Effect of rich resistant starch snack on MCP-1 promoter methylation and triglycerides levels in type 2 diabetes mellitus patients

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is closely related to inflammation. One of inflammation marker in T2DM is monocyte chemoattractant protein-1 (MCP-1). Previous study reported that MCP-1 promoter methylation is associated correlated with plasma triglycerides (TG) levels in T2DM. Furthermore, some studies stated that TG levels in T2DM can be controlled through consumption of diets containing resistant starch. This study aimed to investigate the effect of high resistant starch snack on MCP-1 promoter methylation and TG levels in T2DM patients. This study was a cross-over trial. A total of 19 T2DM patients have been selected with the criteria of fasting blood glucose levels (GDP) >126 mg/dL, aged 40-60 years, and duration of DM at least 1 year. Subject consume snack as much as 32 g/day with the resistant starch content are 4.25 g for 4 weeks. Data collection and blood sample were taken before and after the intervention. The analysis of MCP-1 promoter methylation was performed by methylated specific-PCR (MS-PCR) using DNA samples extracted from mononuclear cells, whereas TG levels analysis was performed by the enzymatic colorimetric method. Statistic analysis was performed by Fisher exact test for methylation data, paired t-test and unpaired t test for TG levels, and Spearman correlation test for the correlation between variabels. Methylation result showed that frequency of methylated (52.6%) and unmethylated (47.4%) status before and after intervention were same ($p > 0.05$). Plasma TG levels after intervention decreased, but not significant ($p > 0.05$). The correlation between MCP-1 promoter methylation and plasma TG levels was significant ($p < 0.05$). In conclusion, the rich resistant starch snacks intervention does not affect MCP-1 promoter methylation changes. This intervention can decrease plasma TG levels T2DM patients, although it is not significant.

ABSTRAK

Diabetes melitus tipe 2 (DMT2) berkaitan erat dengan inflamasi sistemik. Salah satu penanda inflamasi pada DMT2 adalah monocyte chemoattractant protein-1 (MCP-1). Penelitian sebelumnya melaporkan metilasi promotor MCP-1 berkaitan dengan kadar trigliserida (TG) plasma pada DMT2. Selain itu, beberapa penelitian menyebutkan bahwa kadar TG plasma pada DMT2 dapat dikendalikan dengan diet pati resisten. Penelitian ini bertujuan mengkaji pengaruh kue pati resisten tinggi terhadap metilasi promotor MCP-1 dan kadar TG pada pasien DMT2. Penelitian ini merupakan penelitian sama subjek yang

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melibatkan 19 penderita DMT2 yang memenuhi kriteria gula darah puasa > 126 mg/mL, berumur 40-60 tahun, dan menderita DM paling tidak 1 tahun. Subjek mengkonsumsi kue 32 g/hari dengan kandungan pati resisten 4.25 g selama 4 minggu. Data dan sampel darah diambil sebelum dan sesudah intervensi. Analisis metilasi promotor MCP-1 dilakukan PCR spesifik metilasi menggunkan sampel DNA yang diekstraksi dari sel mononuclear, sedangkan analisis kadar TG dilakukan secara enzimatik menggunakan metode kolorimetri. Anaaisis statistic dilakukan menggunakan uji Fisher exact untuk data metilasi, uji t berpasangan dan tidak berpasangan untuk kadar TG dan uji korelasi Spearman untuk hubungan antar variabel. Hasil uji metilasi menunjukkan status metilasi (52.6%) dan tidak termetilasi (47.4%) sebelum dan sesudah intervensi tidak berbeda (p > 0.05). Kadar TG plasma setelah intervensi turun tetapi tidak signifikan (p > 0.05). Teradapat hubungan yang signifikan antara metilasi promotor MCP-1 dan kadar TG plasma (p < 0.05). Dapat disimpulkan, intervensi kue kaya pati resisten tidak mempengaruhi metilasi promotor MCP-1. Intervensi dapat menurunkan kadar TG plasma pasien DMt2 meskipun tidak signifikan.

Keyword: Type 2 diabetes mellitus - resistant starch - DNA methylation - MCP-1 - triglyceride

INTRODUCTION

Diabetes mellitus (DM) is a major health problem in the world due to its prevalence constantly increases. Type 2 diabetes mellitus (T2DM) is a DM type commonly found, at least 90% cases.[1] The T2DM is characterized by high blood glucose levels (hyperglycemia) accompanied by impairment of metabolism of carbohydrates, lipids, and proteins as a result of the body's cells less responsive to insulin or insulin resistance.[2] The T2DM is caused by the interaction between genetic and environment.[3]

Type 2 diabetes mellitus is closely related to inflammatory conditions. One of inflammation marker in T2DM is a chemokine monocyte chemoattractant protein-1 (MCP-1). Regulation of MCP-1 expression involves several mechanisms. The binding of nuclear factor κB (NFκB) in the promoter region of MCP-1 is an important mechanism for the transcription of MCP-1.[4] Epigenetic modifications are another potential mechanism for regulating the expression of MCP-1. The previous research showed that the MCP-1 promoter methylation correlates significantly with the levels of plasma TG in T2DM patients. Increased TG can be a factor or a result of methylation.[5]

Triglyceride levels in T2DM patients may be controlled through the consumption of diets containing resistant starch.[6] Some studies suggest that resistant starch can lowering fasting plasma TG levels.[7,8] Short chain fatty acid as a fermentation results of resistant starch may reduce lipolysis in adipose tissue, resulting circulating free fatty acids or non-esterified fatty acids (NEFA) levels lower. Short chain fatty acids can also increase the β-oxidation of TGs in the liver.[9] In addition, short-chain fatty acids can improve insulin sensitivity, so that it can suppress the production of hepatic VLDL-TG and lower the levels of plasma TG.[10]

Resistant starch consumption needs of each country is different. In Australia with the high consumption habits of resistant starch are recommended to consume 20 g/day.[11] While in developing countries, the average consumption of resistant starch ranged between 3-7 g/day.[12] This study used the intervention snacks with resistant starch content to be consumed per day are 4.25 g (Sunarti *et al.*, Unpublished).

Based on the explanation above, it was assumed that foods high in resistant starch can increase MCP-1 promoter methylation as well as reducing TG levels in T2DM patients. Increased methylation of MCP-1 can suppress the expression of this gene. Therefore, this condition is expected to prevent the vascular complications in T2DM patients.

MATERIALS AND METHODS

Subjects

This study involved 19 T2DM patients from Dr. Sardjito General Hospital, Yogyakarta with the inclusion criteria were fasting blood glucose (FBG) >126 mg/dL, aged 40-60 years, and duration of DM at least 1 year. Patients with T2DM were smokers, pregnant, lactating were excluded from this study. Subject consume snack as much as 32 g/day with the resistant starch content are 4.25 g for 4 weeks. Data collection and blood sampling were conducted before and after the intervention. Data collected with anthropometric measurements included body weight and height. Blood samples were used to analyze the biochemical parameters (fasting blood glucose and TGs) and epigenetic (MCP-1 promoter methylation). This study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada and the Education and Research Section, Dr. Sardjito General Hospital, Yogyakarta.

Biochemical examinations

Fasting blood was collected in EDTA tubes for further separated into blood plasma and buffy coat. Blood plasma was used for FBG examination using the glucose oxydase-p-amino phenazone (GOD-PAP) method and for TGs examination using the enzymatic

colorimetric methods by gliserol-3-fosfat-oksidase (GPO) (DiaSys Kit). Buffy coat was used for MCP-1 methylation analysis.

Gene promoter methylation analysis of MCP-1

The MCP-1 methylation analysis was performed by MS-PCR method. Isolation of peripheral blood mononuclear cells (PBMC) was performed by using Ficoll solution, then the DNA was extracted from mononuclear cells using Wizard® Genomic DNA Purification Kit (Promega). DNA concentration was measured using a Nano Vue Plus, then performed with EZ DNA bisulfite conversion methylation TM Kit (Zymo Research). Furthermore, the amplification was done by using two pairs of primers, which were as follows: U-F 5'-GTG GTT GTT TAA TGA GGT AGG AGT-3 'and 5'-TAA U-R AAA AAA AAA AAA AAT ACA CAA AAC A-3'; M-F 5'-TGG TTT AAG TTG GTA GGT GAA GC-3 'and 5'-M-R AAC AAA AAA AAA AAA AAA CGAA TCA-3' with the product size 234 bp and 228 bp. The CpG sequences located in the promoter region of 2890-3050 bp. The conditions of PCR cycle were: 1 cycle of 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 60 °C (for methylated primer) and 62 °C (for unmethylated primer) for 1 min, 72 °C for 1 min followed by a 10-min extension at 72 °C. The PCR products were run on 1.5% ethidium bromide-stained agarose gel electrophoresis.

Statistical analysis

Normality of data was tested by Saphiro-wilk. Numeric variables were presented as mean values and standard deviation (SD), while categorical data were presented as percentage. Normal distribution data were analyzed using dependent sample t-test for variables between groups and independent

sample t-test for variables in a group. Log transformed data which still not normal was computed using Mann Whitney test. Methylation pattern was classified into two categories (methylated 1, unmethylated 2). Methylation data were compared by Fisher exact test. Correlation between variables were analyzed using Spearman test. A p value <0.05 was considered statistically significant.

RESULTS

Characteristics of subjects

TABLE 1 shows the characteristics of subjects of this study. A total of 19 subjects with 9 male dan 10 female were involved in this study. These subjects had average age 53 years old with the DM duration were 7 years. These T2DM subjects have had a mean fasting blood glucose 169.01 mg/dL and mean TG levels of 144.19 mg/dL.

TABLE 1. Characteristics of subjects (n=19)

Characteristics of subject (n= 19)	Mean ± SD
Age (year)	53.32 ± 3.35
Duration of DM (year)	7.16 ± 6.03
Gender	
- Male [n (%)]	9 (47.4)
- Female [n (%)]	10 (52.6)
Height (cm)	158.67 ± 1.66
Weight (kg)	72.88 ± 1.24
BMI (kg/m^2)	27.54 ± 1.13
FBG (mg/dL)	169.01 ± 59.48
TG (mg/dL)	144.19 ± 55.19

Note: BMI: body mass index; FBG: fast blood glucose; TG: triglyceride

The change of parameters

TABLE 2 shows that mean of body weight, BMI, and FBG levels after the intervention were lower than before the intervention or decrease. However, it was not significant statistically (p>0.05). Mean of TG levels after the intervention was also decrease, but it was not also significant statistically (p>0.05).

TABLE 2. The changes of parameters before and after intervention

Parameters	Pre-intervention	Post-intervention	p*
Height (cm)	160.00 ± 8.19	159.86 ± 8.52	0.518
Weight (kg)	69.87 ± 9.36	69.45± 9.08	0.070
BMI (kg/m^2)	27.33 ± 3.39	27.22 ± 3.34	0.359
FBG levels (mg/dL)	169.01 ± 59.48	159.77 ± 51.23	0.184
TG levels (mg/dL)	144.19 ± 55.19	129.28 ± 56.67	0.059

Data distribution was tested using Saphiro-Wilk test. Data was presented as mean ± SD. BMI: body mass index; FBG: fasting blood glucose; TG: triglyceride. *Dependent sample t-test, p< 0.05 statistically significant

Distribution of MCP-1 promoter methylation status

FIGURE 1 shows images of several representative of MCP-1 promoter methylation pre- and post-intervention. The presence of a visible product in the M lanes indicates the presence of methylated CpG islands (228 bp). The presence of product in U lanes indicates the presence of unmethylated CpG islands (234 bp).
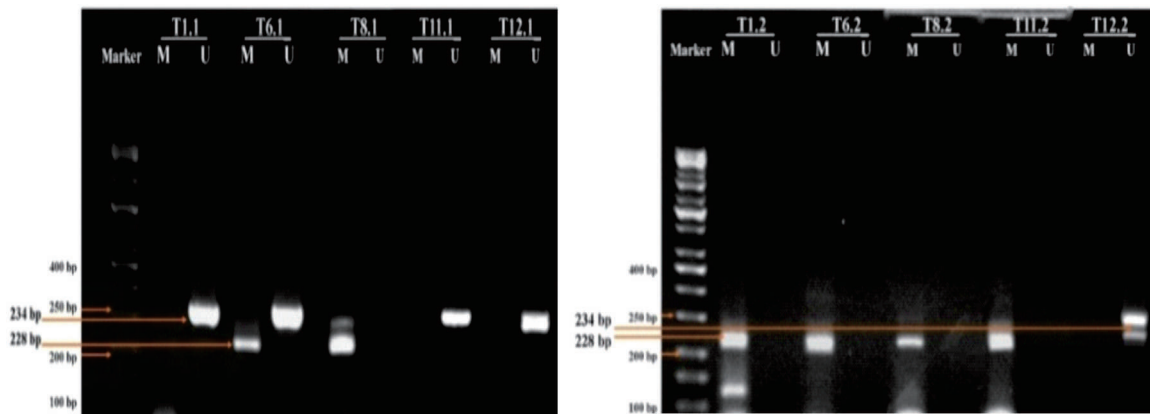
FIGURE 1. Images are several representative of MCP-1 promoter methylation in groups (a) pre-intervention, (b) post-intervention. M: methylation primer; U: unmethylation primer.

Tabulation of MCP-1 promoter methylation status distribution are presented in TABLE 3. This TABLE shows that frequency of methylated and unmethylated status of MCP-1 promoter methylation before and after the intervention were same, so there was no significant difference (p>0.05). But the percentage of status unmethylated (52.6%) higher than methylated (47.4%), before and after intervention.

TABLE 3. Distribution of MCP-1 promoter methylation status

Status	Pre-intervention [n (%)]	Post- intervention [n (%)]	p*	OR (95% CI)
Methylated	9 (47.4%)	9 (47.4%)	1.000	1.00 (0.28-3.57)
Unmethylated	10 (52.6%)	10 (52.6%)		

Data are presented as number (%). Variables between pre- and post- intervention group were compared by Fisher Exact test. OR: odds ratio; CI: confidence interval. *Fisher exact test, p< 0.05 statistically significant

Correlation between MCP-1 promoter methylation status and plasma TG levels

The plasma TG levels of T2DM patients with MCP-1 promoter unmethylation were higher than those with MCP-1 promoter methylation (TABLE 4). However, it was not significant statistically (p>0.05).

TABLE 4. Plasma TG levels between MCP-1 promoter methylation and unmethylation

	Methylation status		p
	Methylated	Unmethylated	
TG levels (mg/dL)	119.34 ± 52.20	153.65 ± 54.74	0.056*

Data distribution was tested using Saphiro-Wilk test. Data was presented as mean ± SD. *Independent t-test, p< 0.05 statistically significant. TG: triglyceride

A significant correlation between MCP-1 promoter methylation and plasma TG levels was observed (p<0.05), although this correlation was weak (r= 0.322) (TABLE 5).

TABLE 5. **Correlation between MCP-1 promoter methylation and plasma TG levels**

		Methylation Status
	r	0.322
TG levels (mg/dL)	p	0.049*
	N	38

* Spearman correlation test, p < 0.05 statistically significant; TG: triglyceride

Snack intake during intervention

The mean of snack intake per day of subjects are 30.77 g. A total snack that must be consumed everyday for intervention time (4 weeks) are 32 g, so the compliance of subjects were 96.16% (TABLE 6).

TABLE 6. Snack intake per day

Classification	Mean ± SD	Min-Max
Male (n = 9)	30.67 ± 1.59	27.43 – 32.00
Female (n = 10)	30.73 ± 0.90	29.72 – 32.00
Total	30.77 ± 1.25	27.43 – 32.00

The mean of resistant starch intake from snack per day of subjects are 4.08 g. This amounts still not fulfill the resistant starch content determined to consume per day, that were 4.25 g (TABLE 7).

TABLE 7. **Mean of nutrients intake from snack per day**

Nutrients	Mean ± SD
Energy (kcal)	142.95 ± 5.82
Protein (g)	0.93 ± 0.04
Fat (g)	5.61 ± 0.23
Carbohydrate (g)	20.15 ± 0.82
Dietary fiber (g)	0.82 ± 0.03
Resistant starch (g)	4.08 ± 0.17

DISCUSSION

No change in methylation status after the intervention was observed in this study. This estimated that the intervention snack affect the MCP-1 promoter methylation yet. However, it needs further study to prove this reason because there was no literature suggest the duration of resistant starch dietary interventions that affect the epigenetic profile. The research about effect of diet on MCP-1 epigenetic profile was performed in animal model with methionine supplementation. This study was performed in mice with a apoliportotein E deficiency (ApoE$^{-/-}$) which induced with high methionine diet for 15 weeks. The results showed the hypomethylation of MCP-1 promoter region significantly. It is estimated as an effect of decreasing activity of nuclear factor-κB (NFκB)/DNA methyltransferase 1 (DNMT1). However, the duration of methionine induction and resistant starch is known to have the same time to influence the MCP-1 methylation yet.[13]

The higher frequency of unmethylated status than methylated status associated with the glycemia condition. Hyperglycemia leads to increased production of reactive oxygen species (ROS) that is associated with increased DNA damage and chromosomal degradation with alteration of hypomethylation of the DNA.[14] Chronic increase of ROS in the cells can also result in lipid peroxidation and generation of a wide range of other reactive products with the potential to damage DNA.[15] Such DNA lesion shave been shown to interfere with the ability of DNA to function as a substrate for the DNA methyltransferases, result in global hypomethylation.[16]

The not significantly decrease in TG levels was probably caused by the lack of resistant starch content in snack that consume of subject. Subject just consumed 4.08 g/day

of resistant starch in this study. This amounts still not fulfill the resistant starch content determined to consume per day, that are 4.25 g. This value was not differ so far from determined value. However, TG levels may decrease significantly if the subjects have a 100% compliance.

Decreasing of TG levels after the intervention was estimated caused by resistant starch content in the snack. Mechanism of decreasing TG levels by resistant starch is caused by the physiological function of SCFA. These SCFA may decreased lipolysis of adipose tissue, then decreasing free fatty acid circulation (FFA) or non-esterified fatty acids (NEFA).[9] Low of free fatty acid circulation may decreased TG hepatic and decreased the secretion of VLDL, so it can control the increasing of plasma TG levels.[17,18] Short chain fatty acid may improve the insulin sensitivity, then inhibit lipolysis of adipose tissue and decreasing TG levels in circulation.[10] This SCFA may also improve the fatty acid oxidation in the liver through the elevation of forkhead transcription factor (Foxa2), PPAR–G coactivator β (PGC-1β), and PPAR-α gene expression.[9]

The results of Spearman correlation test showed that the correlation between MCP-1 promoter methylation and TG levels was statistically significant. Mechanism about the correlation between methylation of MCP-1 and TG levels are still unknown. However, there are two approaches that may explain about this correlation. First, peroxisome proliferator activated receptor (PPAR) activation can inhibit hypermethylation of the inducible nitric oxide synthase (iNOS) gene which implies that fatty acids or TG which is one PPAR ligand could impose epigenetic modifications on critical genes participating in atherogenesis. One of this genes is MCP-1.[19] Second, down-regulation of the S-adenosyl-homocysteine hydrolase (SAH1) expression in yeast leads to accumulation of S-adenosyl-L-homocysteine (AdoHcy) and decreasing *de novo* synthesis of phosphatidylcholine *in vivo*. This decrease followed by an increase in TG levels, demonstrating that SAH1 regulated methylation has a major impact on cellular lipid homeostasis.[20]

CONCLUSION

The rich resistant starch snacks intervention does not affect MCP-1 promoter methylation changes significantly. However, this intervention can decrease plasma TG levels, although it is not significant. In addition, the correlation between plasma TG levels and MCP-1 promoter methylation status are statistically significant.

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