Methylation analysis of Igf2 / H19 Imprinting Control Region (ICR) in Type II Diabetes Mellitus induced mice using Methylation-Specific PCR

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Abstract. The imprinting control region (ICR) is a region that controls imprinted expression of Igf2/H19 so that Igf2 will only be expressed from the paternal allele instead of the maternal allele. This research aimed to reveal if ICR methylation status’ change contributes to the emergence of type II diabetes Mellitus (T2DM) throughout the induction in mice model. The methylation changes were detected by methylation-specific PCR (MSP). The Control group was given a normal diet and buffer vehicle injection while treatment group was given a high fat diet (HFD) and six times 60 mg/kg mice body weight streptozotocin injection. The induction process was conducted for 12 weeks. The insulin tolerance test (ITT) was conducted on week 0, week 9, and week 12. The treatment’s mice group was successfully induced a significantly higher area under the curve (AUC) compared to the control mice (P<0.05). Moreover, ITT showed that diabetic induced mice had higher glucose levels at every time point of the test. Meanwhile, there were no evident ICR methylation changes through week 0 to week 12. Thus, ICR methylation is not proven to contribute to the emergence of T2DM.

Keyword: STZ, Animal, High-Fat, AUC, ITT

1. Introduction

The clinical symptom of diabetes mellitus type II (T2DM) includes when a person experiences an increase in blood sugar without a normal response to lower the levels back. This condition could be preceded by a decrease in insulin receptor sensitivity, which causes insulin resistance. This condition continues to cause pancreatic beta cells' failure to secrete insulin for a prolonged condition of hyperglycemia [1][2]. Pancreatic beta cells can then be damaged [3]. The mechanism of pancreatic beta cells’ susceptibility to damage related to Igf2 gene expression [4].

Igf2 expression depends on the DNA methylation level in an imprinting regulatory region, namely the Imprinting Control Region (ICR). ICR regulates the expression of the Igf2 gene so that it is imprinted on the maternal allele and is expressed from the paternal allele only. Typically, Igf2 is expressed at low levels and plays a role in fetal growth and development [5]. The gene product, namely the IGF2 protein, also has the same affinity as insulin for insulin receptors. Igf2 overexpression could increase pancreatic beta cells to damage, leading to the pathophysiology of T2DM [4]. However, it is unknown whether these changes in T2DM progression cause or effect T2DM.
This study aimed to determine whether there was a change in the CpG site methylation of ICR that correlate with T2DM. Another scientific question to be answered was whether the change is a cause or effect of the emergence of T2DM. Methylation pattern analysis was carried out using methylation-specific PCR (MSP) method to determine the differences in pre- and post-intervention treatment to make T2DM model mice.

2. Material and Method

2.1 Primers
The area selected region for MSP analysis was one CpG site on CpG island (CGI) 1 at the ICR. The Primers for MSP were designed with the help of the online software MethPrimer from Urogene. The inner primer was derived from MSP suggested primer by MethPrimer, while the outer primer was designed from modified bisulfite sequencing primer suggestion. PCR-MSP product specifications were checked using software from BiSearch (http://bisearch.enzim.hu). In silico analysis showed that specific primers produced one PCR product for both outer and inner primers. All primers in the experiment were ordered from Macrogen, South Korea.

| No | Primer Name | Sequence (5' → 3') | Tm (°C) | Amplicon size |
|----|-------------|---------------------|---------|--------------|
| 1  | F_Vel       | GTTTAAAGTAAAAAGGGGATTATT | 59,2    | 449          |
| 2  | R_Vel       | TAAACAAAAAAAACCTAATCTACCTTCC | 57,6    |              |
| 3  | F_V_Met     | ATATGTAGTTATTGTATTGTTATTAC | 54,3    | 122          |
| 4  | F_V_Non     | ATATGTAGTTATTGTATTGTTATTAT | 52,7    |              |
| 5  | R_V_MSP     | TTAATAACTCCTTCAATCTTA | 56      |              |

2.2 Animal Experiment
There were two groups of mice, namely 16 control mice and 16 treatment mice. The induction method refers to[6] with modifications. The treatment group was given a high-fat diet (HFD)[7] and injected with streptozotocin (STZ). The STZ injection was given at a dose of 60 mg/kg body weight of mice [8]. The injection is given six times with a frequency of once a week. The injection is carried out on the 5th to the 10th week of the experiment. The control group of mice was given a standard diet and injected with 0.1 M citrate buffer solution pH 4.5 as a vehicle of STZ. The STZ solution and buffer used for injection are freshly prepared. The blood sugar was measured at weeks 0, 3, 6, 9, and 12 weeks and the insulin tolerance test (UTI) following UTG's significant results [9]. Blood sugar in both tests was measured at 0, 15, 30, 60, and 120 minutes. Blood samples of mice were taken from retroorbital periodically every three weeks, starting from week 0, and used for DNA methylation analysis. The collected blood was stored in an EDTA tube at -20 °C for further use.

2.3 Methyl Specific PCR Analysis
The blood DNA was isolated using the TIANamp Genomic DNA Kit (TianGen) kit with an elution volume of 70μL. The DNA was then bisulfite converted, according to [10], with modifications. The concentration of Na-bisulfite 2.5 M were used. The bisulfite conversion result is in single-strand DNA, then firstly PCR first with outer primer. The first PCR procedure was performed with the following protocol: Five minutes of pre-denaturation step at 94 °C, 40 cycles of denaturation step (94 °C for 30 seconds, annealing of 53 °C for 90 seconds, elongation of 72 °C for 90 seconds), and final elongation of 72 °C for 5 minutes. The result of the first PCR has used for the second step PCR as templates with inner primers. The second PCR was performed using the following step: Five minutes of
predenaturation at 94 ° C, 35 cycles of PCR (denaturation at 94 ° C for 30 seconds, annealing at 49.1 ° C for 90 seconds, and elongation at 72 ° C for 90 seconds), and final elongation at 72 ° C for five minutes.

The PCR results will then be electrophoresed using 2% agarose stained with ethidium bromide. Band visualization was performed using an ultraviolet illuminator with a 100 bp comparison of Promega's DNA ladder markers. Methylation analysis was carried out from the amplicon band visualization results with the help of AlphaEase software, which measures the density of the relative band thickness. The relative band density of the primary amplicon from analyzing methylated or non-methylated amplicon (FMET or FNON) interpreted in the form of integrated density value (IDV).

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\text{IDV} = (\text{AVG pita} - \text{AVG background}) \times \text{Area}
\]

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\% \text{Methylation} = \frac{\text{IDV of FMET Amplicon}}{\text{IDV of FMET Amplicon} + \text{IDV of FNON Amplicon}} \times 100\%
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3. Statistical Analysis
Statistical analyses were performed using IBM SPSS 25.0. Statistical analysis was achieve using the independent t-test to examine differences between the control and treatment groups. The t-test used was two-tailed. Data are presented in mean ± SD. The results were statistically significant if the p-value was <0.05.

4. Results and Discussion
Figure 1 is the result of annealing temperature optimization for MSP settings. The temperature tested for annealing is set on the Techne TC-5000 thermocycler machine at a temperature of 50.2 ° C with a gradient of 23 ° C. PCR test tubes were reacted at block temperatures of 40.6 ° C (A), 43.6 ° C (B), 47.3 ° C (C), and 49, ° C (D). Each was compared with a negative control (-) without a template. At all of the above-mentioned temperature, a specific DNA band was obtained at a size of 122 bp for both (methylated) M and unmethylated (U) primers. In the reaction with an annealing temperature of 47.3 ° C, there was a faint band on the negative control primary M while the band on the negative control was not found. From these results, the optimum temperature used for the next MSP was 49 ° C.

The MSP primers were designed to successfully amplify the template and produce a specific band with the right size. The MSP optimization showed that the primer could be used in a temperature range of 40 ° C to 49 ° C. Negative control is used as a comparison while eliminating amplification interference. The temperature of 49 ° C was chosen for the next MSP of 49 ° C because the negative control results were cleaner than the impurity band.

Figure 2 shows the optimization results of the bisulfite conversion reaction time. The sample used was 18 μL of blood DNA. Incubation with 2.5 M Na-bisulfite reagent was adjusted for 12 hours (D) and 14 hours (E) to determine the best incubation time. The modified isolates were amplified with SS code PDK4 gene primers and with the expected amplicon size of 140 bp, compared to marker (M). The MSP results in these samples showed a specific band in the incubated isolates for 14 hours.

The bisulfite reaction was manually optimized for the incubation duration. The goal was to confirm and to know the conditions of the best conversion reaction results. From the PCR results with different optimization, it was found that the most optimum reaction in the manual method resulted in a specific amplicon band in samples incubated for 12 hours in pancreatic samples only, and 14 and 16 hours in blood samples. In blood samples, no amplicons were found at the 12 hour incubation period. For blood samples, an incubation time of 14 hours was selected.
Figure 1. The visualization results of the annealing temperature optimization. The visualization used a 2% agarose gel with a marker of 100 bp (M). Negative control (-). The annealing temperature used was 40.6 °C (A), 43.6 °C (B), 47.3 °C (C), and 49 °C (D). AM and AU mean the PCR result of annealing temperature 40.6 °C for methylated and methylated primer respectively. The same coding was used for different annealing temperatures.

Figure 2. The results of the concentration optimization of the bisulfite reaction. Incubation with 2.5 M sodium bisulfite reagent was adjusted at 12 hours (D) and 14 hours (E). The result of the modification reaction was amplified with SS code primer for the PDK4 gene with 140 bp amplicon size compared to marker (M).

Figure 3. The AUC bar chart for week 0 to 12 between treatment groups. The comparisons were statistically significant if the P-value was <0.05 (*).
The AUC of the treatment group was significantly lower than the control group at weeks 3 and 6 (figure 3). However, the AUC of the treatment group was then significantly higher at weeks 9 and 12 (P <0.05).

**Figure 4.** The insulin tolerance test for the 0 (A), 9 (B), and 12 (C) weeks of the experiment for control and treatment groups. The results were statistically significant if the P-value <0.05 (*).

The insulin tolerance test for the control and treatment group was the same for week 9. However, the blood glucose level pattern after insulin injection at week nine and week 12 was higher at the treatment group. Moreover, the treatment group's glucose level was significantly higher after 120 minutes of insulin injection at week 9.

**Figure 5.** Bar chart of ICR 1 Igf2 / H19 CGI methylation percentage from week 0 to week 12. The comparison was statistically significant when P <0.05 (*).
According to Skovso (2014), the state of T2DM is characterized by increased fasting blood glucose levels, insulin resistance, and increased glucose intolerance. Glucose intolerance occurs due to the condition of insulin resistance, which can be seen from the delayed biological response compared to the control. The analysis showed that the AUC of the treatment group at week 9 was higher than the control group. The insulin tolerance test also showed that the treatment group's blood glucose level was higher than the control group, at week 9. Based on this, it can be said that the treatment group of mice was successfully induced to experience T2DM conditions in the 9th week. Overexpression of Igf2 in pancreatic beta cells causes cell susceptibility to damage and diabetes[4]. However, these experiments do not explain the pathophysiological sequence of diabetes. Even so, the results of the current study do not mean that Igf2 is not a cause or effect of T2DM. ICR can regulate Igf2 expression. It could be possible that the analyzed CpG site in this study did not have a significant role in regulating Igf2 expression. Besides, there are CGI 2 and CG 3 in ICR, which were not studied in this study. This research can also be developed by considering the CpG site contained in the DMR upstream promoter Igf2[4]. It cannot be determined whether the paternal allele Igf2 promoter undergoes methylation change independently of ICR. Whether or not there is a change in CGI 1 H19 / Igf2 ICR's methylation status can still be explored further in future studies.

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