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Differential DNA methylation of microRNAs within promoters, intergenic and intragenic regions of type 2 diabetic, pre-diabetic and non-diabetic individuals

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abstract

Objective: Accumulating evidence supports the role of epigenetic modifications, and in particular DNA methylation and non-coding RNAs in the pathophysiology of type 2 diabetes. Alterations in methylation patterns within promoter regions are linked with aberrant transcription and pathological gene expression; however the role of methylation within non-promoter regions is not yet fully elucidated.

Design and methods: We performed whole genome methylated DNA immunoprecipitation sequencing (MeDIP-Seq) in peripheral-blood-derived DNA from age-gender-body mass index (BMI)-ethnicity matched type 2 diabetic, pre-diabetic and non-diabetic individuals.

Results: The density of methylation normalized to the average length of the promoter, intergenic and intragenic regions and to CpG count was 3.17, 9.80 and 0.09 for the promoter, intergenic and intragenic regions, respectively. Methylation within these regions varied according to glucose tolerance status and was associated with hypermethylation rather than hypomethylation. MicroRNA-DNA methylation peaks accounted for 4.8% of the total number of peaks detected. Differential DNA methylation of these microRNA peaks was observed during dysglycemia, with the promoter, intergenic and intragenic regions accounting for 2%, 95% and 3% respectively, of the differentially methylated microRNA peaks.

Conclusion: Genome-wide DNA methylation varied according to glucose tolerance. Methylation within non-promoter regions accounted for the majority of differentially methylated peaks identified, thus highlighting the importance of DNA methylation within these non-promoter regions in the pathogenesis of type 2 diabetes. This study suggests that DNA methylation within intergenic regions is a mechanism regulating microRNAs, another increasingly important epigenetic factor, during type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus (T2D) continues to be a major source of morbidity and mortality worldwide [41]. In 2014, an estimated 387 million people worldwide had diabetes mellitus, with the figure projected to increase to 592 million by 2035 [17]. Epigenetics reflect the interplay between genetics and environmental factors, and has attracted considerable interest to explain the missing heritability of T2D [27] or to gain insight into the pathogenesis of the disease [7,38]. These include DNA methylation, histone modifications, chromatin remodeling and small non-coding RNAs, with the former being the most studied [23].

DNA methylation, which refers to the addition of methyl groups to the 5′ position on cytosine nucleotides, primarily in CpG islands in the promoter regions of genes [4], is catalyzed by the enzyme DNA methyltransferase (DNMT). This epigenetic modification alters chromatin structure and regulates gene expression by transcriptional activation or repression of genes, thus affecting phenotype. Aberrant DNA methylation leads to genomic and chromosomal instability and has been demonstrated in many diseases [13,35]. Characterization of altered DNA methylation during disease processes could thus give insight into the pathophysiology of the disease, and reveal novel diagnostic, prognostic and therapeutic targets [23,35].

Recent advances in techniques to study genome-wide methylation patterns have facilitated the identification of significant DNA

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methylation in intergenic and intragenic (genebody) regions [6,19,36]. It is speculated that methylation within these non-promoter regions regulate alternative promoters, RNA processing, transposable elements such as long interspersed elements (LINEs) and short interspersed elements (SINEs), and non-coding RNAs [20].

Non-coding RNAs (ncRNAs) consist of long non-coding (lncRNAs) and microRNAs (miRNAs), and represent another important epigenetic mechanism, with the latter being the most studied. MicroRNAs are approximately 18 to 25 nucleotide RNA molecules that regulate gene expression at the post-transcriptional level by binding to the 3′ untranslated region (UTR) of their target messenger RNA (mRNA) resulting in mRNA degradation or translational repression [12]. Studies show that miRNA expression is dysregulated during disease, thus identifying these epigenetic modifications as key factors in disease pathogenesis [14]. More recently, a link between DNA methylation and miRNA expression was reported [15,22,29,33], aiding our understanding of how these two epigenetic mechanisms interact to regulate gene expression and contribute to disease.

In this study we investigated genome-wide DNA methylation in peripheral blood cell-derived DNA of type 2 diabetic, pre-diabetic and non-diabetic individuals. DNA methylation was characterized according to genomic location (promoter, intergenic, intragenic) and further quantitatively compared between the three groups. MiRNA-differentially methylated regions were further analyzed.

2. Methods

2.1. Study setting and subjects

The study setting has been described elsewhere [25]. Briefly, participants were members of a cohort study conducted in Bellville-South, Cape Town, a mixed ancestry township formed in the late 1950s. The cohort from which three screen detected diabetic, three pre-diabetic and three normo-glycemic age–gender–body mass index (BMI)–duration of residency matched participants were selected for the current study was initiated in April 2014. Participants for this study had no history of doctor diagnosed diabetes mellitus, thus underwent a 75 g oral glucose tolerance test (OGTT) as prescribed by the WHO [1]. Pre-diabetes and diabetes were diagnosed according to World Health Organization (WHO) guidelines [1]. Ethical approval for the study was obtained from the Research Ethics Committees of the Cape Peninsula University of Technology and Stellenbosch University (respectively, NHREC: REC-230 408–014 and N14/01/003).

2.2. DNA extraction

Genomic DNA was extracted from peripheral blood using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, white blood cells were lysed, thereafter cellular proteins were removed by salt precipitation, and high molecular weight genomic DNA left in solution was then concentrated and desalted by isopropanol precipitation. At least 2 μg of DNA (concentrations ranging between 70 ng/μL and 130 ng/μL) with A260/A280 and A260/A230 ratios ≥1.8 was shipped frozen on dry ice, as instructed by Arraystar Inc. (Rockville, MD, USA).

2.3. Sequencing library preparation and sequencing

Methylated DNA immunoprecipitation (MeDIP) was performed by Arraystar Inc. according to Down et al. [10] with minor modifications. Briefly, genomic DNA was sonicated to ~200–900 bp with a Bioruptor sonicator (Diagenode, Denville, NJ, USA). Thereafter, 800 ng of sonicated DNA was end-repaired, A-tailed, and ligated to single-end adapters following the standard Illumina protocol. After agarose size selection to remove unligated adapters, the adaptor-ligated DNA was used for immunoprecipitation using a human monoclonal anti-5-methylcytosine antibody (Diagenode). DNA was heat-denatured at 94 °C for 10 min, rapidly cooled on ice, and immunoprecipitated with 1 μL of primary antibody overnight at 4 °C with rocking agitation in 400 μL of immunoprecipitation buffer (0.5% BSA in PBS). Immunoprecipitated DNA fragments were recovered by adding 100 μL of protein G magnetic beads (Life Technologies, Carlsbad, CA, USA) and incubated for an additional 2 h at 4 °C with agitation. Thereafter, a total of five immunoprecipitation washes with ice-cold immunoprecipitation buffer were performed. As a negative control, nonspecific human IgG immunoprecipitation was performed in parallel to methyl DNA immunoprecipitation. Washed beads were resuspended in TE buffer with 0.25% SDS and 0.25 mg/mL proteinase K for 2 h at 65 °C and allowed to cool down to room temperature. MeDIP and supernatant DNA were purified using Qiagen MinElute columns and eluted in 16 μL EB (Qiagen, Germantown, MD, USA). Fourteen cycles of PCR were performed on 5 μL of the immunoprecipitated DNA using the single-end Illumina PCR primers. Amplicons were purified with Qiagen MinElute columns, after which a final size selection (300–1000 bp) was performed by electrophoresis in 2% agarose. Libraries were quality controlled with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). An aliquot of each library was diluted in EB (Qiagen) to 5 ng/μL and 1 μL was used in real-time PCR reactions to confirm enrichment of methylated region. The enrichment of DNA immunoprecipitation was analyzed by qPCR using specific methylated sites at H19 locus and non-methylated sites at GAPDH. The library was denatured with 0.1 M NaOH to generate single-stranded DNA molecules, and loaded onto channels of the flow cell at 8 pM concentration, amplified in situ using TruSeq Rapid SR Cluster Kit (Illumina, San Diego, CA, USA). Sequencing was performed by running 100 cycles on the Illumina HiSeq 2000 according to the manufacturer's instructions. The Agilent 2100 Bioanalyzer was used for assessment of the quality and concentration of the sequencing library, while the size and concentration of each sample was determined after sequencing library preparation.

2.4. Data analysis

After the sequencing platform generated the sequencing images, the stages of image analysis and base calling were performed using Off-Line Basecaller software (OLB V1.8). After passing Solexa CHASTITY quality filter, the clean reads were aligned to the human genome (UCSC HG19) using BOWTIE software (V2.1.0). MeDIP peaks were identified by MACS2 and differentially methylated regions (DMRs) were identified by MANorm. Statistically significant MeDIP-enriched regions (peaks) detected by MACS2 were identified by comparison to a Poisson background model, using a q-value threshold of 10^{-2}. The peaks in samples were annotated by the nearest gene (the nearest TSS to the center of the peak region) using the newest UCSC RefSeq database (UCSC Genome Browser, University of California, Santa Cruz). Peaks were divided into three classes on the basis of their distances to UCSC RefSeq genes:

1. Promoter peaks: Promoters were defined as 2000 bp upstream and downstream from the transcription start site (TSS). Peaks whose centers were located in these promoter regions were defined as promoter peaks.

2. Intragenic (genebody) peaks: The genebody region was defined as +2000 bp downstream of the transcription start site (TSS) to the transcription termination site (TTS).
3. Intergenic peaks: Intergenic peaks were defined as the other genomic regions not included in the above two regions. Peaks whose centers were located in these intergenic regions were defined as intergenic peaks.

3. Results

3.1. Genome-wide DNA methylation reveals increased DNA methylation in non-promoter regions

The clinical characteristics of subjects are shown in Table 1. After the removal of adapter sequences and low-quality reads from raw reads, the number of sequenced reads were 16,318,686 on average. Of these, 14,115,865 (86.6%) were mapped to the human genome (HG19 GRCh37, UCSC). Mapping rates varied between 84.8% and 93.5% for all individuals. A total of 450,142 statistically significant MeDIP-enriched regions (peaks) were identified in diabetic, pre-diabetic and normoglycemic subjects. The density of methylation normalized to the average length of the promoter, intergenic and intragenic regions and to CpG count was 3.17, 9.80 and 0.09 for the promoter, intergenic and intragenic regions, respectively, illustrating that intergenic DNA was more methylated than promoter DNA, and that intragenic or genebody DNA contained the least methylation. Diabetics had 2.5-fold (260,062) more peaks than controls (102,043), while pre-diabetics had 0.9-fold (88,037) less peaks than controls. In all three groups, the intergenic region contained the most number of peaks (~65%), followed by the intragenic (genebody) (~27%) and lastly the promoter region (~5%), when expressed as a percentage of the total number of methylation peaks identified by MeDIP-Seq, and not the density of normalization (Fig. 1).

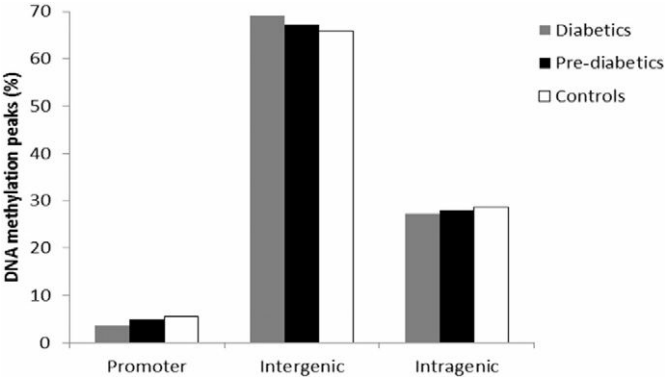

Fig. 1. Statistically significant MeDIP-enriched peaks in the promoter, intergenic and intragenic regions of diabetic, pre-diabetic and non-diabetic individuals. Data are expressed as the percentage of DNA methylation peaks in each genomic location relative to the total number of MeDIP peaks detected by sequencing.

3.2. Differential DNA methylation between diabetics, pre-diabetics and controls is greatest in intergenic regions compared to intragenic and promoter regions

Differentially methylated regions (DMRs) between diabetics and non-diabetic controls, between diabetics and pre-diabetics, and between pre-diabetics and controls were stratified according to gene location. In general, the differences in the number of DMRs in all three gene locations were greatest for diabetics and controls, followed by diabetics and pre-diabetics, and lastly between pre-diabetics and controls. Of the 60,029 DMRs identified between diabetics and controls, 53,254 (88.7%) were hypermethylated compared to 6755 (11.3%) being hypomethylated. Similarly, 43,818 (80.0%) of the 54,741 DMRs between diabetics and prediabetics were hypermethylated compared to 10,923 (20.0%) being hypomethylated. Pre-diabetics had 7913 (67.7%) hypermethylated and 3769 (32.3%) hypomethylated DMRs compared to controls. The greatest number of hypermethylated DMRs in all three comparisons occurred in the intergenic region, followed by the intragenic region and lastly the promoter (Fig. 2A). Similarly, the greatest number of hypomethylated DMRs in all three groups were identified in the intergenic region (Fig. 2B).

3.3. MicroRNA-related differential DNA methylation is predominantly intergenic

Of the 450,142 methylation peaks detected, 21,454 (4.8%) were associated with miRNAs. Of these, 0.2% were in the promoter, 4.5% in the intergenic region and 0.1% were intragenic. The distribution of miRNA-peaks was generally the same across the gene locations for diabetics, pre-diabetics and controls (Fig. 3A).

The percentage of miRNA-DMRs across the three groups was greater in the intergenic regions, followed by intragenic regions and lastly promoters, similar to the general profile of DMRs (Fig. 3B). The distribution of miRNA-DMRs was generally the same across the gene locations for all three group comparisons. The greatest number of miRNA-DMRs was observed between diabetics and controls (3081), followed by diabetics and pre-diabetics (2824), with only 558 miRNA-related changes observed between pre-diabetics and controls.

Next, DMRs were categorized according to their specific miRNAs and analyzed to identify common or uniquely differentially methylated miRNAs between the glucose tolerant groups. Interestingly, a number of miRNAs were hyper-and hypomethylated in diabetics compared to both pre-diabetics and non-diabetic controls, while others were differentially methylated in both diabetics and pre-diabetics compared

Table 1
Clinical characteristics of the subjects.

	Diabetes				Pre-diabetes				Non-diabetic controls			
Case	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
Gender	F	F	F	–	F	F	F	–	F	F	F	–
Fasting plasma glucose (mmol/L)	9.0	8.1	7.8	8.3±0.4	4.7	6.2	6.1	5.7±0.5	5.4	2.8	5.8	4.7±0.9
2-h plasma glucose (mmol/L)	16.4	13.8	5.9	12.0±3.2	7.8	10.3	9.4	9.2±0.7	7.4	5.0	6.1	6.2±0.7
HbA1c (%)	7.2	7.5	6.1	6.9±0.43	5.0	6.1	6.4	5.8±0.43	5.9	5.6	6.1	5.9±0.15
HbA1c (mmol/mol)	55.2	58.5	43.2	52.3±4.65	31.1	43.2	46.4	40.2±4.66	41.0	37.7	43.2	40.6±1.60
Hip circumference (cm)	118.0	124.0	113.0	118.3±3.2	108.0	119.0	129.0	118.7±6.1	127.0	124.0	106.0	119.0±6.6
Waist-to-hip ratio	1.0	0.8	1.0	1.0±0.1	1.0	0.9	0.9	0.9±0.0	0.9	0.8	0.8	0.8±0.0
Systolic blood pressure (mmHg)	153.0	183.0	143.0	159.7±12.0	178.0	164.0	163.0	168.3±4.8	142.0	127.0	137.0	135.3±4.4
Diastolic blood pressure (mmHg)	78.0	130.0	61.0	89.7±20.8	109.0	101.0	78.0	96.0±9.3	72.0	75.0	92.0	79.7±6.2
Insulin (mIU/L) (fasting)	20.7	21.2	–	21.0±0.3	5.7	5.5	11.5	7.6±2.0	10.4	7.6	5.3	7.8±1.5
Insulin (mIU/L) (120 min)	97.9	68.7	47.7	71.4±14.6	77.3	30.0	110.2	72.5±23.3	72.8	20.8	30.1	41.2±16.0
Glucose/insulin	0.4	0.4	–	0.4±0.0	0.8	1.1	0.5	0.8±0.2	0.5	0.4	1.1	0.7±0.1
Total cholesterol (mmol/L)	6.0	6.0	8.3	6.8±0.8	4.9	4.1	5.5	4.8±0.4	3.6	5.2	4.7	4.5±0.5
Triglycerides (mmol/L)	1.5	3.4	2.5	2.5±0.5	2.1	1.2	1.0	1.5±0.3	1.2	1.2	1.3	1.2±0.0
HDL cholesterol (mmol/L)	1.1	1.1	1.9	1.4±0.3	1.1	1.2	1.4	1.2±0.1	1.4	1.1	1.4	1.3±0.1
LDL cholesterol (mmol/L)	3.9	4.2	5.1	4.4±0.4	3.1	2.1	3.5	2.9±0.4	1.6	3.4	2.7	2.6±0.5
Total/HDL cholesterol	5.5	5.5	4.4	5.1±0.3	4.5	3.5	3.9	4.0±0.3	2.7	4.8	3.3	3.6±0.6

Data is presented for each subject as well as the mean ± standard error of the mean (SEM) in each glucose tolerance subgroup.
Hb, Hemoglobin; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

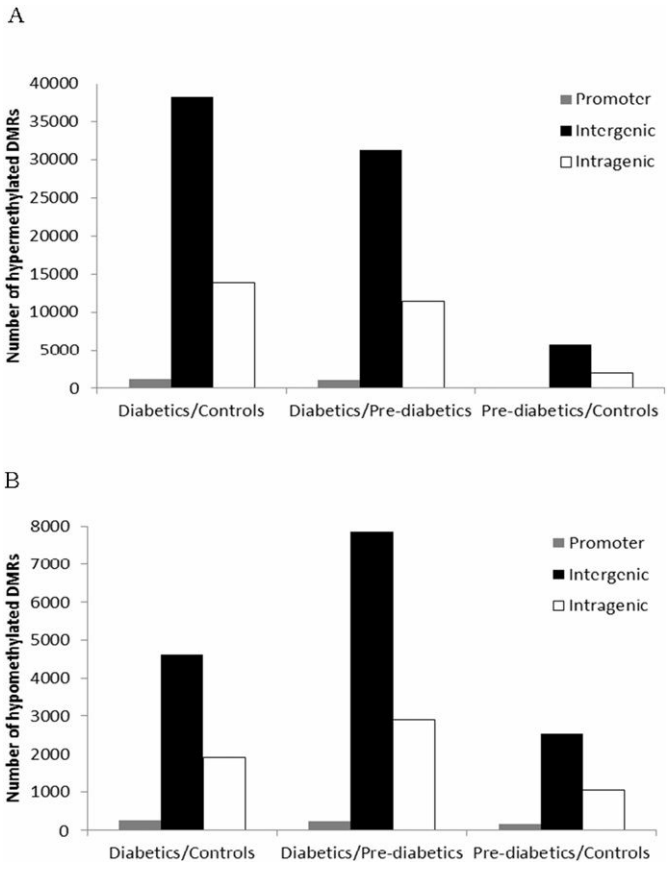

Fig. 2. Differentially hypermethylated (A) and hypomethylated (B) regions according to gene location and diabetic, pre-diabetic and non-diabetic control comparisons.

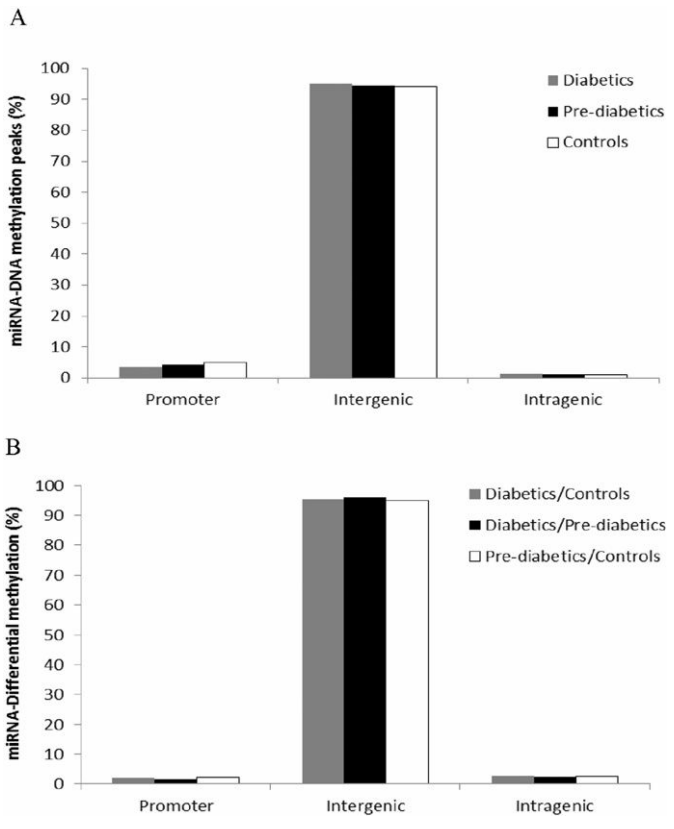

Fig. 3. Gene location of miRNA-DNA methylation peaks and miRNA-related DMRs. MiRNA-DNA methylation peaks in the promoter, intergenic and intragenic region as a percentage of the total number of miRNA-peaks identified in diabetics, pre-diabetics and non-diabetic controls (A), and the percentage of miRNA differential DNA methylation peaks in the group comparisons (B).

to controls, or uniquely methylated in diabetics and pre-diabetics. Many of the differentially methylated miRNAs have previously been shown to be associated with glucose regulation and type 2 diabetes. The miRNAs which were differentially methylated between the groups are listed in Supplementary Tables 1–3.

Stratification according to chromosomal locus, illustrated heterogeneity between the group comparisons and between genome locations, while confirming that the differentially methylated microRNAs were mostly due to hypermethylation rather than hypomethylation (Supplementary Figs. 1–6).

4. Discussion

Genome-wide DNA methylation analysis, facilitated by the improvement of high throughput genomic techniques, has shifted attention towards understanding the role of non-promoter methylation in gene regulation. Epigenome-wide association studies (EWAS) are still in their infancy with only a few studies characterizing the DNA methylome during T2D [7–9,38,39]. In this study, we report DNA methylation of miRNAs detected by MeDIP-Seq in peripheral blood cells of age, gender, BMI and ethnicity-matched type 2 diabetic, pre-diabetic and non-diabetic individuals. Although we did not observe significant differences in the number of miRNA-DMRs across the three groups of glycemic status, the distribution of miRNA-DMRs as a percentage of the total number of peaks detected by MeDIP-Seq was greater in the intergenic regions, followed by intragenic regions and lastly promoters, similar to the general profile of DMRs.

Emerging evidence suggests that in contrast to popular belief, DNA methylation within intragenic and intergenic regions is probably more important in gene regulation than DNA methylation within promoter regions. For example, analysis of 24.7 million CpG sites in human brain demonstrated that intergenic and intragenic methylation accounted for most of the CpG islands, with 5′ promoter methylation representing less than 3% of total methylation [26]. Our study similarly showed that non-promoter methylation, particularly within intergenic regions, was significantly increased compared to promoter methylation, thus agreeing with Maunakea [26]. Moreover, the first comprehensive DNA methylation study conducted in *Arabidopsis thaliana* similarly revealed 5% promoter methylation [40]. Progressive demethylation in the promoters and the intragenic region was associated with disease. For example, promoter and intragenic methylation constituted 5.6% and 28.6%, respectively, of the total methylation in controls, 4.8% and 27.9%, respectively, in pre-diabetics, and 3.7% and 27.2% respectively, in diabetics. In contrast, methylation in the intergenic region was increased with disease progression in controls (65.9%), pre-diabetics (67.2%) and diabetics (69.1%). Quantification of methylation within different gene locations, and their comparison between diabetics, pre-diabetics and controls identified the greatest number of changes between groups in the intergenic region and the least number in the promoter region. In addition differences between groups increased with disease. For example 287, 8300 and 3095 DMRs were identified in the promoter, intergenic and intragenic regions, respectively, when methylated regions of pre-diabetics were compared to that of controls, while 1322, 39,137 and 14,282 DMRs in the promoter, intergenic and intragenic regions, respectively, were observed when diabetics were compared to pre-diabetics. These findings suggest that DNA methylation changes within these non-promoter regions are associated with disease pathogenesis.

Aberrant DNA methylation has been shown to contribute to dysregulation of miRNA expression in various types of cancer [15,29,33]. A number of these dysregulated miRNAs were similarly associated with glycemic status in our study. For example, increased methylation of

miRNA genes, mir-9, mir-34 and mir-124 was observed in diabetics when compared to pre-diabetics and controls [2]. Plaisance et al. demonstrated that mature miR-9 positively regulates glucose-induced insulin secretion in β-cells, supporting our finding of increased DNA methylation in diabetics [31]. MiR-124, another important regulatory miRNA in β-cells [3], was hypermethylated in diabetics compared to pre-diabetics and controls. Increased expression of miR-34 was reported in sera of T2D patients [18] and in the islets of diabetic *db/db* mice [24], thus our contradictory findings of increased DNA methylation and possible decreased gene expression, could suggest a tissue-specific role for this miRNA. A number of other differentially methylated miRNAs were identified and could reveal insight into the pathogenesis of diabetes. For example, mir-1297 was hypermethylated in both diabetics and pre-diabetics compared to non-diabetic controls. Decreased expression of miR-1297 is linked to increased expression of COX-2, an inflammatory gene [5]. Similarly, the gene encoding miR-29a, whose expression was reported to be decreased during hyperglycemia [11], was hypermethylated in diabetics compared to controls.

It is widely reported that DNA methylation is affected by environmental factors including physical activity, smoking, alcohol consumption and diet [21,28,30,32]. Unfortunately, these factors are not known for the study participants, thus presents a limitation of the study. Participants were matched for age, gender, BMI and ethnicity, and it was largely assumed that the environmental factors are similar for all subjects living in this cohort. Another limitation is the use of only females and the small number of study participants. However, we believe that the inclusion of a diabetic and pre-diabetic group adds strength to the study. Moreover, additional analysis of methylation within promoter regions revealed a number of DMRs in T2D causal genes such as glucokinase (GCK) [34], hepatocyte nuclear factor (HNF1A) [37], neurexin 3 (NRXN3) [16] and pancreatic and duodenal homeobox 1 (PDX1) [7]) providing support for our findings. Methylation changes in genes that are involved in the pathogenesis of T2D are currently being validated by bisulfite sequencing.

In conclusion, we demonstrated disease and gene location specific DNA methylation changes in peripheral blood of subjects with type 2 diabetes, pre-diabetes and non-diabetic controls. Further investigation revealed methylation differences in regions associated with miRNAs, thus identifying DNA methylation as a mechanism regulating aberrant miRNA expression during T2D. This study, for the first time, provides evidence for the importance of DNA methylation and miRNAs in the pathogenesis of T2D in South African subjects. Studies to integrate miRNA sequencing and DNA methylation data are currently underway in our laboratory, and will allow further insight into the role of differentially methylated miRNAs in the pathogenesis of T2D.

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Appendix A. Supplementary data

Supplementary Tables 1 to 3 show differentially methylated microRNAs within the promoter region (Table 1), the intergenic region (Table 2) and the intragenic region (Table 3). Differentially hyper- and hypomethylated microRNAs for the following comparisons are illustrated: diabetics vs controls; diabetics vs pre-diabetics and pre-diabetics vs controls.

Supplementary Figs. 1–6 illustrate the chromosomal distribution of the microRNA loci that were differentially methylated between the comparisons. Differentially hyper- and hypomethylated microRNAs for the following comparisons are illustrated: diabetics vs controls; diabetics vs pre-diabetics and pre-diabetics vs controls. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.clinbiochem.2015.11.021.