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ORIGINAL ARTICLE

Elevated histone H3 acetylation is associated with genes involved in T lymphocyte activation and glutamate decarboxylase antibody production in patients with type 1 diabetes

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Keywords
CD4^{+} T lymphocytes, Histone H3 acetylation profile, Type 1 diabetes

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ABSTRACT

Aims/Introduction: Genetic and epigenetic mechanisms have been implicated in the pathogenesis of type 1 diabetes, and histone acetylation is an epigenetic modification pattern that activates gene transcription. However, the genome-wide histone H3 acetylation in new-onset type 1 diabetes patients has not been well described. Accordingly, we aimed to unveil the genome-wide promoter acetylation profile in CD4^{+} T lymphocytes from type 1 diabetes patients, especially for those who are glutamate decarboxylase antibody-positive.

Materials and Methods: A total of 12 patients with new-onset type 1 diabetes who were glutamate decarboxylase antibody-positive were enrolled, and 12 healthy individuals were recruited as controls. The global histone H3 acetylation level of CD4^{+} T lymphocytes from peripheral blood was detected by western blot, with chromatin immunoprecipitation linked to microarrays to characterize the promoter acetylation profile. Furthermore, we validated the results of particular genes from chromatin immunoprecipitation linked to microarrays by using chromatin immunoprecipitation quantitative polymerase chain reaction, and analyzed the transcription level by real-time quantitative polymerase chain reaction.

Results: Elevated global histone H3 acetylation level was observed in type 1 diabetes patients, with 607 differentially acetylated genes identified between type 1 diabetes patients and controls by chromatin immunoprecipitation linked to microarrays. The hyperacetylated genes were enriched in biological processes involved in immune cell activation and inflammatory response. Gene-specific assessments showed that increased transcription of inducible T-cell costimulator was in concordance with the elevated acetylation in its gene promoter, along with positive correlation with glutamate decarboxylase antibody titer in type 1 diabetes patients.

Conclusions: The present study generates a genome-wide histone acetylation profile specific to CD4^{+} T lymphocytes in type 1 diabetes patients who are glutamic acid decarboxylase antibody-positive, which is instrumental in improving our understanding of the epigenetic involvement in autoimmune diabetes.

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INTRODUCTION

Type 1 diabetes is an organ-specific autoimmune disease triggered by immune attack of self-pancreatic β-cells[1]. The disastrous self-destructive manner is mainly caused by T cell-mediated immunity and leads to rapid β-cell dysfunction. As such, patients with type 1 diabetes usually suffer from rapid decay of islet function and require lifelong insulin replacement therapy. In a recent study of type 1 diabetes, Weng *et al.*[2] showed that the incidence of type 1 diabetes in China had increased almost fourfold in children aged <15 years in the past 30 years. However, this situation is going to continually deteriorate, as there is no effective therapy to cure type 1 diabetes so far. It is therefore of great importance to unravel the concealing mechanisms and find potential therapeutic ways to treat type 1 diabetes.

Genetic factors are largely involved in the pathogenesis of type 1 diabetes, especially the human leukocyte antigen (HLA) genes located on chromosome 6, which contribute to 40–50% of the genetic susceptibility[3]. However, numerous studies showed that the genetic factors could not fully explain the progression of type 1 diabetes. A follow-up study of monozygotic twins showed that the onset of type 1 diabetes was not always in concordance, even with an identical genetic background[4], and there is only a small fraction of genetically susceptible individuals that progress to diabetes[5]. In addition, the continual increase of the incidence of type 1 diabetes is also accompanied by rapid social development and lifestyle changes in modern society[2]. All these suggest that there are some factors beyond genetics that are involved in the pathogenesis of type 1 diabetes. In recent years, environmental factors have been found to be able to alter gene expression through epigenetic mechanisms that could regulate gene expression without changes in deoxyribonucleic acid (DNA) sequence, mainly including DNA methylation, histone modification and non-coding ribonucleic acid (RNA)[6]. Extensive evidence has shown that lifestyle change and environmental exposure contribute to the increasing incidence of type 1 diabetes through remodeling the epigenetic modification in particular genes[7].

Histone acetylation is a critical pattern of histone post-translational modification, with histone acetyltransferases and deacetylases modifying the histone acetylation status in the nucleosomal core in a dynamic and reversible manner to regulate the activity of genes by unfolding or condensing the chromatin. Generally, histone acetylation could lead to gene transcriptional activation, whereas histone deacetylation causes gene silencing[8]. Histone acetylation has been found to regulate inflammatory gene expressions and is associated with the progression of autoimmune diseases[9,10]. In autoimmune diabetes, the global histone acetylation has been shown to be elevated in patients[11]. Also, enhanced histone acetylation at promoters has been observed with an increased expression of inflammatory genes in diabetic complications[12]. Remarkably increased histone H3 lysine 9 acetylation, a gene transcription activated marker, has been observed at the promoters of type 1 diabetes susceptible genes in monocytes from type 1 diabetes patients[13].

Type 1 diabetes is characterized by T lymphocytes-mediated destruction of pancreatic β-cells, and $CD4^+$ T lymphocytes are important in recognizing islet autoantigens, especially hybrid insulin peptides[14] and proinsulin[15], and prompting pancreatic infiltration in autoimmune diabetes[16]. Aside from T cell-mediated immunity, the destruction of β-cells also leads to a humoral response with production of antibodies against β-cell autoantigens, with glutamic acid decarboxylase antibody (GADA) being the most common antibody present in autoimmune diabetes[17,18]. Although histone acetylation alteration in a limited set of genes has been investigated in immune cells from type 1 diabetes patients, the genome-wide status of acetylated histone H3 on promoters and its association with gene expression have never been studied in $CD4^+$ T lymphocytes from type 1 diabetes patients, especially for those who are GADA-positive. To this end, the present study aimed to compare the genome-wide histone H3 acetylation (H3Ac) profile of $CD4^+$ T lymphocytes from GADA-positive type 1 diabetes patients and healthy individuals, and to find correlations between histone H3 acetylation and the pathogenesis of type 1 diabetes.

METHODS

Study participants

A total of 12 type 1 diabetes patients were recruited from The Second Xiangya Hospital of Central South University (Changsha, Hunan, China) according to the following criteria: (i) diabetes diagnosed according to World Health Organization criteria in 1999[19]; (ii) acute-onset ketosis or ketoacidosis with immediate insulin replacement therapy; (iii) positive for GADA; (iv) diabetes diagnosed within the past 12 months; and (v) with insulin as the only medication for glucose management. The patients enrolled were free of other autoimmune diseases and did not receive any immunomodulatory drugs. A total of 12 age- and sex-matched healthy controls were enrolled, who showed euglycemia in a standardized 75-g oral glucose tolerance test and had no history of autoimmune-related disease. The study was approved by the Human Ethics Committee of The Second Xiangya Hospital of Central South University. Written informed consent was obtained from all participants. The study was carried out in accordance with the principles of the Helsinki Declaration. Study physicians recorded the participants' height, weight and blood pressure. Fasting venous blood samples were tested for complete blood count, fasting blood sugar, hemoglobin A_{1C}, fasting C-peptide and GADA titer. A standard 466.3 kcal, mixed-meal tolerance test (60.0% of calories as carbohydrate, 26.1% as fat and 13.9% as protein) was administered to type 1 diabetes patients. The 2-h blood sugar and 2-h C-peptide were measured 2 h post the standard meal in patients.

GADA assays

GADA was detected by radioligand assay. The GADA titer of <18 units/mL was defined as positive in a duplicate test. The

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sensitivity and specificity of GADA by this assay were 82% and 97.8%, respectively. The assay was sponsored by the Immunology of Diabetes Society, and with validation by the Islet Autoantibody Standardization Program 2016.

Isolation of CD4$^+$ T lymphocytes

Venous blood samples from participants (60 mL per participant) in the fasting condition were drawn and sodium heparin, an anticoagulant, was added. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Hypaque (GE Healthcare, Pittsburgh, PA, USA) density-gradient centrifugation. Approximately, 60 million PBMCs were collected from 60 mL of venous blood. CD4$^+$ T lymphocytes were isolated by CD4$^+$ selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and 20~25 million CD4$^+$ T lymphocytes were magnetically separated from PBMCs in each participant. The purity of CD4$^+$ T lymphocytes (CD3$^+$ CD4$^+$ cells) was >95%, confirmed by flow cytometry (BD FACSCanto™ II; BD Biosciences, San Jose, CA, USA).

Western blot

Total protein was extracted from 10 million CD4$^+$ T lymphocytes from 12 type 1 diabetes patients and 12 healthy controls separately. Protein concentration was detected using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were then incubated at 4°C overnight with the anti-acetyl-H3 antibody at 1:10,000 dilution (#06-599, Millipore, Billerica, MA, USA) and anti-histone H3 antibody at 1:5,000 dilution (#ab1791; Abcam, Cambridge, UK), followed by incubation with goat anti-rabbit immunoglobulin G. The protein bands were detected using Image Lab™ Software (Bio-Rad Laboratories, Hercules, CA, USA). β-Actin was used as an internal reference for protein loading.

Chromatin immunoprecipitation linked to microarray assay

The DNA immunoprecipitate samples were pooled from four type 1 diabetes patients or five healthy controls in equal quantities of CD4$^+$ T lymphocytes in total. One pooled sample consisting of 3 million mixed CD4$^+$ T lymphocytes was used for sonication. Sonicate cell lysate was divided into three aliquots, and one aliquot contained approximate 1 million cell equivalents of chromatin, which was used for immunoprecipitation with 5.0 μg anti-acetyl histone H3 antibody (#06-599; Millipore). Before the antibody was added, 10 μL of the sonicate cell lysate supernatant was removed as input. The EZ ChIP™ Chromatin Immunoprecipitation Kit (#17-371, Millipore) was used to carry out the chromatin immunoprecipitation (ChIP) assay and DNA purification. DNA was amplified with the Whole Genome Amplification kit from Sigma-Aldrich (Darmstadt, Germany). Fluorescent labeling of the DNA was carried out using the NimbleGen Dual-Color DNA Labeling Kit (F. Hoffman-La Roche Ltd., Basel, Switzerland). Each pooled sample was labeled and hybridized to Roche Nimblegen human 720K RefSeq promoter tiling arrays, with probes designed to cover –3,200–800 bp regions relative to the transcription start sites of 22,542 Refseq genes, to detect sheared DNA pulled down by acetyl-H3 antibody at promoter regions. Hybridizations were carried out by KangChen Bio-tech Inc. (Shanghai, China).

Differentially acetylated genes identification and bioinformatics analysis

To identify differentially enriched regions (also called peaks), the log2 ratio values for each pooled sample (type 1 diabetes [T1D] and healthy controls [HC]) were averaged and the C' value was calculated (C' = average [log2 ChIPT1D / InputT1D] – average [log2 ChIPHC / InputHC]) for each probe. The NimbleScan permutation-based peak-finding algorithm on these data was run to find the differential enrichment peaks. The differential enrichment peaks were filtered according to the following criteria: (i) at least one of the two groups had a median (log2 ChIP / Input) ≥0.3 and median |C'|>0; and (ii) at least half of the probes in a peak might have a coefficient of variability ≤0.8 in both two samples. Multiple testing was carried out to adjust the *P*-value to the false discovery rate, and genes with a false discovery rate ≤0.05 were identified as the significant differentially acetylated genes (DAGs). The DAGs were then submitted to Gene Ontology (GO; http://www.geneontology.org), a community-based bioinformatics resource providing a comprehensive source for functional genomics[20,21], to identify the enriched biological process. The *P*-value denoted the significance of enriched GO terms, and a *P*-value ≤0.05 was potentially significant and interesting.

Acetylation status validation and transcriptional activity detection

ChIP was carried out to pull down the acetylated DNA of CD4$^+$ T lymphocytes in 12 independent type 1 diabetes patients and 12 healthy controls, respectively. Sequencing primers used in ChIP quantitative polymerase chain reaction (qPCR) on the inducible T-cell costimulator (*ICOS*) promoter were designed according to the position of the differential enrichment peak from ChIP linked to microarrays (ChIP-chip) and were as follows: (i) –137/–55 FP 5′-GCATCAAAGAAGAAACACCCC-3′, RP 5′-TGCTGGAAAGGAAGTGGGTT-3′; (ii) –5/+78 FP 5′-ACA ACCGAGAGCCTGAATTC-3′, RP 5′-CCTGACTTCATGTT TGCCAGAA-3′; and (iii) +196/+280 FP 5′-TACGCACC-CAAAAGACAGTG-3′, RP 5′-TGCCATCCACAGTGACATG A-3′. RNA isolation from CD4$^+$ T lymphocytes, complementary DNA synthesis and real-time qPCR were carried out as previously described[22]. Primers for amplifying the *ICOS* transcript were FP 5′-GCCAACTATTACTTCTGCAACCT-3′ and RP 5′-CAACAAAGGCTGCACATCCT-3′. Real-time qPCR was carried out by ABI 7900HT (ABI, Foster City, CA, USA). β-Actin was used as an internal control. Data shown (mean ± standard error of the mean) were from PCRs of 12 independent patient samples with each sample run in triplicate.

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Statistical analysis

Data are presented as mean ± standard error of the mean. Logarithmic transformations were applied for non-normally distributed parameters before statistical tests. Independent samples *t*-test was used for measurement data between two groups. A χ^2-test was used to compare categorical variables. SPSS version 24.0 (IBM Corporation, Chicago, IL, USA) software was used for the statistical analysis. GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) software was used for graphical display. Differences were considered significant at a two-tailed $P < 0.05$.

RESULTS

Elevated histone H3 acetylation level in $CD4^+$ T lymphocytes from type 1 diabetes patients

The general characteristic of type 1 diabetes patients and healthy controls are presented by group in Table 1. Age, sex, body mass index and blood pressure are comparable between two groups. To exclude individuals potentially affected by bacteria or a virus, all participants enrolled in the study had a normal complete blood count including white blood cells, neutrophils and lymphocytes. The western blot result showed that the H3Ac level in $CD4^+$ T lymphocytes was significantly elevated in type 1 diabetes patients compared with healthy controls ($P = 0.008$; Figure 1a,b). In order to ascertain whether this change was secondary to hyperglycemia, subgroup analysis was carried out and no significant difference was found in the global acetylation level between the well- and poorly-controlled type 1 diabetes patients (Figure 1c). In addition, the global acetylation level did not correlate with the GADA titer in type 1 diabetes patients (Figure 1d).

Differentially acetylated genes are involved in critical biological processes

As elevated global H3Ac levels had been observed in type 1 diabetes, we then sought to uncover the genome-wide H3Ac alteration profile in type 1 diabetes patients. The human 720K RefSeq promoter tiling arrays were used to meet our goal and the ChIP-chip experiment flow was carried out, as shown in Figure 2a. From the ChIP-chip assay, we identified 607 DAGs between type 1 diabetes patients and healthy controls (Figure 2b). Among the DAGs, there were 317 hyperacetylated genes and 282 hypoacetylated genes in type 1 diabetes patients compared with controls. The eight genes overlapping in a Venn diagram indicated discordant acetylation alterations in different regions of the gene promoters.

The DAGs were submitted to GO, to statistically highlight the most enriched biological annotation. Fold enrichment score was used to measure the magnitude of enrichment. GO analysis showed that the hyperacetylated genes in type 1 diabetes patients were associated with innate and adaptive immune cell responses, including leukocyte degranulation, mast cell activation and T lymphocyte costimulation, and inflammatory response to antigen or cytokines (Figure 3a). In the annotation terms of hypoacetylated genes (Figure 3b), many genes were linked to other patterns of epigenetic modification, such as genetic imprinting, deubiquitination, methylation and chromatin remodeling. Furthermore, the hypoacetylated genes were clustered to the transforming growth factor beta (TGF-β) production.

Immune-related genes show hyperacetylation in promoters

According to gene function and GO, genes in DAGs closely related to innate and adaptive immunity were selected and

Table 1 | Clinical and laboratory characteristics of participants

	T1D patients (*n* = 12)	Healthy controls (*n* = 12)	*P*-value
Age (years)	27.8 ± 1.6	28.7 ± 1.3	0.694
Sex (male/female)	6/6	6/6	1.000
Duration (months)	4.7 ± 0.6	NA	
BMI (kg/m^2)	19.69 ± 0.70	21.15 ± 0.48	0.100
SBP (mmHg)	108.1 ± 4.0	110.2 ± 2.9	0.676
DBP (mmHg)	71.8 ± 2.8	69.3 ± 1.6	0.641
FBS (mmol/L)	8.11 ± 1.00	4.78 ± 0.13	0.003
2-h BS (mmol/L)	12.09 ± 1.93	5.16 ± 0.31	0.002
HbA1c (%)	8.52 ± 0.80	5.24 ± 0.08	0.001
FCP (pmol/L)	153.07 ± 18.7	346.82 ± 15.14	<0.001
2-h CP (pmol/L)	308.00 ± 50.52	1540.93 ± 143.43	<0.001
GADA (U/mL)	570.28 ± 111.12	NA	
WBC ($\times 10^9$/L)	5.76 ± 0.63	5.48 ± 0.23	0.690
Neutrophil ($\times 10^9$/L)	3.74 ± 0.5	3.39 ± 0.19	0.521
Lymphocyte ($\times 10^9$/L)	1.59 ± 0.12	1.67 ± 0.17	0.722

Data are shown as mean ± standard error of the mean. BMI, body mass index; BS, blood sugar; CP, C-peptide; DBP, diastolic blood pressure; FCP, fasting C-peptide; GADA, glutamic acid decarboxylase; HbA1c, hemoglobin A_{1C}; NA, not appropriate; SBP, systolic blood pressure; T1D, type 1 diabetes; WBC, white blood cells.

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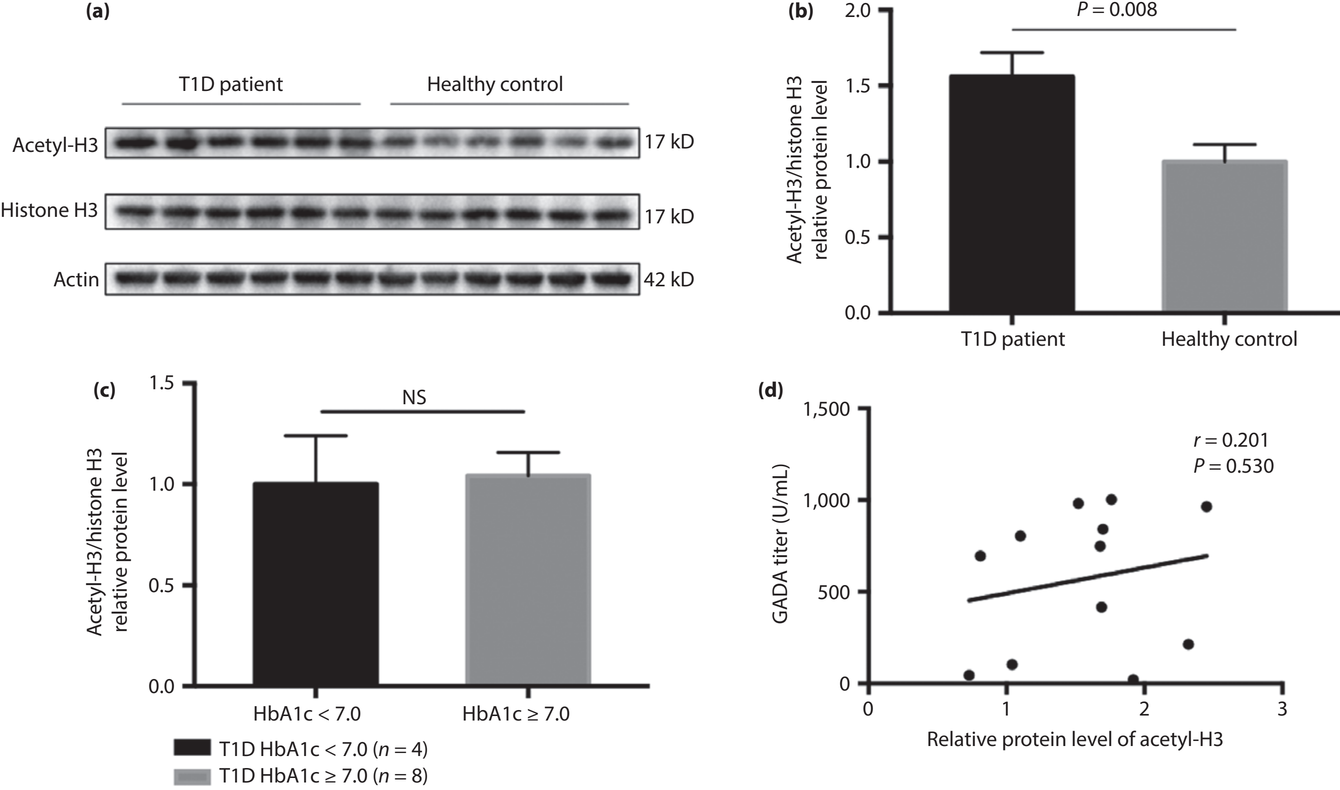

Figure 1 | Histone H3 acetylation was increased in type 1 diabetes (T1D) patients. (a) Representative western blot results of indicated proteins from CD4^{+} T lymphocytes in type 1 diabetes patients and healthy controls (n = 12 in each group). (b) Band intensity analysis showed that acetylated H3 protein levels (normalized to histone H3) were increased in type 1 diabetes patients (n = 12 in each group). β-Actin was used as a control for protein loading. (c) There was no significant difference in the global histone H3 acetylation level between the well- and poorly-controlled type 1 diabetes patients. (d) The global acetylation level was not correlated with the glutamic acid decarboxylase antibody (GADA) titer in type 1 diabetes patients. HbA1c, hemoglobin A_{1C}; NS, not significant.

shown in Table 2, and were ranked by the peak score. Judging from the positive or negative value of the peak score, the number of hyperacetylated genes was significantly greater than that of hypoacetylated genes in type 1 diabetes patients. This result was consistent with the increased H3Ac in type 1 diabetes patients, as shown previously (Figure 1a,b). Among the DAGs list, *TIA1* (TIA1 cytotoxic granule-associated RNA binding), *ICOS*, *HLA-DRB5* (major histocompatibility complex, class II, DR), *FASLG* (fas ligand) and so on are directly related with lymphocyte activation. Other genes from DAGs listed in Table 2 also had functions closely linked to immunity regulation.

Histone H3 acetylation of the *ICOS* promoter is associated with the GADA titer

In the present study, we found that the inducible T-cell costimulator, *ICOS*, showed hyperacetylation in the gene promoter in type 1 diabetes patients, and *ICOS* was enriched in the T lymphocyte costimulation term with the highest fold enrichment. Therefore, we identified *ICOS* as the candidate gene for further study. To gain insight into the relationship between gene expression and acetylation modification, and their impact on type 1 diabetes, we carried out the immunoprecipitation assay and total RNA extraction from CD4^{+} T lymphocytes from 12 type 1 diabetes patients and 12 healthy controls.

The acetylation level of the *ICOS* promoter was shown to be upregulated in type 1 diabetes patients compared with healthy controls, with significantly elevated acetylation occurring in region −137/−55 (1.00 ± 0.20 vs 1.73 ± 0.26, P = 0.036; Figure 4a). Compared with the healthy controls, the expression of *ICOS* messenger RNA (mRNA) was significantly enhanced in type 1 diabetes patients (1.00 ± 0.10 vs 1.85 ± 0.30, P = 0.013; Figure 4b). Correlation analysis showed that the acetylation level at the *ICOS* promoter (region −137/−55) was positively correlated with the mRNA expression (r = 0.655, P = 0.021) in type 1 diabetes patients (Figure 4c). Furthermore, bivariate correlation analysis was carried out between the expression of *ICOS* mRNA and clinical parameters, such as glucose level, hemoglobin A_{1C}, C-peptide, GADA titer or blood pressure. The correlation analysis showed that the GADA titer in type 1 diabetes patients was positively correlated with the expression of *ICOS* mRNA (r = 0.588, P = 0.044; Figure 4d).

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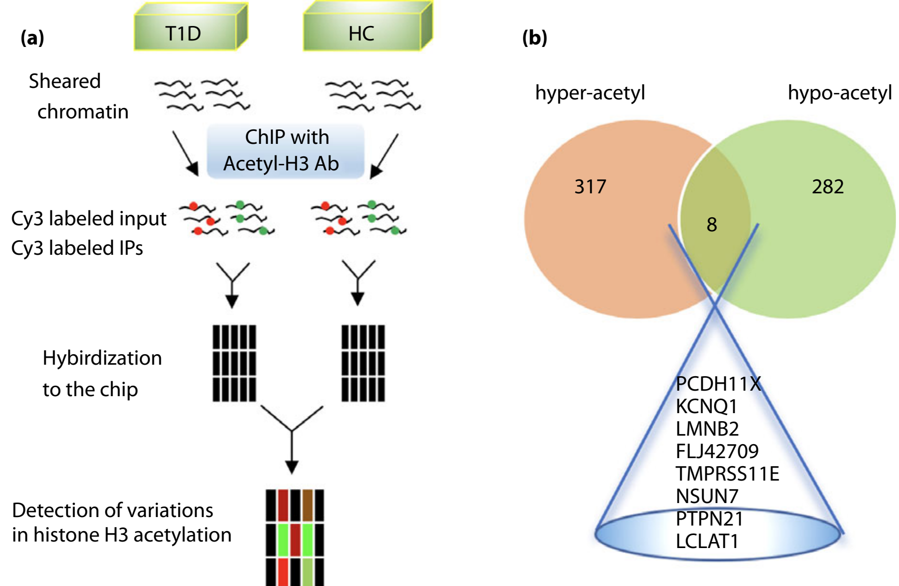

Figure 2 | Histone H3 acetylation profile in CD4+ T lymphocytes from patients with type 1 diabetes (T1D). (a) The schematic diagram of chromatin immunoprecipitation linked to microarrays representing our study flow. (b) Aberrant histone H3 acetylation profile in type 1 diabetes patients. There were 317 genes showing hyperacetylation in promoters, whereas there were 282 genes showing hypoacetylation in type 1 diabetes patients compared with healthy controls. The eight genes overlapping in the Venn diagram were with discordant acetylation alterations in different fragments of gene promoters. Ab, antibody; ChIP, chromatin immunoprecipitation assay; HC, healthy controls; IPs, immunoprecipitates.

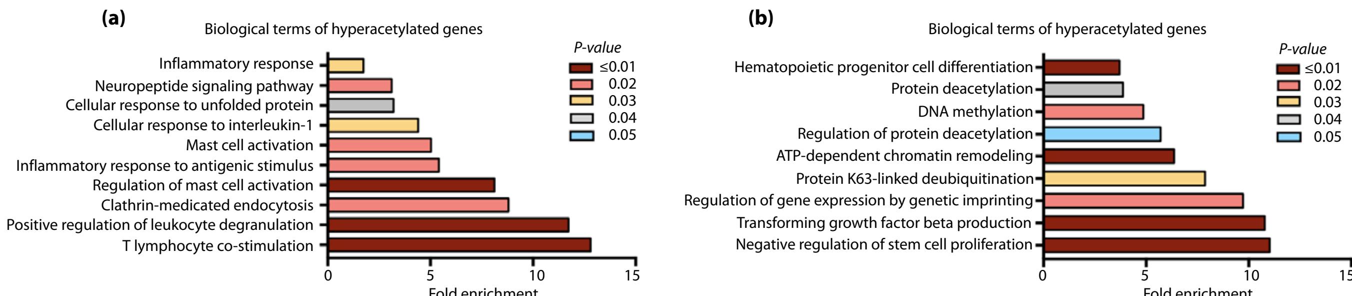

Figure 3 | Gene Ontology analysis of differentially acetylated genes between patients with type 1 diabetes and healthy controls in CD4+ T lymphocytes. (a) Biological terms of hyperacetylated genes. (b) Biological terms of hypoacetylated genes. ATP, adenosine triphosphate; DNA, deoxyribonucleic acid.

DISCUSSION

Epigenetic modifications regulate the expression of genes without changing the DNA sequences, thus affecting important biological processes and disease phenotypes. Accordingly, we used the ChIP-chip method to dissect the whole-genome histone H3 acetylation profile in type 1 diabetes patients. To the best of our knowledge, the present investigation is the first genome-wide epigenetic study applied to purified CD4+ T lymphocytes from GADA-positive type 1 diabetes patients. The present findings generated differentially acetylated gene sets that participated in crucial biological processes, with emphasis on an important T-cell costimulator, *ICOS*.

Given that the histone acetylation is a dynamic and cell-specific process[23], CD4+ T lymphocytes rather than PBMCs mixture were isolated from peripheral blood in the present study. As a wide range of autoimmune disease[10,24] or subtle stimuli[25] have been found to be associated with epigenetic changes, we enrolled patients with new-onset diabetes without complications and with insulin as the only medication. Covariates including age, sex and body mass index were well matched with controls, allowing for a more accurate determination of the acetylation pattern specific to type 1 diabetes rather than physiological causes. We identified evaluated global H3Ac in GADA-positive type 1 diabetes patients in our study, which was consistent with a previous observation in type 1 diabetes patients[11]. However,

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Table 2 | Immune-related differentially acetylated genes sorted by peak score

Gene symbol	Chromosome	Peak score	FDR	Peak length	Peak to TSS	TSS
TIA1†	chr2	1.22	<0.001	285	−699	70329283
IGSF6	chr16	0.95	0.007	266	−3,070	21571473
CASP5	chr11	0.91	0.018	382	−1,422	104399105
IL28RA	chr1	0.88	0.033	137	−1,784	24386338
CCL8†	chr17	0.82	0.009	387	−2,901	29670178
PSMB4	chr1	0.79	0.015	233	−3,033	149638664
HLA-DRB5†	chr6	0.68	0.031	646	−2,931	32605984
BAT4	chr6	−0.75	0.031	242	−2,162	31741142
OTUD5	chrX	−0.75	0.031	288	−1,461	48699837
ICOS†	chr2	0.71	0.029	571	136	204509715
CCL16†	chr17	−0.69	0.033	545	−1,160	31332636
PTPRE	chr10	0.67	0.037	1307	−2,114	129735802
IL1F8	chr2	0.67	0.037	385	246	113526911
FASLG†	chr1	0.57	0.047	240	−2,397	170894807
BAT1	chr6	0.66	0.025	273	−639	31618204
TNFRSF10D	chr8	0.65	0.037	379	−3,036	23077485
NR3C1	chr5	−0.65	0.033	779	−1,626	142795270
SLPI	chr20	−0.65	0.039	264	−1,741	43316620
IRAK2†	chr3	0.64	0.011	857	124	10181562
IL1RL2	chr2	0.64	0.038	376	−1,647	102169864
LENG8	chr19	−0.64	0.039	397	−2,986	59651876
PSMB6	chr17	−0.62	0.043	248	−1,919	4646414
TNFRSF9†	chr1	0.61	0.033	271	−583	7923474
IL1F10	chr2	0.58	0.040	235	−1,247	113542017
NFKB1†	chr4	0.57	0.050	248	−1,832	103641517
TLR5	chr1	−0.57	0.044	551	−13	221383247
FAM19A5	chr22	0.54	0.044	563	−2,030	47263951
LILRA1	chr19	0.53	0.047	1275	−1,812	59796924

†Closely related to lymphocyte activation. Peak score, the log2 ratio of fold change between patients with type 1 diabetes and healthy controls. FDR, false discovery rate; TSS, transcriptional start site.

inconsistency existed in that reduced global H3Ac was observed in patients with latent autoimmune diabetes in adults[26]. That type 1 diabetes is clinically distinct from latent autoimmune diabetes in adults[27] might explain the discrepancy. Also, the latent autoimmune diabetes in adults study included patients with much longer disease duration, and many of them had diabetic complications that might contribute to lower acetylation[26], whereas the present study included only new-onset type 1 diabetes patients. Furthermore, we found that the acetylation of the *ICOS* promoter, rather than global acetylation, was associated with the GADA titer in type 1 diabetes patients.

The present study showed that blood glucose level had no effect on the global acetylation level. Additionally, an epigenetic study in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort showed no difference in the number of hyperacetylated regions in lymphocytes from type 1 diabetes patients between the original Diabetes Control and Complications Trial conventional therapy group and intensive therapy group[28]. Intervention with cytokine without high glucose also brought on marked variations in histone H3 lysine 9 acetylation levels at the promoter regions of type 1 diabetes susceptible genes in an *in vitro* study[13]. This found that changes in global acetylation from lymphocytes might be associated with autoimmune disturbance in type 1 diabetes instead of being secondary to hyperglycemia. ChIP-chip has further identified post-translational modifications in cell models cultured with high glucose[29] or in patients with long enduring type 1 diabetes[28,30]. The present study yielded a bundle of hyperacetylated genes implicated in innate and adoptive immune-related biological processes, which might reflect the initial epigenetic state in new-onset type 1 diabetes patients. Due to the gene transcriptional activation by acetylation, the T lymphocytes transcriptome could be strikingly activated in particular gene sets to exert its pathogenically attacking manner to the islet β-cells.

According to our GO analysis, hyperacetylated DAGs were engaged in T lymphocytes/leukocyte/mast cell activity, inflammatory response and cellular response to interleukin-1. Furthermore, previous studies have discerned that aberrant histone acetylation[26] or dysregulation of gene expression through histone acetylation[31] contributes to the pathogenesis of autoimmune diabetes. Herein, the critical gene *ICOS*, a gene with remarkable

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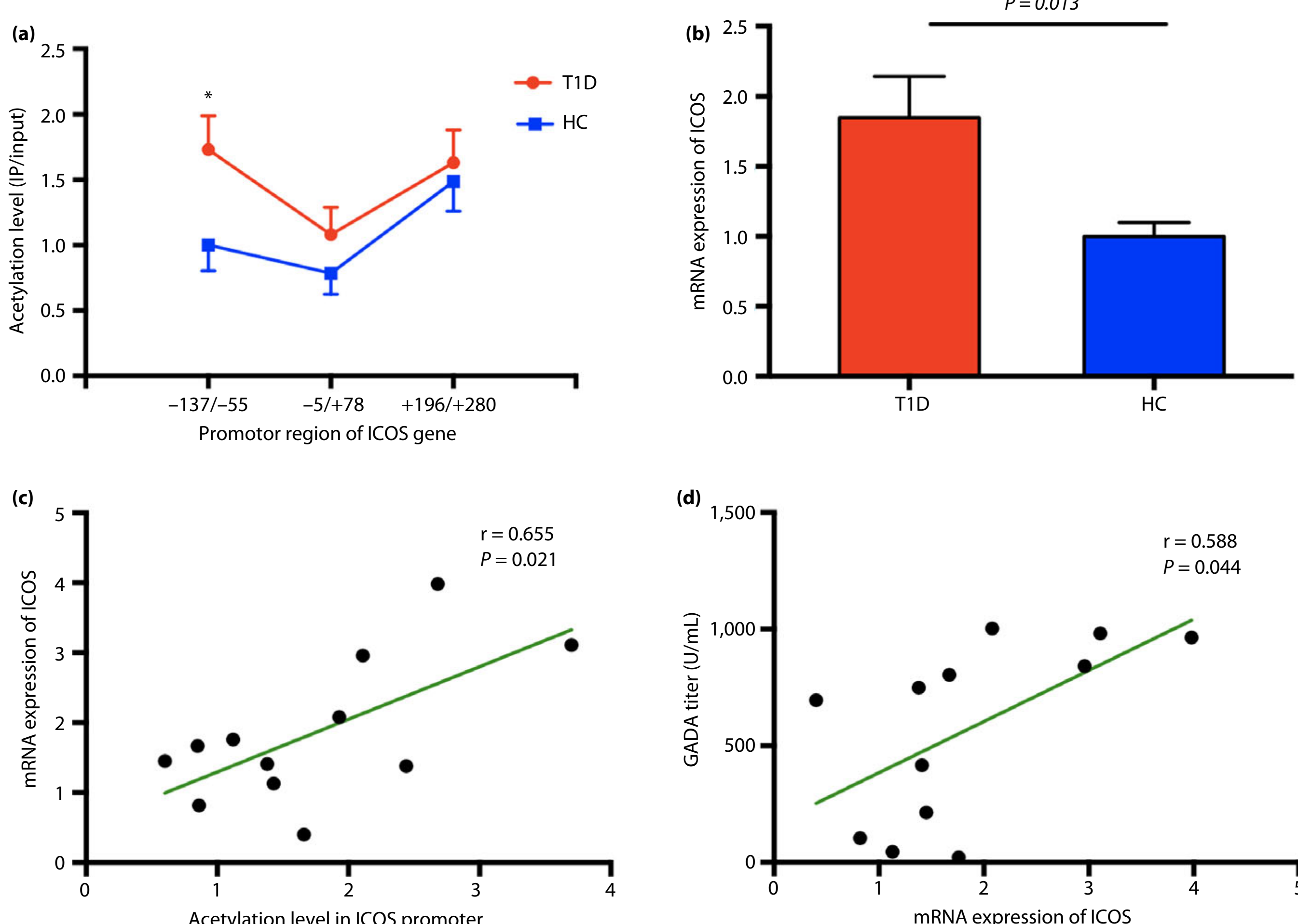

Figure 4 | Histone H3 acetylation of the ICOS promoter was associated with the glutamic acid decarboxylase antibody (GADA) titer in type 1 diabetes patients (T1D). (a) The histone H3 acetylation level of the ICOS promoter was significantly enhanced in type 1 diabetes patients (n = 12) compared with healthy controls (HC; n = 12) in promoter region –137/–55, which was detected by chromatin immunoprecipitation quantitative polymerase chain reaction and data were shown as mean ± standard error of the mean, *$P < 0.05$. (b) Type 1 diabetes patients showed increased ICOS messenger ribonucleic acid (mRNA) level. Total RNAs were prepared from the $CD4^+$ T lymphocytes from 12 individual patients or controls. β-Actin was used as an internal control. Data shown were from quantitative polymerase chain reactions from 12 individual participants with each sample run in triplicate. (c) Correlation analysis showed that histone H3 acetylation in the ICOS promoter (region –137/–55) was positively correlated with the expression of its mRNA in $CD4^+$ T lymphocytes from type 1 diabetes patients. (d) The GADA titer was positively related to the ICOS mRNA level in type 1 diabetes patients.

acetylated alteration and highest fold enrichment in GO analysis, was selected for further study. *ICOS*, an inducible T-cell costimulator, is a member of the T cell-specific cell-surface receptors CD28/CTLA-4 family. The activation of T cells is mediated by the two-signal model[32]: the T-cell receptor recognized the peptide presented by major histocompatibility complex molecule anchoring in the surface of antigen presentation cells, accompanying the ensuing combination of costimulators as a second signal. *ICOS* is expressed de novo after T cells are stimulated, and promotes T-cell activation and differentiation[33]. Previous studies have shown that the ablation of *ICOS* mitigates the severity of insulitis and protects non-obese diabetic mice from spontaneous type 1 diabetes[34,35]. This indicates that *ICOS* is required for insulitis development and the subsequent overt autoimmune diabetes.

Follicular helper T cells (Tfh), a $CD4^+$ T-cell subset expressing *ICOS* and CXCR5, are predominantly important for antibody production by plasma B cells[36]. In systemic lupus erythematosus, the accumulation of pathogenic autoantibodies results in multiple organ dysfunction[37]. With respect to autoimmune diabetes, it is well established that the islet autoantibodies are biomarkers for diagnosis without pathogenicity[38]. Nevertheless, the GADA titer might reflect the strength of immune response[39], and be associated with the decay rate of islet function[39,40]. Many studies have shown that Tfh cells are involved

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in islet antibodies production in type 1 diabetes. The frequency of circulating activated Tfh cells was increased in children with type 1 diabetes positive for multiple autoantibodies[41]. Meanwhile, decreased levels of autoantibodies were found in *ICOS*$^{-/-}$ non-obese diabetic mice[34]. To replenish these observations, the most consistent finding in the present study has been that the expression of *ICOS* and the GADA titer delineate a positive correlation in type 1 diabetes patients. The high GADA titer might partly result from elevated *ICOS*, which is mostly expressed in the Tfh cell, thus prompting T lymphocytes to kill more β-cells, and stimulating more B cells differentiating into plasma cells to produce autoantibodies after binding to *ICOS* ligand on the surface of B cells.

As for the hypoacetylated counterpart, genes engaging in other patterns of epigenetic modification showed reduced acetylation levels in the present study. This could be explained by the well-established theory that epigenetic mechanisms have a mutual effect on each other to regulate gene expression[24], evidenced by the reciprocity of histone deacetylation and DNA methylation on Foxp3 silencing[22,31,42]. In addition, TGF-β maintains the immune homeostasis by inhibiting pro-inflammatory cytokines secretion and controlling peripheral T-cell tolerance[43]. The present finding provided clues that the hypoacetylated alteration on genes linked to the TGF-β production might result in being a causative element for the decreased circulating TGF-β in type 1 diabetes patients[44]. Interestingly, several diabetes susceptible or T-cell activity-related genes – *HLA-DRB5*, *FASLG*, *TNFRSF9* and *NFKB1* – showed altered acetylation levels in the present study. We conjectured that dysregulation of histone acetylation might disequilibrate the immune tolerance and be inclined to activate the diabetogenic T cells in type 1 diabetes.

A genome-wide association study reported a type 1 diabetes-related single-nucleotide polymorphism, rs478222, residing in the intron of gene *EFR3B*[45]. The DAGs in the present study also included the gene, *EFR3B*, with its promoter presenting hypoacetylation in type 1 diabetes patients. As genome-wide association studies continue to identify causative genes implicated in type 1 diabetes[46], the present findings will replenish our knowledge of epigenetic alterations of these genes, and subsequent integrated analysis of genetic and epigenetic association data is moving towards uncovering the disease mechanisms. As it has been reported that the HLA genotype is associated with the GADA titer[47], a limitation of the present study was that it was difficult to carry out association analysis between the HLA genotype and GADA titer in such a small sample size, given the highly polymorphic HLA genotype and large variation of the GADA titer in type 1 diabetes patients.

In summary, we found that various genes are associated with altered histone H3 acetylation in type 1 diabetes patients. More specifically, the genes closely related to the immune system undergo hyperacetylation changes along with upregulation expression of *ICOS*, which is positively correlated with the GADA titer in type 1 diabetes patients. The present study provides an exciting overview and hints for further investigations focusing on identifying epigenetic markers or potential therapeutic targets of type 1 diabetes.

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DISCLOSURE

The authors declare no conflict of interest.

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