

No association between IRS-1 promoter methylation and type 2 diabetes

JIANGBO MA^{1*}, JIA CHENG^{2,3*}, LINGYAN WANG^{4*}, HONGWEI WANG⁵, LEITING XU^{2,3},
PANPAN LIU^{2,3}, SHIZHONG BU³, LINA ZHANG^{2,3}, YANPING LE^{2,3}, MENG YE²,
QINWEN WANG^{2,3}, YUPING SHI⁶ and SHIWEI DUAN^{2,3}

¹Zhejiang Provincial People's Hospital, Hangzhou, Zhejiang 310014; ²The Affiliated Hospital, School of Medicine, Ningbo University, Ningbo, Zhejiang 315000; ³Zhejiang Provincial Key Laboratory of Pathophysiology, Ningbo University, Ningbo, Zhejiang 315211; ⁴Bank of Blood Products, Ningbo No. 2 Hospital, Ningbo, Zhejiang 315010, P.R. China;

⁵Section of Endocrinology, The University of Chicago Pritzker School of Medicine, Chicago, IL 60637, USA;

⁶Second Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou, Zhejiang 310009, P.R. China

Received March 5, 2013; Accepted June 24, 2013

DOI: 10.3892/mmr.2013.1569

Abstract. As a candidate gene for type 2 diabetes (T2D), insulin receptor substrate-1 (*IRS-1*) gene variations were found to be associated with the risk of T2D. The aim of our study was to investigate the contribution of promoter DNA methylation of the *IRS-1* gene to the risk of T2D. Using bisulphite pyrosequencing technology, the DNA methylation levels of 3 CpG dinucleotides within the *IRS-1* gene promoter were measured in 48 T2D patients and 48 age- and gender-matched healthy controls. Our results indicated that there was no significant association between the methylation of the *IRS-1* gene promoter and the risk of T2D ($P>0.1$). A breakdown analysis by gender revealed that *IRS-1* promoter methylation was not associated with an increased risk of T2D for either gender ($P>0.1$), although there were significantly lower methylation levels of CpG1 ($P=0.002$) and CpG2 ($P=0.043$) within the *IRS-1* gene promoter in males than in females.

Introduction

A total of 371 million people worldwide are estimated to have diabetes. Type 2 diabetes (T2D) accounts for 90% of diabetes worldwide, and this epidemic has become a major challenge (1,2). T2D is caused by impaired insulin secretion and/or impaired peripheral insulin resistance (3). T2D complications consist of blindness, cardiovascular disease, neurological and renal abnormalities that are mortiferous and result in disabling problems (4). The most generally accepted hypothesis is that T2D is a polygenic disease resulting from the interaction of several genetic and environmental factors (5). However, little is known regarding the mechanisms for these environmental factors in T2D pathogenesis through the genetic pathway.

As an epigenetic mechanism, the methylation of promoter DNA is capable of stably altering gene expression (5,6). DNA methylation usually suppresses target gene expression (6,7). It has been suggested that DNA methylation contributes to the interaction between environmental stressors and genetic factors (5,6,8). Furthermore, epigenetic changes may provide the connection between environmental exposure and individual genetic background (5). Aberrant DNA methylation of a variety of genes was observed in diabetic islets (5). These epigenetic abnormalities may be involved in the pancreatic β -cell functionality, apoptosis and adaptation to environmental metabolic stress, and eventually lead to T2D (5,6).

The interaction between genetic and environmental factors may be reflected by modifying the methylation of promoter DNA in metabolic disorders (6). Recently, T2D-related epigenetic imbalance of the human genome was found in pancreatic islets (5), skeletal muscle (6), adipose tissues (6) and peripheral blood (9). Fat mass- and obesity-associated (FTO) gene hypomethylation may indicate a significantly increased risk of T2D in young individuals (9). Epigenetic studies on T2D have unlocked the mechanisms for the correlation between individual predisposition and genetic characters (9).

Insulin receptor substrate-1 (*IRS-1*) has a marked effect on the transmission process from insulin and insulin-like growth

Correspondence to: Professor Shiwei Duan, Ningbo University, Ningbo, School of Medicine, 818 Fenghua Road, Jiangbei, Zhejiang 315211, P.R. China
E-mail: duanshiwei@nbu.edu.cn

Dr Yuping Shi, The Second Affiliated Hospital of Zhejiang University, College of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang 310009, P.R. China
E-mail: shiyuping007@hotmail.com

*Contributed equally

Abbreviations: T2D, type 2 diabetes; IRS-1, insulin receptor substrate 1; FTO, fat mass and obesity associated; OR, odd ratios; CI, confidence interval; ALT, alanine aminotransferase; IGT, impaired glucose tolerance

Key words: insulin receptor substrate 1, type 2 diabetes, DNA methylation, promoter

factor-1 receptors to the intracellular signaling pathways of glycometabolism (10). *IRS-1* gene variants have been associated with insulin-resistance as well as with the increased risk of major adverse cardiovascular events in T2D (11,12). IRS proteins have been considered to play broad roles in the common regulatory path to diabetes (10). According to previous findings regarding the contribution of DNA methylation to the risk of T2D, we assessed the differences in the methylation levels of the *IRS-1* gene promoter between T2D patients and their healthy counterparts. These results may clarify the epigenetic role of the *IRS-1* gene in the pathogenesis of T2D.

Materials and methods

Subjects. This study comprised 48 T2D cases and 48 controls from the Zhejiang Provincial People's Hospital and the Affiliated Hospital of Ningbo University. Detailed patient characteristics are provided in Table I. All the individuals are Han Chinese who have resided in Zhejiang for at least three generations. T2D patients were recruited if they had a fasting plasma glucose concentration of ≥ 7.0 mmol/l or plasma glucose levels of ≥ 11.1 mmol/l 2 h after a standard glucose challenge (1). Healthy control individuals were recruited with standard fasting blood glucose levels of < 6.1 mmol/l. None of the controls had a family history of T2D in first-degree relatives or had received any drug therapy. Subjects were excluded from this study if they had hypertension, coronary heart disease, renal inadequacy, drug abuse or a serious disease. The present study was approved by the Ethics Committee of Ningbo University and written informed consent was obtained from all subjects. Blood samples were collected in 3.2% citrate sodium-treated tubes and subsequently stored at -80° for DNA extraction.

Phenotype collection. Blood samples were obtained following a 12-h overnight fast from the antecubital vein into vacutainer tubes containing EDTA. Plasma levels of cholesterol, triglyceride, alanine aminotransferase (ALT), uric acid and glucose were enzymatically measured using a CX7 analyzer (Beckman, Fullerton, CA, USA).

DNA preparation and methylation assay. Human genomic DNA was isolated from peripheral blood samples using the nucleic acid extraction automatic analyzer (Lab-Aid 820, Zeesan, Xiamen, China). DNA was quantified using the PicoGreen® double-strand (dsDNA) DNA quantification kit (Molecular Probes, Inc., Eugene, OR, USA). Bisulphite pyrosequencing technology was used to determine the methylation levels of 3 CpG dinucleotides on the fragment (chr2:227662980-227663002) within the *IRS-1* promoter (Fig. 1). Pyrosequencing assays combined sodium bisulfite DNA conversion chemistry, polymerase chain reaction (PCR) amplification and sequencing by synthesis assay of the target sequence. Sodium bisulfite preferentially deaminated unmethylated cytosine residues to thymines (following PCR amplification), whereas methyl-cytosines remained unmodified. PCR primers were selected by using PyroMark Assay Design software v2.0.1.15. The PCR and pyrosequencing primers for *IRS-1* gene promoter amplification are described in Table II.

Table I. Patient characteristics.

Characteristics	Mean \pm SE	Range
Age (years)	59.2 \pm 7.5	(35.0-69.0)
Gender (M/F)	48/48	
BMI (kg/m ²) ^a	23.71 \pm 3.28	(17.15-42.96)
Total cholesterol (mmol/l)	5.19 \pm 0.96	(2.95-7.90)
Total triglycerides (mmol/l)	1.60 \pm 1.36	(0.40-9.92)
Glucose (mmol/l)	6.76 \pm 2.65	(4.38-22.84)
ALT (IU/l)	21.50 \pm 15.90	(5.00-99.00)
Uric acid (μ mol/l)	294.90 \pm 81.10	(132.00-531.00)
CpG1	83.16 \pm 5.03	(60.00-91.00)
CpG2	84.61 \pm 4.39	(63.00-91.00)
CpG3	92.49 \pm 6.33	(58.00-100.00)

^an=86 (39 males vs. 47 females). SE, standard error; M, male; F, female; ALT, alanine aminotransferase; BMI, body mass index.

Table II. Primers for insulin receptor substrate-1 promoter methylation analysis.

Forward:	5'-AGGTTGGGGAGGATTGAG-3'
Reverse:	5'-Biotin-ATCTAACCCAAACCCTTAACTT-3'
Sequencing:	5'-GGGGAGGATTGAGT-3'

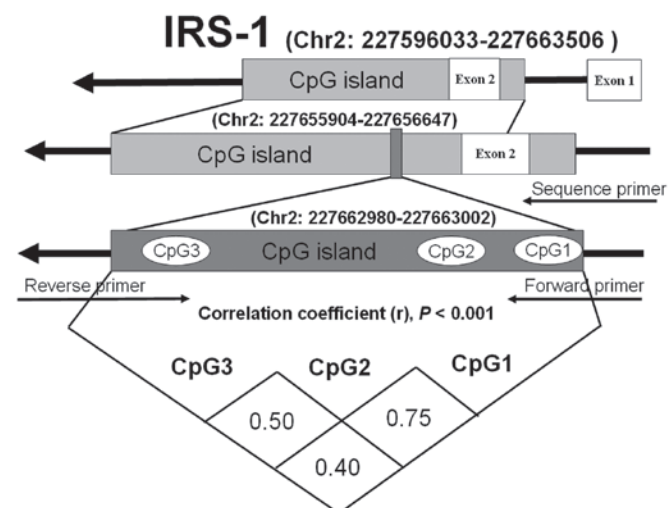


Figure 1. The CpG islands on the fragment within insulin receptor substrate-1 IRS-1 promoter.

Statistical analysis. Pearson's Chi-square test was used to compare categorical variables and mean group differences. Continuous variables were compared using the Student's t-test. Using Pearson's correlation analysis, the association between *IRS-1* DNA methylation and the metabolic features of subjects was determined. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using PASW Statistics 18.0 software (SPSS, Inc., Somers, NY, USA).

Table III. Patient characteristics according to gender.

Characteristics	Males (n=48), mean \pm SE	Females (n=48), mean \pm SE	P-value
Age (years)	59.10 \pm 8.60	59.40 \pm 6.30	0.850
BMI (kg/m ²) ^a	24.12 \pm 4.03	23.37 \pm 2.49	0.292
Total cholesterol (mmol/l)	4.96 \pm 0.94	5.43 \pm 0.93	0.015
Total triglycerides (mmol/l)	1.59 \pm 1.28	1.61 \pm 1.45	0.959
Glucose (mmol/l)	6.77 \pm 3.07	6.76 \pm 2.19	0.995
ALT (IU/l)	25.80 \pm 20.60	17.30 \pm 7.40	0.009
Uric acid (μ mol/l)	325.60 \pm 79.00	264.30 \pm 71.60	0.000
CpG1	81.58 \pm 5.53	84.73 \pm 3.94	0.002
CpG2	83.71 \pm 4.50	85.52 \pm 4.13	0.043
CpG3	92.42 \pm 7.66	92.56 \pm 4.70	0.911

^an=86 (39 males vs. 47 females). SE, standard error; ALT, alanine aminotransferase; BMI, body mass index.

Table IV. Patient characteristics according to the previous history of diabetes (n=96).

Characteristics	Diabetics (n=48), mean \pm SE	Non-diabetics (n=48), mean \pm SE	P-value
Age (years)	59.20 \pm 7.50	59.20 \pm 7.50	1.000
BMI (kg/m ²) ^a	24.17 \pm 4.18	23.18 \pm 1.64	0.146
Total cholesterol (mmol/l)	5.34 \pm 0.83	5.05 \pm 1.06	0.140
Total triglycerides (mmol/l)	1.90 \pm 1.69	1.31 \pm 0.82	0.034
Glucose (mmol/l)	8.31 \pm 2.91	5.22 \pm 0.92	0.000
ALT (IU/l)	25.10 \pm 18.50	18.00 \pm 12.10	0.028
Uric acid (μ mol/l)	289.30 \pm 70.50	300.60 \pm 90.90	0.499
CpG1	82.60 \pm 5.63	83.71 \pm 4.35	0.285
CpG2	83.94 \pm 5.17	85.29 \pm 3.37	0.132
CpG3	92.79 \pm 7.96	92.19 \pm 4.16	0.643

^an=86 (46 diabetics vs. 40 non-diabetics). SE, standard error; ALT, alanine aminotransferase; BMI, body mass index.

Results

A total of 48 cases of T2D and 48 age- and gender-matched controls were recruited in this study. We found 3 CpG dinucleotides within the *IRS-1* gene promoter and correlation of DNA methylation levels among the CpGs is shown in Fig. 1 ($r=0.75$, 0.50 and 0.45). Patient characteristics are shown in Table I. We explored the association between *IRS-1* promoter methylation levels in all the subjects and genders. Our results revealed that CpG1 and CpG2 of the *IRS-1* gene promoter had significantly higher methylation in females compared with males (CpG1, $P=0.002$; CpG2, $P=0.043$) (Table III). Furthermore, the total cholesterol levels in male subjects were significantly lower ($P=0.015$) than in females, and the ALT levels of male subjects were significantly higher than in females ($P=0.009$).

The total triglyceride levels of T2D subjects were higher compared with those of the controls ($P=0.034$). Significant differences in ALT levels were observed between the T2D subjects and the controls ($P=0.028$). No differences were observed for the *IRS-1* gene promoter DNA methylation

(including CpG1-3) between the T2D subjects and healthy controls ($P>0.05$). A breakdown analysis by gender was unable to determine a significant correlation between the occurrence of T2D and DNA methylation of the *IRS-1* promoter (CpG1-3) for either gender (Table V). Notably, there was a significantly higher ALT level in female T2D subjects compared with the healthy female controls ($P=0.019$). By contrast, a similar ALT level was found between T2D cases and healthy controls in the male subgroup ($P=0.119$). This gender-specific difference of ALT levels may be useful in identifying the precise sexual disparity for T2D development and hepatic function for future studies.

Discussion

T2D is a heterogeneous disease with multiple etiological factors such as genetic risk, diet and physical activity (3,12). The interaction of these factors results in pancreatic β -cell failure so that insulin secretion is not capable of compensating for peripheral insulin resistance, insulin secretion

Table V. Patient characteristics by gender.

Characteristics	Diabetic mean \pm SE	Non-diabetic mean \pm SE	P-value
Men (n=48)			
Age (years)	59.10 \pm 8.70	59.10 \pm 8.70	
BMI (kg/m ²) ^a	24.92 \pm 5.17	23.10 \pm 1.21	0.124
Total cholesterol (mmol/l)	5.06 \pm 0.74	4.86 \pm 1.11	0.464
Total triglycerides (mmol/l)	1.81 \pm 1.56	1.38 \pm 0.90	0.254
Glucose (mmol/l)	8.59 \pm 3.49	4.94 \pm 0.34	0.000
ALT (IU/l)	30.40 \pm 23.80	21.10 \pm 15.90	0.119
Uric acid (μ mol/l)	304.70 \pm 70.60	346.50 \pm 82.70	0.066
CpG1	80.42 \pm 6.58	82.75 \pm 4.06	0.146
CpG2	82.96 \pm 5.16	84.46 \pm 3.68	0.253
CpG3	93.08 \pm 9.62	91.75 \pm 5.16	0.553
Women (n=48)			
Age (years)	59.40 \pm 6.40	59.40 \pm 6.40	
BMI (kg/m ²) ^b	23.49 \pm 2.97	23.25 \pm 1.93	0.747
Total cholesterol (mmol/l)	5.62 \pm 0.83	5.24 \pm 1.00	0.161
Total triglycerides (mmol/l)	1.98 \pm 1.85	1.23 \pm 0.75	0.071
Glucose (mmol/l)	8.04 \pm 2.22	5.49 \pm 1.20	0.000
ALT (IU/l)	19.80 \pm 8.60	14.80 \pm 4.80	0.019
Uric acid (μ mol/l)	273.90 \pm 68.30	254.60 \pm 75.00	0.357
CpG1	84.79 \pm 3.39	84.67 \pm 4.50	0.914
CpG2	84.92 \pm 5.09	86.13 \pm 2.86	0.316
CpG3	92.50 \pm 6.07	92.63 \pm 2.89	0.928

^an=39 (22 diabetics vs. 17 non-diabetics), ^bn=47 (24 diabetics vs. 23 non-diabetics). SE, standard error; ALT, alanine aminotransferase; BMI, body mass index.

may be increased further in the impaired glucose tolerance (IGT) stage, however, subsequently, at the diabetic stage, β -cell mass is markedly reduced (10,13). IRS proteins are a common element of the insulin/IGF-signaling pathway for peripheral insulin response and pancreatic β -cell growth and function (10). IRS-1 is important in the insulin action pathway including the activation of related kinases and the subsequent increase in glucose transport (14,15). It has been demonstrated that the unbalanced expression of IRS-1 is involved in the pathogenesis of T2D and obesity (12). In this study, we investigated the role of IRS-1 DNA methylation in T2D. This study provides new information regarding epigenetic regulation of IRS-1 in T2D.

Findings of this study suggest that there was a difference in the DNA methylation level of the *IRS-1* promoter between genders (Table III). The methylation levels of CpG1 and CpG2 were higher in females than in males. The relatively higher DNA methylation status of the *IRS-1* promoter in females may be ascribed to the long-term exposure to estrogen. Recent studies have demonstrated that long-term estrogen exposure was responsible for DNA methylation patterning and epigenetic silencing during tissue development *in vitro*, and in animal models (16-19). In addition, nutritional status has also been demonstrated to be involved in the mechanism for epigenetic regulation (6,20). A chronic, high-fat diet in rat fathers has been

associated with the mutation in the promoter methylation status of their female offspring (6,20).

The aim of this study was to explore the unknown relationship between the CpG methylation levels in the *IRS-1* promoter and the risk of T2D and the exact role of *IRS-1* epigenetics in this metabolic disease. The results showed no significant difference in the methylation levels of the *IRS-1* promoter between the controls and the T2D patients (Table IV). It is notable that although there was a positive gender-specific difference in *IRS-1* promoter methylation levels among total subjects, a negative connection was observed in the gender-dependent analysis (Table V). These results indicated that the DNA methylation mechanism of the gene promoter may not play a significant role in the epigenetic regulation pathway of *IRS-1* expression. It was reported that *IRS-1* gene variations were associated with the risk of T2D (12) and an increased risk of a major adverse cardiovascular event (11,12,21). Furthermore, the expression of *IRS-1* may be target-regulated by miR-126 which inhibits IRS-1 translation (22). These diversified regulatory pathways of IRS-1 biological function may be why the DNA methylation status of this gene promoter exerts no significant effects on the development of T2D.

In conclusion, findings of this study suggest that the DNA methylation levels of the *IRS-1* promoter in blood are not related to the occurrence of T2D. This finding contributes to

the understanding of the epigenetic value of T2D candidate genes such as *IRS-1*. The negative result may be helpful to guide future gene-based treatment strategies and avoid developing non-functional genetic drugs. Additional studies are required in order to identify the epigenetic regulation network of *IRS-1* pathway in T2D.

Acknowledgements

This study was supported by the grants from National Natural Science Foundation of China (31100919), Natural Science Foundation of Zhejiang Province (LR13H020003), Ningbo Social Development Research Projects (2012C50032) and the K.C. Wong Magna Fund in Ningbo University.

References

- Shaw JE, Sicree RA and Zimmet PZ: Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract* 87: 4-14, 2010.
- Travers ME and McCarthy MI: Type 2 diabetes and obesity: genomics and the clinic. *Hum Genet* 130: 41-58, 2011.
- McCarthy MI: Genomics, type 2 diabetes, and obesity. *N Engl J Med* 363: 2339-2350, 2010.
- Stolar M: Glycemic control and complications in type 2 diabetes mellitus. *Am J Med* 123: S3-S11, 2010.
- Volkmar M, Dedeurwaerder S, Cunha DA, *et al*: DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients. *EMBO J* 31: 1405-1426, 2012.
- Barres R and Zierath JR: DNA methylation in metabolic disorders. *Am J Clin Nutr* 93: S897-S900, 2011.
- Bird A: Perceptions of epigenetics. *Nature* 447: 396-398, 2007.
- Villeneuve LM and Natarajan R: The role of epigenetics in the pathology of diabetic complications. *Am J Physiol Renal Physiol* 299: F14-F25, 2010.
- Toperoff G, Aran D, Kark JD, *et al*: Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood. *Hum Mol Genet* 21: 371-383, 2012.
- White MF: IRS proteins and the common path to diabetes. *Am J Physiol Endocrinol Metab* 283: E413-E422, 2002.
- Angiolillo DJ, Bernardo E, Zanoni M, *et al*: Impact of insulin receptor substrate-1 genotypes on platelet reactivity and cardiovascular outcomes in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol* 58: 30-39, 2011.
- Sesti G, Federici M, Hribal ML, *et al*: Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J* 15: 2099-2111, 2001.
- Fernandez-Valverde SL, Taft RJ and Mattick JS: MicroRNAs in β -cell biology, insulin resistance, diabetes and its complications. *Diabetes* 60: 1825-1831, 2011.
- Araki E, Lipes MA, Patti ME, *et al*: Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372: 186-190, 1994.
- Rondinone CM, Wang LM, Lonnroth P, *et al*: Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* 94: 4171-4175, 1997.
- Bredfeldt TG, Greathouse KL, Safe SH, *et al*: Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. *Mol Endocrinol* 24: 993-1006, 2010.
- Christensen BC, Kelsey KT, Zheng S, *et al*: Breast cancer DNA methylation profiles are associated with tumor size and alcohol and folate intake. *PLoS Genet* 6: e1001043, 2010.
- Li L, Lee KM, Han W, *et al*: Estrogen and progesterone receptor status affect genome-wide DNA methylation profile in breast cancer. *Hum Mol Genet* 19: 4273-4277, 2010.
- Starlard-Davenport A, Tryndyak VP, James SR, *et al*: Mechanisms of epigenetic silencing of the *Rassf1a* gene during estrogen-induced breast carcinogenesis in ACI rats. *Carcinogenesis* 31: 376-381, 2010.
- Ng SF, Lin RC, Laybutt DR, *et al*: Chronic high-fat diet in fathers programs β -cell dysfunction in female rat offspring. *Nature* 467: 963-966, 2010.
- Martinez-Gomez LE, Cruz M, Martinez-Nava GA, *et al*: A replication study of the *IRS1*, *CAPN10*, *TCF7L2*, and *PPARG* gene polymorphisms associated with type 2 diabetes in two different populations of Mexico. *Ann Hum Genet* 75: 612-620, 2011.
- Zhang J, Du YY, Lin YF, *et al*: The cell growth suppressor, *mir-126*, targets *IRS-1*. *Biochem Biophys Res Commun* 377: 136-140, 2008.