Elevation of *PTPN1* promoter methylation is a significant risk factor of type 2 diabetes in the Chinese population

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Abstract. The present study aimed to investigate the contribution of DNA methylation of the protein tyrosine phosphatase, non-receptor type 1 (*PTPN1)* gene to the susceptibility to type 2 diabetes (T2D). Peripheral blood mononuclear cells (PBMCs) were collected from 97 patients with T2D and 97 age- and gender-matched controls. DNA methylation of the *PTPN1* gene promoter was evaluated by bisulfite pyrosequencing. Independent sample t-tests were used to compare the differences in the *PTPN1* promoter and other phenotypes between the patients with T2D and the controls. The results indicated a significant correlation between *PTPN1* promoter methylation and the risk of T2D. Additionally, a breakdown analysis by gender revealed that *PTPN1* methylation was associated with an increased risk of T2D in females. Furthermore, low-density lipoprotein (r=-0.183, P=0.046) and total cholesterol (r=-0.310, P=0.001) were inversely associated with *PTPN1* methylation in females. In conclusion, the results indicate that elevated *PTPN1* promoter methylation is a risk factor for T2D in the female Chinese population.

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Abbreviations: PTPN1, protein tyrosine phosphatase, non-receptor type 1; T2D, type 2 diabetes; PBMCs, peripheral blood mononuclear cells; PTP1B, protein-tyrosine phosphatase 1B; IR, insulin receptor; TG, triglyceride; TC, total cholesterol; LDL, low-density lipoprotein; UA, uric acid; CRE, creatinine

Key words: methylation, protein tyrosine phosphatase, non-receptor type 1, promoter, type 2 diabetes

Introduction

Type 2 diabetes (T2D), which accounts for >90% of cases of diabetes, is a complex metabolic disorder, and its prevalence is increasing worldwide. It is estimated by the International Diabetes Federation that the number of patients with T2D will reach 592 million in 2035 (1). Additionally, the high morbidity rate of T2D primarily results from the lack of understanding its pathogenic mechanisms.

Genes, environmental factors and their interactions are hypothesized to contribute to the development of T2D (2). Furthermore, a large number of susceptible genetic loci have been identified by genome wide association studies (3,4). Numerous environmental factors affecting T2D have also been established, including diet (5) and an unhealthy lifestyle (6). However, these observations only explain a small portion of the susceptibility to T2D (7).

Epigenetic modifications are heritable changes in gene function without any change in the nucleotide sequence. The functions of epigenetic modifications on diabetes have been recognized, such as on the origin and progression of diabetes (8-11). Primarily, DNA methylation, which is one of the most studied epigenetic modifications, is affected by several environmental factors, such as the diet (12), hyperglycemia (13) and hyperlipemia (5). There is that aberrant DNA methylation of pancreatic and duodenal homeobox 1 (13), monocyte chemotactic protein 1 (14), B-cell lymphoma/leukemia (*BCL11A)* (15) and glucokinase (*GCK)* (16) genes are associated with the risk of T2D. Furthermore, aberrant DNA methylation can alter gene expression (17). Given these observations, it appears that DNA methylation research may be useful for revealing the pathogenesis of T2D.

Insulin and leptin signaling are important in mammals (18-22). As an insulin signaling pathway gene, the protein tyrosine phosphatase, non-receptor type 1 (*PTPN1)* gene encodes the protein-tyrosine phosphatase 1B (PTP1B) protein (23). Additionally, overexpression of PTP1B hinders the insulin-signaling pathway by decreasing the phosphorylation of the insulin receptor (IR) and/or insulin receptor substrate 1 (20). Conversely, in PTP1B-deficient mice, insulin sensitivity is enhanced, and the phosphorylation of the IR in the liver and muscle is increased and prolonged (24,25). PTP1B

also attenuates leptin action through binding and dephosphorylating the Janus kinase 2-a receptor of leptin (21,22). It is noteworthy that small-molecule inhibitors of PTP1B have gained much progression in the treatment of T2D (26-28).

In the present study, methylation of the *PTPN1* promoter was hypothesized to be capable of altering gene expression. Furthermore, it is expected that testing the association of *PTPN1* methylation with the risk of T2D may provide further information concerning the pathogenic mechanism of T2D.

Materials and methods

Sample collection. A total of 97 T2D cases and 97 age- and gender-matched controls were recruited from 16 Community Health Service Centers, which were affiliated to the Shenzhen Nanshan Center for Chronic Disease Control (Shenzhen, China). The participants were between 50 and 70 years old. The body mass index (BMI) was calculated using the following formula: BMI=weight(kg)/height(m^2). Furthermore, T2D was diagnosed according to the 1999 World Health Organization standard, which defines diabetes as a fasting plasma glucose level ≥7.0 mmol/l, a 2 h oral glucose tolerance test level ≥11.1 mmol/l, or patients receiving antidiabetic medication treatment (29). Additionally, the subjects did not have a history of hypertension, coronary heart disease, drug abuse or other serious diseases. The study was approved by the Ethics Committees of the Affiliated Hospital of Ningbo University and Shenzhen Nanshan Center for Chronic Disease Control, and all participants provided written informed consent forms.

PBMC isolation and DNA extraction. Blood samples were collected in tubes containing EDTA and PBMCs were isolated using Ficoll-Paque (Histopaque 1077; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) by means of density gradient centrifugation (1,000 x g for 30 min at room temperature). Then the PBMCs were washed twice with PBS to prepare for DNA extraction. Human genomic DNA extraction and quantification were performed as described previously (30).

Biochemical and bisulfite pyrosequencing. Plasma triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL) levels, glucose concentration, creatinine (CRE) and uric acid (UA) were all measured using a CX7 Analyzer (Beckman Coulter, Inc., Brea, CA, USA) according to the manufacturer's protocol. The *PTPN1* methylation level was determined using pyrosequencing technology (Pyromark Gold Q24 Reagents; Qiagen China Co., Ltd., Shanghai, China). The primer sequences used were: 5'-biotin-TAGGGGTAGGGGATT GTA-3' (forward) and 5'-CTCCTTTTCCATCTCCATA-3' (reverse), and 5'-TTTTCCATCTCCATAA-3' (sequencing primer). All oligomers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Detailed information on the methylation assay has been provided in previous studies (15,16).

Statistical analysis. Independent sample t-test was used to compare the differences of continuous variables between the patients with T2D and controls. Pearson's correlation analysis was used to analyze the association between *PTPN1* methylation and metabolic characteristics of subjects. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using PASW statistics version 18.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Correlation analysis of the cytosine-guanine dinucleotide site (CpG) methylation of PTPN1 with T2D. As shown in Fig. 1, the bisulfite pyrosequencing assay was performed on a fragment (chr20: 49126618-49127435) in the promoter region of the *PTPN1* gene. A total of eight CpG sites were evaluated in order to explore their association with the risk of T2D. The methylation levels of all eight CpG loci showed a significant strong correlation (mean r>0.47, P<0.001).

Correlation between PTPN1 methylation and T2D. As shown in Table I, the methylation levels of the eight CpGs were significantly elevated in T2D cases compared with the controls (CpG1, P=0.001; CpG2, P=0.002; CpG3, P=0.001; CpG4, P=0.003; CpG5, P=0.007; CpG6, P=0.001; CpG7, P=0.036; CpG8, P=0.001). Increased levels of mean *PTPN1* (CpG1-8) methylation were also observed in T2D cases (P=0.001; Fig. 2). Further gender breakdown analysis demonstrated that the methylation levels of the CpGs (CpG1-8) were significantly elevated in female T2D cases compared with female controls (all P<0.05; Table I). However, no significant differences in *PTPN1* methylation between male T2D cases and male controls were identified (all P>0.05; Table I).

Clinical characteristics of the subjects. The results of the present study indicate that TG (P=0.018) and CRE ($P=6.00x10^{-3}$) levels are significantly different between the patients with T2D and the controls for all subjects (Table II). While the levels of TG (P=0.002), LDL (P=0.040) and UA (P=0.037) were significantly different between the cases and controls in females, the CRE levels were significantly different between the cases and controls in males (P=0.002; Table II). Further correlation analyses between clinical parameters and *PTPN1* methylation were also conducted. Notably, significant correlations of LDL (r=-0.310, P=0.001) and TC (r=-0.183, P=0.046) concentration with *PTPN1* methylation were observed only in females, and age (r=0.275, P=0.017) exhibited a significant correlation with *PTPN1* methylation only in male T2D patients (Fig. 3). Furthermore, no significant correlations between other phenotypes and *PTPN1* methylation were identified (P>0.05).

Discussion

DNA methylation is a common epigenetic modification, and increasing evidence suggests that it is important in T2D (15,16,31). The goal of the present study was to evaluate the contribution of *PTPN1* promoter methylation to the risk of T2D. The results of the present study revealed that T2D cases have a significantly higher methylation level in the *PTPN1* promoter compared with healthy controls, particularly in females. To the best of our knowledge, this is the first study to investigate the association of *PTPN1* promoter methylation with T2D in humans.

PTP1B is a key modulator of energy metabolism, involved in insulin (20), leptin (21) and other signal transduction

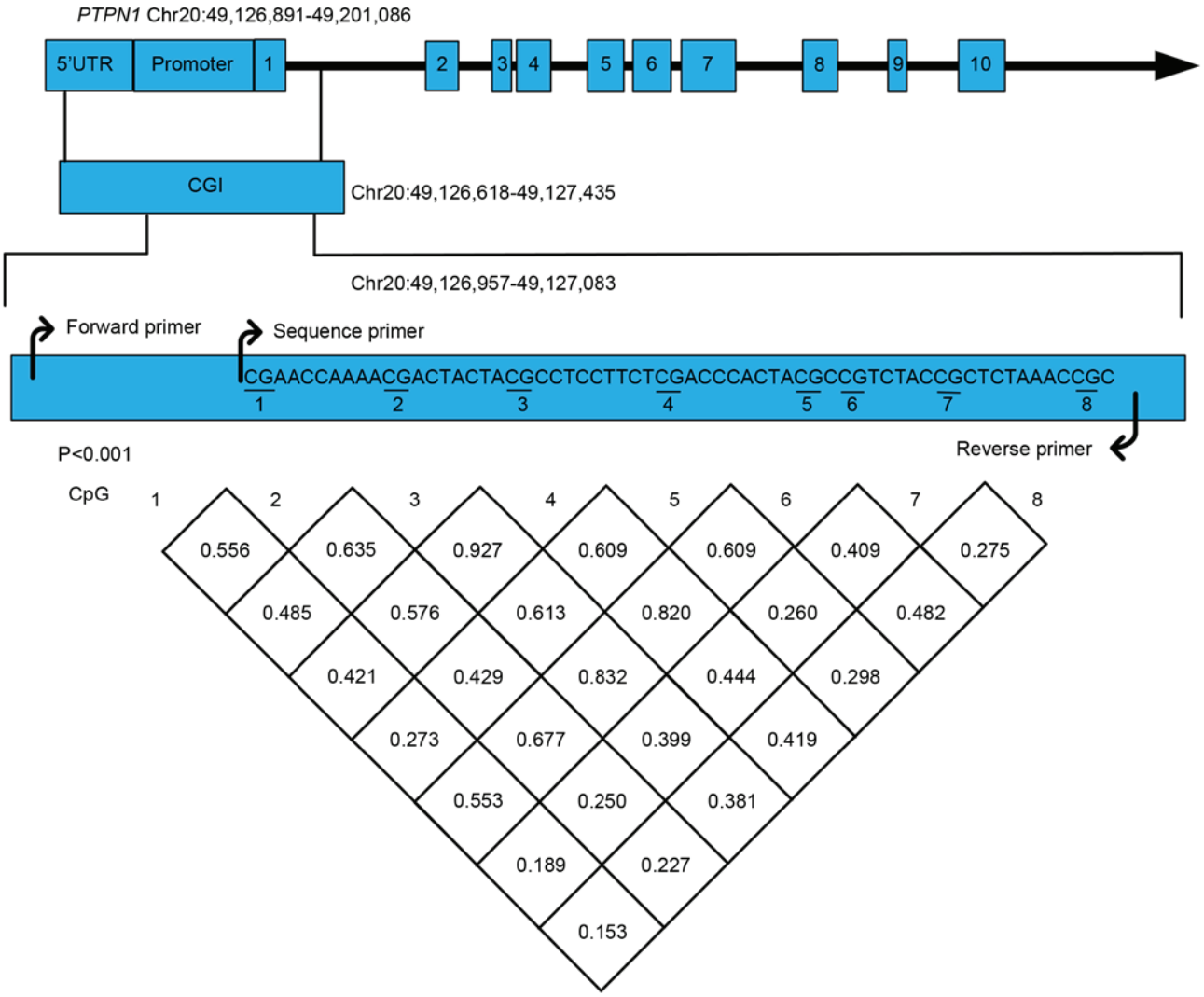

Figure 1. Pairwise correlation among eight methylated cytosine-guanine dinucleotide sites in the *PTPN1* gene promoter. *PTPN1*, protein tyrosine phosphatase, non-receptor type 1; CpG, cytosine-guanine dinucleotide site; UTR, untranslated region.

pathways (32). Insulin resistance is the core mechanism in the development of T2D (20). Previous studies in mice reveal that PTP1B-deficiency increases energy expenditure, tissue-specific insulin sensitivity, obesity resistance (20,24,25) and leptin signaling in the hypothalamus (33). Furthermore, PTP1B antisense oligonucleotide has been shown to improve insulin sensitivity in the liver and fat in diabetic mouse models (20). *PTPN1* 1484insG variation contributes to insulin resistance by upregulating *PTPN1* expression (34). Additionally, *PTPN1* polymorphisms have been shown to be significant risk factors for T2D (35). Murashov *et al* (36) demonstrated that the alteration of mouse *PTPN1* gene expression in the skeletal muscle of offspring may occur as a consequence of paternal long-term exercise, resulting in metabolic disorders, including lower energy expenditure, increased obesity risk, impaired glucose tolerance and elevated insulin levels in the offspring. The present study revealed that elevated *PTPN1* methylation is associated with T2D, providing new information useful for elaborating the role of this gene in the pathogenesis of T2D.

Promoter hypermethylation often downregulates gene transcription (37). Furthermore, DNA methylation has been shown to be important in the development of T2D (38,39). CpG sites in the mouse *Ins2* and human *INS* promoters can suppress insulin promoter-driven reporter gene activity, and thus regulate the insulin expression of pancreatic β cells (39). Our previous studies have suggested that CpG island methylation of *BCL11A* and *GCK* is associated with the risk of T2D in the male Chinese population (15,16). Furthermore, in the present study, *PTPN1* gene methylation was found to predict the risk of T2D in the female Chinese population. Additionally, it may be hypothesized that *PTPN1* methylation alters *PTPN1* gene expression or PTP1B activity, and thus increases the risk of T2D. However, further studies on the mechanism associating *PTPN1* methylation with T2D are required to support this hypothesis.

Previous studies have suggested that gender disparities are widely present in T2D and its complications (15,16,40,41). Sex hormone imbalance has significant association with the high risk of T2D and concomitant complications (42). Sex hormones regulate the level of DNA methylation (43) and their induced epigenetic changes may increase or reduce the disease risk (44). Additionally, the X-chromosome methylation in islets is higher in females than in males (45). Also, sex-specific DNA methylation patterns have been identified

Table I. Comparison of protein tyrosine phosphatase, non-receptor type 1 methylation levels (%) between cases of T2D and controls.

Characteristics	Controls	Cases	P-value
All subjects, n=194[a]			
CpG1	12.00±9.03	15.98±9.18	0.001
CpG2	6.41±3.16	8.68±4.48	0.002
CpG3	5.57±1.88	6.98±3.11	0.001
CpG4	15.46±5.76	18.87±8.09	0.003
CpG5	6.23±1.75	7.51±3.69	0.007
CpG6	4.60±0.87	5.21±1.24	0.001
CpG7	4.84±1.39	5.15±1.60	0.036
CpG8	4.37±0.70	4.89±0.76	0.001
CpG mean	7.43±2.37	9.16±3.02	$1.34x10^{-4}$
Female, n=120[b]			
CpG1	10.77±7.86	16.57±9.33	0.001
CpG2	5.92±2.14	8.77±5.02	0.001
CpG3	5.37±1.88	6.78±3.06	0.013
CpG4	14.80±5.73	18.34±8.25	0.021
CpG5	6.15±1.96	7.18±2.03	0.044
CpG6	4.51±0.92	5.23±1.29	0.006
CpG7	4.59±0.90	5.07±0.94	0.023
CpG8	4.26±0.76	4.84±0.71	$1.00x10^{-3}$
CpG mean	7.05±2.15	9.10±3.12	$4.82x10^{-4}$
Male, n=74[c]			
CpG1	14.00±10.46	15.02±8.96	0.918
CpG2	7.20±4.25	8.53±3.48	0.570
CpG3	5.90±1.86	7.29±3.21	0.067
CpG4	16.51±5.74	19.75±7.85	0.072
CpG5	6.35±1.36	8.06±5.40	0.101
CpG6	4.74±0.78	5.17±1.16	0.128
CpG7	5.24±1.88	5.28±2.32	0.536
CpG8	4.54±0.58	4.96±0.85	0.436
CpG mean	8.06±2.61	9.26±2.89	0.192

[a]DNA methylation in the T2D controls and cases was adjusted for smoking, drinking, TG and CRE in all subjects. [b]DNA methylation in T2D controls and cases was adjusted for drinking, triglyceride, low-density lipoprotein and uric acid in females. [c]DNA methylation in T2D controls and cases was adjusted for drinking, smoking and creatinine in males. Results are expressed as the mean ± standard deviation. CpGs, cytosine-guanine dinucleotide sites; T2D, type 2 diabetes.

in the blood (43), heart muscle (5) and liver (46). The present study indicates that promoter methylation of the *PTPN1* gene may contribute to T2D susceptibility in females, which suggests that *PTPN1* methylation may contribute to the risk of T2D in females by a mechanism involving the regulatory effects of sex hormones.

Aberrant clinical phenotypes are indispensable contributors to the development of T2D. In the present study, *PTPN1* methylation was found to be correlated with TC and LDL in females, although TC and LDL are not able to predict the risk of T2D. Furthermore, it is speculated that *PTPN1* methylation may increase the risk for T2D by influencing TC and LDL metabolism in females.

The present study indicates that *PTPN1* methylation is positively correlated with age in males. In addition to being observed in the peripheral blood, age-associated DNA methylation (47) has been detected in other human tissues, including the skeletal muscle (48), kidney (49) and brain (47). Additionally, age and environmental factors may exert an influence on the risk of diabetes by altering tissue-specific methylation of certain genes (50), and age-dependent gene hypermethylation has also been observed in human skeletal muscle (51).

However, the following limitations of the present study should be noted. First, the sample size calculation indicates that 194 patients will give 97.8% power at α=0.05 to detect a mean difference in DNA methylation (standard deviation, 3.02%) between T2D cases and controls. Further power analysis showed that there was 94.6% power for females and 41.8% power for males. Thus, the observations in the

Table II. Characteristics of the subjects in the T2D case and control groups.

Characteristics	Controls	Cases	P-value
All subjects, n=194			
Age (years)	59.79±9.39	59.79±9.39	1.000
BMI (kg/m^2)	23.19±1.65	23.19±1.61	0.985
TC (mmol/l)	5.15±0.91	5.21±1.25	0.711
TG (mmol/l)	1.49±0.59	2.26±2.89	0.018[d]
LDL (mmol/l)	3.22±0.80	2.98±0.92	0.053
UA (μmol/l)	332.90±88.57	344.28±97.79	0.400
CRE (μmol/l)	69.20±17.80	77.19±24.28	6.00x10^{-3}[d]
Female, n=120			
Age (years)	60.07±8.64	60.07±8.64	1.000
BMI (kg/m^2)	22.86±1.66	22.98±1.61	0.701
TC (mmol/l)	5.28±1.17	5.41±0.87	0.476
TG (mmol/l)	1.52±0.55	2.42±2.20	0.002[a]
LDL (mmol/l)	3.35±0.85	3.01±0.96	0.040
UA (μmol/l)	293.87±70.51	325.18±90.79	0.037
CRE (μmol/l)	63.77±19.07	68.83±23.04	0.084[a]
Male, n=74			
Age (years)	59.35±10.60	59.35±10.60	1.000
BMI (kg/m^2)	23.72±1.51	23.55±1.63	0.633
TC (mmol/l)	4.74±0.82	5.11±1.37	0.162
TG (mmol/l)	1.45±0.66	1.99±3.78	0.969[a]
LDL (mmol/l)	3.01±0.68	2.93±0.88	0.666
UA (μmol/l)	396.19±78.19	375.24±101.96	0.325
CRE (μmol/l)	78.00±10.95	90.73±19.91	0.002[a]

[a]TG and CRE were subjected to log-transformation for comparison. Results are expressed as the mean ± standard deviation. BMI, body mass index; TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; UA, uric acid; CRE, creatinine; T2D, type 2 diabetes.

total samples were reliable; however, the negative results in the males may require confirmation using larger sample sizes in future studies. Secondly, the underlying molecular mechanism explaining why *PTPN1* methylation increases the risk of T2D was not investigated in the present study. Thirdly, epigenetic differences are tissue-specific, and blood samples may not fully represent the contribution of *PTPN1* to T2D in other tissues such as the pancreatic islets.

In conclusion, the present study demonstrates a significant correlation between elevated CpG methylation of *PTPN1* and T2D in females, which may further help to clarify the pathogenesis of T2D. However, further studies with a larger sample size are required to elucidate the underlying mechanisms, as well as to explore the interactions between DNA methylation and environmental factors contributing to the susceptibility of T2D.

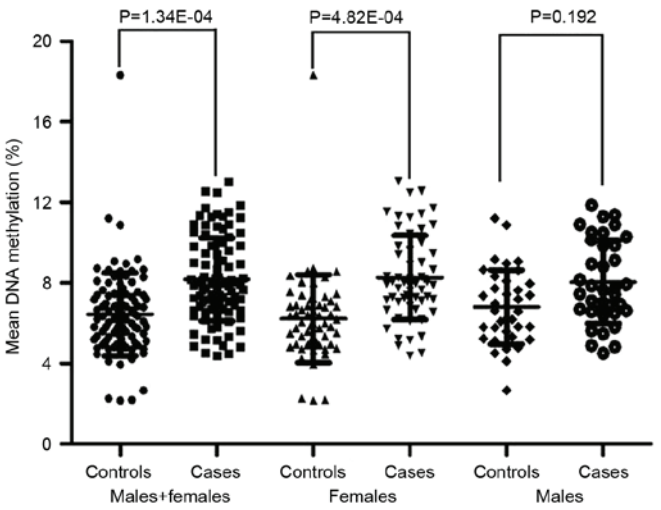

Figure 2. Comparison of the mean DNA methylation levels of the protein tyrosine phosphatase, non-receptor type 1 gene promoter between patients with type 2 diabetes and controls.

Acknowledgements

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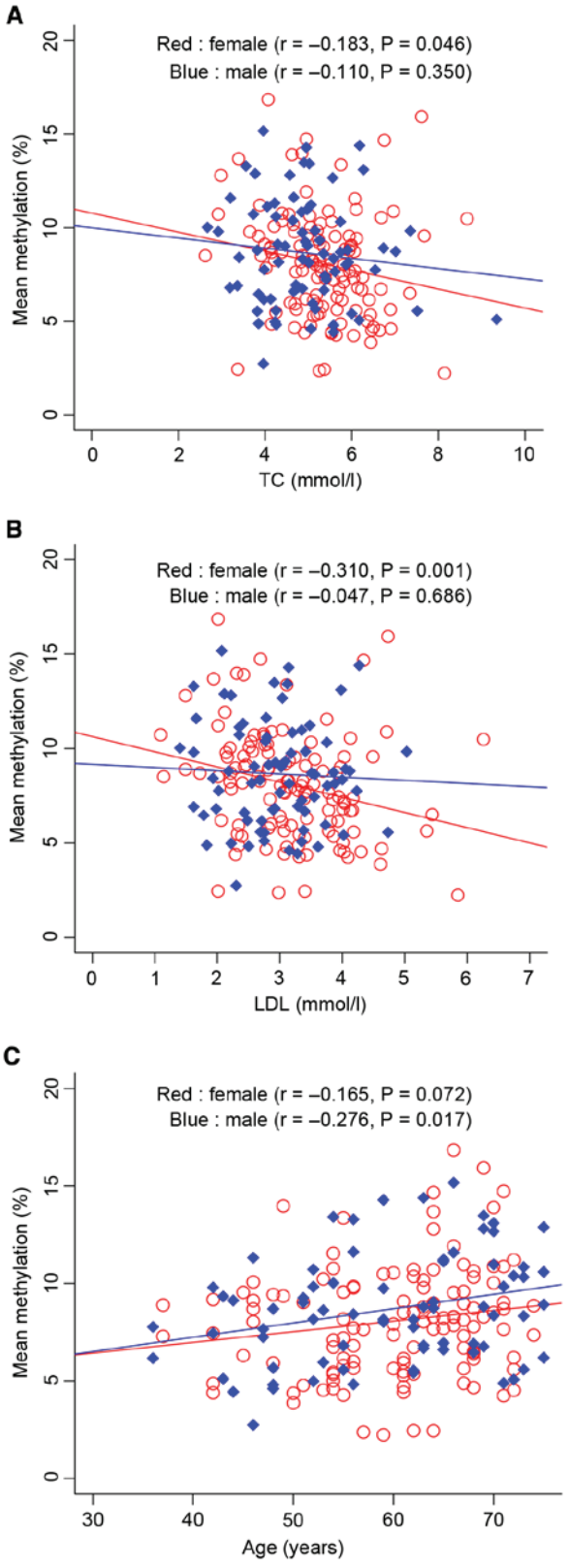

Figure 3. Correlation analyses between the mean protein tyrosine phosphatase, non-receptor type 1 DNA methylation and clinical phenotypes. Correlation analyses between (A) the mean PTPN1 DNA methylation and TC level, and (B) the mean PTPN1 DNA methylation and LDL level. TC, total cholesterol; LDL, low-density lipoprotein.

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