

Aberrant histone modification in CD19⁺ B cells of patients with chronic lymphocytic leukemia

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Abstract: The aim of this study was to detect the alterations in histone methylation and acetylation in patients with chronic lymphocytic leukemia (CLL). Global histone H3/H4 acetylation and H3K4/H3K9 methylation were detected by the EpiQuik™ global histone H3/H4 acetylation and H3K4/H3K9 methylation assay kits. The mRNA expression of selected chromatin modifier genes was measured by real-time polymerase chain reaction (RT-PCR). Our results found that the global histone H3/H4 hypoacetylation in the CD19⁺ B cells of patients with CLL ($P=0.028$ and $P=0.03$, respectively) and the global histone H3K9 methylation in patients with CLL were significantly increased compared with controls ($P=0.02$), while there was no significant difference in the global histone H3K4 methylation between the two groups. The level of SIRT1 and EZH2 mRNA expression was upregulated in patients with CLL ($P=0.03$ and $P=0.02$, respectively), which increased significantly with progression from Binet stage A to stage C ($P=0.015$ and $P=0.01$, respectively) and Rai good to high risk stage ($P=0.007$ and $P=0.008$, respectively). The level of HDAC1 and HDAC7 mRNA expression was significantly increased ($P=0.02$ and $P=0.008$, respectively) and HDAC2 and P300 mRNA expression was reduced in patients with CLL ($P=0.002$ and $P=0.001$, respectively). In conclusion, it is observed that the aberrant histone modification plays an important role in the pathogenesis of CLL.

Keywords: histone methylation, histone acetylation, SIRT1, EZH2, CLL

Introduction

Chronic lymphocytic leukemia (CLL) is a disease characterized by the accumulation of mature, monoclonal B lymphocytes in the peripheral blood, bone marrow and other lymphoid tissues such as spleen, leading to a gradual accumulation of leukemia cells and resulting in cytopenias and/or organomegaly.¹ The clinical course of patients with CLL is heterogeneous, ranging from indolent to aggressive, with survival ranging from a couple of months to several decades.² The mechanisms underlying the aberrant epigenetic changes in CLL are not completely understood, but their effects in the ontogeny of this disease are now well established. As we all known, the growing evidence showed that alterations in chromatin structure are linked to cell cycle regulation, cell death, and overall genome stability. Of the known epigenetic modifications, histone acetylation and histone methylation are the two most documented epigenetic changes.

The histone proteins that package DNA into chromatin play key roles in the regulation of transcription. The N-terminal tails of these proteins are subjected to several posttranslational modifications such as acetylation, deacetylation, and methylation.³ The combination of these covalent modifications gives rise to the so-called “histone code”.⁴ Transcription becomes active when histones are acetylated by histone acetyltransferases (HATs), silenced when histones are deacetylated by

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histone deacetylases (HDACs), and silenced or activated when methylated by histone methyltransferases (HMTs).⁵

The aim of this study was to determine the alterations in global histone H3/H4 methylation/acetylation status and to evaluate the expression profile of HATs, HDACs and HMTs in patients with CLL. Indeed, to our knowledge, no study has correlated expression with the clinical impact of Chinese patients with CLL. Therefore, we carried out the expression analysis of selected members of histone modifier genes: HATs (EP300, CREBBP, PCAF), HDACs (HDAC1, HDAC2, HDAC7, SIRT1), and HMTs (SUV39H1, SUV39H2, EZH2), and further correlated the expression with clinical characteristics.

Patients and methods

Subjects

Peripheral blood samples were collected from 58 newly diagnosed patients with CLL between 2013 and 2015, and 18 healthy controls were enrolled in this study. This study was approved by the ethics committee of the Affiliated Cancer Hospital of Zhengzhou University and conducted according to the principles expressed in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Written informed consent was obtained from all the subjects.

The diagnosis of CLL required a persistent B lymphocytosis of $>5.0 \times 10^9/L$ and a typical CD19⁺, CD5⁺, CD23⁺, immunoglobulin (Ig) light chain (κ or λ)-restricted immunophenotype as revealed by the flow cytometry of peripheral blood cells, which was according to the guidelines of the International Workshop Chronic Lymphocytic Leukemia/National Cancer Institute (iwCLL/NCI).⁶ Complete clinical profiles were obtained from all the subjects. All the patients were grouped by age, sex, lactate dehydrogenase (LDH) level, β_2 microglobulin (β_2 -MG) level, Binet stage, and Rai risk stage. High LDH was defined as >245 U/L and β_2 -MG as >3.5 mg/L.

Cell preparation, RNA isolation, and reverse transcriptase

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. CD19⁺ B cells were isolated by magnetic beads, according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the B cell was $>98\%$, which was detected by the flow cytometry method. Total RNA was isolated by Trizol reagent (Invitrogen), quantified by Nanodrop, and a total of 10 ng of RNA was subsequently used to synthesize

complementary DNAs using the Reverse Transcription Kit (Applied Biosystems).

Real-time polymerase chain reaction (RT-PCR)

For PCR amplification, an initial denaturation at 94°C for 10 min was followed by 40 cycles of denaturation at 94°C for 15 s, and extension at 60°C for 1 min. The relative quantification of gene expression was obtained by comparing with the relative expression of β -actin using $2^{-\Delta\Delta C_t}$ method. Each experiment was repeated three times, and the averages were calculated. Selected chromatin modifier genes were used. The primer sequences are shown in Table 1.

Detection of global histone H3/H4 acetylation and global H3K4/H3K9 methylation

Histone extraction and the detection of global histone H3/H4 acetylation and global H3K4/H3K9 methylation were performed using the EpiQuik™ global histone H3/H4 acetylation and H3K4/H3K9 methylation assay kits, according to the manufacturer's instructions (Epigentek, Brooklyn, NY, USA). In brief, histone proteins (1 mg) were stably spotted on the strip wells. Acetylated histone H3K4/H3K9 was detected with a high-affinity antibody, and the ratios and amounts of acetylated histone H3K4/H3K9 were quantified with a horseradish peroxidase-conjugated secondary

Table 1 Primer sequences

Name	Sequence 5'–3'
SIRT1 forward	TAC CGA GAT AAC CTT CTG
SIRT1 reverse	TCC AGT CAC TAG AGC TTG
HDAC1 forward	CAA GCT CCA CAT CAG TCC TTC C
HDAC1 reverse	TGC GGC AGC ATT CTA AGG TT
HDAC2 forward	AGT CAA GGA GGC GGC AAA A
HDAC2 reverse	TGC GGA TTC TAT GAG GCT TCA
HDAC7 forward	CTT CTC CAC AAG GAC AAG
HDAC7 reverse	CTC CAG GGT TCT GTA GG
PCAF forward	ATG AAT ATG CAA TTG GAT AC
PCAF reverse	CTC CTT CAT AAT CCT TGA TA
CREBBP forward	CTG CAC ACG ACA TGA CT
CREBBP reverse	GAA GTG GCA TTC TGT TG
P300 forward	CTG TAT GTG CTC CAG AAC
P300 reverse	GAC AAA AAG GCA GTT CC
EZH2 forward	ACATCCTGACTTCTGTGAG
EZH2 reverse	GGAGACCAAGAATACATTA
SUV39H1 forward	AAGAAGATCCGCGAACAGGAA
SUV39H1 reverse	GGAAGTGCTTGAGGATACGCAC
SUV39H2 forward	ATCCACCTGGTACTCCCATCT
SUV39H2 reverse	GCAAAGCGAATACTGTGTGCC
β -actin forward	CCTGGCACCCAGCACAAAT
β -actin reverse	GGGCCGGACTCGTCATAC

antibody color development system. Color was measured by absorbance at 450 nm.

Statistical analysis

SPSS18.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The results were presented as mean \pm SD. One-way analysis of variance (ANOVA) or the nonparametric Mann–Whitney *U*-test was used. A *P*-value <0.05 was considered to be statistically significant.

Results

Patient characteristics

This study included 34 males (60.7%) and 22 females (39.3%) with a median age of 63 years (range, 60–80 years). The median number of white blood cells (WBCs) was $26 \times 10^9/L$ (range from $11.5 \times 10^9/L$ to $280 \times 10^9/L$). The clinical parameters of 58 patients are summarized in Table 2.

Table 2 Demographic and clinical characteristics of patients with CLL (n=58)

Characteristics	No of patients (%)
Sex	
Male	34 (58.6)
Female	24 (41.4)
Age (years)	
≤ 60	22 (37.9)
> 60	36 (62.1)
Binet stage	
A	15 (25.9)
B	20 (34.4)
C	23 (39.6)
Rai risk stage	
Good	3 (5.2)
Intermediate	34 (58.6)
High	21 (36.2)
LDH	
Normal	21 (36.2)
Elevated	37 (63.8)
$\beta 2$ -MG	
Normal	23 (39.7)
Elevated	35 (60.3)
IgVH	
Mutated	36 (62.1)
Unmutated	22 (37.9)
ZAP-70	
Negative	48 (82.8)
Positive	10 (17.2)
CD38	
Negative	47 (81.0)
Positive	11 (19.0)
FISH cytogenetics	
No aberration or sole del(13q)	32 (55.2)
Del(17p) or del(11q) or trisomy 12	26 (44.8)

Abbreviations: CLL, chronic lymphocytic leukemia; FISH, fluorescence in situ hybridization; LDH, lactate dehydrogenase; $\beta 2$ -MG, $\beta 2$ microglobulin.

Global histone H3/H4 acetylation and H3K9 methylation in patients with CLL and controls

Histone acetylation and methylation were detected in 15 patients with CLL and 15 healthy controls. Our data showed that the global histone H3/H4 acetylation was significantly decreased in the CD19⁺ B cells of patients with CLL, when compared with controls ($P=0.028$ and $P=0.03$, respectively) as shown in Figure 1A and B. There was no significant difference in the global histone H3K4 methylation between the two groups, as shown in Figure 1C, but the global histone H3K9 methylation in patients with CLL was significantly higher than that of healthy controls ($P=0.02$), as shown in Figure 1D.

The mRNA expression of chromatin modifier genes in patients with CLL and controls

It was shown that the expression level of SIRT1 and EZH2 mRNA in patients with CLL was significantly higher than that of healthy controls ($P=0.03$ and $P=0.02$, respectively), as shown in Figures 2A and 3A. We also investigated whether SIRT1 and EZH2 expression was associated with subgroups of CLL with different clinical characteristics such as Binet stage, Rai risk stage, LDH, $\beta 2$ -MG, ZAP-70, CD38, and FISH cytogenetic. It showed that the level of SIRT1 and EZH2 mRNA expression increased significantly with progression from Binet stage A to stage C ($P=0.015$ and $P=0.01$, respectively), as shown in Figures 2B and 3B, and Rai good to high risk stage ($P=0.007$ and $P=0.008$, respectively), as shown in Figures 2C and 3C. The level of EZH2 mRNA expression in the $\beta 2$ -MG⁺ subgroup was increased significantly compared with $\beta 2$ -MG[−] subgroup ($P=0.029$), as shown in Figure 3D.

Moreover, it was found that the expression level of HDAC1 and HDAC7 mRNA in patients with CLL were significantly higher than that of healthy controls ($P=0.02$ and $P=0.008$, respectively), as shown in Figure 4A and C, while the level of HDAC2 and P300 mRNA expression was reduced significantly in patients with CLL ($P=0.002$ and $P=0.001$, respectively), as shown in Figure 4B and D. There was no significant difference in the expression of PCAF, CREBBP, SUV39H1, and SUV39H2 mRNA between patients with CLL and controls, and between any subgroups (Figure 5).

Discussion

CLL is the most common leukemia in adult populations of Western countries. The exact mechanism of the pathogenesis

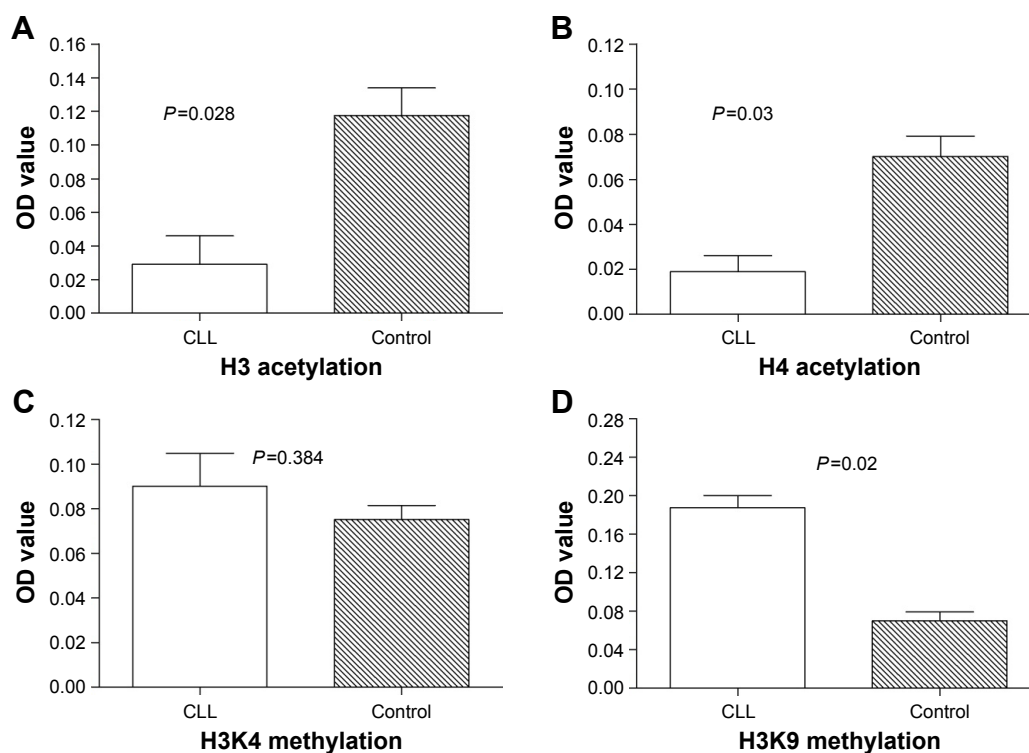


Figure 1 Global histone H3/H4 acetylation and H3K9 methylation in subjects.

Notes: The global histone H3/H4 hypoacetylation in the CD19⁺ B cells of patients with CLL compared with controls (**A** and **B**) ($P=0.028$ and $P=0.03$, respectively). No significant difference in the global histone H3K4 methylation between the two groups (**C**). The global histone H3K9 methylation in patients with CLL was significantly higher than that of healthy controls (**D**) ($P=0.02$). The bars are presented as mean \pm SD; OD units at 450 nm.

Abbreviations: CLL, chronic lymphocytic leukemia; OD, optical density; SD, standard deviation.

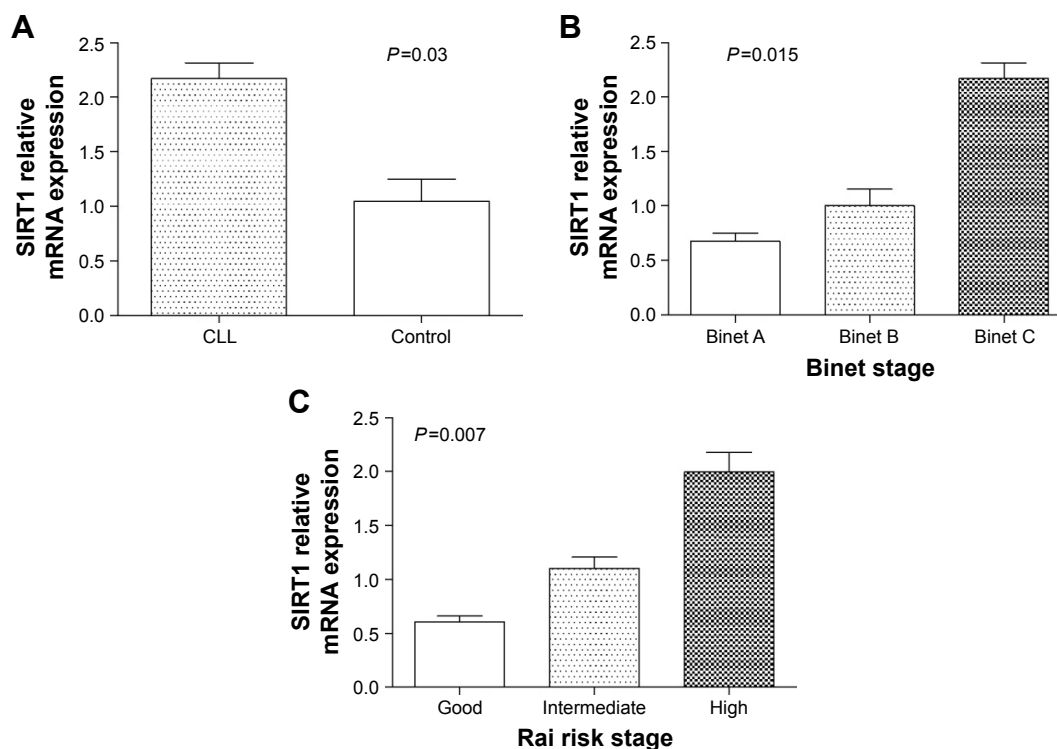


Figure 2 The SIRT1 mRNA expression in subjects.

Notes: The expression level of SIRT1 mRNA in patients with CLL was significantly higher than that of healthy controls ($P=0.03$) (**A**). The level of SIRT1 mRNA expression increased significantly with progression from Binet stage A to stage C ($P=0.015$) (**B**) and Rai good to high risk stage ($P=0.007$) (**C**). The bars indicate the relative mRNA levels of SIRT1 (mean \pm SD).

Abbreviations: CLL, chronic lymphocytic leukemia; SD, standard deviation.

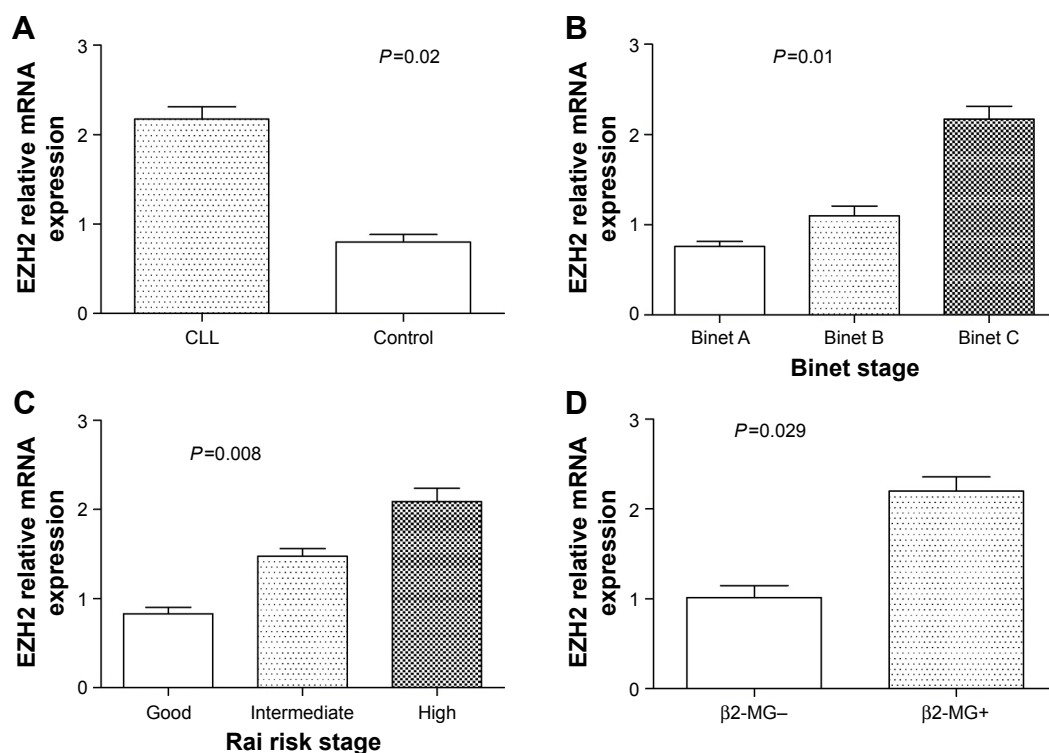


Figure 3 The EZH2 mRNA expression in subjects.

Notes: The expression level of EZH2 mRNA in patients with CLL was significantly higher than that of healthy controls ($P=0.02$) (A). The level of EZH2 mRNA expression increased significantly with progression from Binet stage A to stage C ($P=0.01$) (B) and Rai good to high risk stage ($P=0.008$) (C). The level of EZH2 mRNA expression increased significantly in the $\beta 2$ -MG+ subgroup compared with that in the $\beta 2$ -MG- subgroup ($P=0.029$) (D). The bars indicate the relative mRNA levels of SIRT1 (mean \pm SD). $\beta 2$ -MG+, defined as $\beta 2$ -MG >3.5 mg/L; $\beta 2$ -MG-, defined as $\beta 2$ -MG ≤ 3.5 mg/L.

Abbreviations: CLL, chronic lymphocytic leukemia; $\beta 2$ -MG, $\beta 2$ microglobulin; SD, standard deviation.

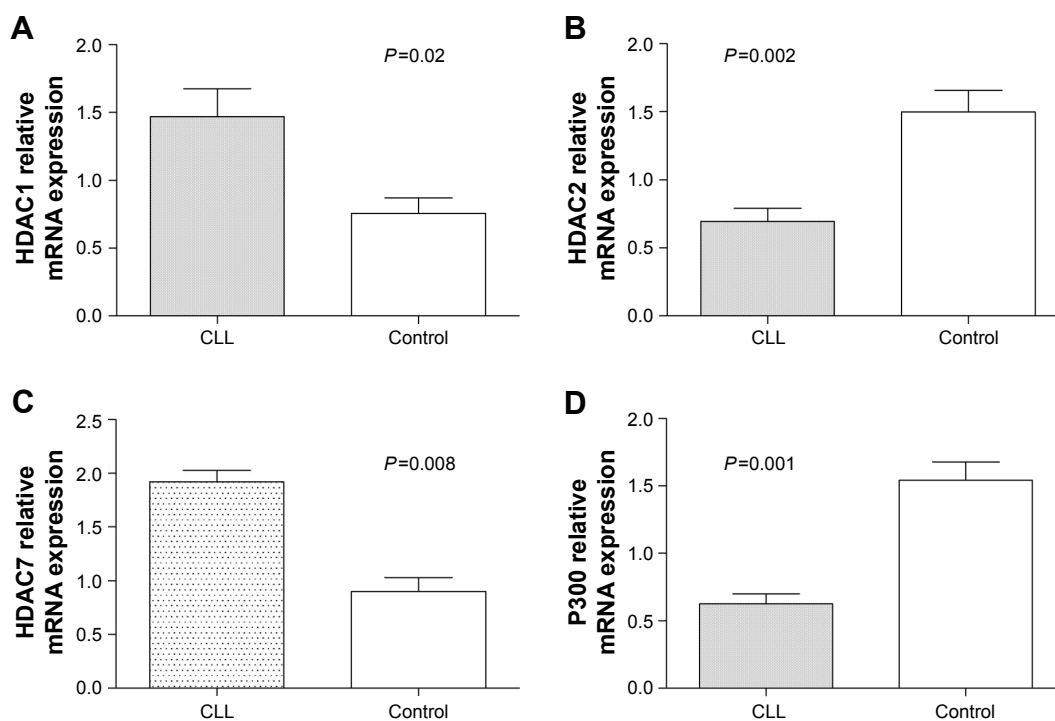


Figure 4 The mRNA expression of selected chromatin modifier genes in subjects.

Notes: The expression level of HDAC1 mRNA in patients with CLL was significantly higher than that of healthy controls ($P=0.02$) (A). The level of HDAC2 mRNA expression was reduced significantly in patients with CLL ($P=0.002$) (B), HDAC7 mRNA expression level was increased in patients with CLL ($P=0.008$) (C). The level of P300 mRNA expression was reduced significantly in patients with CLL ($P=0.001$) (D). The bars indicate the relative mRNA levels of SIRT1 (mean \pm SD).

Abbreviations: CLL, chronic lymphocytic leukemia; SD, standard deviation.

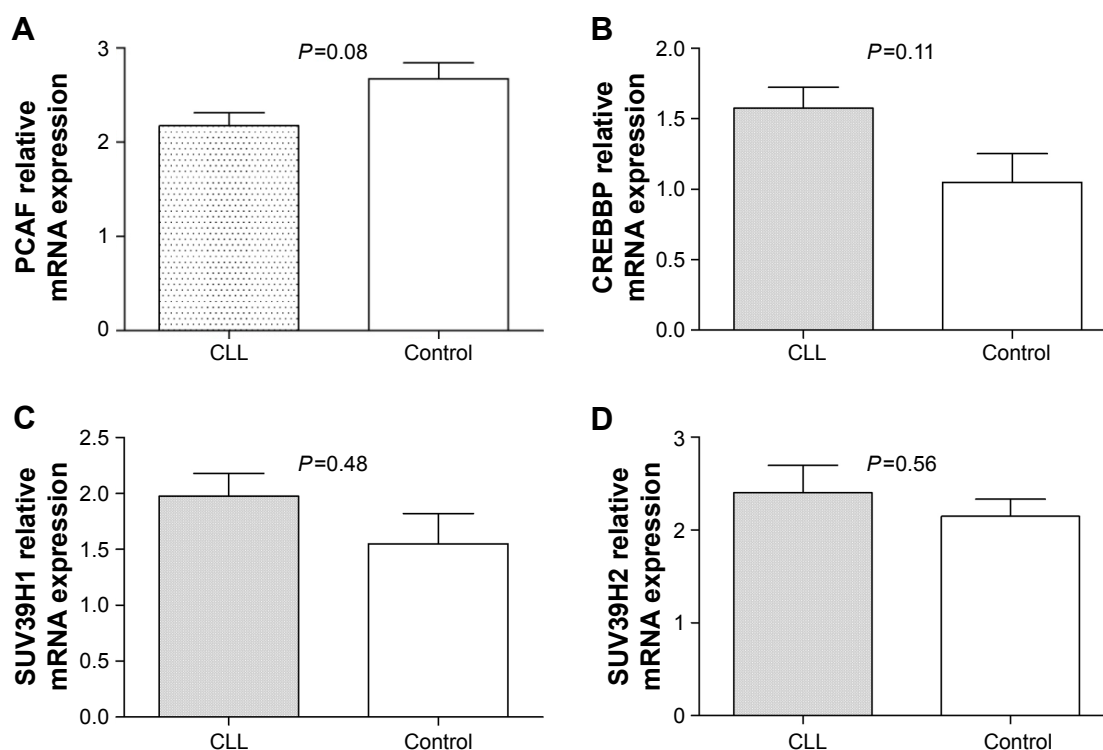


Figure 5 The mRNA expression of selected chromatin modifier genes in subjects.

Note: There was no significant difference in the expression of PCAF (A), CREBBP (B), SUV39H1 (C), and SUV39H2 (D) mRNA between patients with CLL and controls, and between any subgroups.

Abbreviation: CLL, chronic lymphocytic leukemia.

of CLL still has to be determined. In the current study, we focus on the change in histone modification in CD19⁺ B cells of the patients with CLL.

Our data showed that the global histone H3/H4 acetylation was significantly decreased in the CD19⁺ B cells of patients with CLL, compared with controls, which was in line with the results of Aron's data,⁷ in which depsipectide, an HDAC inhibition, can increase the histone H3 and H4 acetylation to induce apoptosis in CLL B cells, indicating that the downexpression of histone acetylation involves in the pathogenesis of CLL. SIRT1 is a class III nuclear deacetylase that can activate or repress genetic programs by modifying histones and transcription factors. SIRT1 is upregulated in several human tumor types.⁸ Interestingly, in this study, it was shown that the expression level of SIRT1 mRNA in patients with CLL was significantly higher than that of controls. Furthermore, the level of SIRT1 mRNA expression increased significantly with progression from Binet stage A to stage C and Rai good to high risk stage; this is keeping with independent observations linking increased expression of the enzyme to tumor transformation.⁹ Furthermore, it was shown that the expression level of HDAC1 and HDAC7 mRNA in patients with CLL was significantly increased than that of

healthy controls, while the level of HDAC2 and P300 mRNA expression was reduced significantly in patients with CLL, suggesting that the abnormal expression of those HDACs may play a vital role in the pathogenesis of CLL.

It was found that the global histone H3K9 methylation in patients with CLL was significantly higher than that of controls, but there was no significant difference in the global histone H3K4 methylation between the two groups, suggesting that the dysregulation of histone methylation plays an important role in the etiology of CLL. EZH2 is overexpressed in several cancer types, both solid tumors and hematopoietic malignancies,^{10,11} and its overexpression is correlated with disease aggressiveness.^{12–15} In acute myeloid leukemia, EZH2 inhibited differentiation programs in leukemic stem cells, increasing their leukemogenic activity.¹⁴ EZH2 was found to be aberrantly overexpressed in natural killer/T-cell lymphoma (NKTCL), conferring a poor prognosis.¹⁵ In CLL, it is reported that EZH2 overexpression is related to a poor prognosis of CLL and could be a useful tool to assess its aggressiveness.^{16,17} In this study, it was found that the expression level of EZH2 mRNA in patients with CLL was significantly higher than that of controls, and EZH2 mRNA expression increased significantly with

progression from Binet stage A to stage C and Rai good to high risk stage. The level of EZH2 mRNA expression in the $\beta 2$ -MG+ subgroup was increased significantly compared with $\beta 2$ -MG- subgroup. Those data were in accordance with other's results,^{18,19} implicating EZH2 in the pathophysiology of CLL with remarkable clinical and biological heterogeneity. But, the complex mechanisms leading to EZH2 overexpression still have to be better understood.

Conclusion

In this study, the global histone H3/H4 hypoacetylation, H3K9 hypermethylation, and overexpression of SIRT1 and EZH2 were detected in the CD19⁺ B cells of patients with CLL, indicating that the aberrant histone modification was involved in the pathogenesis of CLL.

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Disclosure

The authors report no conflicts of interest in this work.

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