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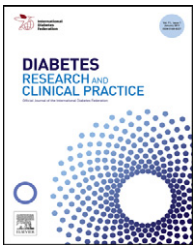

Abnormal DNA methylation in CD4+ T cells from people with latent autoimmune diabetes in adults

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ABSTRACT

Aberrant DNA methylation in T cells has been linked to pathogenesis of autoimmune diseases. To investigate genomic and gene-specific DNA methylation levels in CD4+ T cells from patients with latent autoimmune diabetes in adults (LADA), and to investigate changes in the expression of genes that regulate methylation as well as the autoimmune-related gene FOXP3 in these patients. Global CD4+ T cell DNA methylation was measured in 15 LADA patients and 11 healthy controls using a methylation quantification kit. mRNA levels of DNA methytransferases (DNMTs), methyl-DNA binding domain proteins (MBDs) and FOXP3 were measured by real time PCR. Methylation of a FOXP3 regulatory element region was determined by bisulphite genomic sequencing. Genomic DNA methylation in CD4+ T cells from LADA patients was significantly increased compared to controls. DNMT3b mRNA levels were higher in CD4+ T cells from LADA patients than in controls. DNMT3b expression positively correlated with global DNA methylation in LADA CD4+ T cells. FOXP3 expression was decreased, and the FOXP3 promoter region was hypermethylated in CD4+ T cells from LADA patients compared with controls. DNA methylation levels are altered in CD4+ T cells from LADA patients, which may contribute to disease onset and progression by affecting the expression of autoimmune-related genes.

1. Introduction

Epidemiological studies suggest that latent autoimmune diabetes in adults (LADA) may account for 2–12% of all cases of diabetes [1,2,3]. The presence of autoantibodies along with islet-reactive T cells in LADA provides strong evidence that the disease process is autoimmune [4,5,6]. LADA is thought to be a subgroup of type 1 diabetes, which has a slow procession of autoimmune destruction of β-cells. CD4+ regulatory T cells are reduced and the expression of forkhead box P3 (FOXP3) in CD4+ T cells is decreased in LADA patients [7], suggesting that defects in immunological tolerance could contribute to the development of LADA. Although there is evidence to show that genetic factors such as human leucocyte antigen (HLA) can confer an increased risk of LADA [8], a low twin concordance for adult-onset type 1diabetes implies that the genetic impact in adult-onset diabetes is limited, favoring a substantial impact of non-genetic factors which may include epigenetics

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[9]. Epigenetic modifications, including the addition or removal of methyl moieties on cytosines in CpG dinucleotides (5-methylcytosine), cause heritable effects on transcriptional regulation without changing the sequence of the genome. The level and pattern of 5-methylcytosines within regulatory DNA sequences (i.e., the methylation status of the DNA) affects the binding of transcription factors and thus the rate of gene transcription within the region. The regulation of DNA methylation is critical for a diversity of biological events, including embryonic development, X chromosome inactivation, genomic ''imprinting'', chromatin condensation and the silencing of endogenous retroviruses [10,11,12].

Three enzymes determine the methylation status of DNA in humams: DNA methyltransferase 1 (DNMT1) is involved in maintaining methylation patterns after DNA synthesis and cell division, whereas DNA methyl transferase 3a (DNMT3a) and DNA methyl transferase 3b (DNMT3b) catalyze *de novo* methylation [13]. In general, methylated DNA results in the down-regulation of gene transcription by recruiting transcriptional co-repressors belonging to the methyl-DNA binding domain family of proteins (MBDs) [14].

In a number of autoimmune disorders, including systemic lupus erythematosus (SLE) and rheumatoid arthritis, the methylation status of genomic DNA and specific autoimmunity-related genes is altered, increasing expression of genes and leading to autoreactivity. These changes have been shown to be associated with changes in the expression of DNMTs and MBDs [15,16,17]. In this study, we investigated global methylation patterns and the expression of DNMTs and MBDs in $CD4^+$ T cells of patients with LADA. We also assessed the methylation status of the FOXP3 gene in these cells and correlated FOXP3 expression with methylation status.

2. Materials and methods

2.1. Patients and healthy control subjects

15 patients with LADA (3 females and 12 males; mean ± SD age = 41 ± 6 years) with less than one year of disease history were enrolled in the study. The diagnostic criteria of LADA proposed by the Immunology of Diabetes Society were used for this study. All patients had no other autoimmune diseases and did not receive any immunomodulatory drugs. An age- and ethnicity-matched healthy control group was also included in the study (n = 11, 3 females, 8 males; mean ± SD age = 39 ± 8 years). All healthy controls had no personal or family history of autoimmune diseases and diabetes. This work was approved by the Institutional Review Board at the Second Xiangya Hospital. Written informed consent was obtained from all study subjects.

2.2. Isolation of $CD4^+$ T cells

A total of 60 ml of venous peripheral blood from each patient and control subject was collected in Heparinum tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll gradients. $CD4^+$ T cells were separated by positive selection using magnetic beads, according to the protocol provided by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, the PBMCs freshly isolated from peripheral blood were resuspended in buffer (PBS, pH 7.2, 0.5% BSA and 2mMEDTA) and mixed with anti-CD4 magnetic beads at 4 °C for 15 min. Cells were washed by adding buffer and centrifuging at 300 × g for 10 min. The supernatant was discarded and cells were resuspended in buffer. The cell suspension was applied to the column, after having prepared the column by rinsing with buffer in the magnetic field of a suitable separator. The column was then washed three times with 3 ml buffer, and then removed from the separator and placed on a collection tube. Bead-labeled cells were immediately flushed out with buffer by firmly pushing the plunger into the column. The purity of $CD4^+$ T cells was generally higher than 95%, as determined by flow cytometry.

2.3. Genomic DNA extraction and measurement of global DNA methylation

Genomic DNA extraction was carried out with the TIANamp Genomic DNA blood kit (Tiangen Biotech, Beijing, China). Global DNA methylation was measured using the MethylampTM Global DNA methylation Quantification Kit (Epigenetek Group Inc, Brooklyn, NY, U.S.A.). This kit yields accurate measures of methylcytosine content as a percentage of total cytosine content. Briefly, DNA was immobilized on a strip well specifically treated to have a high affinity for DNA. DNA methylation levels were then quantified by an enzyme-linked immunosorbent assay-like reaction using a 5-methylcytosine antibody. The amount of methylated DNA is proportional to the optical density (OD), and from this value the degree of DNA methylation can be calculated using the following formula:

$$\text{Methylation}\,\% = \frac{\text{OD(sample} - \text{NC)}/X}{\text{OD(positivecontrol} - \text{NC)} \times 10} \times 100\%$$

in which NC is the negative control, positive control is the methylated control DNA, and X is the GC content of any species' DNA (41% for human genomic DNA).

2.4. RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was isolated from $CD4^+$ T cells using the RNeasy mini Kit (Qiagen, Valencia, CA, U.S.A.) and used to prepare cDNA. cDNA synthesis was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada) and 1 μg of total input RNA according to the manufacturer's instructions. Real-time quantitative PCR was performed using a Rotor-Gene 3000 (Corbett Research, Sydney, NSW, Australia) and mRNA levels were quantified using the SYBR Premix Ex Taq™ real-time PCR Kit (Takara Biotech Co., Dalian, China). A dilution series of sample cDNA was also included to generate a standard curve used to calculate relative concentrations of transcript in each cDNA sample. β-Actin was also amplified and used as a loading control. Primers used are listed in Table 1.

2.5. Genomic bisulfite sequencing

Bisulfite conversion of genomic DNA was performed using the EpiTect Bisulfite Kit (Qiagen). The 217 bp fragment FOXP3