DNA methylation level of the gene encoding thioredoxin-interacting protein in peripheral blood cells is associated with metabolic syndrome in the Japanese general population

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Abstract. Metabolic syndrome (MetS) is cluster of metabolic diseases, including abdominal obesity, hyperglycemia, high blood pressure, and dyslipidemia, that directly escalate the risk of type 2 diabetes, heart disease, and stroke. Thioredoxin-interacting protein (TXNIP) is a binding protein for thioredoxin, a molecule that is a key inhibitor of cellular oxidation, and thus regulates the cellular redox state. Epigenetic alteration of the TXNIP-encoding locus has been associated with components of MetS. In the present study, we sought to determine whether the level of TXNIP methylation in blood is associated with MetS in the general Japanese population. DNA was extracted from the peripheral blood cells of 37 subjects with and 392 subjects without MetS. The level of TXNIP methylation at cg19693031 was assessed by the bisulfite-pyrosequencing method. We observed that TXNIP methylation levels were lower in MetS subjects (median 74.9%, range 71.7–78.4%) than in non-MetS subjects (median 77.7%, range 74.4–80.5%; $p = 0.0024$). Calculation of the confounding factor-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for hypomethylation revealed that subjects with MetS exhibited significantly higher ORs for hypomethylation than did those without MetS (OR, 2.92; 95% CI, 1.33–6.62; $p = 0.009$). Our findings indicated that lower levels of TXNIP methylation are associated with MetS in the general Japanese population. Altered levels of DNA methylation in TXNIP at cg19693031 might play an important role in the pathogenesis of MetS.

Key words: Metabolic disorder, Obesity, Thioredoxin-interacting protein (TXNIP), DNA methylation, Pyrosequencing

METABOLIC SYNDROME (MetS) has become a serious problem in developed countries, with an estimated prevalence of 25% in the world population [1]. MetS is a cluster of conditions that includes abdominal obesity, elevated glucose levels, high blood pressure, elevated triglycerides, and reduced levels of high-density

lipoproteins. It is well accepted that MetS is a highly prevalent condition reflecting the cumulative effect of a series of factors, and that MetS increases the risk of type 2 diabetes mellitus (T2DM) and cardiovascular disease [2]. Indeed, MetS is considered one of the largest causes of morbidity and mortality worldwide.

There has been a recent explosion of interest in the notion that chronic low-grade inflammation is associated closely with MetS [3]. In addition, MetS is thought to be promoted by oxidative stress reflecting an imbalance in the oxidant/antioxidant state of the cell, resulting in turn from the excessive production of reactive oxygen species (ROS) [4, 5]. Thioredoxin-interacting protein (TXNIP) is

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a binding protein for thioredoxin, a key inhibitor of cellular oxidation, and thus regulates the cellular redox state, leading to oxidative stress [6, 7]. In response to ROS accumulation and the development of oxidative stress, TXNIP also plays an important role in inflammatory responses [8, 9]. Depending on the generation of oxidative stress, TXNIP can serve as an activator of the nucleotide-binding oligomerization domain-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome, thereby regulating inflammation [10]. Thus, the association between dysregulation of TXNIP and the development of MetS has been the focus of recent work.

DNA methylation is an epigenetic modification that results in the placement of a methyl group on cytosines at CpG sites of DNA; this modification has been shown to alter the regulation of gene expression [11, 12]. Recently, there is growing evidence of the association between TXNIP DNA methylation and metabolic dysfunction. A recent study examined methylation at cg19693031, which is located at base 145,441,552 (hg19) of chromosome 1, within a DNA sequence corresponding to the 3'-untranslated region (3'-UTR) of the TXNIP mRNA. Particularly in white blood cells, decreased methylation at cg19693031 has been shown to be closely associated with the levels of fasting blood glucose, glycated hemoglobin A1C (HbA1c), homeostasis model assessment of insulin resistance (HOMA-IR) values, and triglycerides, and the prevalence of T2DM [13-17]. Depending on the decreased methylation level at cg19693031, the levels of TXNIP mRNA in white blood cells are increased, which may result in the induction of inflammation [18]. TXNIP methylation in white blood cells may play a critical role in the pathogenesis of metabolic dysfunction, given that white blood cells themselves are responsible for oxidative stress and inflammation. Notably, Nuotio *et al.* recently reported that TXNIP methylation at cg19693031 in peripheral white blood cells is associated with MetS and its components [19]. Thus, methylation at cg19693031 in the TXNIP gene appears to be a candidate contributor to the pathogenesis of MetS.

Lifestyle includes different factors such as nutrition, behavior, stress, physical activity, working habits, smoking, and alcohol consumption. Increasing evidence shows that these lifestyle factors may influence epigenetic mechanisms, such as DNA methylation [20, 21]. Differences in culture, climate, and geography mean that lifestyle habits vary greatly between people of various regions and ethnicities. People in the United States and Canada, for example, consume more than 30% of their total calories from fat [22, 23]. In contrast, people in Asia, such as the Japanese and Chinese, consume far less than 30% of their total energy intake in fat [24, 25]. The

distinct lifestyles of Western and Asian populations suggest the need to clarify the relationship between MetS and DNA methylation in various regional and ethnic groups. However, the relationship between MetS and TXNIP methylation has been reported (to our knowledge) only in a study examining European-derived populations [19].

Therefore, in this study, we investigated whether differential TXNIP methylation in peripheral blood cells was present in MetS compared to control subjects in the Japanese general population.

Materials and Methods

Study subjects

Since 1982, annual public health check-ups have been conducted for residents of Yakumo-cho, Hokkaido, Japan, aged 39 years or older. Various cross-sectional and longitudinal investigations of lifestyle-related diseases have enrolled individuals who participated in these health examinations [26-29]. We have collected epidemiological data at these annual health checkups, and have used these data as the basis of the Yakumo Study. The present cross-sectional study was undertaken in 525 individuals who participated in health examinations in 2015 as part of the Yakumo study. We excluded 96 subjects who had a clinical history of cancer or did not provide informed consent for the current study, or for whom the obtained blood samples were not adequate for the measurement of DNA methylation levels. The remaining 429 eligible individuals (189 men and 240 women) were included in the analysis described in the current report. The protocol for this study was approved by the Ethics Committee of Fujita Health University (Approval No. HG19-069).

Collection of participant data

Health information for each participant, including smoking habits (current, ever, or never), alcohol consumption (current, ever, or never), and a history of major illnesses (yes/no) were obtained by a trained public health nurse using self-administered questionnaires during the health examination. Height, weight, waist circumference, and blood pressure were measured during the health examination. The body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2). Serum parameters were analyzed using an auto analyzer in the Yakumo Town Hospital laboratory.

Assessment of metabolic syndrome

The individuals diagnosed with MetS in the current study had central obesity, as defined by waist circumference (≥ 85 cm in men and ≥ 90 cm in women) plus at least

two of the following three factors that define MetS; (1) elevated triglycerides (≥ 150 mg/dL) or decreased high-density lipoprotein cholesterol (HDL-C) (< 40 mg/dL), (2) elevated blood pressure (systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg), or (3) elevated fasting glucose (≥ 110 mg/dL) [30]. Individuals without central obesity were classified as MetS component 0, while those with central obesity were classified as MetS component 1. MetS components 2 to 4 were defined by the number of factors of either dyslipidemia, hypertension, or hyperglycemia, in addition to central obesity.

Measurement of *TXNIP* DNA methylation

DNA methylation analysis was conducted as described previously [31, 32]. Briefly, peripheral blood obtained under fasting conditions was collected into tubes containing ethylenediaminetetraacetic acid (EDTA)-2Na, and the buffy coat was obtained by centrifugation of these samples. DNA extracted from the buffy coat was bisulfite converted using the EpiTect Fast DNA Bisulfite Kit (QIAGEN, Hilden, Germany). We performed PCR using bisulfite-treated DNA (10 ng/ μ L) with the TaKaRa EpiTaq HS (for bisulfate-treated DNA; Takara, Otsu, Japan). Using PCR products, quantitative CpG methylation analysis was carried out on a PyroMark Q24 Advanced (QIAGEN) system with PyroMark Q24 Advanced Reagents (QIAGEN) in accordance with the manufacturer's instructions. We selected the following PCR and sequencing primers, as described in the previous study [33]: *TXNIP*: forward: 5'-TGTTTGGTGGATG GGT T T A A A A T A A T T-3', reverse: 5'-biotin-AAACCT C C A A A A A C C T T A A A A A C T T-3', and sequencing: 5'-GGGTTAGGTAAATGG-3'. The results of CpG methylation level were analyzed using the PyroMark Q24 Advanced software (QIAGEN).

Statistical analyses

Statistical analyses were performed using JMP v.14.0 (SAS Institute, Cary, NC, USA). The data for subject characteristics according to metabolic syndrome status are shown as mean $\pm$ standard deviation (SD) or percentage. Analysis of differences between subjects with and without MetS were performed by two-tailed non-paired Student's *t* tests. The DNA methylation levels of *TXNIP* were represented as geometric means and 25th–75th percentile ranges. The Cochran–Armitage test for trend was used to assess whether there was a monotonically decreasing trend in the *TXNIP* methylation level depending on the number of MetS components. We defined the hypomethylation group as the first tertile of the *TXNIP* DNA methylation levels (range 53.9%–75.6%). Multivariate adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for hypomethylation were calculated by

logistic regression analysis according to the MetS and MetS components. We used age, sex, body mass index, smoking status, alcohol consumption, neutrophil percentage, and antihyperlipidemic or antihypertensive or anti-diabetic medication use (user/non-user) as confounding factors. Probability values less than 0.05 were considered statistically significant.

Results

The characteristics of the subjects are presented in Table 1. A total of 37 cases with MetS (28 male, 9 female) and 392 subjects without MetS (161 male, 231 female) were enrolled in this study. No difference was observed in mean age between the two groups. By definition, subjects with MetS in this study had significantly higher BMI, waist circumference, triglycerides, systolic (SBP) and diastolic (DBP) blood pressure, and glucose,

Table 1 Subject characteristics according to metabolic syndrome status

	Non-MetS (N = 392)	MetS (N = 37)	p-value
Age (years)	62.9 $\pm$ 9.9	61.7 $\pm$ 10.4	0.50
Male/Female (%)	41.1/58.9	75.7/24.3	<0.001
Abdominal obesity			
BMI (kg/m ²)	23.2 $\pm$ 3.2	27.3 $\pm$ 2.9	<0.001
Waist circumference (cm)	80.9 $\pm$ 9.8	92.3 $\pm$ 5.8	<0.001
Dyslipidemia			
Triglycerides (mg/dL)	98.0 $\pm$ 57.4	205.9 $\pm$ 123.1	<0.001
HDL cholesterol (mg/dL)	60.1 $\pm$ 13.8	48.2 $\pm$ 10.7	<0.001
Hypertension			
SBP (mmHg)	130.0 $\pm$ 20.7	144.2 $\pm$ 15.4	<0.001
DBP (mmHg)	75.2 $\pm$ 13.8	85.7 $\pm$ 11.4	<0.001
Hyperglycemia			
Glucose (mg/dL)	88.3 $\pm$ 14.2	102.6 $\pm$ 25.7	<0.001
Metabolic components (%)			
0	74.2	0	
1	7.7	0	
2	18.1	0	
3	0	91.9	
4	0	8.1	

Data are shown as mean $\pm$ SD or as percentage.

Analysis of differences between groups performed by two-tailed Student's *t* test.

Abbreviations: MetS, metabolic syndrome; BMI, body mass index; HDL, high density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure.

and significantly lower HDL-C (Table 1). Among subjects with MetS, 91.9% had three of the MetS components, and 8.1% had four.

Methylation site cg19693031 in the *TXNIP* gene previously has been shown to be associated with various metabolic dysfunctions such as T2DM. Therefore, we analyzed the methylation level of cg19693031 using the bisulfite-pyrosequencing method. We observed that the methylation level at cg19693031 was lower in leukocyte samples from subjects with MetS (median 74.9%, range 71.7–78.4%) compared to subjects without MetS (median 77.7%, range 74.4–80.5%; $p < 0.01$) (Fig. 1A). As shown in Fig 1B, a trend in the measured *TXNIP* methylation level was observed as the number of MetS components increased, although the difference fell short of statistical significance (p for trend = 0.08).

To explore these results further, a logistic regression analysis was employed to verify the ability of *TXNIP* methylation level to discriminate MetS from non-MetS. The confounding factor-adjusted ORs and 95% CIs for hypomethylation were calculated, and significantly higher ORs for hypomethylation were observed in subjects with MetS than in subjects without MetS (Table 2). For MetS components, there was a pattern of increasing ORs from one toward three or four MetS components, and subjects with three or four MetS components had statistically significant higher ORs for hypomethylation than did those with no MetS components (Table 3). As a next step, we divided the subjects by the type of MetS

components, and calculated the confounding factor-adjusted ORs and 95% CIs for hypomethylation. We observed no significant difference in ORs between the subjects with and without abdominal obesity or hypertension. For dyslipidemia and hyperglycemia, significantly higher ORs for hypomethylation were observed in the subjects with either of these components compared to those lacking these components (Table 4).

Discussion

In the present study, we explored the link between MetS and *TXNIP* DNA methylation levels in white blood cells of the general Japanese population. We found a significant decrease in *TXNIP* DNA methylation level in our subjects with MetS compared to those without. Interestingly, a significant trend in the measured *TXNIP* DNA methylation level was observed with an increase in the number of MetS components: the DNA methylation was found to be lower in subjects with worse metabolic profiles. In addition, subjects situated in the tertiles with lower *TXNIP* methylation levels exhibited significantly greater risk of being affected by MetS when adjusted for sex, age, smoking habits, alcohol consumption, BMI, medication use, and percentage of neutrophils. Based on these results, we suggest that decreased levels of DNA methylation at cg19693031 in leukocytes is related to MetS in the general population of Japan.

The chronic low-grade inflammatory state associated

Fig. 1 DNA methylation levels of *TXNIP* in peripheral blood from subjects with and without MetS. (A) Boxplot showing distribution of methylation levels at cg19693031 (*TXNIP*) in non-MetS and MetS subjects, as measured by pyrosequencing. The horizontal middle lines show the medians of the data, while the boxes show the 25th to 75th percentiles (Q1 and Q3). The whiskers represent maxima and minima. Two-tailed Student's *t* tests were performed to test for differences between the two groups. (B) Comparison of *TXNIP* methylation level among subjects ranked by the number of MetS components is shown by a boxplot. The Cochran–Armitage test for trend was performed to assess whether there was a monotonically decreasing trend in the *TXNIP* methylation level depending on the number of MetS components.

Table 2 Multivariate adjusted ORs and 95% CIs for *TXNIP* DNA hypomethylation according to MetS

	Hypomethylation (%)	Adjusted ORs (95% CI)	<i>p</i> -value
Non-MetS	122 (31.1%)	Reference	
MetS	22 (59.5%)	2.92 (1.33–6.62)	<0.01

Adjusted for age, sex, body mass index, smoking status, alcohol consumption, neutrophil percentage, and medication use

Table 3 Multivariate adjusted ORs and 95% CIs for *TXNIP* DNA hypomethylation according to the number of MetS components

MetS components	Hypomethylation (%)	Adjusted ORs (95% CI)	<i>p</i> -value
0	91 (31.3%)	Reference	
1	7 (23.3%)	0.41 (0.15–1.13)	0.08
2	24 (33.8%)	0.93 (0.44–1.96)	0.85
3 or 4	22 (59.5%)	2.47 (1.00–6.11)	<0.05

Adjusted for age, sex, body mass index, smoking status, alcohol consumption, neutrophil percentage, and medication use.

Table 4 Multivariate adjusted ORs and 95% CIs for *TXNIP* DNA hypomethylation according to the type of MetS components

MetS components	Hypomethylation (%)	Adjusted ORs (95% CI)	<i>p</i> -value
Abdominal obesity			
No	91 (31.3%)	Reference	0.79
Yes	53 (38.4%)	0.92 (0.49–1.71)	
Dyslipidemia			
No	105 (30.1%)	Reference	<0.05
Yes	39 (48.8%)	1.90 (1.10–3.30)	
Hypertension			
No	65 (31.9%)	Reference	0.53
Yes	79 (35.1%)	1.15 (0.74–1.81)	
Hyperglycemia			
No	125 (31.7%)	Reference	<0.05
Yes	19 (54.3%)	2.47 (1.12–5.58)	

Adjusted for age, sex, body mass index, smoking status, alcohol consumption, neutrophil percentage, and medication use

with MetS has been implicated as a major factor in the establishment of MetS [34, 35]. However, the causes of the inflammatory conditions associated with MetS are not fully understood. *TXNIP* is an important redox regulator and plays an important role in inflammatory processes such as the activation of NLRP3 and the secretion of interleukin (IL)-1 β [10, 36]. The up-regulation of *TXNIP* expression, which is influenced by the demethylation of cg19693031, orients peripheral blood cells toward an inflammatory activated state [18]. In addition, *TXNIP*-mediated inflammation is closely associated with the hyperglycemic state and lipid accumulation, which are risk factors for MetS [8, 36]. Thus, inflammatory pathways resulting from *TXNIP* methylation also may be involved in the pathogenesis of MetS. In fact, our data indicated that the level of cg19693031 methylation is decreased in subjects with MetS compared to those without MetS, a result that is consistent with the inference that *TXNIP* methylation is a strong candidate marker for MetS pathogenesis [19].

Our data indicated that *TXNIP* hypomethylation is associated with dyslipidemia and hyperglycemia among the MetS components. The methylation site cg19693031 in the gene *TXNIP* has been reported to be associated with lipid and glucose metabolism [37, 38], suggesting that these abnormalities might be the cause of *TXNIP* hypomethylation. However, logistic regression analysis

showed that the methylation level of *TXNIP* was clearly different between MetS and non-MetS. Importantly, there was no significant difference in the OR for *TXNIP* hypomethylation between subjects with one or two MetS components and those without MetS components. The appearance of *TXNIP* hypomethylation is clearly distinguishable between MetS and non-MetS patients, suggesting that changes in *TXNIP* methylation levels might be caused by the development of MetS.

Nuotio *et al.* reported an association between the methylation of cg19693031 and MetS in European-derived populations [19]. However, for several reasons, that evidence was expected to have only limited application for the prediction of MetS in other geographic/ethnic groups. First, the diagnostic criteria for MetS differ among various countries [39]. For example, the waist circumference threshold for men in Japan is ≥ 85 cm, while that in the USA and Canada is ≥ 102 cm. Second, global leukocyte DNA methylation differs by geography and ethnicity, and these variables need to be taken into consideration in studies of global DNA methylation as an epigenetic marker for disease [40]. Finally, the fact that dietary habits, which significantly influence both the development of MetS and DNA methylation, differ among regions suggests the critical importance of validating the association between cg19693031 and MetS in non-European populations. In the present study, we found an association between DNA methylation of *TXNIP* and MetS, in agreement with the results reported by Nuotio *et al.* [19], despite the use of a distinct (Japanese) population compared to that (Europeans) used in the previous study. Our work also differed from the previous study in that we employed a pyrosequencing method. Pyrosequencing can detect and quantify even small changes in methylation level at selected CpG sites in specific target genes, and has been established as the “gold standard” for DNA methylation analysis [41]. Our findings indicated that cg19693031 in *TXNIP* may play an important role in the pathogenesis of MetS.

DNA methylation can be influenced by lifestyle factors such as diet, smoking, and stress. In fact, we recently reported that nutritional status has a significant impact on methylation patterns in blood [42–44]. We also have shown an association between DNA methylation level at cg19693031 and smoking behavior [33]. In this way, DNA methylation status in blood incorporates the influences of multiple lifestyle factors. Similarly, the relationship between MetS and long-term exposure to unsuitable habits also is recognized. Therefore, it seems possible that *TXNIP* hypomethylation occurs in response to adverse lifestyle habits, leading in turn to MetS.

DNA methylation has a number of advantages as a biomarker. First, DNA methylation patterns are robust,

since this modification reflects the covalent attachment of methyl groups to DNA [45]. Second, DNA methylation analysis can be focused on specific and small regions of interest, given that aberrant DNA methylation occurs only in limited regions that encompass CpG sites. Third, as shown in the present study, analysis of methylation in DNA from peripheral blood samples is possible, enabling noninvasive testing. Taken together, these observations suggest that further technical advances and clinical studies may facilitate the successful implementation of epigenetic assays for the diagnosis of diseases, including MetS.

It is well established that MetS increases the incidence of T2DM. However, limited evidence is available regarding factors that may be predictive for the incidence of T2DM among subjects with MetS. In the present study, we showed that hypomethylation at cg19693031 is associated with MetS. Although epigenetic alterations at cg19693031 have been reported in subjects with T2DM [15, 16], most of our MetS subjects had not been diagnosed as overtly diabetic patients. Recently, growing evidence has suggested that epigenetic modifications, especially DNA methylation, may predispose humans to disease [46, 47]. Based on these findings, we speculate that the level of methylation at cg19693031 may serve as a marker for T2DM risk assessment among subjects with MetS.

The frequency of MetS in elderly Japanese is expected to be approximately 20% [48, 49]. In this study, less than 10% of the subjects were classified as MetS. One of the reasons is that those taking medications for metabolic diseases were excluded from the MetS group. Since medication use has a significant impact on DNA methylation status [50, 51], those who use medications were not classified as MetS. It is necessary to note that our results are likely to be susceptible to selection bias in that each metabolic abnormality may not be so severe.

The present study has several limitations. First, we did not examine the relationship between mRNA level and DNA methylation status of *TXNIP* in peripheral blood cells. Rong *et al.* reported the relationship between decreased methylation level of *TXNIP* and increased mRNA expression using a human monocytic cell line [18]. Furthermore, analyses using human peripheral blood leukocytes have shown a negative correlation between *TXNIP* methylation levels and *TXNIP* mRNA expression [52]. Therefore, *TXNIP* hypomethylation in the peripheral blood of subjects with MetS is predicted to result in increased *TXNIP* gene expression. Second, it is unclear whether the subtle methylation changes in the *TXNIP* gene observed in the present study induce the MetS phenotype. However, in recent years, it has become increasingly evident that small (<10%) changes

in DNA methylation levels are associated with complex disease phenotypes [53]. As subtle changes in methylation levels increasingly are being recognized as an epigenetic feature of complex disease phenotypes, changes in *TXNIP* methylation of even a few percent are expected to play an important role in the pathogenesis of MetS. Third, we did not analyze our subjects for any inflammatory biomarkers such as serum tumor necrosis factor- α or C-reactive protein. Fourth, the current study analyzed only the relationship between MetS components and *TXNIP* hypomethylation. To further understand the role of *TXNIP* methylation on the pathology of MetS, *TXNIP*'s relationship to insulin resistance needs to be analyzed. Fifth, the analysis of *TXNIP* DNA methylation was adjusted for the proportion of neutrophils. The methylation levels of peripheral blood cells are influenced by the differences resulting from varying proportions of white blood cell types [54]. Therefore, it remains possible that changes in *TXNIP* methylation rates in peripheral blood are influenced by cell composition. Finally, given that this work was a cross-sectional study, it is not clear whether the *TXNIP* hypomethylation observed in subjects with MetS is a cause or consequence of the development of MetS. DNA methylation abnormalities have been suggested to predispose to a variety of diseases. Moreover, DNA methylation is highly influenced by environmental factors, particularly lifestyle. These results lead us to speculate that hypomethylation of *TXNIP* gene is a cause of MetS development. To address these limitations, future studies will be needed to elucidate the relationship between *TXNIP* hypomethylation and MetS in more detail.

In summary, this study revealed that lower *TXNIP* methylation levels in peripheral blood cells are associated with MetS in subjects from the general Japanese population. As a result, altered levels of DNA methylation at cg19693031 may play an important role in the pathogenesis of MetS. *TXNIP* hypomethylation may serve as a biomarker or therapeutic target for MetS, although additional research will be required to further strengthen these observations.

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Disclosure

None of the authors have any potential conflicts of

interest associated with this research.

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