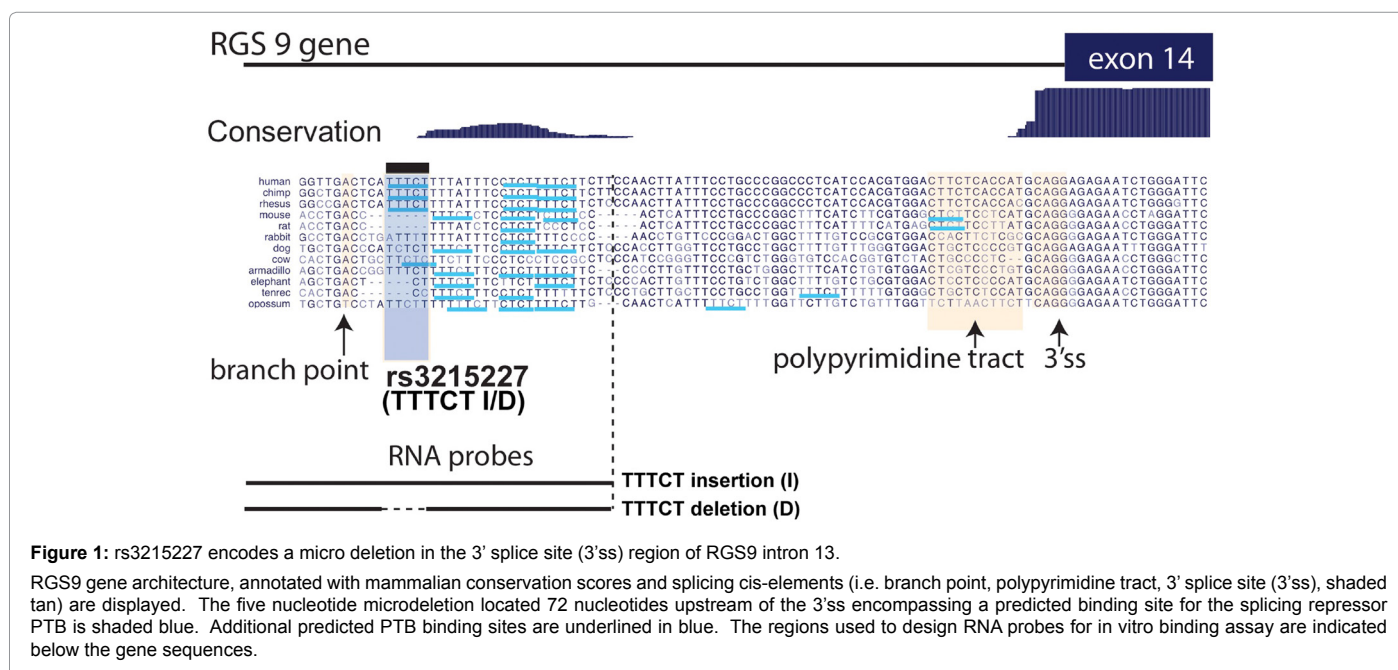### Splicing reporter mini-gene assay

The reporter system and assay used to screen for the possible actions of PTB binding on the splicing of gene segments containing the RGS9 I/D polymorphism has been previously described and a schematic of the minigene construct is shown in this Supplement section, Figure S1A [18]. The splicing reporter was derived from a plasmid vector containing a 3 exon minigene under the control of the human cytomegalovirus promoter that has previously been described

by Wang and colleagues [22]. The small constitutively spliced central exon of the original splicing reporter contains two restriction cloning sites, Xho1 and Apa1, into which we inserted a synthesized (Epoch Life Sciences, Missouri City, TX) fragment of the human RGS9 gene [18]. The inserted RGS9 gene fragment consisted of the final 57 base pairs of exon 13 (Supplement Figure S1A, grey box,) followed by a fusion of the first 100 and the last 243 base pairs in the adjacent intron 13 (Supplement Figure S1A, dashed grey bar). The insertion of the regions from intron 13 of the RGS9 gene effectively splits the central exon of the original minigene into 2 separate exons thus creating a new minigene construct with 4 exons (Figure S1A). Two splicing reporters were constructed, one in which the inserted RGS9 gene fragment was synthesized with TTTCT insertion (I) and another one in which the inserted RGS9 gene fragment was synthesized with TTTCT deletion (D) at the appropriate intron 13 site.

Human embryonic kidney cells (HEK293, American Type Culture Collection, and Manassas, VA) were separately co-transfected with a splicing reporter minigene constructs containing either the RGS9 TTTCT I or D allele's deletion positive and cDNA encoding for either PTB or nPTB. The transient transfections were performed using LTX transfection reagent (according to manufacturer's instructions Invitrogen, Carlsbad, CA). After 48 hr the cells were harvested and total cellular RNA was isolated using TRIzol reagent (according to manufacturer's instructions, Invitrogen). Next, the RNA was reverse transcribed (reverse transcriptase enzyme, Sigma Aldrich) at 37°C for 50 min in a reaction that was primed with random hexamer nucleotides and contained dNTP mixture and RNase inhibitor (Ambion) and buffer provided by the enzyme supplier.

Identification of RNA binding proteins by UV-cross linking

All templates for transcription were ordered as custom DNA oligonucleotides containing T7 promoters from IDT (idtdna.com). An oligo complimentary to the T7 promoter was also purchased, and the two were mixed in equimolar ratios, heated to 95°C and allowed to cool slowly at room temperature. These mixtures served as transcription templates for a T7 polymerase reaction using the MEGA short script

Figure 1: rs3215227 encodes a micro deletion in the 3' splice site (3'ss) region of RGS9 intron 13.

RGS9 gene architecture, annotated with mammalian conservation scores and splicing cis-elements (i.e. branch point, polypyrimidine tract, 3' splice site (3'ss), shaded tan) are displayed. The five nucleotide microdeletion located 72 nucleotides upstream of the 3'ss encompassing a predicted binding site for the splicing repressor PTB is shaded blue. Additional predicted PTB binding sites are underlined in blue. The regions used to design RNA probes for in vitro binding assay are indicated below the gene sequences.

transcription kit (Ambion). Unlabeled cold competitors were prepared following the protocol supplied with the kit, whereas radiolabeled probe for UV-crosslinking experiments was prepared with the addition of 32P UTP to a final concentration of 10 µCi. All samples were purified using the phenol-chloroform extraction and ethanol precipitation method and visualized on 7 M urea polyacrylamide gels and quantified via phosphor imaging (Image Quant; GE Healthcare) and/or UV spectrometry (Nanodrop).

For each reaction, the radiolabeled probe (wt/R9 RNA and D/R9 RNA in 1, 5 or 10 molar excess for either of the RNA probe) was added to 30 µg of HeLa cell nuclear extract. Volume was raised to 80 µL with Buffer E (20 mM Tris pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT) and incubated at 25°C for 30 min. Reactions were exposed to UV in a UV-crosslinker FB-UVXL-1000 (Fisher Scientific) for 15 min at 120 mJ/cm2, 5 cm from the UV source. RNA was digested using RNAse A/T1 mix (Ambion) for 1 hr at 37°C. For competition assay, 200 ng (0.01 nM) of radiolabeled probe (S11) were added to 30 µg of HeLa cell nuclear extract with an increasing amount (0, 5, or 50 fold molar excess) of unlabeled competitor RNA. The rest of the protocol is same as described above for UV cross linking experiment. All samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE), dried at 80°C for 25 min, and imaged using a GE Healthcare storage phosphor screen and scanned on a Typhoon 9410 variable mode imager and packaged software.

Statistical analyses

The power of the genetic association study (cases and controls) was calculated with the software of Power and Sample Size Calculation (Dupont WD, Plummer WD: http://biostat.mc.vanderbilt.edu/ twiki/bin/view/Main/Power Sample Size). At the 5% significance levels, we had 82% power to detect OR of 0.8 or 1.25 with a MAF D allele of 0.25 in adult obesity population for generating the association hypothesis of RGS9 I/D polymorphism and obesity in this study. For the association analysis of RGS9 I/D polymorphism and T2D, the Jiangsu population has over 90% power to detect OR of 0.8 or 1.2 with a MAF D allele of 0.28 in control population.

Among controls, genotype frequencies were analyzed by Fisher's exact test for Hardy-Weinberg equilibrium (HWE). The allele frequencies and genotype distributions between cases and controls were compared by the Chi-square (χ2) test and a P-value of 0.05 was defined to be statistically significant. Odds Ratios (OR) and 95% Confidence Intervals (CI) were calculated to test for association. For the stratification analysis, a multiple unconditional logistic regression was applied to evaluate the association. The adjustment for covariates including age and sex was done in the analyses of allelic association with obesity, while the adjustment for age and BMI was used in the analyses of the association with T2D in males and females, respectively. All analyses were performed using IBM SPSS Statistics (Version 20, SPSS Inc, and Chicago, USA).

In addition, a mata-analysis was used to systematically evaluate the association of RGS9 I/D polymorphism and T2D for Chinese Jiangsu population, Beijing population and Malaysia population. Heterogeneity chi-squared test was used to detect the variation in estimation attributable to heterogeneity and if I-squared is less 50.0%, random model would be selected to calculate the pooled OR of the association between RGS9 I/D polymorphism and T2D. The analyses were performed using Stata 10.0 (StataCorp LP, College Station, TX).

Results

The RGS9 gene in human consists of 19 exons and the RGS9 I/D (TTTCT insertion/deletion) polymorphism (rs3215227 in dbSNP) is located in intron 13. Comparison with other primate species suggests that the polymorphism arose in the human lineage as a TTTCT deletion at some point after the human-chimpanzee divergence (Figure 1).

We performed a genetic association study of the RGS9 I/D polymorphism with obesity and T2D in a larger sample set of Chinese and Malaysian subjects. The D allele frequency was present at 23.8% and 28.5%, respectively, in these two Asian populations. The genotype distributions of the RGS9 I/D polymorphism in the studied populations were kept in HWE and the results of the study are summarized in Table 2. The genotype distributions of the RGS9 I/D polymorphism in the studied populations were kept in Hardy-Weinberg equilibrium. In the Chinese population, women who were obese (BMI ≥ 28) had a significantly (P=0.003, OR=0.757, 95% CI 0.631-0.909) lower frequency (23.7 vs 29.0%) of the D allele compared to the women who were lean (BMI 18.5-23.). The D allele frequency was not different between lean and obese Chinese men (26.2% vs. 26.6%, P=0.877). In Chinese children, the D allele was found to be strongly associated with a decreased risk for obesity in girls (25.5% vs. 36.3%, P=0.002, OR=0.600, 95% CI 0.436-0.828) and moderately associated with a decreased risk for obesity in boys (24.3% vs. 30.5%, P=0.039, OR=0.730, 95% CI 0.541-0.984).

Further stratification analyses for the association of the RGS9 I/D polymorphism with obesity were performed separately in women and men and also adjusted for covariates including age, BMI, daily diet and levels of physical activities. Results of the study are summarized in Table 3. In the Chinese population, women who were obese had a significantly (23.7 vs 29.0%, P=0.003, OR=0.753, 95% CI 0.625-0.906) lower frequency

Region/Country Population	Group	Genotype			Allele D Frequency	P-value OR (95% CI) [a]
		I/I	I/D	D/D		
Jiangsu/China Chinese	Adults, lean BMI 18.5-23.9	566	452	82	0.28	0.019[a]
	Adults, obese BMI ≥ 28	463	310	47	0.25	0.838 (0.723-0.972) [a]
Jiangsu/China Chinese	Children BMI p20-50	204	194	53	0.33	3.1×10−4 [a]
	Children BMI p>80	202	139	20	0.25	0.667 (0.535-0.831) [a]
Jiangsu/China Chinese	Non-diabetic	1035	814	141	0.28	0.820[b]
	T2DM	678	509	92	0.27	1.014 (0.870-1.147) [b]
Beijing/China Chinese	Non-diabetic	537	372	95	0.28	0.999[b]
	T2DM	555	397	97	0.28	1.000 (0.876-1.141) [b]
Penisular/Malaysia Malaysian	Non-diabetic	331	241	57	0.28	0.741[b]
	T2DM	295	239	50	0.29	0.966 (0.785-1.188) [b]

Tests were done with an additive model; BMI: Body Mass Index; T2DM: Type 2 Diabetes; a: Adjustment for Age and Sex; b: Adjustment for Age, Sex and BMI.

Table 2: The allele D frequency of the RGS9 I/D polymorphism and its association with obesity.

Region/Country Population	Gender	Group	Genotype			Allele D Frequency	P-value OR (95% CI)
			I/I	I/D	D/D		
Jiangsu/China Chinese	Women	Adults, lean BMI 18.5-23.9 Adults, obese BMI ≥ 28	342 316	291 194	54 31	0.290 0.237	0.003[a] 0.753 (0.625-0.906) [a]
Jiangsu/China Chinese	Men	Adults, lean BMI 18.5-23.9 Adults, obese BMI ≥ 28	224 147	162 116	27 16	0.262 0.265	0.965[a] 1.006 (0.782-1.293) [a]
Jiangsu/China Chinese	Girls	Children BMI p20-50 Children BMI p>80	91 85	92 64	32 8	0.363 0.255	0.002[a] 0.604 (0.437-0.835) [a]
Jiangsu/China Chinese	Boys	Children BMI p20-50 Children BMI p>80	113 117	102 75	21 12	0.305 0.243	0.038[a] 0.724 (0.533-0.983)[a]
Jiangsu/China Chinese	Women	Non-diabetic BMI<28 T2DM BMI<28	656 450	511 325	97 60	0.286 0.266	0.669[b] 0.967 (831-1.126)[b]
Jiangsu/China Chinese	Men	Non-diabetic BMI<28 T2DM BMI<28	379 228	303 184	44 32	0.269 0.279	0.317[b] 1.113 (0.902-1.372)[b]
Beijing/China Chinese	Women	Non-diabetic BMI<28 T2DM BMI<28	281 263	193 197	46 36	0.274 0.271	0.815[b] 0.977 (0.805-1.186)[b]
Beijing/China Chinese	Men	Non-diabetic BMI<28 T2DM BMI<28	256 292	179 200	49 61	0.286 0.291	0.833[b] 1.020 (0.850-1.223)[b]
Penisular Malaysia Malaysian	Women	Non-diabetic BMI<28 T2DM BMI<28	142 158	102 126	24 27	0.280 0.289	0.168[b] 0.809 (0.599-1.094)[b]
Penisular Malaysia Malaysian	Men	Non-diabetic BMI<28 T2DM BMI<28	158 172	120 133	29 27	0.290 0.282	0.395[b] 1.135 (0.848-1.517)[b]

Tests were done with an additive model; BMI: Body Mass Index; T2D: Type 2 Diabetes; a: Adjustment for Age; b: Adjustment for Age and BMI

Table 3: Stratification analysis on the association of RGS9 I/D polymorphism with obesity.

of the D allele compared to the women who were lean (23.7 vs. 29.0%, P=0.003, OR=0.753, 95% CI 0.625-0.906). The D allele frequency was not different between lean and obese Chinese men (26.2% vs. 26.6%, P=0.965, OR=1.006, 95% CI 0.782-1.293). In Chinese children, the D allele was found to be strongly associated with a decreased risk for obesity in girls (25.5% vs. 36.3%, P=0.002, OR=0.604, 95% CI 0.437-0.835) and moderately associated with a decreased risk for obesity in boys (24.3% vs. 30.5%, P=0.038, OR=0.724, 95% CI 0.53-0.983).

In order to ask if there was any association between the RGS9 I/D polymorphism and T2D, we selected two cohorts of Chinese (from Jiangsu province and Beijing, respectively) and one cohort of Malaysian adults with and without T2D for analyses and those patients were not obese. We found no significant difference in the D allele frequency between non-diabetic controls and T2D patients in either the Chinese or Malaysian populations (Table 2) although the analyses were stratified by gender (Table 3). In addition, a mata-analysis was used to systematically evaluate the association of RGS9 I/D polymorphism and T2D for Chinese Jiangsu population, Beijing population and Malaysia population. Heterogeneity test indicated that there is no heterogeneity among the study populations ($\chi2$=0.15, P=0.930) and $I2$ was 0.0%, random model was selected to calculate the association and pooled OR (95%CI) was 1.000 (0.917-1.090) for the association between RGS9 I/D polymorphism and T2D (z=0.00, P=0.997).

Since the RGS9 I/D polymorphism was strongly associated with obesity and matched the consensus binding motif for the RNA binding Polypyrimidine Tract Binding Protein (PTB) [23], we tested if the polymorphism could modulate RGS9 gene function through a mechanism of allele specific interactions with PTB. In our previous study, we had shown that processing of the RGS9 gene transcript was altered by the I/D polymorphism, but we did not report on the binding of PTB to the RNA alleles or the effect of PTB expression transcriptional processing of these RNA alleles. Exons 13 and 14, which flank the polymorphism, code for the conserved and functionally important RGS domain. Therefore, alterations in splicing, such as exon skipping, at this site, produced as a result of reduced PTB binding to the D allele, will lead to alterations in the levels of functional RGS9 protein. Altered PTB binding could also result in allele specific changes in RNA polyadenylation, stability or structure [23].

We assayed the RGS9 I/D RNA alleles for differences in binding to PTB in cell nuclear extracts. The binding affinity of PTB to these allelic RNA substrates was determined using a standard Ultraviolet (UV) cross-linking reaction. Briefly, this assay captures RNA-protein binding events through a label transfer strategy. RNA corresponding to the 2 alleles is transcribed utilizing 32P-radio-labeled nucleotides and incubated with a nuclear extract from cells to allow RNA-protein complexes to form. The internally radio-labeled RNA is covalently cross-linked to bound protein using UV light. RNAses are used to "trim" the RNA that is covalently attached to protein to a few nucleotides and the trimmed RNA effectively serves as a radiolabel for the interacting proteins. The mobility of these labeled RNA-binding proteins can be measured by Poly-Acrylamide Gel Electrophoresis (PAGE) and their identity confirmed by immunoprecipitation.
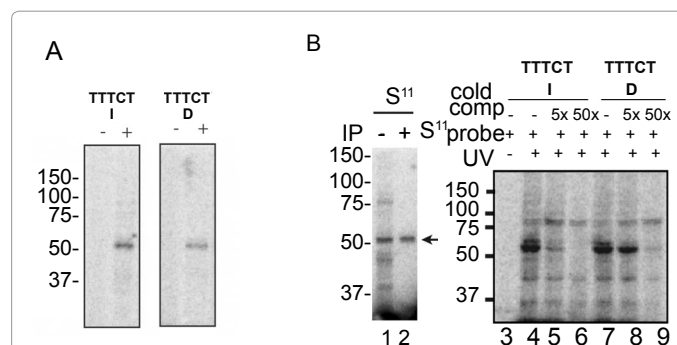
Figure 2: PTB binds the RGS9 TTTCT insertion (I) allele with 10-fold higher affinity than the deletion (D) allele.

RNA oligos corresponding to the I and D alleles and the previously characterized PTB ligand, S11, were radiolabelled, incubated in HeLa extract and UV crosslinked to interacting proteins. Radiolabeled crosslinked proteins were visualized by SDS-PAGE/autoradiography. A. A prominent 58 kD band corresponding to the molecular weight of PTB can be detected on both the I and D allelic probes. B. The 58 kD band is also detected with the S11 ligand (lane 1) and can be immunoprecipitated with antibodies against PTB (lane 2). The relative strength of the I or D RNA allele to bind PTB is inferred from the ability of unlabeled RNA probes (cold comp), corresponding to these alleles, to compete with the radiolabeled S11 ligand for PTB binding (lanes 3-9).

Both the I and D RNA alleles bound a 58 kD protein, that corresponded to the molecular weight of PTB, with the I RNA allele appearing to bind more strongly (Figure 2A). The same 58 kD protein was also detected using a previously characterized RNA ligand for PTB, S11 (Figure 2B, left panel) [5]. Crosslinking of radiolabeled S11 with cell nuclear extracts followed by immunoprecipitation of PTB confirmed that PTB is the dominant 58 kD binding protein for S11 (Figure 2B, right panel, lanes 4 and 7). The crosslinking efficiency and degree of radiolabel incorporation can vary from one RNA substrate to another so that relative signals obtained for the 58 kD protein in Figure 2A do not necessarily represent the relative level of binding of the I and D RNA alleles to that protein. Therefore, a more accurate representation of the level of binding of the RNA alleles to PTB was achieved by examining the ability of each of the unlabeled alleles to compete with the binding of PTB to the previously characterized radiolabeled RNA ligand for PTB, S11 [14]. We performed competition binding experiments to PTB with radiolabeled S11 and progressively higher concentrations (5-50 molar excess) of unlabeled I and D variants of the RGS9 RNA, respectively (Figure 2B, lanes 3-9). The relative strength of the I or D RNA allele to bind PTB was inferred from the ability of unlabeled RNA probes, corresponding to these alleles, to compete with the radiolabeled S11 ligand for PTB binding. The results show that a 10-fold higher concentration of the D variant is required to achieve the same amount of competition with S11 at PTB as the I allele (Figure 2B, right panel, lanes 3-9) and indicate that the I RNA allele does indeed bind PTB with much higher affinity than the D variant.

We then utilized a splicing reporter assay to examine whether the difference in interaction between PTB and the two RNA alleles could alter how the alleles were functionally processed in cells. Since RGS9

transcripts are primarily expressed either in the brain or the retina we also tested the effects of a neuronal paralog of PTB, nPTB. Supplemental Figure S1A represents a schematic of the RGS9 mini-gene construct composed of four exons with the I/D polymorphism-containing sequence from intron 13 of RGS9 inserted between experimental exons 2 and 3 which was transfected into cells.

RNA isolated post-transfection was analyzed by reverse-transcriptase (RT)-PCR. Correct constitutive splicing of all 4 exons corresponded to a 451 nucleotide (nt) product ("a" in Figure 3A and Supplemental Figure S1B). Coexpression of PTB or nPTB led to the repression of correct splicing and the detection of two additional PCR products corresponding to distinct alternatively spliced mRNA products. The splicing of experimental exons 1 and 2, but skipping of the exon immediately downstream of the polymorphism, exon 3, leads to splicing of exon 2 and 4 and results in an ~400 nt band ("b" band). Splicing of exon 1 and 4 due to skipping of exons 2 and 3 was also detected ("c" band).
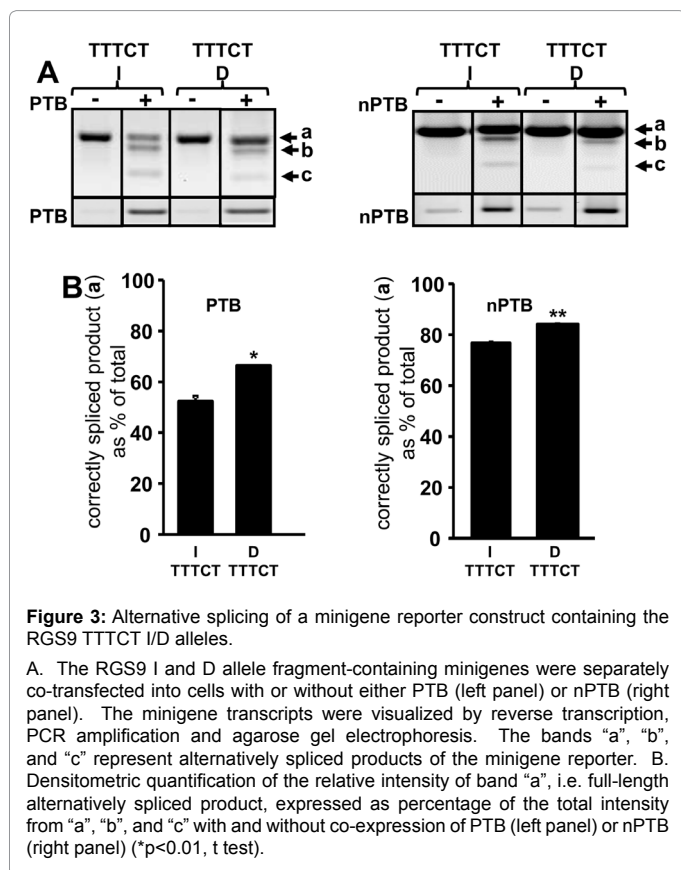
The repression of splicing produced by co-expression of either PTB or nPTB was biased to the RGS9 TTTCT I allele (Figure 3) and the fraction of transcript that included all 4 exons ("a" band) produced with the I variant was significantly less than with the D variant, i.e. the repressive effect of PTB or nPTB was significantly stronger on the I allele. We confirmed preferential PTB binding to the I allele in vivo by repeating the UV crosslinking in cell culture and coimmunoprecipitating the crosslinked PTB/pre-mRNA complexes (Supplemental Figure S2). The latter confirmed that the I allele binds more strongly to PTB in vivo and thus has a greater potential to be modulated by PTB than the D allele.

Discussion

Here, we have present genetic and biochemical data to support a role for the RGS9 TTTCT I/D polymorphism in affecting obesity in humans. These findings appear to parallel those us previously observed in rodents where RGS9 expression levels are correlated with adiposity/body weight [18].

Men and women differ in body composition, patterns of weight gain, hormone biology, metabolic markers and the manner in which social, ethnic and environmental factors contribute to the obesity phenotype [24-26]. In this context, our observation of a sexually dimorphic association of a polymorphism in the RGS9 gene is significant as it is one of few studies that have demonstrated sex-specific effects of weight-influencing gene variants. Our previous mouse studies had produced similar results: targeted disruption of the rgs9 gene resulted in a more pronounced increase in body weight in female mice and the divergence in body weight between the knockout mice and wild-type mice occurred at an earlier age in females [18]. The mechanisms underlying the sex-specific effects of obesity and eating behavior are not well understood and this study establishes RGS9 as one starting point to study some of these mechanisms.

Human genetic studies have linked variations in two other R7 RGS proteins, RGS6 and RGS7 to obesity [27]. These studies provide a precedent for R7 RGS family proteins regulating body-weight through their expression in the brain since the protein products of the R7 RGS gene family have been reliably detected only in the brain or in excitable tissue such as retina and heart [5,9]. Further support for a role for R7 RGS proteins in regulating body weight and adiposity has been provided through studies with Gβ5, a unique G protein β subunit. It has been reported that mice with a targeted deletion of one copy of

Figure 3: Alternative splicing of a minigene reporter construct containing the RGS9 TTTCT I/D alleles.

A. The RGS9 I and D allele fragment-containing minigenes were separately co-transfected into cells with or without either PTB (left panel) or nPTB (right panel). The minigene transcripts were visualized by reverse transcription, PCR amplification and agarose gel electrophoresis. The bands "a", "b", and "c" represent alternatively spliced products of the minigene reporter. B. Densitometric quantification of the relative intensity of band "a", i.e. full-length alternatively spliced product, expressed as percentage of the total intensity from "a", "b", and "c" with and without co-expression of PTB (left panel) or nPTB (right panel) (*p<0.01, t test).

Gβ5, are heavier and have increased adiposity when compared to their wild-type counterparts [28]. Gβ5 protects R7 RGS family proteins from proteolysis and expression of R7 RGS family proteins, including RGS9, is eliminated or hugely reduced in the absence of Gβ5 [5,29].

According to the information from dbSNP database and our recent study with a smaller set of Asian subjects [25], the RGS9 I/D polymorphism is also common in Japanese and Koreans, which suggests that the polymorphism may have utility in biomarker panels for predicting obesity in Asian populations. It was more practicable to investigate the association in this population versus in Caucasians in whom the occurrence of the D allele is quite rare. In fact, our previous results with rodents indicate that RGS9 contributes to body weight set-points in multiple species and therefore is likely to contribute to regulating body-weight in humans regardless of ethnicity [25]. Furthermore, most of T2D patients included in this study were not obese. Inclusion of T2D patients with lean for the study may allow ascertaining whether the RGS9 I/D polymorphism are associated with T2D or not.

In conclusion, we present genetic and biochemical data to support a genetic role of RGS9 in obesity but unlikely in T2D. The RGS9 I/D polymorphism influences the post-transcriptional processing of the gene through an altered affinity for the splicing factor PTB and are associated with obesity.

Acknowledgements

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