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Original Article

Quantitative DNA hypomethylation of PTEN in Uyghur patients with type 2 diabetes mellitus

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Abstract: Background: PTEN antagonises the action of PI3K, a key kinase upstream of AKT in the insulin signalling cascade. Recently, important roles of epigenetic mechanisms have been identified in the pathogenesis of type 2 diabete (T2DM). The objectives of this study were to reveal whether aberrant PTEN methylation occurs in Uyghur patients with T2DM by MassARRAY Spectrometry. Results: The methylation levels of the PTEN gene were significantly lower in Uyghur patients with T2DM (3.12%) than those in normal controls (4.20%), which was not present in IFG/IGT (3.96%) compared with NGTs. In addition, two CpG units showed a statistically significant difference between Uyghur T2DMs and NGTs, which was not present in IFG/IGT. Conclusions: The study implicates for the first time that promoter methylation of the PTEN as an important event in the pathogenesis of T2DM. The aberrant methylation in CpG sites within the PTEN promoter may potentially serve as a candidate biomarker for Uyghur patients with T2DM.

Keywords: PTEN, promoter methylation, type 2 diabetes mellitus, Uyghur, MassARRAY Spectrometry

Introduction

Type 2 diabetes mellitus (T2DM) is a complex polygenic disease, commonly resulting from defects in insulin secretion and/or diminished sensitivity of target tissues to insulin, which is becoming one of the major causes of premature illness and death in most countries [1]. Thus, understanding the molecular pathogenesis of T2DM may provide clues in developing therapeutic regimens for T2DM, yet the information is largely unrecognized.

The Uyghurs are a majority in the Xinjiang Uyghur Autonomous Region (XUAR) of Western China. The prevalence of T2DM in Uyghur was significantly higher than that in other ethinc groups [2]. Recently, evidence is emerging about the importance of epigenetic regulatory mechanisms in the pathogenesis of T2DM [3]. Our previous study demonstrated that the miR-375 promoter is hypomethylated, in Kazak patients with T2DM, which may regulate the expression of miR-375 and contribute to the pathogenesis of T2DM [4]. However, the molecular pathogenesis of T2DM remains unclear.

Phosphatase and tension homolog deleted on chromosome 10 (PTEN) is a tumor suppressor and involves in basic cellular functions such as adhesion, migration, proliferation and cell survival [5]. Although PTEN was first identified as a candidate tumor suppressor gene, early studies suggest PTEN antagonises the action of PI3K, a key kinase upstream of AKT in the insulin signalling cascade, and plays an important role in glucose metabolism [6, 7]. However, it is important to note that only 25% of cancer patients portray a correlation between the loss of PTEN protein and its mRNA level, which emphasizes the importance of PTEN regulation at the post-transcriptional and post-translational levels [8]. In the tumor area, epigenetic regulation of PTEN gene within or near CpG islands in their promoter regions can result in aberrant expressions of PTEN. Our laboratory has previously shown that the promoter hypermethylation of the PTEN gene is a common event in STSs which may play a role in the oncogenesis of soft tissue sarcomas [9]. These findings suggest the importance of PTEN regulation at the epigentic level, but the exact role of the methylation of PTEN gene in T2DM has yet to be elucidated.

Table 1. Sequences of MassARRAY primers and positions relative to the translational start codon for the assays used to analyze DNA methylation of PTEN

	Primer Name	Sequence (5'→3')	Position (bp)
Amplicon	PTEN-F	aggaagagagTGAGTTTTAGGTTTTAGTTTTTTGGTTTGT	-2,515 to -2,186
	PTEN-R	cagtaatacgactcactatagggagaaggctTTAAAAAACTTTCCAAATTCCCACTCC	

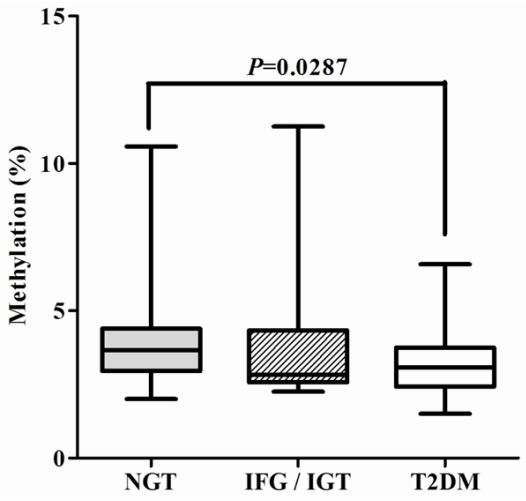

Figure 1. The overall methylation levels are displayed within amplicon as box plots in IGT/IFGs and T2DMs compared with NGT samples.

Therefore, the aim of the present study is to quantitatively evaluate methylation status of CpGs within the PTEN promoter using MassARRAY Spectrometry and to determine whether aberrant PTEN methylation patterns occurs in Uyghur T2DM, and furthermore, whether any of these alterations has potential values serving as a novel biomarker and the potential clinical importance in Uyghur patients with T2DM.

Materials and method

Clinical samples

Ninty-four Chinese Uyghur subjects with IFG/IGT (n = 17), 54 patients with T2DMs and a group of 23 age- and BMI-matched subjects with NGT were enrolled from the Department of Endocrinology and Metabolism at the First Affiliated Hospital of Shihezi University School of Medicine between 2014 and 2015. Glucose tolerance was studied during oral glucose tolerance test (OGTT) and 2006 WHO criteria were applied. Any individuals who may have had an infectious disease prior to or during the recruitment were excluded from this study, as well as patients with autoimmune diseases. Written informed consent was obtained from all participating patients before enrollment in the study. This study was approved by the institutional ethics committee at the First Affiliated Hospital of Shihezi University School of Medicine and conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Nucleic acid isolation

Genomic DNA was isolated from blood cells using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to manufacturer's instruction. The nucleic acid samples were quantized by measuring their absorption at 260 nm.

MassARRAY Spectrometry methylation analysis

The sequence of CpG island (CGI) was identified by the use of the UCSC genome browser (http://genome.ucsc.edu/), (chr10:89621773-89624128 %GC = 58.1 and Obs/Exp CpG = 0.86). The targets of the promoter regions were one amplicon, as previously reported [10-12]. We designed two primer sets for methylation analysis of the PTEN promoter region by EpiDesigner software (http://epidesigner.com; **Table 1**). For each reverse primer, an additional T7 promoter tag was added for in vivo transcription, and a 10-mer tag was added to the forward primer to adjust for the melting temperature differences. The primers used in the present study detected specifically the promoter sequence of the PTEN gene rather than that of the PTEN pseudogene (**Figure 2**) [13].

To quantify methylation levels of the PTEN CpG islands in the clinical samples, the high-throughput MassARRAY platform (Sequenom, San Diego, USA) was carried out as described previously [14]. Briefly, primers for the miR-375 CpG island were used to amply bisulfite treated DNA and then the PCR products were spotted on a 384-pad SpectroCHIP (Sequenom, San Diego, USA), followed by spectral acquisition on a MassARRAY Analyzer. Methylation data of

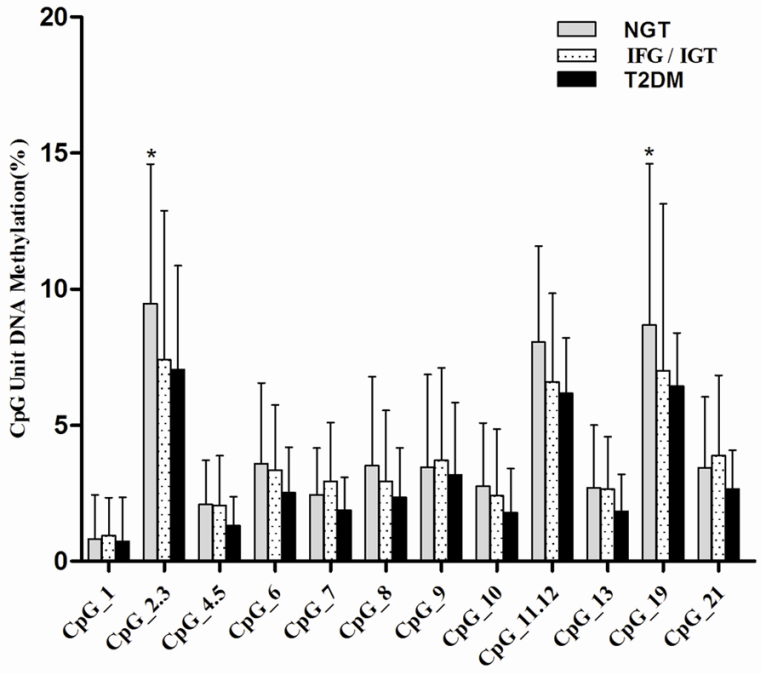

Figure 2. Comparison of PTEN methylation among T2DMs, IGT/IFGs and NGTs. The average methylation of the CpG units of amplicon is presented for IGT/IFGs, T2DMs and NGTs. T2DMs or IGT/IFGs carrier compared with NGTs: **P*<0.05. Error bars represent standard error.

individual units (one to three CpG sites per unit) were generated by the EpiTyper v1.0.5 sotware (Sequenom, San Diego, USA).

Statistical analysis

A Student *t*-test and an analysis of variance (ANOVA) were used to detect differences in the mean values of the variables. Fisher's exact test was used appropriately to analyze differences in the rate of each variable. *P*<0.05 was taken as significant. The distances between CpG methylation sites to transcription start sites were calculated by using the RMySQL package and the SQL database version of the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Two dimensional clustering was determined by the heatmap. 2 function in the gregmisc package. Classical multidimensional scaling had been performed by using the cmdscale function, and visualization was done through the scatter plot3d function in the same package. Power calculations were done using the software of Power and Sample Size Calculation 3.0 (USA). Tests for statistical significance had been used with standard function in R statistical environment.

Results

Aberrant promoter methylation of PTEN

We carried to detect methylation Patterns of PTEN CpG island and performed quantitative high throughput analysis of DNA methylation by the MassARRAY system within the PTEN-2, 515 bp to -2, 186 bp relative to the translation initiation site, which contains 21 CpG sites (**Table 1**). The methylation status of PTEN promoters were studied in all the samples collected between NGT (n = 23), IFG /IGT (n = 17) and T2DM (n = 54). A 330 bp region of the PTEN promoter containing 21 CpG sites which could be divided into 15 CpG units were examined by MassARRAY system. Among these units, 3 CpG units (6 CpG sites) did not yield successful measurements. Ninty-four (94) samples had good results for >90% of the samples. The final data set consisted of 12 CpG units from 94 samples. The average DNA methylation frequency ranged from 2.0% to 10.58% in NGTs and from 2.25% to 11.25% in IFG/IGTs and from 1.5% to 6.58% in T2DMs.

Aberrant methylation was significantly lower within amplicon of the PTEN promoter in T2DMs and IFG/IGTs than in NGTs, an average of 3.12%, 3.96% and 4.20% (**Figure 1**), respectively. There were significant methylation changes observed within amplicon in T2DMs than in NGTs (P = 0.0287, **Figure 1**). In contrast, there were no significant methylation changes observed within amplicon in IFGs and/or IGTs than in NGTs (P>0.05). No association between PTEN methylation and clinical characteristics of the patients was found. These data demonstrate the occurrence of aberrant promoter methylation of PTEN in T2DM samples.

Methylation levels at individual CpG sites along PTEN promoter region

We next examined the methylation status of individual CpG sites within each promoter

region (**Figure 2**). For PTEN, there was some variability in the methylation level of individual CpG sites within the different samples. The methylation level of every CpG units was numerically lower in samples of T2DMs than in NGTs, CpG_2.3 and CpG_19 were significantly different between these two groups ($P<0.05$). There were no differences in the methylation level of individual CpG sites within amplicon in IFGs and/or IGTs than in NGTs.

Discussion

Epigenetic modifications of DNA, such as methylation, have been suggested to have a key role in T2DM progression [15]. Some recent studies show that DNA methylation contributes to downregulation of PTEN during tumorigenesis [16, 17]. However, it is not clear whether PTEN promoter methylation plays a role in T2DM. In the present study, we hypothesized that aberrant PTEN methylation patterns occur in Uyghur T2DM.

In the current study and for the first time, we have employed MALDI-TOF MS to evaluate methylation patterns at multiple CpG sites within the promoter region of PTEN in T2DM. The core promoter of PTEN located at positions -2013 to -2549 was found to be capable of governing the maximum promoter activity [18]. So we turned our attention to the upstream region located 2.190 to 2.542 kilobases from the translation start site ATG. Although our results did not show significant aberrant methylation of PTEN between IFG/IGTs and NGTs, we found that aberrant methylation of PTEN is significantly lower in T2DM than in NGTs. These results suggest that promoter hypomethylation of the PTEN gene is a common event in T2DMs which may play a role in the pathogenesis of T2DMs.

We have further evaluated aberrant methylation status of CpG units that may be used as novel biomarkers for T2DM. Previous studies suggest that quantitative cytosine methylation profiling can be used to identify molecular markers in tumors [19, 20]. These studies have revealed specific hypermethylated CpG sites that are useful in diagnosis of cancers. Our previous study demonstrated a possibility that the methylation frequency at individual CpG units may serve as novel diagnostic biomarkers in patients with T2DM [21]. In this study, we have shown that significant differences in the frequency of methylation at individual CpG units between T2DM and NGTs. The methylation level of every CpG units was numerically lower in samples of T2DMs than in NGTs, CpG units (CpG_2.3 and CpG_19) are significantly hypomethylated in T2DM cases as compared with NGTs. There were no differences in the methylation level of individual CpG sites within amplicon in IFGs and/or IGTs than in NGTs. Thus, these observations suggest a possibility that the methylation frequency at individual CpG units might serve as novel diagnostic biomarkers capable of distinguishing among T2DMs and NGTs. In this study, however, these subjects cannot represent the whole province population for only one region was chosen. However, the current investigation has paved the way for studying PTEN gene methylation using MALDI-TOF MS technology and warrant further studies using larger sample sizes of T2DMs.

This is the first report analyzing PTEN promoter methylation in T2DM patients using MALDI-TOF MS technology and the results implicate for the first time that promoter methylation of the PTEN as an important event in the pathogenesis of T2DM. The aberrant methylation in CpG sites within the PTEN promoter may potentially serve as a candidate biomarker for Uyghur patients with T2DM.

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Disclosure of conflict of interest

None.

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