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

Chong Shen

Meenakshi Sharma
University of Rhode Island

Daniel C. Reid

Pengtao Li

Jeremy Celver

See next page for additional authors

Follow this and additional works at: https://digitalcommons.uri.edu/bps_facpubs

Citation/Publisher Attribution
Shen C, Sharma M, Reid DC, Li P, Celver J, et al. (2014) A Polymorphic Microdeletion in the RGS9 Gene Suppresses PTB Binding and Associates with Obesity. J Diabetes Metab 5:437. doi: 10.4172/2155-6156.1000437
Available at: http://dx.doi.org/10.4172/2155-6156.1000437

This Article is brought to you for free and open access by the Biomedical and Pharmaceutical Sciences at DigitalCommons@URI. It has been accepted for inclusion in Biomedical and Pharmaceutical Sciences Faculty Publications by an authorized administrator of DigitalCommons@URI. For more information, please contact digitalcommons-group@uri.edu.
A Polymorphic Microdeletion in the RGS9 Gene Suppresses PTB Binding and Associates with Obesity

Creative Commons License

This work is licensed under a Creative Commons Attribution 4.0 License.

Authors

Chong Shen, Meenakshi Sharma, Daniel C. Reid, Pengtao Li, Jeremy Celver, Norhashimah Abu Seman, Jinfeng Chen, Senthil K. Vasan, Hairu Wang, Tianwei Gu, Ying Liu, Wan Nazaimoon Wan Mohamud, Hongbing Shen, Kerstin Brismar, William G. Fairbrother, Abraham Kovoor, and Harvest F. Gu

This article is available at DigitalCommons@URI: https://digitalcommons.uri.edu/bps_facpubs/25
A Polymorphic Microdeletion in the RGS9 Gene Suppresses PTB Binding and Associates with Obesity

Chong Shen1*, Meenakshi Sharma2*, Daniel C. Reid3*, Pengtao Li4, Jeremy Celver3, Norhashimah Abu Seman5,6, Jinfeng Chen1, Senthil K. Vasan5, Hairu Wang1, Tianwei Gu1, Ying Liu1, Wan Nazaimoon Wan Mohamad2, Hongbing Shen1, Kerstin Brismar5, William G. Fairbrother3#, Abraham Kovoor2#, Harvest F Gu2#

1Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University, Nanjing, P. R. China
2Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, Rhode Island, USA
3Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island, USA
4National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences; School of Basic Medicine, Peking Union Medical College, Beijing, P. R. China
5Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden
6Cardiovascular, Diabetes and Nutrition Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia

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

*These authors contributed equally to the work.

#Corresponding authors: Harvest F Gu, Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska University Hospital, Karolinska Institutet, Stockholm, Se-17176, Sweden, Tel: +46 851779515; E-mail: harvest.gu@ki.se.

Abraham Kovoor, Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island, RI 02881 United States of America, Tel: +1 401 874 4727; E-mail: abekovoor@uri.edu.

William Fairbrother, Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912, United States of America, Tel: +1 401 863 6215; E-mail: fairbrother@brown.edu.

Received August 20, 2014; Accepted September 18, 2014; Published September 24, 2014

Citation: Shen C, Sharma M, Reid DC, Li P, Celver J, et al. (2014) A Polymorphic Microdeletion in the RGS9 Gene Suppresses PTB Binding and Associates with Obesity. J Diabetes Metab 5: 437 doi:10.4172/2155-6156.1000437

Copyright: © 2014 Shen C, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
consensus high affinity binding motif for the ubiquitously expressed RNA binding Polypyrimidine Tract Binding Protein (PTB) [14]. PTB and its neuronal 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 overweight: 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 |
| Sample 2 | Chinese | Jiangsu, China | Adults with obese | 820 (66.0) | 59.9 ± 10.4 | 29.1 ± 2.0* | 6.0 ± 2.2* |
| Sample 3 | Chinese | Jiangsu, China | Children without overweight | 451 (43.6) | 10.1 ± 2.9 | 16.4 ± 1.5 | 4.2 ± 1.0 |
| Sample 4 | Chinese | Beijing, China | Children with overweight | 361 (47.3) | 10.1 ± 3.0 | 21.3 ± 3.8* | 4.3 ± 1.0 |
| Sample 5 | Malaysian | Peninsular, Malaysia | Non-diabetic controls | 1990 (63.5) | 60.1 ± 10.3 | 22.5 ± 2.9 | 4.4 ± 0.4 |
| Sample 4 | Chinese | Beijing, China | Type 2 diabetes | 1279 (65.3) | 60.2 ± 9.5 | 25.4 ± 3.7* | 8.7 ± 3.3* |
| Sample 5 | Malaysian | Peninsular, Malaysia | Non-diabetic controls | 1004 (51.7) | 55.6 ± 9.6 | 25.3 ± 3.6 | 5.0 ± 0.3 |
| Sample 4 | Chinese | Beijing, China | Type 2 diabetes | 1049 (47.3) | 55.8 ± 10.1 | 25.3 ± 3.4 | 9.0 ± 3.1* |
| Sample 5 | Malaysian | Peninsular, Malaysia | Non-diabetic controls | 575 (49.6) | 45.8 ± 15.1 | 26.0 ± 5.1 | 5.6 ± 0.7 |
| Sample 4 | Chinese | Beijing, China | Type 2 diabetes | 643 (51.7) | 56.3 ± 10.4 | 27.8 ± 5.2 | 8.6 ± 3.1* |

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: AHO12R) was designed and then synthesized by Life Technologies (Grand Island, USA). In addition, a specific PCR protocol was included RGS9delF1 (5’-agttggaagaagaaaagaggaaat-3’), RGS9delF2 (5’-agttggaagaagaaaaggggaaataaaagaaa-3’) and RGS9delR (5’-agttggaagaagaaaaggggaaataaaagaaa-3’). The PCR program was 95˚C, 5min; (95˚C, 30sec; 54˚C, 30sec; 72˚C,30 sec) x 31; 72˚C, 8 min. PCR templates for a T7 polymerase reaction using the MEGA short script slow at room temperature. These mixtures served as transcription templates for a T7 polymerase reaction using the MEGA short script and contained dNTP mixture and RNase inhibitor (Ambion) and buffer provided by the enzyme supplier.

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 been previously described by Wang and colleagues [22]. The small constitutively spliced central exon of the original splicing reporter contains two restriction cloning sites, XhoI and ApaI, 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 TTTTCT 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 TTTTCT 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.](image-url)

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 a 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, 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 dodecy1-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/PowerSampleSize). 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 (X²) 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 meta-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 1. 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=0.753, 95% CI 0.625-0.906) lower frequency (23.7 vs 29.0%, P=0.003, OR=0.753, 95% CI 0.625-0.906) than 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) |
|----------------------------|----------------|----------|--------------------|--------------------|
| Shenzhen/China             | Adults, lean BMI 18.5-23.9 | I/I | 566 | 452 | 82 | 0.28 | 0.019* |
|                            | Adults, obese BMI ≥ 28 | I/D | 463 | 310 | 47 | 0.25 | 0.838 (0.723-0.972)* |
| Beijing/China              | Children BMI p>80 | I/D | 202 | 139 | 20 | 0.25 | 3.1×10** |
| Shanghai/China             | Non-diabetic | I/D | 1035 | 814 | 141 | 0.28 | 0.820* |
|                            | T2DM           | D/D | 678 | 509 | 92 | 0.27 | 1.014 (0.870-1.147)* |
| Malaysian                  | Non-diabetic | I/D | 537 | 372 | 95 | 0.28 | 0.999 |
|                            | T2DM           | D/D | 555 | 397 | 97 | 0.28 | 1.000 (0.876-1.141)* |
| Malay                      | Non-diabetic | I/D | 331 | 241 | 57 | 0.28 | 0.741 |
|                            | T2DM           | D/D | 295 | 239 | 50 | 0.29 | 0.968 (0.785-1.188)* |

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.
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=0.669, 95% CI 0.437-0.935) and moderately associated with a decreased risk for obesity in boys (24.3% vs. 30.5%, P=0.03, OR=0.724, 95% CI 0.53-0.958).

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 meta-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 Poly-Acrylamide Gel Electrophoresis (PAGE) and their proteins. The mobility of these labeled RNA-binding proteins can be measured by Poly-Acrylamide Gel Electrophoresis (PAGE) and their identity confirmed by immunoprecipitation.

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 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 corresponding to the molecular weight of PTB. radiolabeled RNA oligos corresponding to the I and D alleles and the previously characterized PTB ligand, S11, were radiolabeled, 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).

Figure 2: PTB binds the RGS9 TTTCT insertion (I) allele with 10-fold higher affinity than the deletion (D) allele.

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

| Region/Country Population | Gender | Group | Genotype | Allele D Frequency | P-value OR (95% CI) |
|---------------------------|--------|-------|----------|-------------------|-------------------|
| Jiangsu/China Chinese     | Women  | Adults, lean BMI 18.5-23.9 | I/I  | 0.290            | 0.003*            |
|                           |        | Adults, obese BMI ≥ 28     | I/D  | 0.237            | 0.753 (0.625-0.906)* |
|                           | Men    | Adults, lean BMI 18.5-23.9 | I/I  | 0.262            | 1.060 (0.782-1.293)* |
|                           |        | Adults, obese BMI ≥ 28     | I/D  | 0.286            | 0.669 (0.437-0.835)* |
| Jiangsu/China Chinese     | Girls  | Children BMI p20-50        | I/I  | 0.363            | 0.002*            |
|                           |        | Children BMI p>80         | I/D  | 0.255            | 0.604 (0.437-0.835)* |
| Jiangsu/China Chinese     | Boys   | Children BMI p20-50        | I/I  | 0.305            | 0.038*            |
|                           |        | Children BMI p>80         | I/D  | 0.243            | 0.724 (0.533-0.983)* |
| Jiangsu/China Chinese     | Women  | Non-diabetic BMI<28        | I/I  | 0.266            | 0.669*            |
|                           |        | T2DM BMI=28                | I/D  | 0.266            | 0.967 (831-1.126)* |
| Jiangsu/China Chinese     | Men    | Non-diabetic BMI<28        | I/I  | 0.317*            | 1.113 (0.902-1.372)* |
|                           |        | T2DM BMI=28                | I/D  | 0.279            | 1.135 (0.848-1.517)* |
| Beijing/China Chinese     | Women  | Non-diabetic BMI<28        | I/I  | 0.274            | 0.815*            |
|                           |        | T2DM BMI=28                | I/D  | 0.271            | 0.977 (0.805-1.168)* |
| Beijing/China Chinese     | Men    | Non-diabetic BMI<28        | I/I  | 0.286            | 0.833 (1.020-1.223)* |
|                           |        | T2DM BMI=28                | I/D  | 0.291            | 0.828 (0.599-1.094)* |
| Peninsular Malaysia Asian | Women  | Non-diabetic BMI<28        | I/I  | 0.280            | 0.168*            |
|                           |        | T2DM BMI=28                | I/D  | 0.289            | 0.809 (0.599-1.094)* |
| Peninsular Malaysia Asian | Men    | Non-diabetic BMI<28        | I/I  | 0.290            | 0.239*            |
|                           |        | T2DM BMI=28                | I/D  | 0.282            | 0.959 (0.848-1.517)* |

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.
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 by 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 we 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, RG66 and RG7 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
Gβ5, are heavier and have increased adiposity when compared to their wild-type counterparts [28]. Gβ5 protects R7 GFS family proteins from proteolysis and expression of R7 GFS 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 is 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

The authors wish to thank all subjects for their participation. The project described was supported by the grants from National Natural Science Foundation of China (No. 81273185 and 30800947), the Natural Science Foundation of Jiangsu Province (BK2011776), Science & Technology Program of Jiangsu Province (BE2009613) and the Priority Academic Program Development for the Higher Education Institutions (SC), Family Erling-Persson Foundation (KB), Young Elite Scientist Sponsorship Program of CAS (YESS), Key Science & Technology Program of Jiangsu Province (BK2011776), and Science & Technology Program of Jiangsu Province (BE2009613). The authors wish to thank all subjects for their participation.

References

1. Visscher TL, Seidell JC (2001) The public health impact of obesity. Annu Rev Public Health 22: 355-375.
2. Campos P, Saguy A, Ernsberger P, Oliver E, Gaesser G (2006) The epidemiology of overweight and obesity: public health crisis or moral panic? Int J Epidemiol 35: 55-60.
3. Wang Y, Qi J, Shan XY, Wang QJ, Ge KY (2007) Is China facing an obesity epidemic and the consequences? The trends in obesity and chronic disease in China. Int J Obes (Lond) 31: 177-188.
4. Yang W, Lu J, Weng J, Jia W, Li J, et al. (2010) Prevalence of diabetes among men and women in China. N Engl J Med 362: 1090-1101.
5. Anderson GR, Posokhova E, Martemyanov KA (2009) The R7 GFS protein family: multi-subunit regulators of neuronal G protein signaling. Cell Biochem Biophys 54: 33-46.
6. Vries L, Zheng B, Fischer T, Elenko E, Farquhar MG (2000) The regulator of G protein signaling family: multi-subunit regulators of neuronal G protein signaling. Cell Biochem Biophys 54: 33-46.
7. Rahman Z, Gold SJ, Potenza MN, Cowan CW, Ni YG, et al. (1999) Cloning and characterization of RGS9-2, a striatal-enriched alternatively spliced product of the RGS9 gene. J Neurosci 19: 2016-2026.
8. Zhang K, Howes KA, He W, Bronson JD, Pettenati MJ, et al. (1999) Structure, alternative splicing, and expression of the human RGS9 gene. Gene 240: 23-34.
9. Stiepak VZ (2009) Structure, function, and localization of Gβγ-RGS complexes. Prog Mol Biol Trans Sci 86: 157-203.
10. Kelley AE, Baldo BA, Pratt WE, Will MJ (2005) Corticotropin-hypothalamic circuitry and food motivation: integration of energy, action, and reward. Physiol Behav 86: 773-795.
11. Berndt KC, Ho CY, Richard JM, Difeliceantonio AG (2010) The tempted brain eats: pleasure and desire circuits in obesity and eating disorders. Brain Res 1350: 43-64.
12. Sice E, Spoor S, Ng J, Zaid DH (2009) Relation of obesity to consummatory and anticipatory food reward. Physiol Behav 97: 551-560.
13. http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=3215227
14. Reid DC, Chang BL, Gunderson SJ, Alpert L, Thompson WA, et al. (2009) Next-generation SELEX identifies sequence and structural determinants of splicing factor binding in human pre-mRNA sequence. RNA 15: 2385-2397.
15. Spellman R, Lorian M, Smith CW (2007) Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1. Mol Cell 27: 420-434.
16. Sawicka K, Bushell M, Sriggska KA, Willis AE (2008) Polypyrindimine-tract-binding protein: a multifunctional RNA-binding protein. Biochem Soc Trans 36: 641-647.
17. Anderson GR, Lujan R, Semenov A, Prawetorini M, Posokhova EK, et al. (2007) Expression and localization of RGS9-2/G 5/R7BP complex in vivo is set by dynamic control of its constitutive degradation by cellular cytosine proteases. J Neurosci 27: 14117-14127.
18. Waugh JL, Celver J, Sharma M, Dufresne RL, Terzic D, et al. (2011) Association between regulator of G protein signaling 9 and body weight. PLoS One 6: e27994.
19. Ji CY; Working Group on Obesity in China (WGOC) (2007) Report on childhood obesity in China (4) prevalence and trends of overweight and obesity in Chinese urban school-age children and adolescents, 1985-2000. Biomed Environ Sci 20: 1-10.
20. Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15: 539-553.
21. Liu Y, Niu N, Zhu X, Du T, Wang X, et al. (2008) Genetic variation and association analyses of the nuclear respiratory factor 1 (nrf1) gene in Chinese patients with type 2 diabetes. Diabetes 57: 777-782.
22. Wang Z, Rolish ME, Yeo G, Tung V, Mawson M, et al. (2004) Systematic identification and analysis of exonic splicing silencers. Cell 119: 831-845.
23. Sawicka K, Bushell M, Sriggska KA, Willis AE (2008) Polypyrindimine-tract-binding protein: a multifunctional RNA-binding protein. Biochem Soc Trans 36: 641-647.
24. Zappia M, Annesi G, Niccolitti G, Arabia G, Annesi F, et al. (2005) Sex differences in clinical and genetic determinants of levodopa peak-dose dyskinesias in Parkinson disease: an exploratory study. Arch Neurol 62: 601-605.
25. Lovejoy JC, Sainsbury A; Stock Conference 2008 Working Group (2009) Sex differences in obesity and the regulation of energy homeostasis. Obes Rev 10: 154-167.
26. Meyer MR, Clegg DJ, Prossnitz ER, Barton M (2011) Obesity, insulin resistance and diabetes: sex differences and role of oestrogen receptors. Acta Physiol (Oxf) 203: 259-269.
27. Aissani B, Perusse L, Lapointe G, Chagnon YC, Bouchard L, et al. (2006) A quantitative trait locus for body fat on chromosome 1q43 in French Canadians: linkage and association studies. Obesity (Silver Spring) 14: 1605-1615.
28. Wang Q, Levay K, Chanturiya T, Dvoriantchikova G, Anderson KL, et al. (2011) Targeted deletion of one or two copies of the G protein β subunit G5/γ gene has distinct effects on body weight and behavior in mice. FASEB J 25: 3949-3957.
29. Chen CK, Eversole-Cire P, Zhang H, Mancino V, Chen YJ, et al. (2003) Instability of GGL domain-containing RGS proteins in mice lacking the G protein beta-subsunit Gβ5. Proc Natl Acad Sci U S A 100: 6604-6609.

Citation: Shen C, Sharma M, Reid DC, Li P, Celver J, et al. (2014) A Polymorphic Microdeletion in the RGS9 Gene Suppresses PTB Binding and Associates with Obesity. J Diabetes Metab 5: 437 doi:10.4172/2155-6156.1000437