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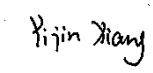

Yijin Xiang

04.15.2020

Yijin Xiang

Date

Diabetes-related DNA Methylation of TXNIP Independently Associated with Inflammation

By

Yijin Xiang
Degree to be awarded: MPH

Department of Epidemiology

_____ [Chair's signature]

Yan Sun, Ph.D.
Committee Chair

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By

Yijin Xiang

B.S.N
Shanghai Jiao Tong University
2018

Thesis Committee Chair: Yan Sun, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Department of Epidemiology
2020

Abstract

Diabetes-related DNA Methylation of TXNIP Independently Associated with Inflammation

By Yijin Xiang

Background: Thioredoxin-interacting protein (txnip) plays a key role in diabetes development and prognosis through pancreatic β-cell dysfunction and death as well as upregulating inflammatory response in hyperglycemia condition. However, the role of inflammation biomarkers in the association between *TXNIP* DNA methylation (DNAm) and type 2 diabetes (T2D) remains unclear.

Methods: We conducted an epigenetic association study of T2D-related traits and a panel of inflammatory biomarkers among 218 middle-aged male twins from the Emory Twin Study. DNA methylation and inflammation biomarkers were assessed in blood samples. Age in years, current smoking status and BMI were adjusted as covariates. Linear mixed models (LMM) were performed to model shared (between-twin effect) and unshared (within-twin effect) epigenetic associations of co-twins, as well as the batch effect due to laboratory assays. The significance threshold was corrected due to multiple testing. In addition, a stepwise generalized estimating equations (GEE) method was performed to assess the effect of inflammatory biomarkers on *TXNIP*-cg19693031 association with T2D.

Results: Hypomethylation of *TXNIP*-cg19693031 were significantly associated with diabetes traits and inflammation biomarkers (VCAM-1, ICAM-1, MMP-2, sRAGE, and sP-selectin) after multiple testing correction. Within twin pairs, 1% increase in beta-values was associated with 13.84 ng/ml ($P=4.3\times10^{-5}$), 36.03 ng/ml ($P=2.8\times10^{-4}$), 2.97 ng/ml ($P=9.5\times10^{-4}$), and 1.87 ng/ml (P=.003), decrease in ICAM-1, VCAM-1, MMP-2, and sP-selectin. Meanwhile, 1% increase of beta-values between twin pairs was associated with 23.65 ng/ml ($P=6.0\times10^{-5}$), 2.21 ng/ml (P=.001) and 26.17 pg/mL (P=.003) decrease in VCAM-1, MMP-2 and sRAGE. In the adjusted GEE models, within-twin association indicated that 1% increase of DNAm in *TXNIP*-cg19693031 was associated with the 24% decrease of T2D (OR=0.76, 95% CI 0.61, 0.95). Meanwhile, 1% increase of shared DNAm of twin pairs in *TXNIP*-cg19693031 was associated with the 18% decrease of T2D (OR=0.82, 95% CI 0.71, 0.94). The epigenetic association between *TXNIP*-cg19693031and T2D was independent from inflammatory biomarkers including VCAM-1, ICAM-1, MMP-2 and sRAGE.

Conclusion: Our results suggest that hypomethylation of *TXNIP*-cg19693031 is strongly associated with both Type 2 diabetes and higher level of inflammation biomarkers whereas the relationship between *TXNIP*-cg19693031 and T2D is independent from the inflammation biomarkers.

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Acknowledgments

I wish to express my sincere appreciation to my supervisor, Dr. Yan Sun, who really helps me building a research path in Epigenetics and developing critical thinking as well as is always patient and reachable. Without his persistent help, I might not fully reach the goal of this project. I also want to thank Zeyuan Wang, who initiates this project and continuously providing help during the process.

I wish to acknowledge the support and great love of my family, my mother, Donglin; my father, Yang; my aunts, Hui. They kept me going on and this work would not have been possible without their input. In addition, I wish to show my gratitude to the friends I met during my graduate study, Lili, David, Daniel, Yichun, and Jiandong. They helped me go through all tough time and provide me with accompany and help.

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Introduction

Thioredoxin interacting protein (TXNIP) is a ubiquitously expressed protein that inhibits a major cellular antioxidant protein thioredoxin, and thus regulates the redox-dependent signal pathways, mediates oxidative stress, suppress cell growth and induces cell apoptosis (Chen, Saxena, Mungrue, Lusis, & Shalev, 2008; Shalev, 2014; Yoshihara et al., 2014). Txnip protein has been shown to prohibit cell proliferation and induce cell-cycle arrest at G0/G1 phase, increasing cell susceptibility to apoptosis (Han et al., 2003; Junn et al., 2000).

Hyperglycemia (HG) is considered as a primary environmental risk factor for diabetes and its complications (Chen et al., 2008; Mortuza, Chen, Feng, Sen, & Chakrabarti, 2013). The prevailing mechanism leading to diabetes is an increase of the metabolic input into cells and subsequent induction of oxidative stress. The subsequent reaction is via increase in reactive oxygen species (ROS) formation (Mortuza et al., 2013), NLRP3 inflammasome activity and apoptotic pathway via mTOR pathway which are pathways where *TXNIP* plays a major roles (Kumar & Mittal, 2018). Upregulated protein level of txnip is found in pancreatic islets from diabetic mouse (Minn, Hafele, & Shalev, 2005) and its association with pancreatic Beta cells apoptosis are also identified in human islet microarray study(Minn et al., 2005). According to recent study, TXNIP has been demonstrated as a pathogenic factor in the development of both type 1 and type 2 diabetes (T2D) (Chen et al., 2008; Hui et al., 2008; Khodneva, Shalev, Frank, Carson, & Safford, 2016; Yin, Kuo, Chang, Chen, & Wang, 2017). TXINP silencing leads to improved insulin sensitivity and glucose tolerance in mice model (Hui et al., 2008) and

attenuated HG-induced pancreatic beta-cell apoptosis (Chen et al., 2008). Downregulation of *TXNIP* via administrating calcium channel blocker (Xu, Chen, Jing, & Shalev, 2012), such as widely used hypertension medication, verapamil, is revealed to be associated with lower fasting blood glucose levels in diabetic participants (Khodneva et al., 2016; Xu et al., 2012) and significantly reduced incidence of T2D in patients older that 65 years (Yin et al., 2017).

Epigenome-wide associations studies (EWAS) of T2D as well as HbA1c identified differentially methylated DNA methylation (DNAm) site cg1969031 on gene *TXNIP* (Chambers et al., 2015; Mathur et al., 2019; Meeks et al., 2019). In a case-control study among sub-Saharan Africans (Meeks et al., 2019), hypomethylation of DNAm site *TXNIP*-cg19693031was associated with T2D cases not on glucose-lowering medication after adjusted BMI (OR= 0.87, 95%CI 0.82, 0.90). In the same study, decrease in DNAm level was associated with higher levels of HbA1c (2.5% decrease in DNAm level per 1 ml/mol increase in HbA1c). Similar results were also reported in other blood-based EWAS (Florath et al., 2016; Kulkarni et al., 2015; Soriano-Tarraga et al., 2016; Walaszczyk et al., 2018), regardless ethnic, social and environmental difference. The result was also replicated in HIV infected individuals, among which T2D cases were with lower level of methylation of DNAm site *TXNIP*-cg19693031 (Mathur et al., 2019). In addition, CpG sites *TXNIP*-cg19693031 was significantly associated with development of incident T2D. After 8-year follow-up, the relative risk of developing T2D was 1.09 (p-value of 1.2×10^{-17}) per 1% decrease in methylation for *TXNIP*-cg19693031(Chambers et al., 2015).

Several studies focusing on the association between *TXNIP* and diabetic retinopathy reported that overexpression of *TXNIP* was detected in diabetic rats' retina and is also related to retinal cells inflammation and development of diabetic retinopathy (Perrone, Devi, Hosoya, Terasaki, & Singh, 2009; Singh, Devi, & Yumnamcha, 2017). The study by Perrone demonstrated that receptor for advanced glycation end products (RAGE) induces *TXNIP* expression, while *TXNIP* itself can induce RAGE expression in return (Perrone et al., 2009). There is also evidence that increased expression of *TXNIP* potentially contributes to endothelial inflammation and progression of diabetes (Perrone et al., 2009). TXNIP protein can activate gene expression for intercellular adhesion molecule 1 (ICAM-1) (Wongeakin, Bhattarakosol, & Patumraj, 2014), interleukin 6 (IL-6) and vascular cell adhesion protein 1 (VCAM-1) (Y. Li et al., 2015), which contribute to the risk of developing diabetes. However, the relationship between the inflammation biomarkers and *TXNIP* DNAm has not been studied thoroughly at population level. The present study investigates the association between T2D-associated DNAm site *TXNIP*-cg19693031 and inflammatory biomarkers, and whether the association between *TXNIP* methylation and diabetes is affected by these inflammatory biomarkers.

Materials and Methods

Study sample

The Emory Twin Study (ETS) consists of 307 middle-aged male Caucasian monozygotic (MZ) and dizygotic (DZ) twin pairs from the Vietnam Era Twin Registry (Goldberg,

Curran, Vitek, Henderson, & Boyko, 2002) who were on active duty in the US military during the Vietnam conflict era (1964-1975) (V. Vaccarino, Brennan, et al., 2008; V. Vaccarino, Lampert, et al., 2008). For the baseline visit, all twins were examined in pairs at the Emory University General Clinical Research Center between 2002 and 2010. Twins were given the same diet the night before the assessments and instructed to refrain from smoking. All measurements were performed in the morning after an overnight fast, and both twin pairs were tested at the same time. All medications were held for approximately 24 hours prior to testing. Biochemical assays for each twin pair were processed in the same analytical run. A medical history and a physical exam were obtained from all twins. Venous blood samples were drawn for the collection of plasma and peripheral blood leukocytes (PBL), and stored at -80°C until the biomedical assay. Information on zygosity was determined by DNA analysis. A subgroup of 220 twins with sufficient amount of genomic DNA were successfully epityped using the Illumina HumanMethylation450 (450K) Beadchip. The ETS was approved by the Emory Institutional Review Board, and all participants signed an informed consent.

Phenotypes

Weight and height were measured and used to calculate body mass index (BMI). Waist circumference and hip circumference was measured in centimeters at the level of the umbilicus. Cigarette smoking status were self-reported and classified into current smokers (any number of cigarettes) versus non-current smokers (i.e., never or past smokers). Any medication for diabetes and history of chronic heart disease were classified into yes versus no. As previously described (Viola Vaccarino et al., 2014),

physical activity was categorized as a 3-level ordinal variable of "no physical activity", "moderate physical activity" (participated in at least one moderate every day activity, such as walking short distance, climbing stairs, etc.), and "vigorous physical activity" (performed regularly in at least one intense exercise for at least the last 3 months, such as jogging, biking, swimming, etc.). Plasma levels of VCAM-1, ICAM-1, Interleukin 6 (IL-6), P-selectin (sP-selectin), high-sensitivity c-reactive protein (hs-CRP), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), sRAGE, and fibrinogen were assessed using commercially available ELISA kits from R and D Systems (Minneapolis, Minnesota). Venous blood samples were drawn for the measurements of plasma glucose. Total triglycerides and cholesterol were determined by enzymatic methods (Beckman Coulter Diagnostics, Fullerton, CA). Levels of blood glucose, insulin, and HbA1c in fasting plasma were measured on the Beckman CX7 chemistry auto analyzer (Beckman Coulter Diagnostics). Diabetes mellitus was defined as having a fasting glucose level of >126 mg/dl or being treated with antidiabetic medications.

DNA methylation profiling and data processing

As previously described (Huang et al., 2018), bisulfite-convert genomic DNA samples from PBLs were whole-genome amplified, enzymatically fragmented, and purified. Following standard protocol, samples were then hybridized to the 450K BeadChip, which were then fluorescently stained, scanned, and assessed for fluorescence intensities at each bead site. Each DNAm site was quantified using probe intensities and beta (β)-values, which represent the ratio of fluorescence intensity of the methylated and unmethylated

sites (ranging from 0 to 1). Using the detection *p*-value threshold of 0.001, two individual samples with a missing rate above 5% were excluded, resulting in 218 eligible twins in the following analyses. No sample was detected with control probe values greater than 4 standard deviations from its mean value. We further performed data normalization and batch effects correction using the preprocessSWAN function of the "minfi" package (Aryee et al., 2014) available in R.

Statistical analysis

To compare the characteristics of diabetes and non-diabetes participants, student's t test was used for continuous variables, and Chi-square or Fisher's exact test (cells having counts less than 5) for categorial variables. We used linear mixed models to identify the epigenetic association between DNA methylation of *TXNIP*-cg19693031 and diabetes-related traits as well as inflammatory biomarkers. Age in years, current smoking status and BMI were adjusted as covariates. Random effects were modeled to address the chip/batch effect and the heterogeneity between twins. Fixed effect of twin-pair average methylation level at each CpG site and individual deviation from the twin-pair average methylation level at each CpG site were included in the model (Carlin, Gurrin, Sterne, Morley, & Dwyer, 2005). The estimate for the latter term represented the differential methylation caused by unshared environmental factors. As DNAm was measured in PBLs, differential methylation in leukocyte subtypes (Baron et al., 2006) and various proportions of leukocyte subtypes in individual PBL samples may cause spurious epigenetic associations. We adjusted the proportions of PBL subtypes (B cells,

granulocytes, monocytes, NK cells, and T cells) using the method developed by Houseman et al (Houseman et al., 2012).

To understand the effect of inflammation biomarkers on the association between *TXNIP*-cg19693031 and Type 2 diabetes, we used a stepwise generalized estimating equations (GEE) method by including inflammation biomarkers in the epigenetic association model of twins. GEE was applied to account for the correlation between observations within twin pairs. Proportions of PBL subtypes were adjusted to address the potential confounding effect.

All analyses were conducted in R studio with R version 3.4.3. R package *nlme* and *geepack* was used to implement linear mixed effect model and generalized estimating equations, respectively. To correct for multiple testing, we used a Bonferroni threshold for statistical significance (nominal p-value<0.004, correcting for 14 tested biomarkers).

Results

Table 1 summarized the characteristics of 218 ETS participants in the present study, stratified by the status of T2D. The mean age of T2D group (n=24) was 56.08, not significantly different from which of non-DM group (n=194, mean age of 55.52). Compared to non-T2D group, T2D group had higher BMI, waist circumference, and hip circumference, as well as higher level of diabetes-related indicators including HbA1c, fasting glucose, and insulin. Participants with T2D had higher prevalence of coronary heart disease comorbidity than participants without T2D (33% versus 16%). All

comparison but insulin and systolic BP were significantly different (p-value < 0.05) between two groups. Levels of inflammatory biomarkers were not statistically different between participants with and without T2D. Similar to previous studies, participants having T2D had lower DNAm level in *TXNIP*-cg19693031.

To examine the associations of DNAm levels of *TXNIP*-cg19693031 with T2D-related traits and inflammatory biomarkers, we applied linear mixed models adjusting for age, BMI, current smoking status, and proportions of PBL subtypes. T2D-related traits including HbA1c, fasting glucose and insulin were significantly associated with DNAm levels of *TXNIP*-cg19693031 after multiple testing correction (Table 2). Within twin pairs, 1% increase of beta-values was associated with 0.06 unit decrease in HbA1c (p-value of 9.8×10^{-4}). Meanwhile, 1% increase of beta-values between twin pairs were associated with 0.09 mIU/L (p-value=0.001) and 1.83 mg/dL (p-value of 6.6×10^{-5}) decrease in insulin and glucose, respectively. Among nine tested inflammatory biomarkers, VCAM-1, ICAM-1, MMP-2, sRAGE, and sP-selectin were significantly associated with DNAm levels of *TXNIP*-cg19693031 (Table 2). Within twin pairs, 1% increase in beta-values was associated with 13.84 ng/ml (p-value of 4.3×10^{-5}), 36.03 ng/ml (p-value of 2.8×10^{-4}), 2.97 ng/ml (p-value of 9.5×10^{-4}), and 1.87 ng/ml (p-value of 0.003), decrease in ICAM-1, VCAM-1, MMP-2, and sP-selectin. Meanwhile, 1% increase of beta-values between twin pairs was associated with 23.65 ng/ml (p-value of 6.0×10^{-5}), 2.21 ng/ml (p-value of 0.001) and 26.17 pg/mL (p-value of 0.003) decrease in VCAM-1, MMP-2 and sRAGE, respectively.

We then examined the effect of inflammatory biomarkers on the association between DNAm of *TXNIP*-cg19693031 and T2D using twin-specific GEE models. Treating T2D as the outcome, the basic model included age, BMI, current smoking status, and proportions of PBL subtypes as covariates. We modeled between-twin pair (i.e., mean DNAm level of a twin pair) and within twin pair DNAm levels (difference between a twin's DNAm level and the mean of a twin pair) to examine the shared and unshared epigenetic associations simultaneously. Both shared and unshared DNAm of *TXNIP*-cg19693031 were statistically significantly associated with the prevalence of T2D. The test for within-twin association (i.e., unshared effect) indicated that 1% increase of DNAm in *TXNIP*-cg19693031 was associated with the 24% decrease of T2D (OR=0.76, 95% CI 0.61, 0.95). Meanwhile, 1% increase of shared DNAm of twin pairs in *TXNIP*-cg19693031 was associated with the 18% decrease of T2D (OR=0.82, 95% CI 0.71, 0.94). Each of the five inflammatory biomarkers significantly associated with DNAm of *TXNIP*-cg19693031 (i.e., ICAM-1, VCAM-1, MMP-2, sRAGE, and sP-selectin) was included in the basic model separately. The associations between DNAm of *TXNIP*-cg19693031 and T2D had little changes after adjusting for individual inflammatory biomarkers (Table 3). Both shared and unshared epigenetic association between *TXNIP*-cg19693031 and T2D remained significant except for the model adjusting for VCAM-1. Plasma level of VCAM-1 was independently associated with T2D (OR=1.54, 95% CI 1.03, 2.31), which reduced the within-twin pair association to OR of 0.8 (95% CI 0.62-1.03, p-value of 0.086).

Discussion

Epigenetic variations can be determined by genetic, shared and unshared environmental factors. Previous studies have bridged epigenetic modification, environmental exposures and human diseases. However, the influence of shared and unshared factors on identified epigenetic associations remain mostly unclear limited by sampling unrelated participants. In the present study, we took advantage of twin participants and a twin-specific statistical model to implement both within-twin and between-twin terms to represent the DNA methylation changes due to unshared environment and DNA methylation changes due to shared environmental/familial factors, respectively (Carlin et al., 2005). This study shows that hypomethylation of *TXNIP*-cg19693031 is associated with higher odds of T2D and higher plasma level of five inflammatory biomarkers (i.e., VCAM-1, ICAM-1, MMP-2, sRAGE, and sP-selectin). The epigenetic associations between *TXNIP*-cg19693031 and T2D and related traits (HbA1c, fasting glucose and insulin) are consistent with previous findings (Chambers et al., 2015; Meeks et al., 2019). However, the associations with glucose and insulin are mostly driven by shared between-twin effects, while the association with HbA1c is driven by both unshared within-twin and shared between-twin effects using the twin-specific modeling. Our discovery of epigenetic association of *TXNIP*-cg19693031 with inflammatory biomarkers suggests the epigenetic linkage between *TXNIP* and endothelial function. These inflammatory biomarkers have little effects on modifying the epigenetic association between *TXNIP*-cg19693031 and T2D.

DNA methylation is also called cytosine methylation, an epigenetic modification on 5-C position of cytosine sidechain of cytosine-phosphate-guanine dinucleotides (CpG). CpG

sites at active gene promoters normally have lower level of methylation, allowing for transcription. Hypomethylation at specific promotors may correlate with higher level of gene expression and can trigger some aberrant pathways, such as tumor cell differentiation (Portela & Esteller, 2010). TXNIP, as a regulator of the cellular redox state, shows early response to high concentration of glucose in diabetes development. *TXNIP* transcription is largely induced by glucose via the activation of carbohydrate response element-binding protein (ChREBP), which binds to the TXNIP promotor (Cha-Molstad, Saxena, Chen, & Shalev, 2009). Overexpression of *TXNIP* subsequently upregulate oxidative stress response causing the β-cell apoptosis (Minn et al., 2005). Study shows that cg19693031 located in the 3'UTR of *TXNIP* (site associated with glucose import and protein biding sites) and methylation differences in this locus may affect the function of methylation-sensitive transcription regulators (Soriano-Tarraga et al., 2016). This is consistent with the association between higher odds of T2D and hypomethylation in *TXNIP*-cg19693031 found in our study.

TXNIP also plays significant roles in inflammation responses besides cell apoptosis (Perrone et al., 2009; Rong et al.; Szpigel et al., 2018; Wongeakin et al., 2014). In response to oxidative stress, TXNIP could bind to NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome and active NLRP3-inflammasomee to regulate inflammation (Rong et al.). In addition, TXNIP was found to induce NF-kB nuclear translocation and increase inflammatory gene expression for COX2 (Perrone et al., 2009). The NF-kB is known to be involved in the expression of ICAM-1 and VEGF in diabetes (Nagai et al., 2007). In the present study, we found that inflammatory

biomarkers (VCAM-1, ICAM-1, MMP-2, sRAGE, and sP-selectin) were negatively associated with DNA methylation level of *TXNIP*-cg19693031. Both ICAM-1 and VCAM-1 are pro-inflammatory markers, which are expressed on endothelial cells and subsequently upregulated during inflammation (X. Li et al., 2017). MMP-2 belongs to the matrix metalloproteinases family, responsible for cellular proliferation, angiogenesis and inflammation (Lewandowski et al., 2008). MMP-2 deficient mice were found exacerbates hepatic inflammatory conditions compared to wild-type mice (Kato, Duarte, Liu, Busuttil, & Coito, 2015). RAGE, as an endogenous pattern recognition receptor, plays important roles in immune disease and inflammatory disorders (Hou, Yang, & Yin, 2019; Sterenczak et al., 2009). sRAGE is generated through cleavage of RAGE and shown to antagonize RAGE signaling pathway and deregulation of which is associated with several disease (Vazzana, Santilli, Cuccurullo, & Davi, 2009). such as Alzheimer's disease (Yan et al., 2007), cardiovascular disease (Falcone et al., 2005; Lindsey et al., 2009), arthritis (Hofmann et al., 2002) and diabetes (Basta et al., 2006). P-selectin is an adhesion receptor for leukocytes, stored in membranes of α granules of platelets and Weibel–Palade bodies of endothelial cells, the distribution of which will change to the plasma membrane due to the activation of those cells via mediators such as thrombin or histamine (Ushiyama, Laue, Moore, Erickson, & McEver, 1993). P-selectin are shown to initiate leukocyte recruitment corresponding to inflammation action (Varga-Szabo, Pleines, & Nieswandt, 2008). Elevated level of sP-selection are discovered in patient with a variety of cardiovascular disorders (i.e. coronary artery disease, hypertension, and atrial fibrillation) (Blann, Nadar, & Lip, 2003) and it is concluded as a consequence rather than a cause of cardiovascular disease in transgenic mice (Panicker et al., 2017).

Endothelial dysfunction is considered a major cause of diabetes complication, which can be measured by relevant inflammatory biomarkers such as VCAM-1 and ICAM-1. Although DNAm of *TXNIP*-cg19693031 is strongly associated with plasma level of VCAM-1, ICAM-1, MMP-2, sRAGE and sP-selectin, the associations between *TXNIP*-cg19693031 and T2D were little changed after adjustment for inflammation biomarkers (ICAM-1, MMP-2, sRAGE and sP-selectin). These results suggest that the epigenetic association between *TXNIP*-cg19693031and T2D may not be mediated via inflammatory response. The relationship between DNAm of *TXNIP*-cg19693031, inflammation and T2D may improve the understanding the role of epigenetic regulation in multiple pathophysiological processes underlying T2D development.

This study addresses some limitations of previous epigenetic association studies. In addition, we used stepwise modeling strategy to test the direct effect of inflammation biomarkers on the association between DNA methylation of *TXNIP*-cg19693031 and T2D. However, we acknowledge several limitations. The power and precision of estimates can be limited by a small number of T2D cases. Further, the homogeneity of the study cohort, similar life experience, all middle age and male, may lead to lack of generalizability of our study result to other populations with different characteristics. The present study used blood cells for DNAm profiling, which may not accurately represent the epigenetic modifications in other tissues directly related to T2D (e.g., pancreatic cells and adipose tissues) or inflammation (e.g., liver). Future studies in other disease-related tissues would be ideal but may not always be practical. Large reference panel of

epigenetic markers across many different tissues or cell types from the same individuals may provide evidence of correlated epigenetic profile for trans-tissue effects, and improve interpretability of blood-based epigenetic studies. Additionally, the lack of longitudinal epigenetic and phonotypic data, particularly the lack of diabetes incidence data, limits our ability to establish the causal relationship between DNAm, inflammation and T2D, and to formally test the mediation of inflammatory biomarkers.

Epigenetics is an important molecular mechanism to understand many aspects of disease development and etiology. With some successes in initial round of EWAS of complex diseases including T2D, we have identified strong epigenetic associations among relatively small sample size. Different from genetic association studies and genome-wide association studies, epigenetic associations are prone to confounding and reverse causation. Therefore, it is critical to conduct in depth follow-up analyses of epigenetic markers to understand their biological roles, causal pathways, and determinants to further evaluate their clinical utilities. The present study of *TXNIP*-cg19693031, inflammation and T2D among twins provides a proof of concept, which may lead to proper design, evaluation and application in future epigenetic epidemiologic studies of T2D.

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Tables

Table 1. Descriptive characteristics of the male participants from Emory Twin Study stratified by type 2 diabetes status (n=218)

	DM (n=24)	Non-DM (n=194)	P-value
Age (yrs)	56.08 (3.2)	55.52 (3.3)	0.421
Body Mass Index (kg/m2)	32.22 (3.79)	28.95 (4.54)	**0.001**
waist Circumference (cm)	107.71 (8.79)	98.3 (11.64)	**<0.001**
Hip Circumference (cm)	106.67 (6.58)	103.27 (11.07)	**0.035**
HbA1c (%)	7.01 (1.29)	5.49 (0.51)	**<0.001**
Insulin (mIU/L)	21.1 (42.39)	8.47 (6.97)	0.159
Glucose(mg/dL)	132.46 (56.87)	99.23 (12.96)	**0.009**
ICAM-1 (ng/ml)	904.11 (741.46)	627.51 (231.29)	0.082
VCAM-1 (ng/ml)	404.18 (253.53)	318.46 (104.34)	0.114
IL-6 (pg/mL)	2.4 (2.17)	2.09 (2.15)	0.526
sP-selectin (ng/ml)	98.53 (39.74)	100.59 (29.56)	0.807
High-sensitivity CRP (mg/L)	2.69 (2.65)	2.95 (6.02)	0.721
MMP-2 (ng/ml)	189.15 (61.98)	165.81 (29.04)	0.081
MMP-9 (ng/ml)	33.79 (15.67)	33.45 (18.08)	0.922
sRAGE (pg/mL)	995.69 (520.56)	976.49 (434.96)	0.864
Fibrinogen (mg/dL)	357.41 (77.51)	350.52 (88.03)	0.702
TXNIP (cg19693031)	0.69 (0.06)	0.74 (0.05)	**0.005**
Any Medication for Diabetes,	21 (0.88)	---	---
Current Smoker	8 (0.33)	56 (0.29)	0.683
History of Chronic Heart Disease	8 (0.33)	31 (0.16)	**0.048**
Hypertension	8 (0.33)	64 (0.33)	0.973
Physical Activity (3 categories)			0.902
Moderate Physical Activity	15 (0.62)	120 (0.62)	
Vigorous Physical Activity	7 (0.29)	50 (0.26)	

Data are shown as mean (SD) for continuous variables and as number of individuals (%) for categorical variables

p-value are calculated from Student's t test for continuous variables and Chi-square or Fisher's exact test (more than 20% of cells have expected frequencies<5) for categorial variables

Table 2. Summary of between-pair/within-pair association between diabetes, inflammation and TXNIP (cg19693031)

Phenotype	N	Within-Pair			Between-Pair		
		Beta	SE	P-value	Beta	SE	P-value
HbA1c (%)	215	-0.06	0.02	**9.8×10^{-4}**	-0.04	0.01	0.010
Insulin (mIU/L)	217	0.87	0.49	0.079	-0.90	0.26	**0.001**
Glucose(mg/dL)	217	0.57	0.64	0.372	-1.83	0.44	**6.6×10^{-5}**
Hip Circumference (cm)	217	-0.18	0.25	0.475	0.03	0.15	0.851
waist Circumference (cm)	217	-0.05	0.16	0.747	0.04	0.11	0.684
ICAM-1 (ng/ml)	215	-13.84	3.23	**4.3×10^{-5}**	-5.45	2.20	0.015
VCAM-1 (ng/ml)	215	-36.03	9.55	**2.8×10^{-4}**	-23.65	5.61	**6.0×10^{-5}**
MMP-2 (ng/ml)	215	-2.97	0.87	**9.5×10^{-4}**	-2.21	0.64	**0.001**
sP-selectin (ng/ml)	215	-1.87	0.61	**0.003**	-0.53	0.62	0.396
IL-6 (pg/mL)	212	0.10	0.07	0.151	-0.01	0.04	0.742
High-sensitivity CRP (mg/L)	214	0.18	0.18	0.330	-0.05	0.10	0.599
sRAGE (pg/mL)	215	-9.67	9.93	0.332	-26.17	8.69	**0.003**
Fibrinogen (mg/dL)	198	-1.96	2.31	0.399	2.04	1.70	0.233
MMP-9 (ng/ml)	215	3.54	5.31	0.507	-4.35	3.17	0.173

The analysis is controlled for BMI, Current smoking status, Age and Proportions of PBL subtypes (B cells, granulocytes, monocytes, NK cells, and T cells).
Table is sorted on within-pair comparison associated P values among each category. Bonferroni correction for multiple testing were used (p-value <0.004).
The effect size is based on 0.01 unit change in DNA methylation (%).

Table 3. Summary of association analysis between diabetes, age, BMI, current smoking, within-pair, between-pair, and inflammatory biomarker

Variable	Model 1		Model 2		Model 3		Model 4		Model 5		Model 6		Model 7	
	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value
Age	1.05 (0.88, 1.25)	0.578	1.07 (0.86, 1.32)	0.548	1.05 (0.85, 1.29)	0.669	1.06 (0.86, 1.31)	0.578	1.04 (0.84, 1.29)	0.714	1.07 (0.86,1.32)	0.542	1.07 (0.87,1.32)	0.514
BMI	1.15 (1.01, 1.32)	0.031	1.17 (1.05, 1.31)	0.004	1.18 (1.06, 1.32)	0.004	1.17 (1.04, 1.31)	0.007	1.17 (1.05, 1.31)	0.004	1.17 (1.03,1.32)	0.013	1.18 (1.06,1.31)	0.003
Current Smoking	1.26 (0.41, 3.83)	0.689	1.98 (0.55, 7.12)	0.295	1.66 (0.42, 6.65)	0.471	1.6 (0.37, 6.95)	0.534	1.98 (0.52, 7.61)	0.318	1.97 (0.58,6.76)	0.279	2.22 (0.53,9.29)	0.276
Within-Pair			0.76 (0.61, 0.95)	0.017	0.8 (0.62, 1.03)	0.086	0.78 (0.61, 0.99)	0.042	0.77 (0.6, 0.99)	0.042	0.76 (0.61,0.95)	0.016	0.76 (0.6,0.95)	0.015
Between-Pair			0.82 (0.71, 0.94)	0.004	0.83 (0.72, 0.97)	0.017	0.83 (0.72, 0.95)	0.009	0.83 (0.72, 0.97)	0.018	0.81 (0.68,0.95)	0.012	0.81 (0.71,0.93)	0.003
VCAM1					1.54 (1.03, 2.31)	0.036								
ICAM1							1.16 (0.86, 1.58)	0.328						
MMP-2									1.37 (0.86, 2.2)	0.183				
sRAGE											0.85 (0.45,1.6)	0.607		
sP-selectin													0.84 (0.49,1.44)	0.538

OR – Odds ratio; CI – Confidence interval; Within-Pair – Effect of non-shared measured influences; Between-Pair – Effect of shared measured influences
The analysis controlled for proportions of PBL subtypes (B cells, granulocytes, monocytes, NK cells, and T cells) to address the batch effect.
Odds ratios of diabetes are per 1% increase in DNA methylation and per 1 unit of standard deviation increase in inflammatory biomarkers