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Investigation of KLF14 Promoter Methylation in Patients with Type 2 Diabetes. A Preliminary Analysis

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Abstract

Background and Objectives: Krüppel-Like Factor 14 (KLF14) is defined as a master regulator of gene expression in adipose tissue and has been associated with body mass index (BMI) and type 2 diabetes (T2D). Genome-wide association studies aim to find predictive biomarkers for the development of type 2 diabetes, taking into account epigenetic changes affected by environmental factors. DNA methylation plays a decisive role in the regulation and control of gene expression. The objective of this work was to identify the percentage of methylation in the promoter region of the KLF14 gene in patients with type 2 diabetes. and controls and to reveal the contribution of the KLF14 to the development of Type 2 diabetes. **Methods:** A total of 20 volunteers participated in this study (10 patients with type 2 diabetes and 10 controls), between 35 and 70 years of age, and were evaluated for KLF14 promoter methylation. Genomic DNA was extracted from peripheral blood using the blood DNA extraction kit (*Qiagen, Inc., Valencia, CA*). Genomic DNA was used for bisulfite conversion by the Epitect Bisulfite kit (Qiagen). The methylation status was assessed by bisulfite sequencing. The calculation of the methylation percentage is performed by measuring the signal (peak height) of cytosine and thymine by applying the formula "a" = [Peak Height C/(Peak Height C + Peak Height T)] × 100. **Results:** By sequencing analysis, we found methylation in 26 of the 35 CpG sites analyzed in the total sample (T2D and controls). Our results show increased methylation levels in the KLF14 gene promoter in diabetic individuals compared to control subjects [54.56% ± 17.89% vs. 35.77% ± 16.67% vs. respectively; RR = 1.9 (1.236 - 2.963), p = 0.001]. Further analysis revealed that specific CpG sites in *KLF14* gene display increased methylation in T2D patients. A hierarchical cluster analysis was used to classify a sample in a ranking

list, based on relative methylation percentage over or under the mean standardized scores of individual variables. The study identified three groups of dinucleotide CpGs in the total sample, Promoter region analysis identified three GGGCGG boxes of the Sp1 transcription factor. **Interpretation and Conclusions:** The present investigation, carried out in an Argentine population, demonstrated a higher methylation status in the KLF14 promoter is associated with type 2 diabetes. We postulate that increased methylation in the GGGCGG boxes, could interfere with Sp1 factor binding, and consequently silence/decrease KLF14 transcription in individuals with T2DM. We propose that the methylation status of specific CpG sites of the KLF14 promoter could be used as an epigenetic predictor of type 2 diabetes development.

Subject Areas

Diabetes & Endocrinology, Genetics

Keywords

Argentina, CpG Sites, DNA Methylation, Epigenetic, San Luis, Type 2 Diabetes

1. Introduction

In 2024, the prevalence of diabetes reached 540 million adults worldwide (https://diabetesatlas.org/atlas/tenth-edition/). The most common diabetes, around 90%, is type 2 diabetes (T2D). It is characterized by being a long-term metabolic disorder accompanied by hyperglycemia and insulin resistance. Diabetic complications include heart disease, stroke, and diabetic retinopathy.

It is known that genetic, epigenetic and non-genetic factors influence the pathogenesis of type 2 diabetes [1]. Age, physical inactivity and obesity are non-genetic risk factors for type 2 diabetes. It has been shown by genome-wide association studies (GWAS) in large case-control cohorts and family studies that the risk of type 2 diabetes is influenced by genetics. Although more than one hundred genetic variants have been identified that are associated with the risk of type 2 diabetes, they can only explain a modest portion of the heritability. [2]. Furthermore, alterations in epigenetic patterns could explain the heritability of type 2 diabetes.

DNA and histone methylation, as well as non-coding RNA (ncRNA), are included in epigenetic modifications. DNA methylation, in differentiated mammalian cells, occurs mainly at cytosines followed by a guanine, called CpG sites.

Epigenetic modifications, such as DNA and histone methylation, play a fundamental role in homeostasis and adaptation to the environment. Furthermore, in humans, the association between epigenome diversity and disease incidence has been demonstrated. [3]. The identification of the genetic and environmental mechanisms that contribute to the risks of type 2 diabetes would be of great

importance for the prevention/treatment of the disease. One of the molecular mechanisms explaining type 2 diabetes could be the levels of DNA methylation of particular sites, depending on aging in humans [4]-[7], suggesting that epigenetic change associated with aging could be the cause of the T2D.

There are modifications in the DNA that do not change its sequence but can affect genetic activity. These modifications are known as epigenetic changes. A common type of epigenetic modification is known as DNA methylation. Methylation at the 5'-carbon of cytosine, typically in the promoter region of a gene, does not alter the DNA sequence but affects interactions that play a critical role in gene expression regulation.

The methylation of the 5'-carbon of cytosine, often in a gene promoter, is a form of epigenetic modification that does not affect the primary DNA sequences but affects secondary interactions that play a critical role in the regulation of gene expression [8] [9].

DNA methylation levels are usually analyzed in the promoter region of a gene, at CpG sites that are used as indicators of epigenetic effects [10]. A CpG island is defined as a region containing 300 to 3000 base pairs with a CG content exceeding 0.6. Methylation plays a key role in gene activation regulation along with ubiquitination, acetylation, and phosphorylation [11].

Kitajima and Yamamoto (2012) conducted an epigenomic analysis using the Illumina Human Methylation 450 K BeadChip and blood DNA. The results identified genes where DNA methylation levels were statistically correlated with individuals' chronological age [12]. The study corresponds to the gene Krüppel-Like Factor 14 (KLF14), which is a master regulator of gene expression in adipose tissue, and has been associated with BMI and T2D in a GWAS study [13]-[15].

KLF14 belongs to the family of zinc finger transcription factors capable of binding to sequences rich in GC, regulating the transcription of various genes. For this reason, KLF14 is associated with coronary heart disease, hypercholesterolemia, and type 2 diabetes [13] [14].

The present study aimed to evaluate the correlation between KLF14 and the development of type 2 diabetes. The level of methylation in the promoter of the KLF14 gene was determined in blood from diabetic patients. The results may provide useful information for assessing the epigenetic effects of the KLF14 gene in type 2 diabetes.

2. Material and Methods

2.1. Study Population

The present study was carried out in accordance with the guidelines of the Declaration of Helsinki and the protocol was approved by the Ethics Committee of Hospital San Luis, Argentina. A total of 20 volunteers participated in this study (10 patients with type 2 diabetes and 10 controls), between 35 and 70 years of age, residing in San Luis, Argentina. Diagnosis of type 2 diabetes was made according to the criteria of the Diabetes Mellitus Classification and Diagnostic Criteria

Committee [16].

All study participants signed an informed consent prior to data collection. The following were considered: Individuals with type 1 diabetes, renal, hepatic, mental, cerebrovascular or endocrine disorders, pregnant women or women on estrogen therapy, use of lipid-lowering drugs that can affect glucose metabolism and/or increase insulin resistance. Consumption of hypoglycemic medications, in addition to metformin. Alcohol abuse and the use of illicit drugs.

All participants were recruited consecutively between March 2018 and March 2019.

2.2. DNA Analysis

Genomic DNA was extracted from peripheral anti-coagulated blood (EDTA) using the blood DNA extraction kit (*Qiagen, Inc., Valencia, CA*) following the manufacturer's instructions. DNA concentration was quantified using a NanoDrop spectrophotometer (ND-1000 Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). An optical density (at 260/280 ratio) of >1.8 is acceptable for DNA purity and PCR. 400 ng of gDNA were used for bisulfite conversion by the Epitect Bisulfite kit (Qiagen) following the manufacturer's instruction and cleanup of bisulfite-converted DNA was done. The methylation study was carried out on the 35 CpG sites on the *KLF14* promoter region (*GeneBank* NG_016152, position −409/+59) and primers for bisulfite sequencing PCR (BSP) were predicted through the online platform MethPrimer (http://www.urogene.org/methprimer/) (**Figure 1**).

TGCAACCCAGAAGTTCCGACTGGGGAGTTTCGCTCTGTTACCATTACCTGGCTCGCCGGCAGAAG
AAAGAACGCGGAGACAAGATAATTTCTGAGGCTGTTAAACATGACTTAGCCGGGGGCCGCGCGTTCCGAGGGG
GTGTCCTGCGGGCCGGGGCGGGTCTCTGCCGCCCCCGCGGGCTCCGGTGCGTCAGGGGCGCGTCAGGCGGGGC
GGGCTCCGCGCGGGCGGCGGCGGCAGCGGCGGCTGCGGCGGCGGCGGCGGCAGCAGGCGGCAGGCGGCGAG
CACCCGGCCTCCTGCTTCTCGCTCCGAGGCTGCGGGACGGACGCTCCCGGGAACTCCGTCGCCCGCGGCCGGCCG
CGTTCGGACGGCGCTCGCCGGCGGCCGGGCGGTCCCAGCCAGCATGTCGGCCGCCGTGGCGTGCCTGGACTACT
TCGCCGCCGAGTGCCTGGTGTCCATGT

Figure 1. 466 bp fragment belonging to the KLF14 gene promoter sequence, indicating the location of the forward and reverse primers for BPS. Highlighted in yellow are CpGs dinucleotides. The transcription start site is indicated in red.

A fragment of 466 bp was amplified by PCR from bisulfite-treated DNA (60 ng) using primers forward 5'AGGTTGTTGTAATTTAGAT3' and reverse 5'AACATAAACACCAAACAC3'. PCR master mix includes 10 units of HotStart Taq Platinum polymerase (Invitrogen) and optimized buffer Green G ($MgCl_2$ included) 1X containing 250 pM of dNTPs and 0.25 mM of each primer. The template bisulfite-treated DNA was denatured for 5 minutes at 95˚C before undergoing 45 cycles of denaturation for 30 seconds at 94˚C, primer annealing for 1 minute at 54˚C and extension for 1 minute at 72˚C, and final extension at 72˚C for 10

minutes. PCR control containing unmethylated DNA was included in experimental design as technical control.

The methylation status was assessed by bisulfite sequencing, which is considered to be the gold standard for methylation evaluation. PCR amplified fragments were purified with PCR QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and sequenced by MACROGEN service.

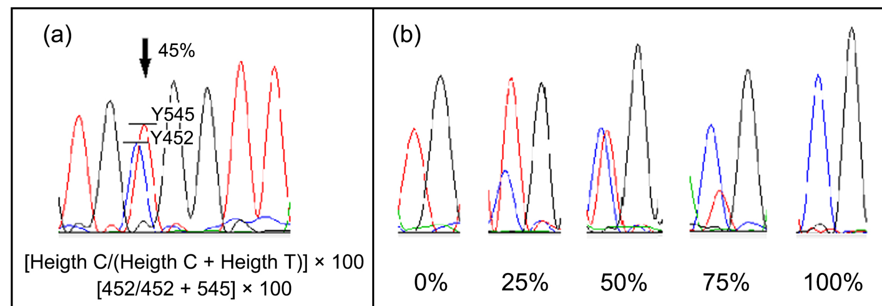

Figure 2. Partial chromatogram was obtained using the direct sequencing method. (a) Exemplification of the cytosine percentage calculation. Through a visualization program, the relative height (Y) of the peaks corresponding to cytosine (blue) and thymine (red) is obtained; These values are applied in the detailed formula to obtain the methylation percentage. (b) Images for CpG dinucleotide peaks with 0%, 25%, 50%, 75% and 100% methylation. The black peak corresponds to guanine.

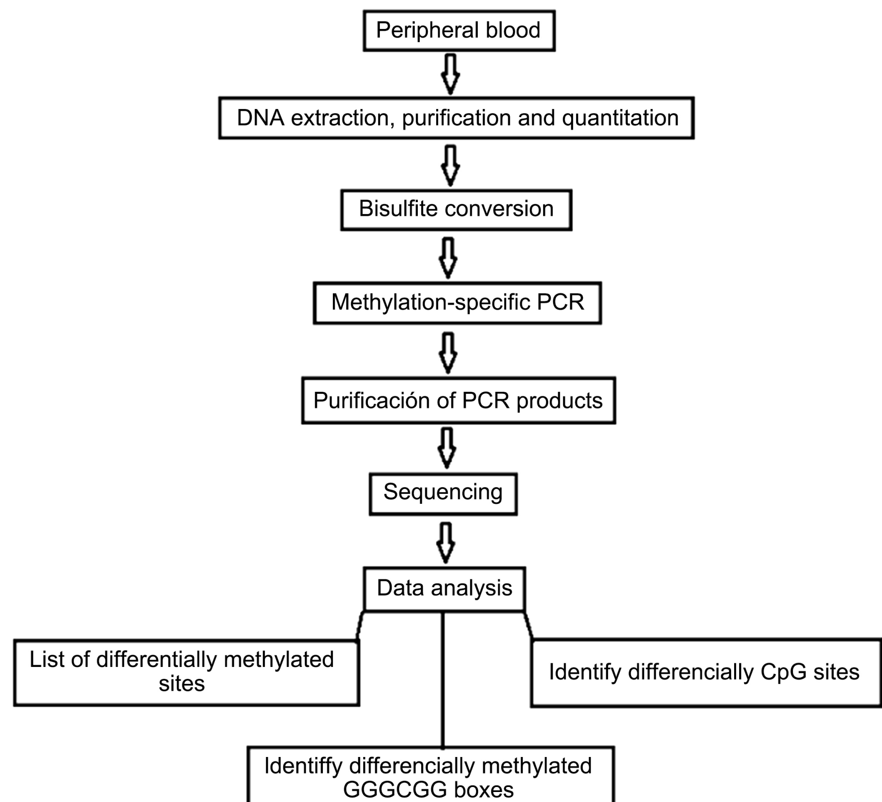

Figure 3. Workflow for analysis of DNA methylation using data from bisulfite sequencing experiments.

The direct sequencing method was used to determine the percentage of methylation [17] [18]. Interpretation of the bisulfite DNA sequencing data was performed using the chromatogram extracted from the Chromas program Lite 2.1.1. A single C at the corresponding CpG site was considered as 100% methylation, a single T as no methylation and overlapping C and T as partial methylatión (0% - 100%). The methylation percentage was performed by measuring the signal (peak height) of cytosine and thymine applying the formula "a" = [Peak height C/(Peak height C + Peak height T)] × 100 [17]-To better understand this strategy, an example is shown in **Figure 2**.

Tests for comparison of continuous variables between groups were assessed by unpaired t-test or one-way ANOVA followed with Tukey's post hoc test. Data were given as the means ± SD. P-value less than 0.05 was considered significant. Statistical calculations were performed by using InfoStat/L Statistic. **Figure 3** shows the workflow of the investigation process.

3. Results

The methylation analysis presented a clear resolution of an area spanning from positions −409 to −142, upstream of the ATG site of the KLF14 gene, including 35 CpGs dinucleotides (**Figure 4**).

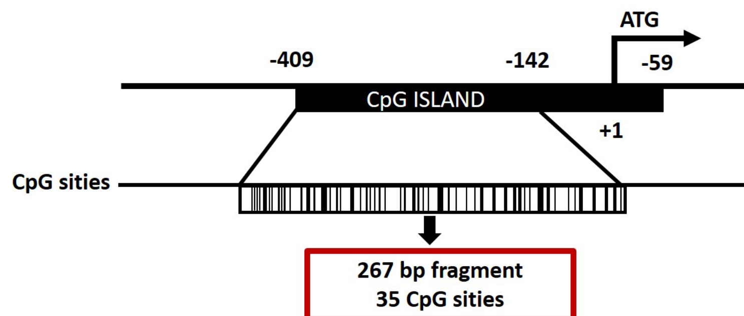

Figure 4. Illustrative map of the KLF14 promoter region. The vertical lines represent the area of the analyzed CpG dinucleotides.

The position of each 35 CpGs dinucleotides was determined according to the distance at which they are, upstream, from the transcription start site. (**Figure 5**).

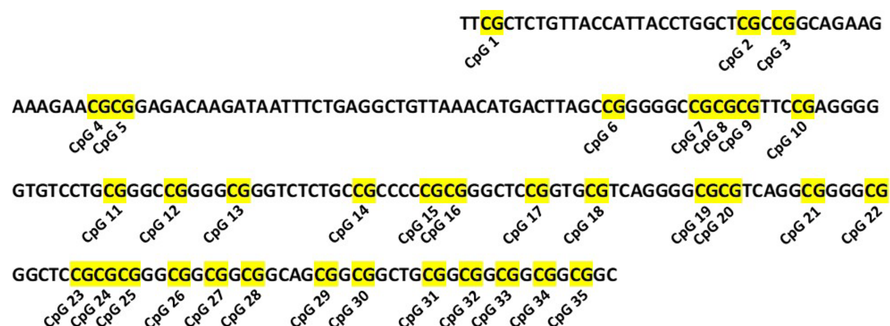

Figure 5. Position of each 35 CpGs dinucleotides analyzed.

By sequencing analysis, we found methylation in 26 of the 35 CpG sites analyzed in the total sample (T2D and controls). Figure 6 shows that promoter methylation levels were increased in T2D compared to controls [54.56% ± 17.89% vs. 35.77% ± 16.67% vs. respectively; RR = 1.9 (1.236 - 2.963), p = 0.001].

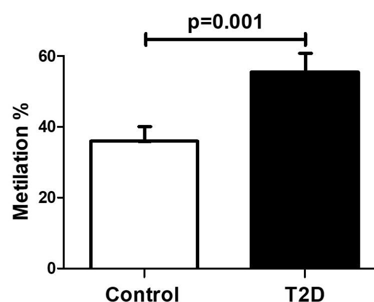

Figure 6. KLF14 gene promoter methylation levels in controls and T2D. The values express the mean ± SEM.

The descriptive analysis of the 26 CpG dinucleotides showed that seven CpG dinucleotides showed methylation only in DMT2 [CpG8 (−276) 48.3%; CpG11 (−254) 30.7%; CpG16 (−225) 33.1%; CpG21 (−195) 31.08%; CpG23 (−183) 45.85%; CpG26 (−175) 44.16% and CpG28 (−169) 62.2%] and only two in controls [CpG10 (−269) 58.6% and CpG19 (−204) 39.85%]. In the remaining 17 CpG dinucleotides, methylation was observed in both groups, showing significant differences between diabetic patients and controls (Table 1).

Table 1. Methylation percentage average of single CpG at the *KLF14* promoter in controls and T2D patients.

CpG sites[a]	Position[b] (pb)	Controls (%) (n = 10)	T2D (%) (n = 10)	*P*
CpG3	−344	24.76	89.5	<0.0001
CpG4	−329	20.33	59.37	<0.0001
CpG5	−327	35.37	58.5	0.0018
CpG6	−285	42.8	83.1	<0.0001
CpG12	−249	53.4	73.95	0.0033
CpG20	−202	28.03	31.77	0.6434
CpG22	−190	34.8	53.45	0.0030
CpG24	−181	22.5	54.77	<0.0001
CpG25	−179	28.2	6.8	<0.0001
CpG27	−172	60.2	79.86	0.0034
CpG29	−163	15.46	84.9	<0.0001
CpG30	−160	79.8	30.06	<0.0001

Continued

CpG31	−154	23.87	48.8	0.0004
CpG32	−151	32.7	57.9	0.0007
CpG33	−148	24.5	52.5	<0.0001
CpG34	−145	20.9	46.08	0.0003
CpG35	−142	33.56	43.67	0.1920

[a]The CpGs dinucleotides were named in relation to the ATG site; [b]The position of the CpGs dinucleotides has been determined according to the position of the ATG site of the KLF14 gene (GeneBank NG_016152).

This study detects two differentially methylated CpG nucleotides groups based on principal component analysis that explained most of the variation in methylation, both in controls and in T2D. The percentage of methylation was calculated in group 1 (which encompasses CpGs dinucleotides at positions between −142 to −172) and in group 2 (which encompasses CpGs dinucleotides at positions between −285 to −344), observing higher percentages of methylation in diabetics compared to controls, in both groups (group 1: 56.84% ± 19.92% vs. 38.86% ± 24.79% and group 2: 67.39% ± 18.36% vs. 31.15% ± 8.99%; respectively) (Figure 7)

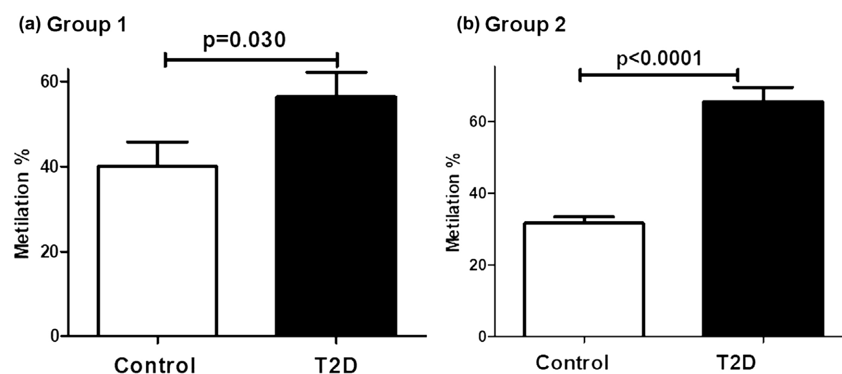

Figure 7. KLF14 gene promoter methylation levels in controls and T2D. (a) Group 1: CpGs dinucleotides spanning positions −142 to −172, starting from the ATG site. (b) Group 2: CpGs dinucleotides spanning positions −285 to −344, starting from the ATG site. The values express the mean ± SEM.

We used hierarchical clustering to further explore how our principal component analysis describes the relationships between samples. A hierarchical cluster analysis was used to classify a sample in a ranking list, based on relative methylation percentage over or under the mean standardized scores of individual variables. To reveal the natural internal relationship between samples, we first computed the nearest neighbor hierarchical clustering similarities of the most highly variable probes in the full dataset. Hierarchical cluster analysis identified three groups of dinucleotide CpGs in the total sample, where Group 3 had the highest percentage of methylation in the KLF14 promoter (Figure 8).

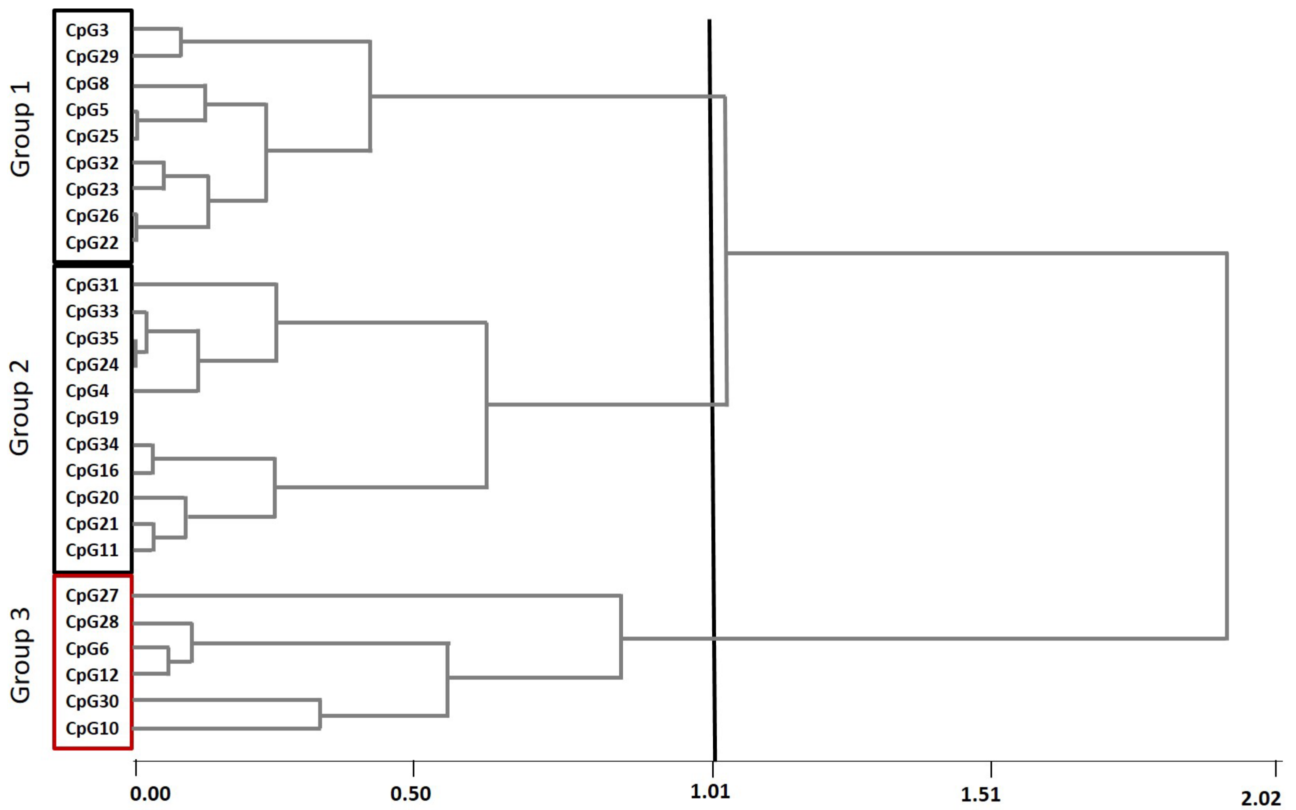

Figure 8. Cluster analysis of the CpG island in the promoter region of the KLF14 gene in the total of the analyzed sample.

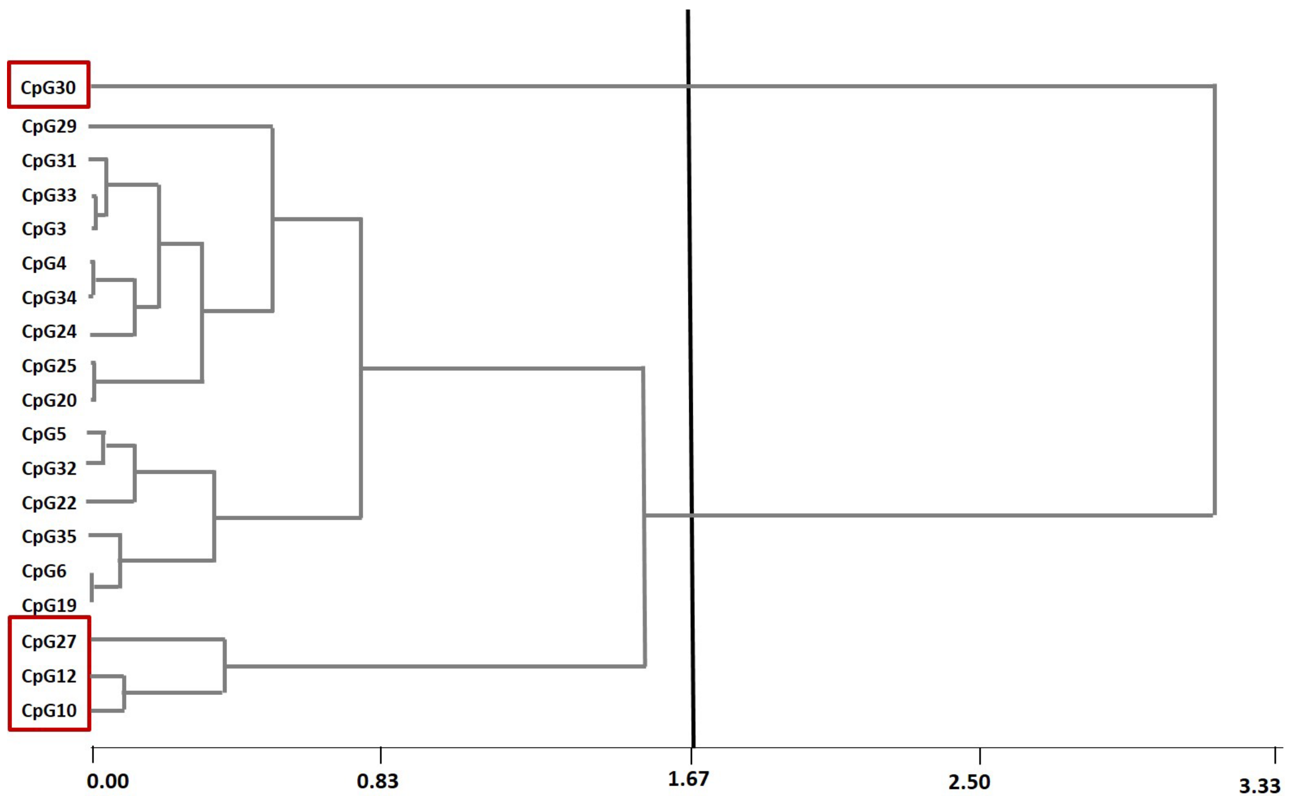

Figure 9. Cluster analysis of the CpG island in the promoter region of the KLF14 gene in controls.

When hierarchical cluster analysis was performed on controls, two groups with higher percentage of methylation were identified: the first group included the CpG30 dinucleotide and the second group: the CpG27, CpG12 and CpG 10 dinucleotides (Figure 9).

The same type of analysis performed in diabetic patients allowed the identification of two groups with higher percentage of methylation: the first group included CpG3, CpG6, CpG29, CpG27 and CpG12 dinucleotides and the second group: CpG28 and CpG25 dinucleotides (Figure 10).

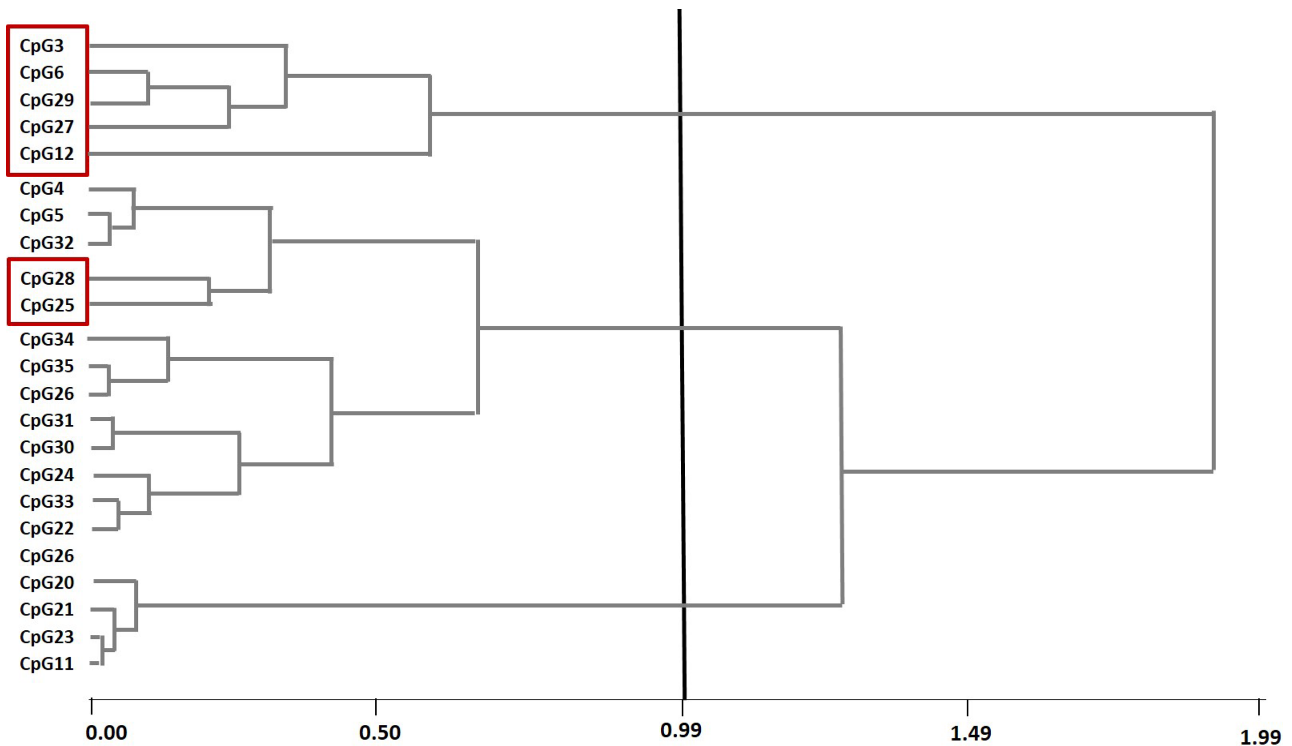

Figure 10. Cluster analysis of the CpG island in the promoter region of the KLF14 gene in T2D.

(a) GAGGGGGGGCGAGGCTGCTGCAACCCAGAAGTTCCGACTGGGGAGTTTCGCTCTGTTACCATTA
CCTGGCTCGCCGGCAGAAGAAAGAACGCGGAGACAAGATAATTTCTGAGGCTGTTAAACATGAC
TTAGCCGGGGGCCGCGCGTTCCGAGGGGGTGTCCTGCGGGCCGGGGCGGGTCTCTGCCGCCCCC
GCGGGCTCCGGTGCGTCAGGGGCGCGTCAGGCGGGGCGGGCTCCGCGCGGGCGGCGGCGGCA
GCGGCGGCTGCGGCGGCGGCGGCGGCAGCAGGCGGCAGGCGGCGAGCACCCGGCCTCCTGCTT
CTCGCTCCGAGGCTGCGGGACGGACGCTCCCGGGAACTCCGTCGCCCGCGGCCGGCCGCGTTCGG
ACGGCGCTCGCCGGCGGCCGGGCGGTCCCAGCCAGCATG

(b) GAGGGGGGGCGAGGCTGCTGCAACCCAGAAGTTCCGACTGGGGAGTTTCGCTCTGTTACCATTA
CCTGGCTCGCCGGCAGAAGAAAGAACGCGGAGACAAGATAATTTCTGAGGCTGTTAAACATGAC
TTAGCCGGGGGCCGCGCGTTCCGAGGGGGTGTCCTGCGGGCCGGGGCGGGTCTCTGCCGCCCCC
GCGGGCTCCGGTGCGTCAGGGGCGCGTCAGGCGGGGCGGGCTCCGCGCGGGCGGCGGCGGCA
GCGGCGGCTGCGGCGGCGGCGGCGGCAGCAGGCGGCAGGCGGCGAGCACCCGGCCTCCTGCTT
CTCGCTCCGAGGCTGCGGGACGGACGCTCCCGGGAACTCCGTCGCCCGCGGCCGGCCGCGTTCGG
ACGGCGCTCGCCGGCGGCCGGGCGGTCCCAGCCAGCATG

Figure 11. KLF14 gene promoter sequence showing the CpG dinucleotides and GGGCGG Boxes of Sp1 transcription factor in (a) T2D and (b) controls.

Promoter region analysis identified three GGGCGG boxes of the Sp1 transcription factor. The biological significance of this finding will be discussed below. The sequence of the KLF14 gene promoter is shown below, where the CpGs dinucleotides are indicated, highlighting in yellow the dinucleotides in which both groups showed methylation, in purple the dinucleotides with the highest percentage of methylation in each group, and the dinucleotides only methylated in T2D are indicated in turquoise and in controls in green (**Figure 11**).

4. Discussion

We have investigated DNA methylation levels of the KLF14 gene in an Argentinean population. Our main findings include an island of 35 CpG sites in the promoter region of the FLF14 gene and increased DNA methylation levels of this gene are associated with T2D. The present study is a clinical observation. Although we have no samples of pancreatic islets/adipose tissue available for analysis, the epigenetic study with blood samples is clinically accessible. Our findings are unlikely to be false positive because the average DNA methylation of the KLF14 gene is high.

DNA methylation analysis can be performed in the scales of the global genome or specific gene region and in peripheral blood with mixed cell types or specific tissues [19].

A recent report has indicated that both approaches of whole-blood DNA methylation profiling and adipose tissue-specific methylation analysis for study of epigenetic changes are related to body mass index (BMI) and suggested that the analysis of blood DNA methylation is worthwhile and can reflect changes in relevant tissues for a phenotype [10].

Studies have shown a correlation between DNA methylation profile in various tissues and peripheral blood [20] [21]. The identification of new biomarkers in blood could be used to predict and/or control the progression of T2D is of public health interest.

Dayeh *et al.* (2014) analyzed DNA methylation throughout the genome and in pancreatic islets, from diabetic subjects and non-diabetic controls, identifying multiple genes with altered methylation and expression in the islets of diabetic individuals, including the KLF14 gene. In the same study, the association between DNA methylation pattern and insulin secretion was demonstrated. The methylation status of a single CpG dinucleotide (cg08097417) was analyzed, establishing that a 1% increase in DNA methylation is associated with increased insulin secretion, which is a risk factor for T2D [22].

The study by Kananen *et al.* (2016) in non-diabetic subjects between 40 - 49 years old, report increased methylation in two CpGs dinucleotides of the KLF14 gene (cg08097417 - cg09499629), establishing that age is a factor influencing the degree of methylation. It should be noted, however, that the region that these authors studied is downstream (3') of the KLF14 gene and we analyzed the promoter area of the gene [23]. In turn, two studies analyzed the methylation profile in the

coding region of the KLF14 gene, in non-diabetic individuals, observing an increase in the percentage of methylation at older ages [7] [24].

The published methylation studies are confined to different areas of the KLF14 gene, and are mainly age-related in non-diabetic individuals. To our knowledge, no study has been performed to analyze the methylation profile of the KLF14 gene promoter in individuals with T2DM.

DNA methylation is frequently described as a "silencing" epigenetic mark, and indeed this function of 5-methylcytosine was originally proposed in the 1970s. Now, thanks to improved genome-scale mapping of methylation, we can evaluate DNA methylation in different genomic contexts: transcriptional start sites, in gene bodies, at regulatory elements and at repeat sequences. The emerging picture is that the function of DNA methylation seems to vary with context. DNA methylation in proximal promoter and/or enhancer regions is thought to have silencing effects on gene transcription, whereas DNA methylation within the coding region of the gene could stimulate transcriptional elongation and contribute to alternative splicing events [8].

In the analysis of the promoter area studied, we found three GGGCGG boxes that bind to the Sp1 transcription factor. NCBI database analysis identified putative transcription factors involved in the regulation in trans of KLF14, including the Sp1 transcription factor [25]. In our work, two of these boxes presented CpGs dinucleotides with increased methylation percentage in diabetic patients. This information gives us the basis to postulate that increased methylation in the GGGCGG boxes could interfere with Sp1 factor binding, and consequently silence/decrease KLF14 transcription in individuals with T2DM.

In recent years, several groups including our report have begun to investigate the epigenetic effects in T2D. From these reports, we have learned that DNA methylation levels between non-diabetic control subjects and T2D patients differ significantly [10] [19] [26] [27].

There are a number of genes contributing to genetic and epigenetic effects to the disease, and the contribution of each gene may be minor. In this case, it is of important to analyze the accumulating genetic and epigenetic effects in T2D. Data from the present study demonstrate that the KLF14 gene DNA methylation is high and implies that the association of increased KLF14 DNA methylation levels with T2D should be included into the accumulating analyses. Improving our understanding of the functions of DNA methylation is necessary for interpreting changes in this mark that are observed in diseases such as T2D.

Limitations

The data provide the basic information for further epigenetic analysis of the KLF14 gene in the present study. However, the major limitation of this study is the limited number of cases and controls enrolled in the study. This does not allow us to reach definitive conclusions. Additional investigation with large cohorts is necessary to confirm the association of the KLF14 gene methylation with T2D.

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Conflicts of Interest

The authors declare no conflicts of interest.

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