MASTERARBEIT

Titel der Masterarbeit

„Epigenetic changes of Toll like receptor 4 (TLR4) and Long interspersed nuclear element 1 (LINE1) in obese and diabetic patients after an intervention with a calorie restricted diet combined with an antidiabetic medicine Victoza"

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ZUSAMMENFASSUNG

HINTERGRUND

Adipositas und Diabetes sind zwei häufige metabolische Krankheiten, die beide normalerweise mit einem erhöhten inflammatorischen Zustand in den Patienten assoziiert sind(Carey N. Lumeng, 2011). Unterschiedliche Zustände von epigenetischen Markern in Adipositas als auch in Kontroll Gruppe wurden berichtet (Paul W Franks, 2011). Das Ziel von dem vorliegenden Projekt war zu untersuchen, ob es Veränderungen von epigenetischen Markern bei Patienten mit metabolischem Syndrom nach einer Intervention mit einer kalorie-reduzierte Ernährung gibt. Veränderungen der epigenetischen Markern wurden auch für ihre Folgen von den Entzündung begleitenden metabolischen Syndrome analysiert .

Epigenetische Biomarker von relevanten Gene und ihre Signalwege sind besonders bei der Prävention, Kontrolle und Behandlung von mit Entzündung-verbundenem metabolischen Syndrome wie Diabetes Typ 2 melitus wichtig. Von Toll-like-Rezeptoren, insbesondere TLR4, wurde berichtet, dass er zu Insulinresistenz und chronische Entzündung in Adipositas führen könnte (Jane J. Kim , 2010). Das Ziel der vorliegenden Studie war die Analyse epigenetischer Veränderungen von TLR4 und LINE-1 (globale Methylierung) bei übergewichtigen Patienten mit Diabetes nach einer Intervention von einer kalorie-begrenzten Ernährung mit einer antidiabetischen Medizin Victoza . Auch die Beziehungen zwischen Alter, Gewicht, HbA1c-Wert, Bauchumfang und Methylierungen von TLR4 und LINE1 wurden untersucht.

METHODEN

Genomische DNA von 42 Teilnehmern zu drei Zeitpunkten wurden mit PAX-Gen Blut-DNA-Kits (22 in der adipösen Diabetes Gruppe und 13 in der Kontrollgruppe, sowie 7 in der adipösen Kontrollgruppe) extrahiert und untersucht. T1 war vor der Intervention, T2 war ca. einer Monat mit der Intervention, T3 war ca. vier Monate mit der Intervention. DNA-Methylierungen wurden mit Pyrosequenzierung der gereinigten genomischen DNA nach bisufite Conversion quantifiziert.

ERGEBNISSE

Die sequenzierte Region von TLR4 lag am Begin des Exons. Es gab signifikante negative Assoziationen zwischen BMI, Bauchumfang und TLR4-Methylierungen bei drei Zeitpunkten, sowie positive Assoziationen zwischen HbA1c-Wert, Bauchumfang und LINE-1 Methylierungen bei drei Zeitpunkten. Darüber hinaus könnten BMI- und Bauchumfang Veränderungen auch mit DNA-Methylierung Veränderungen gezeigt werden. Gewichtszunahme korrilierte mit den TLR4-Methylierungen, während Gewichts-verlust die TLR4-Methylierungen in der ersten intervention Zeitraum erhöhte. Auch Bauchumfang-szunahme korreliert mit den LINE1 Methylierungen, während sich mit Abnahme des Bauchumfang die LINE1 Methylierungen in dem ersten Zeitraum reduzierte. Aber während des zweiten Zeitraum wurde einen positiven Zusammenhang zwischen den Bauchumfang-Veränderungen und den TLR4-Methylierung Veränderungen gefunden, was darauf hinwies, dass ein verminderter Bauchumfang mit der TLR4-Methylierung zusammenhängte. Die DNA Methylierungs-Veränderungen von TLR4 und LINE1 nach der Intervention waren nicht so signifikant im Vergleich mit den anderen zwei Kontrollgruppen.

SCHLUSSFOLGERUNGEN

Höherer BMI und grösser Bauchumfang sind bei Adipositas mit einem erhöhten Entzündungszustand verbunden. Dadurch ist die TLR4 Methylierungen erhöht, und Patienten mit höherem HbA1c-Wert und grösserem Bauchumfang haben auch höhere LINE1 Methylierungen. In dem ersten Intervention Zeitraum wurde ein negativer Zusammenhang zwischen den Veränderungen von Gewicht und Bauchumfang und TLR4-Methylierungen gezeigt, sowie einer positive Assoziation zwischen Bauchumfang und LINE1 Methylierungen.

Die Ergebnisse von dem ersten Zeitraum waren identisch mit den vor der Intervention erhielten Ergebnissen, während in der zweiten Interventionszeitraum, Eine Bauchumfang-sabnahme könnte eine verminderte TLR4 Methylierungen induzieren. DNA-Methylierungen könnten von verschiedenen Faktoren wie BMI, Bauchumfang, HbA1c, Medikamente, Ernährung, individuelle Empfindlichkeit gegen-über der Intervention, etc beeinflusst werden. Die Auswirkungen der Intervention auf DNA Methylierungen könnten in einem langfristigen interventions-Zeitraum verändert oder modifiziert werden .

ABSTRACT

BACKGROUND

Obesity and Diabetes are two common dieases, both of which are usually associated with a low-grade inflammatory status in patients (Carey N. Lumeng, 2011). Different performances of epigenetic markers have been reported in obesity as in lean control group (Paul W Franks, 2011). The present project wants to explore whether epigenetic changes of markers can be seen in patients with metabolic syndrome and in patients undergoing caloric restriction diet. Potentially changes of epigenetic markers will be analyzed for their consequences for the inflammation accompanying metabolic syndrome.

The project focuses on the establishment of epigenetic biomarkers relevant for the monitoring and treatment of metabolic syndrome. These biomarkers might be of interest to predict the outcome of an intervention. For example, promoter methylation of TNF-alpha in weight loss response is presently under discussion as a first epigenetic marker.

Established epigenetic biomarkers of inflammatory genes and pathways is important, especially in the prevention, control, and treatment of inflammation-associated comorbidities of metabolic syndrome such as diabetes melitus type 2 (Jane J. Kim, 2010). Toll like receptors, as one main group of pattern recognition receptors to activate the innate immune system to respond to invading pathogens, play a role in a variety of chronic inflammatory diseases like obesity and diabetes (Caroline Ospelt, 2009). TLR4 was the first of human TLRs to be identified, it has been reported to contribute to insulin resistance and enhanced chronic inflammation in obesity (Jane J. Kim, 2010) (Seung-Jin Kima, 2012). The aim of the present study was to explore whether epigenetic changes of TLR4 and LINE-1 (global methylation) can be seen in obese patients with Diabetes after a intervention of calorie restricted diet combined with an antidiabetic medicine called Victoza. And the relationship between age, weight, HbA1c level, waist circumference, and methylations of these two markers will also be discussed.

METHODS

Genomic DNA of 42 participaints with three timepoints were extracted with PAX gene blood DNA kit (22 in the obese diabetes group, 13 in the lean control group, 7 in the obese control group). T1 is before the intervention, T2 is about one month with the intervention, T3 is about four months with the intervention. DNA methylation was quantified by using pyrosequencing after bisufite conversion of the purified genomic DNA.

RESULTS

The sequencing region of TLR4 was not in the promoter region, but in the exon region (+ 215 to + 250bp) with 4 CpGs. We found significant negative associations between BMI, waist circumference and TLR4 methylation levels of three timepoints, while positive associations between HbA1c level, wasit circumference and LINE1 methylation levels of three timepoints were indicated. In addition, BMI and waist circumference change might also be related with DNA methylation alterations. Weight gain was indicated to decrease the TLR4 methylation levels, while weight loss could increase the TLR4 methylation levels in the first period. And waist circumference gain could increase the LINE1 methylation levels, while waist circumference loss could decrease the LINE1 methylation levels in the first period. However, during the second period, a positive association between waist circumference change and the alteration of TLR4 methylation levels was found. And the DNA alterations of methylation levels of TLR4 and LINE1 after the intervention were not so significant compared with other two control groups.

CONCLUSION

In conclusion, higher BMI and wider waist circumference in obesity with higher inflammation levels, were supposed to induce lower TLR4 methylation levels, and higher HbA1c level and also wider waist circumference in the obese diabetes group could increase the LINE1 methylation levels. In the first intervention period, negative association between the weight change, waist circumference change and TLR4 methylation levels was indicated, while positive association between waist circumference and LINE1 methylation levels was indicated. The results

received after the first intervention period were identical with the associations found before the intervention, while in the second intervention period, decreased waist circumference could induce a decreased TLR4 methylation levels. We supposed, that the DNA methylation levels could be influenced by various factors including BMI, waist circumference, HbA1c, medication, diet, individual sensitivity to the intervention, etc, and the effects of the intervention on DNA methylations might be altered or modified as the intervention continued.

INTRODUCTION

EPIGENETICS

Epigenetics is the study of heritable gene modifications, which can alter the gene expression without changing the DNA sequence. Examples of epigenetic changes include DNA methylation, histone modification and microRNAs, which can contribute to gene expression regulation together (J. A. McKay, 2011).

The most well-studied epigenetic mechanism is the methylation of cytosine on the CpG positions. DNA methylation in a promoter region is usually resulted in a change of gene expression. In the promoter of the genes, the CpGs are usually hypomethylated in healthy individuals, while in the gene region are relative highly methylated. Aberrant DNA methylations in both promoter and gene regions are generally related with metabolic disorders and cancers (Manel Esteller, 2002). Additionally, DNA methylation status is also tissue-specific. For example, TLR4 has been reported to be hypermethylated in large intestine cells, while it was hypomethylated in splenic cells. And different methylation status also lead to different gene expressions in this study(Kyoko Takahashi, 2011).

Histone modifications also play a crucial role in epigenetic regulation. Histone acetylation and modification are two most common forms of histone modifications, which can influence the chromatin functions. It has been demonstrated that the contacts between histones and DNA can ben interrupted by the histone modifications, and those histone marks are necessary for recruiting cofactors, which are important in regulating the downstream responses.

It has been recently reported, that the basic DNA methylation can be mediated by the histone modification during the early development. For example, binding to Histone H3 can make the DNMT3L lose the function to recruit the methylatransferases, which play crucial roles in de novo DNA methylation. In that case, the de novo DNA methylation can be inhibited because of the lack of methyltransferases, when the H-3K4 has been methylated.

In contrast, Histone modification can also be affected by DNA methylation by bringing more acetylated or non-acetylated histones. (Howard Cedar, 2009) .

MicroRNAs are very small RNAs with an average of 22 bp. MicroRNAs can bind to the RNA specifically, thus regulating the transcription. Almost all the genetic pathways can be influenced by MicroRNAs. (J. A. McKay, 2011).

In brief, all of these three distincts work indenpendently or interact with each other, repressing or activating gene transcription. There are two modes in DNA methylations, which can interfere with transcription. The first is that the methyl group can inhibit the binding of transcriptional factors to the DNA sequence. The second is that the methyl group can attract the transcriptional factors (Adrian, 2011). Activation and Inactivation of gene transcriptions are directly associated with the gene expression, which plays a crucial role in developing metabolic dieases and cancers. It has been long known, that hypermethylation of Tumor suppressor genes with low expressions of certain proteins are related with tumor development or progression (Hoi-Hung Cheung, 2009).

Some DNA methylation inhibitors and also histone deacetylation inhibitors have been developed to reverse the epigenetic changes including DNA methylations and histone decetylations in cancer therapy. For example, demethylation of tumor suppressor gene is a potential treatment of cancer. However, it is also critical to the normal functions of cells (Feng-Feng Cai , 2011).

Epigenetic changes caused by different environmental factors including stress, physical and mental abuse, diet, medication and etc, have been reported to be associated with disease susceptibility. A monozygotic twin study can explain the different susceptibilities to diseases in monozygotic twins with identical genetics because of differential epigenetic changes. A longitudinal-developmental twin study indicated that DNA methylation status in monozygotic twins were already apparent different in early childhood, and the individual differences are instable over time because of the continued influences from the environmental factors(Chloe Chung Yi Wong , 2010).

Dietary exposures are considered to play a crucial role in epigenetics. Common complex diseases and also cancer have been reported to be associated with diet. Additionally, a dietary control is also recommended in a therapy to the patients with chronic diseases, especially for Typ II Diabetes and Obesity, which are usually induced by excessive food intake. Caloric restriction is recommended for the treatment of obesity, and both of DNA methylations and gene expressions are responsive to caloric restriction. However, Significant different DNA methylations and gene expressions could influence the weight-loss responses in patients (Luigi Bouchard, 2010).

Bioactive food components like polyphenols and nutrients like folate, vitamin B12, methionine and etc, have been also reported to be able to reverse or change the epigenetic phenomena such as histone modifications and DNA methylations. Physiologic and pathologic processes can be also modified by nutrients through epigenetic mechanisms during our life time. Additionally, specific nutrients can prevent the development of complex diseases and the progression of cancers by modulating the epigenetic phenomena (Friso, 2010) .

PUBLICATION DRAFT

INTRODUCTION

The incidence of obesity and diabetes has increased rapidly in the last few decades. And there is usually a overlap of these two chronic diseases. The dimension of inflammatory activation in obesity and diabetes is not large and so it is often called “low-grade” chronic inflammation, which has been reported to be related with characteristic patterns of intestinal microorganisms (Herbert Tilg , 2011).

The intestinal microbiota plays an important role in immune development and homeostasis. T-he assemblages of bacteria resident in the intestinal tract provide a first line of denfence that can exclude invading pathogens, allergens and LPS. Human immune functions are affected by several bacterial groups, especially SCFA-(acetate,propionate and butyrate) producing consituents of the human microbiota, which are of importance for underlying low grade inflammation.

The mechanisms, which have been reported to link microbiota with obesity and diatetes, are based on the fact that the obesity and insulin resistance are associated with low-grade chronic systemic inflammation. Endotoxemia like LPS, triggers low grade inflammation, which contributes to induce obesity and diabetes. Patients with type 2 diabetes also show higher LPS level than the control group without diabetes.

Analysis of fecal gut microbiota during a weight loss programme indicated different patterns of microbiota in obese patients as in lean control group, and the amount of certain bacteria was also changed after the weight loss. It was supposed that the inflammatory status could be altered after the weight loss by intestinal bacteria alteration .

Toll like receptors have been reported to play a key role in innate immune system. Here, TL-R4 is the responder to LPS from Gram-negative bacteria and is thus especially important in t-he low-grade inflammation. In addition, elevated levels of free fatty acids in obesity has also been shown to be related with insulin resistance, and recent studies have demonstrated that free fatty acids can activate TLR4, and the downstream transcriptional factor NF-kb may play an important role in insulin resistance. Increased expression and activity of TLR4 have been reported in both T1DM and T2DM (Hang Shi, 2006).

In our study, a intervention of a calorie restricted diet combined with an antidiabetic medicine Victoza has been carried out in the obese patients with diabetes. Microbiota changes have been reported in the participants after the intervention, the aim of the study is to find the effect of the intervention on the epigenetic changes of TLR4, which was supposed to be related with the inflammatory status in obesity and also Diabetes, and its methylation status was also supposed to be influenced by the altered inflammatory status induced by the intervention.

The relationship between epigenetics and metabolic disorders have been widely discussed. It is also well known, that epigenetic marks can be modified by environmental factors like dietary exposure, life style, medication and also aging. Calorie restriction diet, as an important environmental intervention, has been reported to influence the aging process by recovering the aging induced aberrant DNA methylation, but by specific loci control rather than globally (Marı´a Berdasco , 2012 ;Yuanyuan Li, 2011). In our study, DNA Methylation of Line1 (long intersperd nuclear element 1) was also investigated as a control for the alteration of global methylation.

Recent studies have demonstrated, that LINE1 hypomethylation was usually related with higher incidence of tumors (Charlotte S. Wilhelm, 2010). It has also been reported, that global LINE-1 DNA methylation was positive associated with blood glycaemic and lipid profiles (Mark S Pearce, 2012). Global DNA methylation in peripheral blood leukocytes was also demonstrated to be significant positive associated with insulin resistance in a MZ twin study, independent of established risk factors (Jinying Zhao, 2011).

In our study, a intervention of calorie restricted diet combined with an antidiabetic medicine Victoza has been carried out as a therapy for diabetes. The intestinal microbiota and other parameters like BMI, HbAc1 could be changed after the intervention, and the DNA methylations of TLR4 and also LINE1 were expected to be influenced after the intervention. In addition, the associations between these variables and methylation status of TLR4 and LINE1 were also investigated.

MATERIALS AND METHODS

STUDY DESIGN

This study was one part of the epigenetic project conducted at the department of nutritional science of the university of Vienna between January 2011 and june 2013.

We analyzed the DNA methylation of TLR4 and LINE-1 at three different time points: T1 (before the intervention, T2 (after one month of intervention), T3 (after four months of the intervention). The subjects were recruited for three groups: A obese diabetic group (n=21), a lean control group (n=13), and a obese control group (n=7). The OD group used a formula diet to lose weight accompanied with a medicine called Victozo, while the other two groups were requested not to change their lifestyle, especially their nutrition and sport habits.

Table 1. Characteristics in the referents and intervention group

	Referents		**Intervention**
	Obese control (n =7)	Lean control (n = 13)	Obese and diabetic patients (n =22)
Mean age, y (min-max)	26 (24-31)	55(49-59)	59 (40-76)
Gender, n (%)			
Male	5 (71)	2 (15)	13 (59)
Female	2 (29)	11(85)	9 (41)
Height, (min-max)	175,6 (166-191)	169,2 (160-184)	172 (156-189)
Mean BMI, (min-max)	32,4 (24,8-38,3)	21,1 (18,1-24,9)	38,5 (33,2-56,2)
Waist circum. (min-max)			123 (110-149)
Mean HbA1c/ T1, (min-max)			8,3 (6,3-10,3)

INCLUSION CRITERIA INTERVENTION AND OBESE CONTROL GROUP

Patients with Diabetes mellitus type II, impaired glucose tolerance, impaired fasting glucose or insulin resistance and at least two of the following: blood pressure: > 140/90mmHg, dyslipidaema: TG> 1,695 mmol/L and HLD-C< 0,9mmol/L by male, <1 mmol/L by female. BMI> 30, were recruited in the hosiptial of Burgenland.

SAMPLE COLLECTION

Blood was sampled at T1 (before the intervention), T2 (after one month of the intervention) and T3 (after four months of the intervention) using PAXgene Blood DNA tubes (Qiagen, Hilden, Germany). The samples were stored until preparation at -20°C.

DNA ISOLATION AND BISULFITE CONVERSION

DNA was extracted using the PAXgene Blood DNA Kit (Qiagen, Hilden, Germany) according to manufactures' instructions. For methylation analysis, all samples were bisulfite - converted with the Epi TectBisulfite Kit (Qiagen, Hilden, Germany), resulting in the deamination of un-methylated cytocines to urcil, whereas methylated cytosines remain unchanged. 2ul converted DNA was amplified using the EpiTect Whole Bisulfitome Kit (Qiagen, Hilden, Germany). The concentration of DNA was measured on a Pico 100 (Picodrop Limited). All reactions were carried out according to manufacturer's protocols. Samples were stored at -20°C.

PCR CONDITIONS AND GEL ELECTROPHORESIS

Primer design software was used to find the suitable primers (Qiagen, Hilden, Germany) for the bisulfite converted DNA to amplify the analyzed regions of TLR4. The PCR was carried out in 25 ul reaction mixtures, which contains 12,5ul Pyromark 2X PCR master mix, 12,5 pmol of each primer, 2,5 ul Coraload Concentrate 10X (Qiagen, Hilden, Germany), and 5 ng (TLR4) or 10ng (LINE1) bisulfite converted DNA. PCR Conditions were 95 °C for 15 min, 45 cycles of 94°C for 30s, 59°C for 45s, and 72°C for 45 s, and a final elongation at 72 °C for 10 min for TLR4, while the PCR conditions were 95 °C for 15 min, 45 cycles of 94°C for 30s, 50 °C for 30s, 72°C for 30 s, and a final elongation at 72 °C for 10 min. After PCR, 5 ul of each product inclusive the control without samples were checked by 2% agarose gel electrophoresis. The products should be identical and without byproducts, the control should also be clean.Then the rest of 20ul PCR products can be used for the Pyrosequencing.

QUANTITATIVE GENE METHYLATION ANALYSIS OF TLR4 BY PYROSEQUENCIN

A mixture of 20 ul PCR product, 3 ul streptavidin-coated Sepharose beads (GE Healthcare, Uppsala, Sweden), 40 binding puffer,and 17ul distilled water was prepared for pyrosequencing using a Pyromark Q24 System (Qiagen, Hilden, Germany). The Biotinylated end of ampflied PCR Products could bind to the beads, which were sucked by the vaccum Prep Tool. The PCR products were washed and denatured using 70% Alcohol, 0,2M NaOH solution and washing puffer.The purified single-stranded PCR products were released to the annealing buffer (Qiagen, Hilden, Germany), which contains corresponding pyrosequencing primer(10ng). The buffer with diluted PCR products was used for the Pyrosequencing. The Pyrosequencing assay was designed with the Pyromark Q24 Software (Qiagen, Hilden, Germany), The CpG-cytosines were shown in C and T in the dispensation, the DNA methylation was indicated in the ratio of C/T. all non-CpG cytosines were converted to T, A single Cytosine was inserted after or before the T, which were converted from non-CpG cytosines, as a internal control for the effectiveness of the bisdulfite DNA conversion.

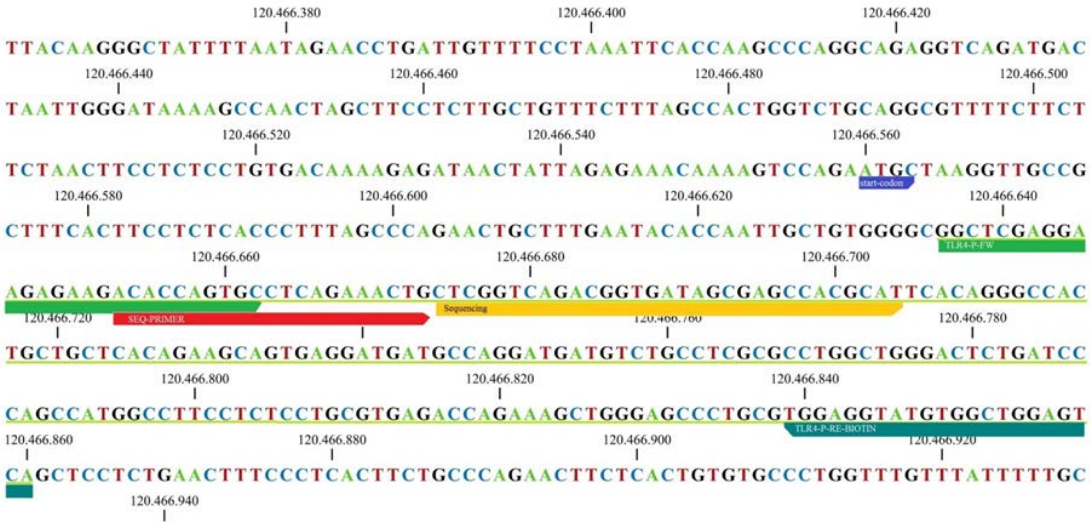
Table 2 Primers used for PCR and Pyrosequencing

Name	Sequence 5'- 3'	Size	Application	AT
TLR4 (F)	GGTTAGAGGAAGAGATATTAGTGT	27	225 bp	59°C
TLR4 (R)	Biotin-TAACTCCAACCACATACCTCCA	22		59°C
TLR4 (S)	ATATTAGTGTTTTAGAAATTG	21		
LINE1 (F)	TTTTGAGTTAGGTGTGGGATATA	23		50°C
LINE1 (R)	Biotin-AAAATCAAAAAATTCCCTTTC	21		50°C
LINE1 (S)	AGTTAGGTGTGGGATATAGT	20		

Table 3 Analyzed sequence of TLR4 and LINE1

Assay	Sequence to analyze 5'-3'	Nr of CpGs
TLR4-P	CTCGGTCAGACGGTGATAGCGAGCCACGCAT	4
LINE1-P	TTCGTGGTGCGTCGTTT	3

Figure 1 Bisulfite converted TLR4 gene sequence region used for the pyrosequencing



STATISTICAL ANALYSIS

Methylation levels of TLR4 and LINE1 were analyzed with SPSS 20 (IBM, Armonk, NY). Boxplot was used to show the methylation levels of different groups at three timepoints, spearson test was used to determine the correlations between different relevant parameters and the methylation levels. A p value of $< 0,05$ was considered statistically significant.

RESULTS

DNA methylations of TLR4 and LINE1 with three timepoints were analyzed to investigate th-e effect of the intervention. A lean and a obese group are recruited as controls. The informati-on of the participants including age, sex, height, weight, waist circumference, HbA1c level, etc was also collected to evaluate the assciations between these variables and Methylation leve-ls of these two markers.

PYROSEQUENCING OF THE TLR4 GENE REGION

Quantitative analysis of these 4 CpGs in a 36 bp sequence, which is not located in the promot-er, but at the very beginning of gene region of TLR4(+215 to +250 bp).

Figure 2 Transcription factors located in TLR4 sequencing region

In the sequencing region of TLR4, we found tree transcription factors inclusive GATA2, GATA3, and ZNF354C, which locate near or at the CpGs positions of this region.

Figure 3 TLR4-P assay with 4 analyzed CpGs for Blood samples. Two bisulfite treatment controls (6,28) were used to confirm the complete conversion of unmethylated cytosine residues.

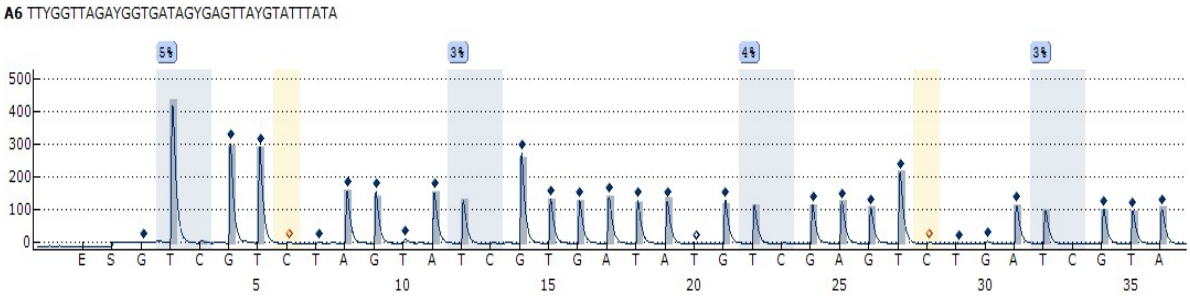


Table 4 Methylation levels of 4 CpGs of TLR4 at T1,T2,and T3

OD	CpG1	CpG2	CpG3	CpG4	LC	CpG1	CpG2	CpG3	CpG4
OD1/T1	8,91	8,89	6,48	5,57	LC1/T1	7,59	6,45	6,07	6,42
OD1/T2	7,17	6,52	6,0	4,95	LC1/T3	7,41	6,7	6,91	6,24
OD1/T3	6,88	6,22	5,64	4,99	LC2/T1	6,64	6,05	5,82	4,83
OD2/T1	9,05	8,73	9,41	7,68	LC2/T2	4,91	3,74	3,97	3,83
OD2/T2	9,23	9,0	9,08	7,83	LC2/T3	6,49	4.81	6,17	4,20
OD2/T3	7,98	7,36	7,89	6,59	LC3/T1	5,61	5,27	4,97	4,8
OD3/T1	6,31	5,22	5,12	4,47	LC3/T2	6,39	5,21	5,77	4,39
OD3/T2	6,99	6,38	6,91	6,86	LC5/T1	7,25	5,13	4,82	4,73
OD3/T3	6,68	6,05	6,61	6,67	LC5/T3	6,62	5,5	6,03	5,87
OD4/T1	5,68	5,07	5,17	4,38	LC6/T1	7,16	6,45	4,92	4,8
OD4/T2	8,68	5,89	6,45	6,97	LC6/T2	5,19	4,87	5,18	5,02
OD4/T3	5,64	5,93	5,13	3,73	LC6/T3	5,29	4,08	5,82	3,41
OD6/T1	4,75	4,38	4,86	3,96	LC7/T1	4,49	4,76	4,41	3,14
OD6/T2	6,14	4,98	4,94	4,48	LC7/T2	4,89	4,6	4,3	3,41
OD6/T3	5,69	4,7	4,44	4,18	LC7/T3	7,07	6,08	5,5	4,37
OD7/T1	5,85	5,49	4,44	4,36	LC9/T1	5,78	5,21	5,09	4,6
OD7/T2	6,47	5,42	4,75	4,04	LC9/T2	7,2	4,87	5,22	5,08
OD7/T3	6,22	4,88	4,55	4,38	LC9/T3	4,55	3,82	4,36	3,74
OD8/T1	5,55	6,96	5,38	5,49	LC10/T1	6,81	3,91	4,53	4,05
OD8/T2	4,57	3,54	3,14	3,11	LC10/T2	6,67	4,14	4,29	3,87
OD8/T3	4,74	3,65	4,19	3,76	LC10/T3	6,29	4,15	4,24	3,82
OD9/T1	7,22	5,77	5,09	4,53	LC11/T1	6,4	4,06	4,78	3,86
OD9/T2	6,29	5,33	4,23	4,59	LC11/T2	6,84	5,19	6,36	4,79
OD9/T3	6,56	5,63	4,91	4,16	LC11/T3	6,72	4,84	4,78	4,87
OD10/T1	9,41	6,9	6,4	4,8	LC12/T1	6,40	4,08	3,67	4,22
OD10/T2	5,3	4,66	4,61	3,94	LC12/T2	6,52	5,17	5,98	4,44
OD10/T3	6,3	6,13	5,17	4,64	LC12/T3	7,82	6,2	5,84	5,39
OD12/T1	5,28	3,17	2,69	2,69	LC13/T1	7,62	5,74	4,95	4,58
OD12/T2	4,36	3,68	3,34	1,77	LC13/T2	5,66	4,75	5,46	4,9
OD12/T3	4,59	4,29	3,48	3,22	LC13/T3	5,65	5,04	5,11	3,91
OD13/T1	6,89	5,3	5,48	5,06	LC15/T1	4,17	3,52	4,09	3,5

OD13/T3	6,24	5,19	5,8	4,51
OD14/T1	6,16	5,12	5,79	5,44
OD14/T2	5,75	5,03	4,39	4,33
OD14/T3	7,89	6,59	6,20	5,72
OD15/T1	5,74	3,98	4,76	4,98
OD15/T2	7,26	5,69	5,27	5,14
OD15/T3	6,3	6,78	5,29	4,47
OD16/T1	7,75	5,43	5,5	4,21
OD16/T2	6,27	5,47	5,54	4,42
OD16/T3	7,25	6,6	5,53	5,22
OD18/T1	5,25	4,27	4,41	4,11
OD18/T2	4,48	4,34	4,18	2,98
OD18/T3	6,4	4,48	3,33	3,25
OD19/T1	6,47	4,4	3,83	3,42
OD19/T2	5,97	4,32	5,07	4,45
OD20/T1	8,7	5,01	4,22	3,63
OD20/T2	6,63	4,97	4,59	3,57
OD21/T1	5,86	4,9	4,79	3,74
OD21/T2	6,63	4,22	4,51	4,07
OD21/T3	5,4	5,03	4,41	3,89
OD22/T1	7,78	7,19	5,89	4,81
OD22/T2	6,27	5,53	4,46	3,81
OD22/T3	6,36	5,10	4,55	3,52
OD23/T1	5,11	4,52	4,16	3,26
OD23/T2	6,16	6,57	4,35	4,89
OD23/T3	6,26	5,71	4,58	4,28
OD24/T1	8,35	5,63	6,19	4,16
OD24/T2	7,0	3,81	4,46	3,93
OD24/T3	8,6	5,56	4,61	4,18
OD25/T1	7,53	4,99	4,5	5,4
OD25/T2	5,51	4,39	4,91	3,66
OD25/T3	9,18	6,06	4,88	4,76

LC15/T3	5,97	4,57	5,38	5,21

OC	CpG1	CpG2	CpG3	CpG4
OC1/T1	6,49	4,39	5,0	4,77
OC1/T2	5,41	4,01	4,13	2,24
OC2/T1	5,23	4,43	4,07	3,71
OC2/T2	4,09	3,66	4,05	4,29
OC2/T3	5,13	4,93	4,33	4,33
OC3/T1	3,85	3,11	3,2	3,18
OC3/T2	3,77	3,19	3,58	3,1
OC3/T3	3,98	3,75	3,43	3,3
OC4/T1	7,89	3,76	3,84	3,27
OC4/T2	8,24	3,96	3,61	3,22
OC4/T3	7,47	3,95	3,66	3,46
OC5/T1	4,21	3,85	2,92	4,91
OC5/T2	5,70	5,22	5,07	4,26
OC5/T3	4,95	4,52	4,34	4,17
OC6/T1	5,24	3,15	4,3	3,56
OC6/T2	4,49	3,89	3,18	2,62
OC8/T1	5,55	4,53	3,92	3,57
OC8/T2	5,81	4,11	4,16	3,57
OC8/T3	5,81	3,47	2,02	3,49

Quantitative analysis of these 3 CpGs in a 17 bp sequence (Qiagen, Hilden, Germany).

Figure 4 LINE1-P assay with 3 analyzed CpGs for blood samples. One bisulfite treatment control (1) was used to confirm the complete conversion of unmethylated cytosine residues.

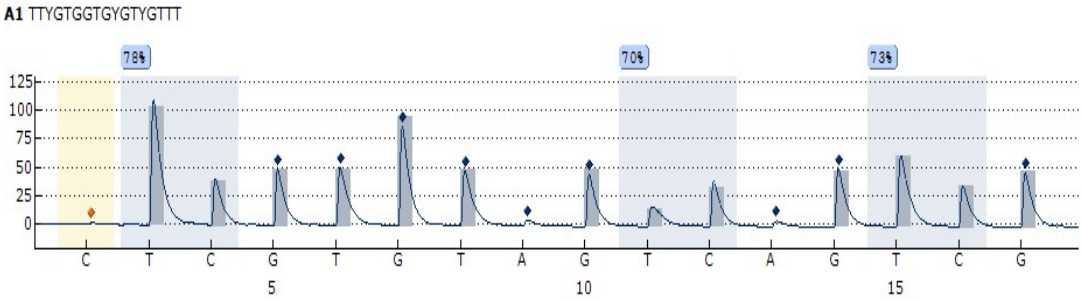


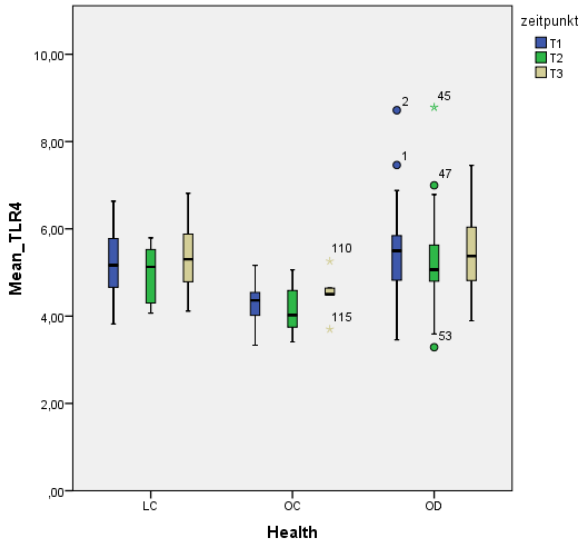
Table 5 Methylation states of 3 CpGs of LINE1 at T1,T2,and T3

LC	CpG1	CpG2	CpG3
LC2/T1	67,02	51,72	55,43
LC2/T2	71,38	63,45	63,1
LC2/T3	66,57	53,49	55,31
LC8/T1	66,7	57,04	58,6
LC8/T2	66,66	61,51	61,06
LC8/T3	69,83	63,62	60,56
LC9/T1	76,1	63,4	66,71
LC9/T2	72,25	61,08	62,93
LC9/T3	70,81	61,90	63,37
LC10/T1	74,77	64,89	65,48
LC10/T2	74,79	64,62	65,18
LC10/T3	73,8	64,24	64,99
LC11/T1	76,2	64,48	66,89
LC11/T2	76,11	64,1	66,87
LC11/T3	75,2	63,62	66,1
LC12/T1	74,07	61,87	63,89
LC12/T2	75,51	64,25	67,67
LC12/T3	76,23	64,25	67,54
LC15/T1	68,3	61,86	60,71
LC15/T2	68,83	59,48	58,92
LC15/T3	71,45	59,14	59,62
LC18/T1	71,87	63,63	64,09
LC18/T2	69,86	62,9	62,48
LC18/T3	70,29	61,18	61,75

OC	CpG1	CpG2	CpG3
OC1/T1	70,43	58,89	61,32
OC1/T2	73,43	62,48	64,01
OC2/T1	64,5	55,43	55,4
OC2/T2	59,54	59,89	59,86
OC2/T3	60,33	59,09	57,15
OC3/T1	68,21	60,01	58,16
OC3/T2	63,63	59,66	56,78
OC3/T3	66,04	58,52	56,01
OC4/T1	68,05	61,69	60,76
OC4/T2	68,74	62,47	61,67
OC4/T3	70	62,29	62,19
OC5/T1	75,58	64,53	64,65
OC5/T2	74,28	63,92	65,59
OC5/T3	72,87	62,5	63,46
OC6/T1	72,65	62,78	63,47
OC6/T2	73,43	63,69	65,03
OC8/T1	70,19	63,22	62,96
OC8/T2	73,29	64,61	65,51
OC8/T3	72,06	62,9	64,59

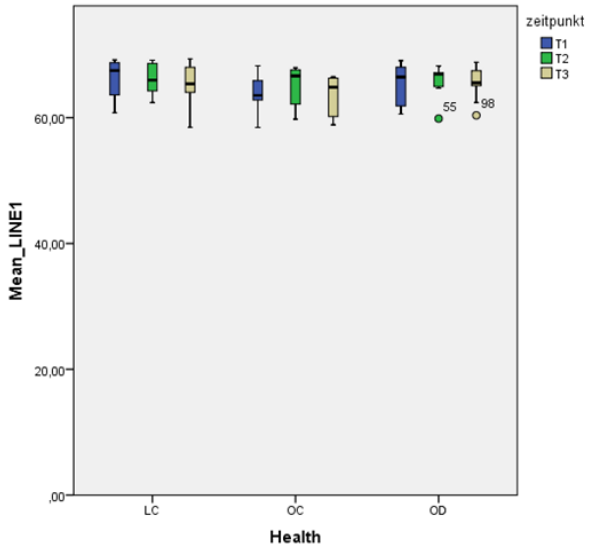
OD	CpG1	CpG2	CpG3
OD2/T1	70,37	62,78	63,44
OD2/T2	69,66	62,72	62,49
OD2/T3	69,65	63,75	62,58
OD4/T1	75,41	65,55	66,68
OD4/T2	73,47	63,49	64,22
OD4/T3	74,34	65,15	66,31
OD7/T1	73,21	65,27	65,76
OD7/T2	71,78	64,21	64,41
OD7/T3	75,42	64,81	66,22
OD12/T1	71,01	55,68	56,03
OD12/T2	71,41	61,32	61,36
OD12/T3	72,40	61,57	61,6
OD13/T1	68,01	59,73	59,18
OD13/T2	72,67	63,37	64,84
OD13/T3	71,6	61,56	62,26
OD14/T1	65,38	58,68	57,07
OD14/T2	65,6	57,36	56,59
OD14/T3	63,32	59,38	58,59
OD16/T1	66,9	60,02	59,62
OD16/T2	72,33	63,05	64,14
OD16/T3	67,39	59,86	60,08
OD21/T1	74,17	63,82	64,76
OD21/T2	75,31	63,35	65,99
OD21/T3	74,26	63,3	64,88
OD24/T1	76,58	63,75	65,32
OD24/T2	75,87	63,43	65,2
OD24/T3	72,91	61,84	62,71
OD25/T1	73,6	64,35	66,19
OD25/T2	73,61	62,87	64,9
OD25/T3	71,43	62,18	64,03

Figure 5 Mean methylation levels of 4 CpGs of TLR4 of different groups at three timepoints



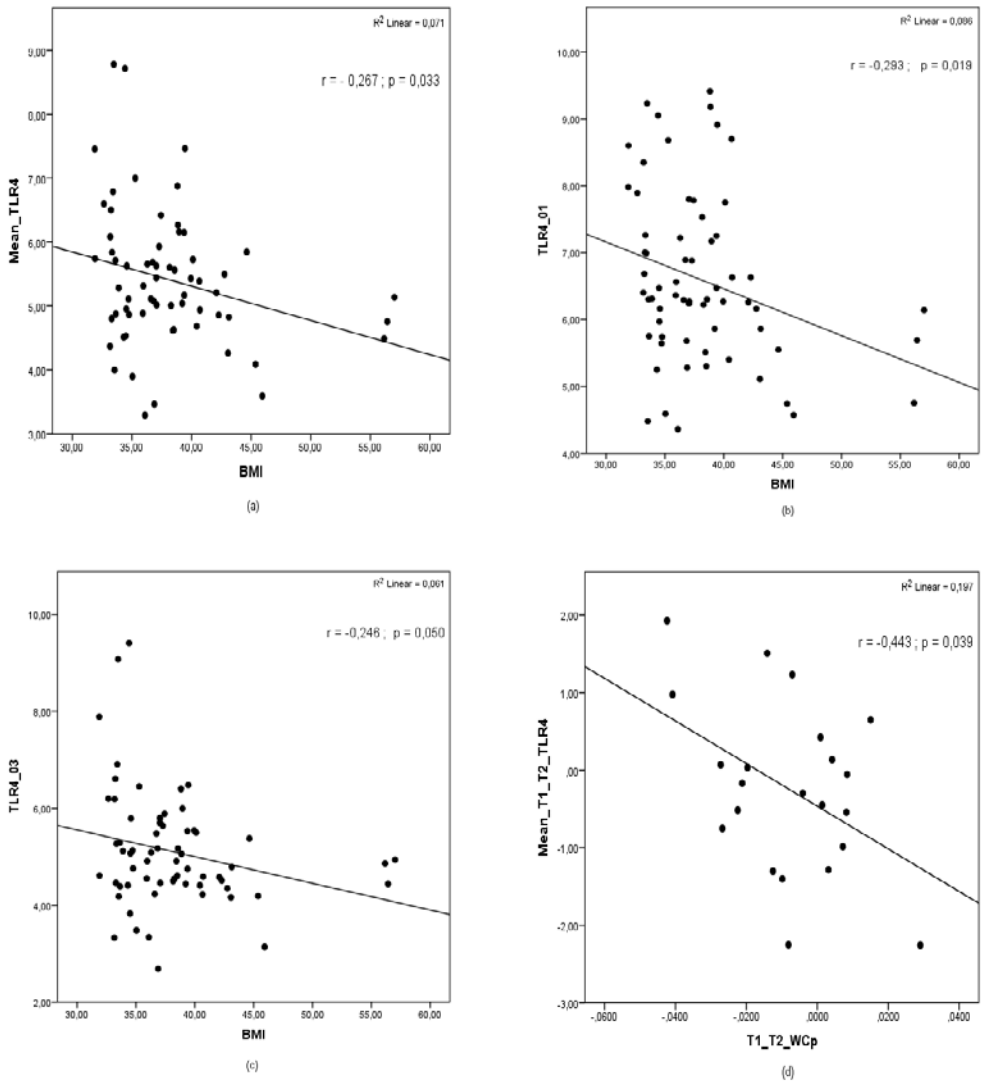
From the figure above, we can observe that the methylation levels of TLR4 with 3% to 8% are not very high. The obese control group shows relative lower mean methylation levels than the intervention and lean control group at three timepoints. However, there was no significant differences of the TLR4 mean methylations between the three timepoints in all three groups. Deficiency of the samples in obese control group might contribute to the lower mean methylation levels.

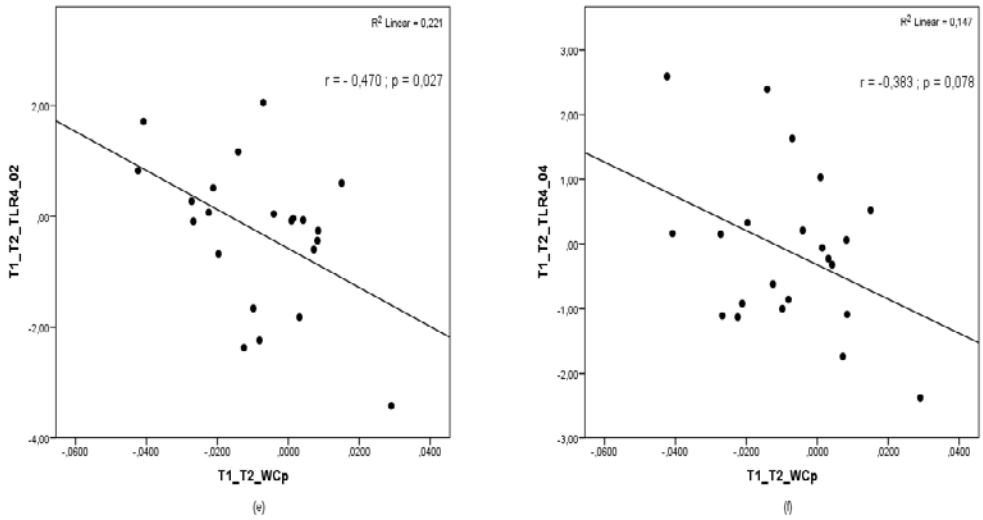
Figure 6 Mean methylation levels of 3 CpGs of LINE1 of different groups at three timepoints



From the figure above, the mean methylation of LINE1 is about 70% in this analyzed region, and no significant differences of the LINE1 mean methylation levels between the three groups are observed. In addition, the differences of LINE1 mean methylation are also very slight at three timepoints in all three groups.

Figure 7 Associations between BMI, weight change percentage and DNA methylation levels of TLR4 in OD group.

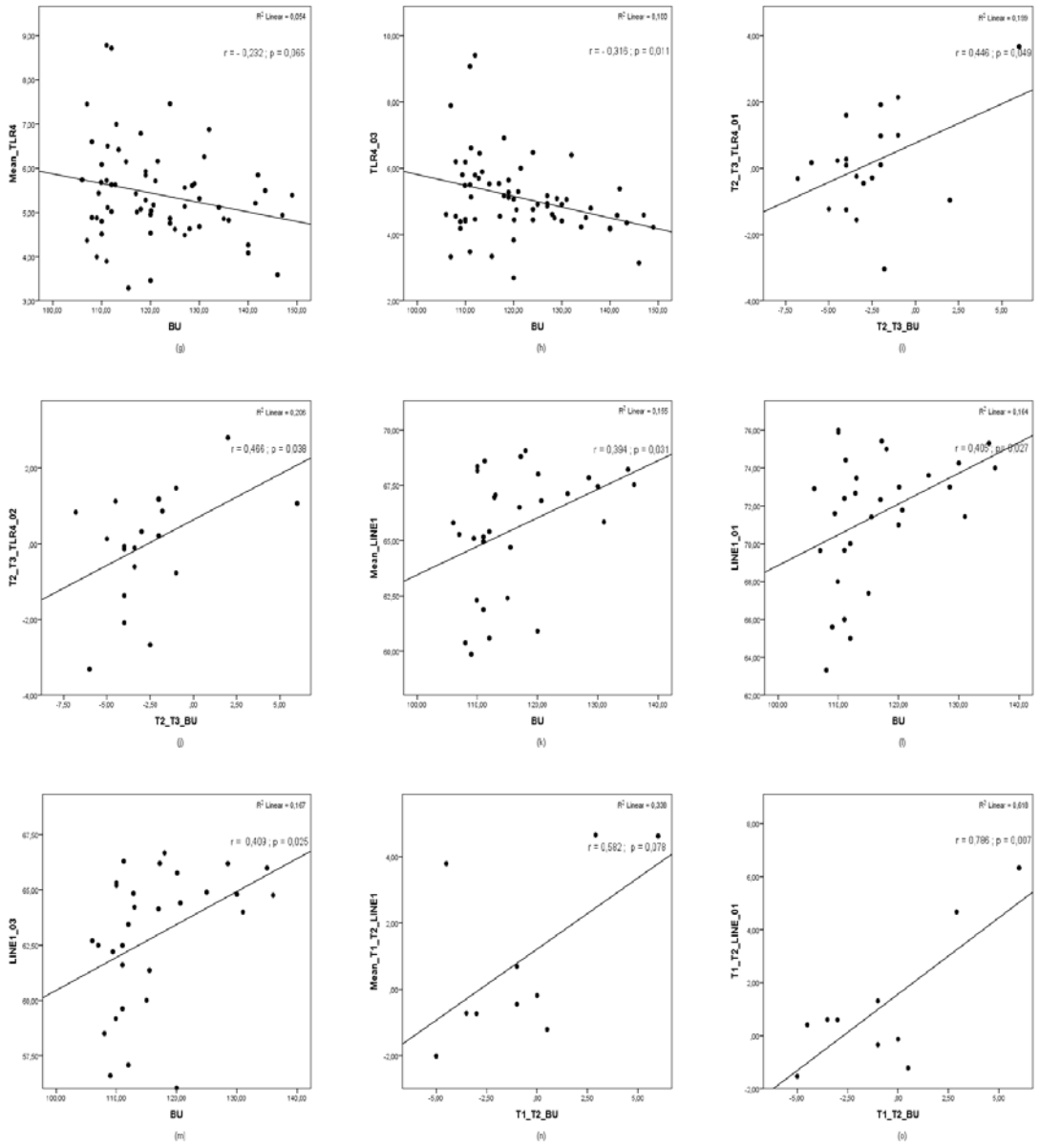




a) Signifcant negative association between BMI and TLR4 mean methylation. b)Significant negative association between BMI and TLR4 methylation at position 1. c)Significant negative association between BMI and TLR4 methylation at position 3. d)Significant negative association between weight change percentage and TLR4 mean methylation alteration during the first intervention month (T1-T2). e)Significant negative association between weight change percentage and TLR4 methylation alteration at postion 2 during the first intervention month (T1-T2). f)Significant negative association between weight change percentage and TLR4 methylation alteration at position 4 during the first intervention month (T1-T2).

The relationships between the variables including sex, age, height, waist circumference, HbA1-c, etc and the methylation levels of TLR4 were investigated. No significant correlation was found between sex, height and TLR4 methylation levels, while significant negative associati-ons were indicated between BMI and TLR4 methylation level at position 1 and position 3, as well as the TLR4 mean methylation. In addition, significant negative association between weight change percentage and TLR4 methylation was also indicated at position 2 and position 4, as well as the TLR4 mean methylation alterations. It was indicated, that higher BMI led to lower TLR4 methylation levels, and weight loss might increase the TLR4 methylation levels, while the weight gain might decrease the TLR4 methylation levels.

Figure 8 Association between waist circumference and DNA methylation levels of TLR4 and LINE1 in OD group.



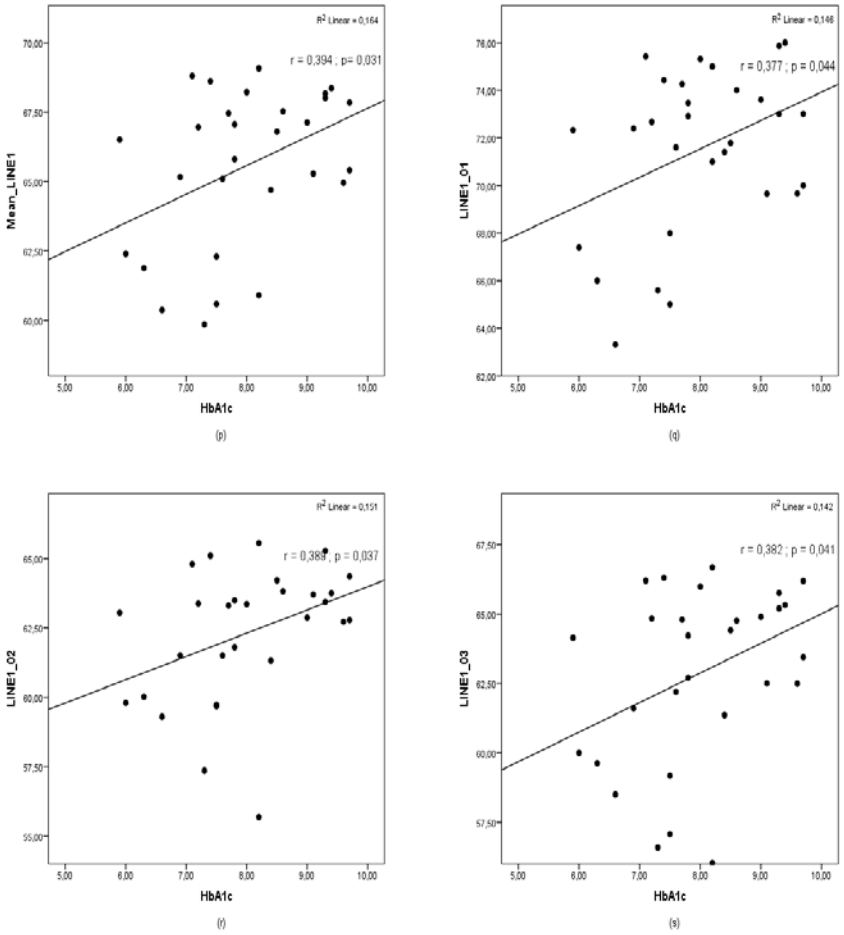
q) Significant negative association between waist circumference and mean TLR4 methylation levels. h) Significa-nt negative association between wasit circumference and TLR4 methylation levels at position 3. i)Significant po-sitive association between waist circumference change and TLR4 methylation level alteration at position 1 during the second intervention period (T2-T3). J) Significnat positive association between waist circumference change a-nd TLR4 methylation alteration at position 2 during the second intervention period(T2-T3). k) Significant positi-ve association between waist circumference and LINE 1 mean methylation. l) Significant positive association bet-ween waist circumference change and LINE 1 methylation levels at position 1. m)Significant positive association between waist circumference and LINE 1 methylaiton levels at position 2. n) Significant positive association bet-ween waist circumference change and LINE 1 mean methylation level

alteration during the first intervention peri-od (T1-T2). o) Significant positive association between waist circustance change and LINE 1 methylation altera-tion at position 1 during the first intervention period (T1-T2).

Waist circumference , as an important parameter for obesity and metabolic disorders, plays al-so a role in the DNA methylation of TLR4 and LINE1. Significant negative association betw-een waist circumference and TLR4 methylation level at position 3, and also the TLR4 mean methylation level was indicated , while there were significant positive associations between waist circumference and LINE1 methylation level at position 1 , position 2, and also the mean methylation level. Waist circumference change caused by weight loss was also associated with the alterations of DNA methylation of TLR4 and LINE1. Significant positive associations be-tween waist circumference change and TLR4 methylation alteration were indicated at position 1 and position 2 during the second intervention period, while significant positive associations between waist circumference and LINE 1 methylation alteration at position 1, as well as the m-ean methylation level were indicated in the first intervention period.

In brief, wider waist circumference could induce a lower TLR4 and higher LINE1 methylation levels, and decreased waist circumference could induce decreased LINE1 methylation level during the first intervention period, while the increased waist circumference could increase the LINE1 methylation level. In the second intervention period, a positive association was also in-dicated between waist circumference and TLR4 methylation levels. This result was not identic-al as the association between weight change percentage and TLR4 methylation levels in the f-irst period, indicating that the effect of the intervention on TLR4 methylation levels might be altered or modified by various factors in the second period as in the first period.

Figure 9 Association between HbA1c level and LINE1 methylation levels in OD group at three timepoints.



p) Significant positive association between HbA1c level and LINE1 mean methylation levels in OD group at three timepoints. q) Significant positive association between HbA1c level and LINE 1 mean methylation levels at position 1 in OD group at three timepoints. r) Significant positive association between HbA1c level and LINE1 mean methylation level at position 2 in OD group at three timepoints. s) Significant positive association between HbA1c level and LINE 1 mean methylation level at position 3 in OD group at three timepoints.

Glycated hemoglobin (hemoglobin A1c, HbA1c, A1C, or Hb1c; sometimes also HbA1c) is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. It is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose. Normal levels of glucose produce a normal amount

of glycated hemoglobin. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin increases in a predictable way. This serves as a marker for average blood glucose levels over the previous months prior to the measurement. In our study, significant positive association has also been found between the HbA1c level and LINE1 methylation levels at all three positions, as well as the LINE1 mean methylation level in OD group at three timepoints. It was indicated, that higher HbA1c level could induce higher LINE1 methylation level. And it has been also reported, that there was positive association between LINE1 methylation and fasting glucose level.

DISCUSSION

In our study, we analyzed the CpGs of TLR4 in exon region, which contains relative more CpGs compared with the promoter in TLR4. The results showed, that the alterations of mean methylation levels at three timepoints differed slightly in different groups. However, the TLR4 mean methylation levels of the obese control group were relative lower than the intervention and lean control groups, we supposed that, the deficiency of samples in obese control group might be the contributor .

Visceral obesity has been reported to be an important risk factor for low-grade inflammation, which can increase the risk of diabetes and cardiovascular diease, thus waist circumference, as the main indicator of visceral fat, is considered to play a crucial role in metabolic dieases (B. Strasser1, 2012). In our study, BMI and Waist circumference were indicated to be negative associated with TLR4 methylation levels. In addition, Weight change percentage during the first intervention period was also negative correlated with the TLR4 methylation level alteration, while significant positive association between waist circumference and TLR4 methylation alteration has also been found during the second intervention period, but not during the first intervention period. It was indicated, that higher BMI and wider waist circumference could induce lower TLR4 methylation level, and weight gain could decrease TLR4 methylation level, while weight loss could increase TLR4 methylation level during the first intervention period. This result was not identical as the association indicated between the waist circumference change and the alteration of TLR4 methylation level during the second period. It was supposed that, the TLR4 methylation levels and its alteration might be effected by various factors including BMI, waist circumference, weight change, individual sensitivity to the intervention, etc. And its responses to the intervention might be altered and modified as the intervention continued.

Generally, Hypermethylation in the promoter and hypomethlyation in exon region are considered to be aberrant DNA methylations, which are usually shown in patients with cancers (YOON HEE CHO, 2010) . TLR4 as the responder to LPS, which produced by negative-Gram bacteria, plays an important role in the connection of the inflammation caused by the microbiota in the intestine and the innate immune system (Arunan S. Vamadevan, 2010). It is known, that the intestinal epithelial cells express low levels of TLR4 and are hyporesponsive to commensal bacteria. A mice study showed this low expression of TLR4 is associated with

a high methylation of TLR4 in large intestine. Relative lower methylation of TLR4 with relative higher TLR4 expression was observed in small intestine, while in splenic cells, a very low methylation of TLR4 with high expression was indicated. In this mice study, DNA methylation of 7 CpG motifs in the 5' region (nucleotides - 102/+202) was analyzed, which includes both promoter and gene region of TLR4 (Kyoko Takahashi, 2011). As we know that the human TLR4 gene region is approximately 80% identical with the mouse, we supposed that there was also a identical relationship between the TLR4 methlyation and expression in our study. In the intervention group, a significant negative association between BMI, waist circumference and TLR4 methylation level indicated that obesity might be related with lower TLR4 methylation level with a higher expression. As we mentioned before, BMI and wasit circumference are two main indicators for obesity and metabolic diseases, the current results showed, that lower TLR4 methylation level with a higher expression level caused by the enhanced inflammation level, which might be induced by increased BMI and wasit circumference. However, we found no significant correlation between the TLR4 methylation level and the HbA1c level, which was considered to be related with the TLR4 activation.

In the sequenced region of TLR4 with 4 analyzed CpGs, we found three transcription factors inclusive GATA-2, GATA-3, and ZNF345C near or at the CpG sites. The GATA family of transcription factors, which contain zinc fingers in their DNA binding domain, have emerged as candidate regulators of gene expression in hematopoietic cells (Carmen Vicente , 2011). GATA factors have also been reported to play a key role in adipogensis. GATA-2 and GATA-3 are specially expressed in murine preadipocytes, but not mature adipocytes. Expression of GATA factors can inhibit the maturation of preadipocyte cell lines (Orkin, 2012) . In addition, reduced GATA-3 level has been reported to induce insulin resistance by increased expression of T-bet in breast cancer cells (Kasi McCune1 and Badve2 2010). Thus we supposed, that expression of GATA might inhibit the development of obesity and other metabolic dieases by prompting or blocking the recruitment of RNA polymerase to specific genes like TLR4. ZNF354C, as one of the Zinc finger transcription factors, can also control the expression of certain genes. In our study, ZNF354C binding site contains a CpG site, the methylation of Cytosine at this position was supposed to influence the binding of transcription factor ZNF354C. Lower TLR4 methylation levels was demonstrated to be related with higher inflammation status and also a higher TLR4 expression in our study. Thus the decreased Methylation of TLR4 might promote the binding of GATA, which can promote the RNA transcription of TLR4. The role of transcription factor ZNF354 C is still unknown.

LINE1 methylation as a control of global methylation was also investigated in our study. LINE1 hypomethylation was usually reported to be related with a higher incidence of cancer (Valentina Bollati, 2007; Kenichiro Saito, 2010). In a recent study, positive association between fasting glucose, Triglyceride and LINE1 methylation has been reported (Mark S Pearce and Relton, 2012). In our study, the LINE1 methylation levels of three groups were very similar, and the alterations of LINE 1 mean methylation levels of different timepoints in three groups were also very slight. It was indicated, that LINE1 as a control for global methylation was relative stable compared with the specific gene markers. In the invention group, higher HbA1c level and wider circumference could increase LINE1 mehthylation levels. In addition, waist circumference gain could increase the LINE1 methylation level, while waist circumference loss could decrease the LINE1 methylation level during the first intervention period.

In our study, Pyrosequencing was used to investigate the DNA methylation conditions. This method showed very similar results of the same products after one end-point PCR in one run. In order to get accurate results of the methylation alterations ,we also put all three time-points of one participiant in one run.

The intervention in this project consists of a calorie-restricted diet and an antidiabetic medicine Victoza, both of which can affect the DNA methylation levels. We did not have a control group, which only recieved Victoza. A effective therapy for Diabetes is ususally a combination of medication and a improved lifestyle. Our study focused on the investigation of the effects of an antidiabetic therapy on epigenetic changes of TLR4 and LINE1.

Victoza, which was used in our study is a new developed injected antidiabetic medicine. It is usually applied to control the blood sugar level in type II diabetes, and a combination with Metformin or a Sulfonylharnstoff is usually recommended. Common side effects of Victoza including nausea, diarrhea, etc might also contribute to extra inflammation in the intervention group, affecting the DNA methylation levels.

CONCLUSION

Analysis of fecal gut micfrobiota during a weight loss programme indicated different patterns of microbiota in obese patients as in lean control group, and the weight loss could change the amount of certain bacteria, which were considered to be responsible for the low-grade inflammation in metabolic dieases. TLR4, which reponds to the LPS produced by negative-gram bacteria in the intestine, has been reported to be related with obesity and insulin resistance in Typ II diabetes. From the results of our study, we concluded that, the lower TLR4 methylation could be induced by higher BMI and wider waist circumference in obesity than in the lean control group, while there was no significant correlation between the HbA1c level and the TLR4 methylation levels. The relative lower TLR4 methylation caused in obesity with a supposed higher TLR4 expression was considered to be associated with a higher inflammatory status.

LINE 1 methylation was significant positive correlated with HbAc1level and waist circumference in the intervention group. Higher HbAc1 level could induce higher LINE 1 methylation. In addition, increased waist circumference could also increase the LINE1 methylation in the first period. We supposed that LINE1 methylation was poitive associated with the severity of metabolic syndromes.

In brief, Bacteria patterns and also cells could be affected by metabolic syndromes on epigenetics. And the intervention with a calorie restricted diet and Victoza as a therapy could improve the epigenetic markers by improving several parameters like BMI, waist circumference and HbAc1.

ATTACHMENT

DETECTION AND PYROSEQUENCING OF HUMAN COLONIC EPITHELIAL DNA IN FECES

INTRODUCTION

It has been estimated that approximately 1,5 million human colonic epithelial cells can be isolated per gram of human stool. Thus, exfoliated cells in human stool offer excellent opportunities to non-invasively detect molecular markers associated with colorectal dieases, especially for cancer screening, because it is easier and more sensitive than fecal occult blood testing. A pilot study of Ikuko Kato and etc investigated the feasibility of determining human DNA methylation in stool specimens. The results showed that less than 10% of the samples falied in individual assays. We supposed that stool samples offered also excellent opportunities to evaluate the epigenetic changes of certain genes like TLRs, FFAR3 in human colonic epithelial cells, which might be more sensitive to the changes of intestinal environment like microbiota compared with blood cells.

MATERIALS AND METHODS

STUDY DESIGN

12 stool DNA samples of lean control group, 8 of obese control group, and 34 of obese diabetes group were selected. Human DNA amount in each sample was detected by end-point PCR, and only samples with adequate amount of DNA were bisulfite converted for Pyroseqeuncing.

PCR CONDITIONS AND GEL ELECTROPHORESIS

Primer design software was used to find suitable primer (Qiagen, Hilden, Germany) for the unconverted DNA to amplify the TLR4 region, which we were interested in. The PCR was carried out in 25 ul reaction mixtures, which contains 12,5 ul Pyromark master mix, 62,5pmol of each primer, 2,5 ul Coraload Concentrate 10X (Qiagen, Hilden, Germany), and 2 ul unconverted fecal DNA. PCR Conditions were 95 °C for 15 min, 45 cycles of 94°C for 30s, 56°C for 45s, and 72°C for 45 s, and a final elongation at 72 °C for 10 min. After PCR, 7ul of each product inclusive the control without samples were checked by 2% agarose gel electrophoresis. Samples with TLR4 products were selected because of adequate amount of human colonic epithelial DNA.

BISULFITE CONVERSION AND PYROSEQUENCING

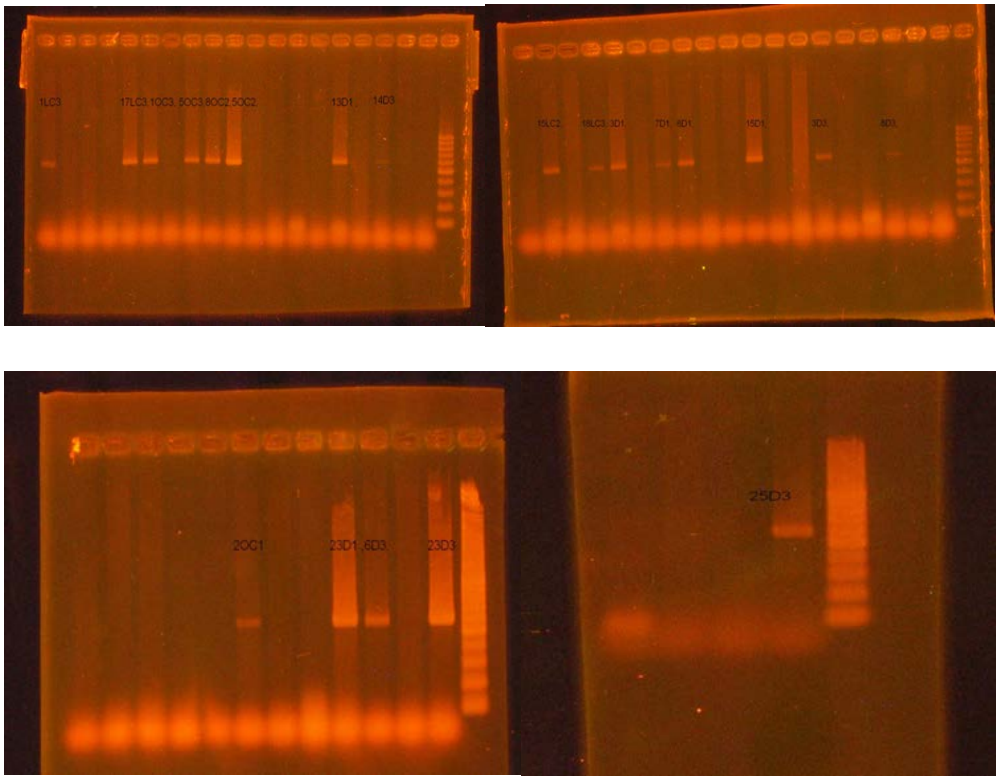
Seclected Samples were bisulfite converted, and the converted DNA were sequenced after the PCR with special primers for TLR4 bisulfite converted region.

Table 6 Primers used in PCR for detecting human DNA

Name	Sequence 5'- 3'	Size	AT
TLR4 (F)	ATTTAAAGAAATTAGGCTTCATAAGCT	27	56°C
TLR4 (R)	CCAAGAAGTTTGAACTCATGGTAA	24	56°C

RESULTS

Figure 10 Gelectrophoresis for detection of human colonic epithelial DNA in feces in TLR4 region

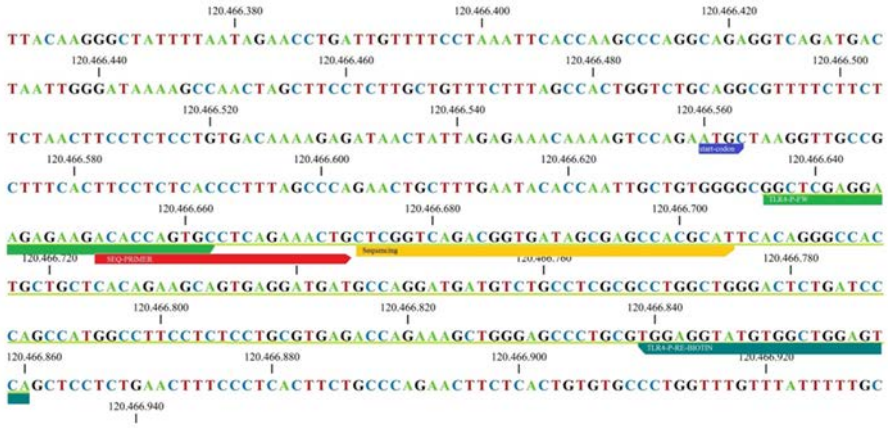


In our try, only 22 in 54 samples were indicated with adequate amount of human DNA, while the other samples had a very little amount of human DNA.

RESULTS AFTER BISULFITE CONVERSION

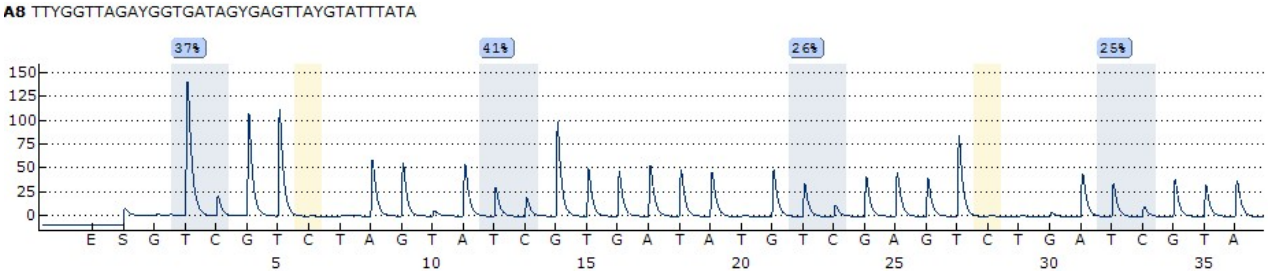
The bisulfite conversion was effective to only a small amount of DNA, and much more volume of samples were required for the PCR, in order to get enough amount of products for Pyrosequencing.

Figure 1　Pyrosequencing of the TLR4 gene region



Quantitative analysis of these 4 CpGs in a 36 bp sequence ,which is not located in the promoter, but at the very beginning of gene region of TLR4.

Figure11　TLR4-P assay with 4 analyzed CpGs for human colonic epithelial cells. Two bisulfite treatment controls(6,28) were used to confirm the complete conversion of unmethylated cyt-osine residues.



DISCUSSION

The results of TLR4 methylation in human colonic epithelical DNA were much higher than in blood samples, and the differences between three groups were also significant. It was indicated, that epigenetic status of TLR4 in colonic epithelical cells were more sensitive to the environment than in the blood. However, most of the samples failed because of their limited amount of human DNA.

In our study, we used the QIAamp DNA Stool Mini Kit (Qiagen) to isolate the fecal DN-A from the feces. It was a common method, which was also used in the study of Ikuko Kato, etc and the study of Wai K.Leung, etc. The extracted fecal DNA consisted of a great amount bacterial DNA and a very small amount of human colonic epithelical DNA, which needed a sufficient amplication. In the study of Ikuko Kato etc, a nested PCR was used to increase the specificity and sufficient amplication of this human DNA target at the promoter region of ESR1 after the bisulfite conversion. The PCR products of target DNA region was purified and sequenced, and the prensence of methylation of each CpG site was scored as methylated or non-methylated according to the presence of C or T respectively, compared with the reference human genomic sequence. Methylated and unmethylated status instead of the methylation ratio of each position were shown in this study. In the study of Wai K.Leung etc, a methylation-specific PCR (MSP) was used for dectecting the presence of methylated DNA.

In brief, a nest PCR might be a nice method to get more target DNA, especially for samples with a small human DNA amount. A combination of a nested PCR and Pyrosequening should be discussed in the future research for the epigenetic study using fecal DNA.

CONCLUSION

Fecal DNA offers excellent opportunities for the epigenetic researchs. However, the DNA of human colonic epithelial cells is difficult to be separated from the bacterial DNA, which might influence the PCR reactions for amplification of certain gene region of human DNA. Purification and amplification of the target DNA are very necessary to get adequate PCR products for Pyrosequencing.

PROTOCOLS

GENOMIC DNA PURIFICAITON OF HUMAN WHOLE BLOOD

NOTES

- 8,5 ml of human whole blood are collected in the PAXgene Blood DNA Tubes.
- Samples should be centrifugated at room temperature.
- Frozen PAXgene Blood DNA tubes should be thawed at 18-25°C for about 2 hours or in a water bath at 37°C.Then invert the thawed Blood tubes 10 times carefully before the procedure.
- Dissolved PreAnalytiX Protease should be stored in aliquots at -20°C.
- Thaw frozen PAXgene Blood DNA Tubes in a wire rack at ambient temperature for about 2 hours or at 37°C in a water bath for about 15 minutes. Carefully invert the thawed PAXgene Blood DNA tubes 10 times before beginning the procedure.

PROCEDURE

1. Blood is poured from each tube into a Processing Tube with 25ml of BG1 Buffer. Do not close the tube too tight to avoid the cracking of the processing tubes. Invert the tube 5 times carefully to mix the blood.
2. Use a swing-out rotor to centrifuge the blood at 2500xg for 5 min.
3. The supernatant should be discarded carefully and slowly.
4. Add 5ml of Buffer BG2 and close the tube. Vortex for 5 s.
5. Centrifuge at 2500x g in a swing-out rotor for about 3 min.
6. The supernatant should be discarded carefully and slowly .
7. Add 5 ml of Buffer BG3 and close the tube, vortex for 20 s.
8. The tube will be placed in a water bath at 65°C for 10 min to incubate till the color turns to light green.
9. Vortex the tube again for 5 s .

10. Invert the tube more than 20 times after adding 5ml isopropanol (100%) until the white DNA strands appear.
11. Centrifuge again at 2500xg for 3 min.
12. The supernatant can be discarded and the tube can be left inverted on a absorbent paper for about 1 min.
13. vortex for 1 s after adding 5ml 70%(v/v)ethanol.
14. Centrifuge again at 2500xg for 3 min.
15. The supernatant can be discarded and the tube can be left inverted on a absorbent paper for about 5 min.
16. Dissolve the DNA with 1 ml Buffer BG4 by incubating in a water bath at 65°C for 1 hr, and folled by overnight incubation at room temperature.

SODIUM BISULFITE CONVERSION OF UNMETHYLATED CYTOSINES IN DNA

NOTES

- This standard protocol is suitable for DNA amounts for 1ng to 2 ug in a volume of up to 20ul.
- Samples should be centrifugated at room temperature.
- One Bisulfite Mix is for 8 reactions.
- Buffer BW should be added with 30 ml ethanol (96 -100 %) .Store the BW Buffer at room temperature (15 -25°C). Invert the bottle several times before the starting of the procedure.
- Buffer BD should be added with 27 ml ethanol (96 -100 %) . Store the BD Buffer at 2-8°C. Invert the bottle several times before the starting of the procedure. Close the bottle immediately afte the use,for the BD buffer is very sensitive to the carbon dioxide.
- Carrier RNA(310ug) should be dissolved in 310 ul RNase free water by vortexing before the use. Carrier RNA is not necessary ,when more than 100 ng DNA is used.
- Dissolved carrier RNA is added to Buffer BL. Calculation of the volume of both are needed before the processes.
- Example for Carrier RNA and Buffer BL volumes: 1 sample is with 620ul BL Buffer and 6,2ul Carrier-RNA, and 4 samples are with 2,5ml BL Buffer and 25ul Carrier-RNA, and 24 samples are with 15ml BL Buffer and 150ul Carrier-RNA.

PROCEDURE

1. 800 ul RNase-free water is added to each aliquot of Bisulfite Mix. Vortex for about 5 min till it is completely dissolved.
2. Prepare the bisulfite reactions in 200 ul PCR tubes according to the process below: The total volume is 140ul with maximum 20ul DNA solution (1 ng -2 ug), variable volume of RNase-free water, 85ul Bisulfite Mix(dissolved), and 35ul DNA Protect Buffer.
3. Close the tubes and mix the bisulfite reactions. The bisulfite DNA conversion is carried out in a thermal cycler, which should take about 5 h including Denaturation(95°C,5min), Incubation (60°C, 25min), Denaturation(95°C, 5min), Incubation (60°C, 85min), Denaturation (95°C, 5min), and a final Incubation (60°C, 175min),
4. The PCR tubes are placed into the thermal cycler. Then the thermal cycling incubation can be started.
5. Centrifuge the PCR tubes after the reactions. Transfer the bisulfite reactions to microcentrifuge tubes with 1,5 ml volume.
6. 560 ul Buffer BL with 10 ug/ml carrier RNA is added to each sample. Vortex and centrifuge.
7. Transfer each mixture into the prepared EpiTect spin coulumn.
8. Centrifuge the EpiTect spin coulumn at high speed for 1 min. Place the spin columns back after discarding the flow- through.
9. 500 ul Buffer BW is added to each spin column. Centrifuge at high speed for about 1 min. Place the spin columns back after discarding the flow- through.
10. 500 ul Buffer BD is added to each spin column, 15 min is needed for the incubation at room temperature. Do not forget to close the tubes before the incubation.
11. Centrifuge the spin columns at high speed for about 1 min. Place the spin columns back after discarding the flow -through.
12. 500 ul Buffer BW is added to each spin column. Centrifuge at high speed for about 1 min. Place the spin columns back after discarding the flow through.
13. Repeat step 12 once.
14. The spin columns are placed into new 2 ml collection tubes,and will be centrifuged at high speed for about 1 min.
15. Incubation for 5 min at 56°C in a heating block for the spin columns with clean 1,5 ml microcentrifuge tubes is recommended .

16. 20 ul Buffer EB is Dispensed onto the center of each membrane. The purified DNA can be Eluted by centrifugation for 1 min at high speed. And the purified DNA can be stored at -20°C.

PROTOCOL USING PYROMARK PCR MASTER MIX

NOTES

- One primer must be biotinylated at 5' end to prepare a single –stranded PCR product for the Pyrosequencing procedure.
- PyroMark Assay Design Software 2.0 is recommend for primer design.
- The optimal PCR amplicon length for Pyrosequencing is between 80 and 200 bp, with experience length from 60 to 90 bp work well in human blood DNA.
- An activation step of 15 min at 95°C is required for HotStarTaq DNA Polymerase .
- A final concentration of 1,5 mM MgCl2 in PyroMark PCR Master Mix can produce satisfactory results.

PROCEDURE

1. PyroMark PCR Master Mix,Coraload Concentrate,primer solutions are thawed at room temperature.
2. Set up the reactions according to Table 1.

Table 1: Example for reaction composition using PyroMark PCR Master Mix

Component	**Volume/reaction**	**Final concentration**
Reaction mix PCR Master Mix, 2x	12,5ul	Contains HotStarTaq DNA Polymerase,1x PyroMark PCR Buffer,*and dNTPs
CL Concentrate, 10x	2,5ul	1x
Primer A	1ul(12,5pmol/ul)	0,5pmol/ul
Primer B	1ul(12,5pmol/ul)	0,5pmol/ul
RNase-free water	6ul	-
Template DNA	2ul (10ng/ul)	10 -20 ng bisulfite converted DNA
Total volume	25ul	

3. Optimized PCR Program for PyroMark Master Mix :Activation (15min, 95°C), 45 “3-step” cycles including Denaturation(30s, 94°C), Annealing (30s, 56°C), and Extension (30s, 72°C) and with a final Elongation(10min, 72°C).

4. The PCR tubes will be placed in the thermal cycler and the cycling program can be started.

(all procedures are from the handbooks of Qiagen company)

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POSTERS

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