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SHORT COMMUNICATION

Unraveling *KDM4* histone demethylase expression and its association with adverse cytogenetic findings in chronic lymphocytic leukemia

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Abstract

The acquisition of complex karyotypes is related to the progression of chronic lymphocytic leukemia (CLL) and patients with this condition have a poor prognosis. Despite recent advances in the classification of prognosis in CLL patients, understanding of the molecular mechanisms that lead to genomic instability and progression of this disease remains inadequate. Interestingly, dysregulated expression of *KDM4* members is involved in the progression of several cancer types and plays a role in the DNA damage response; however, the gene expression profile and the importance of *KDM4* members in CLL are still unknown. Here, we assessed the gene expression profile of *KDM4A, KDM4B*, and *KDM4C* in 59 CLL samples and investigated whether these histone demethylases have any influence on the prognostic markers of this leukemia. *KDM4A* gene expression was higher in CLL patients as compared with control samples. In contrast, CLL samples showed decreased levels of the *KDM4B* transcript in relation to control cases, and no difference was detected in *KDM4C* expression. Furthermore, patients with positive expression of ZAP-70 had lower expression of *KDM4B* and *KDM4C* as compared with ZAP-70-negative patients. More importantly, patients with low expression of these histone demethylases had higher leukemic cell numbers and displayed adverse cytogenetic findings and the acquisition of a complex karyotype. The present data clearly show that the expression of *KDM4* members is dysregulated in CLL and impact the prognosis of this leukemia. These findings are useful for a better understanding of the impact of epigenetics on CLL progression.

Keywords *KDM4* · Chronic lymphocytic leukemia · Prognostic factors · ZAP-70 · Karyotype

Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder characterized by the accumulation of mature CD5+ B-lymphocytes in the blood, bone marrow, lymph nodes, and/or other lymphoid tissues. There is a broad spectrum of clinical presentation in CLL, in which some patients present an indolent disease pattern, living for many years without treatment, while others have an aggressive disease with a very poor prognosis. Given this heterogeneous clinical course of CLL, it is of fundamental importance to establish an effective tool for the classification of patients

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according to their prognosis. The Rai and Binet staging systems, developed in the 1970s, were used for decades to stratify CLL patients by their clinical characteristics, but these systems do not allow identification of the risk of progression. More recently, other prognostic parameters have begun to be used for the classification of CLL patients, including the mutational status of immunoglobulin heavy-chain variable region (IgVH) and the expression of CD38 and ZAP-70. Knowledge regarding chromosomal aberrations and genetic mutations in CLL has allowed refinement of the prognostic subgroups of this disease [1, 2]. Accordingly, it has been shown that the acquisition of complex karyotypes is related to the progression of CLL and that such patients have a poor prognosis [3, 4]. Despite advances in the classification of prognosis in CLL patients, understanding of the molecular mechanisms that lead to genomic instability and progression of this disease remains inadequate.

In recent years, the field of epigenetics has developed dramatically and it is now well known that the altered function of histone methyltransferases and demethylases contributes to cancer development and progression [5]. Dysregulation of histone demethylases in cancer can promote the activation of oncogenes, repression of tumor suppressors, alteration of DNA mismatch repair, and disruption of chromosomal stability. Specifically, aberrant expression of the histone demethylase members *KDM4A/JMJD2A, KDM4B/JMJD2B*, and *KDM4C/JMJD2C*, which catalyze the removal of H3K9 and H3K36 di- and trimethyl marks, has been detected in several cancer types, such as breast, colorectal, lung, prostate, esophageal, and lymphoma [6, 7]. More specifically, it has been shown that *KDM4* members play a role in the DNA damage response [8, 9].

To date, the gene expression profile and importance of *KDM4* members in CLL remain unknown. Here, we assessed the gene expression profile of *KDM4A, KDM4B*, and *KDM4C* in 59 CLL samples and investigated whether these histone demethylases have any influence on the prognostic markers of this leukemia.

Methods

Sample collection

Peripheral blood samples were collected from 59 consecutive CLL patients diagnosed according to Matutes score [10] and treated at the University Hospital of the Medical School of Ribeirao Preto (University of São Paulo, Brazil). In addition, peripheral blood samples from 10 age-matched healthy donors were obtained as a control. The present study was approved by the Local Ethical Committee and informed consent was obtained from all patients and healthy donors.

Isolation of peripheral blood mononuclear cells and immunomagnetic selection of CD19+ B-cells

Peripheral blood mononuclear cells (PBMCs) from CLL patients and healthy donors were obtained following centrifugation of blood samples through Ficoll-Paque PLUS density gradient media (Amersham Biosciences). Subsequently, magnetic beads coupled to CD19 were employed for the immunomagnetic selection of control B-cells, following the manufacturer's recommendations (Miltenyi Biotec). All isolated CD19+ cells had a purity greater than 90% as determined by flow cytometry.

Determination of ZAP-70 protein expression by flow cytometry

Following identification of the CD19+CD5+ population, the protein expression of ZAP-70 was measured using a PE-conjugated anti-ZAP-70 antibody (Dako). The percentage of ZAP-70-positive cells was determined by comparison with an isotype control (cut-off, 20%). All samples were evaluated using a FACSCalibur Flow Cytometer (BD Biosciences), and data analysis was performed with the Cell Quest software (BD Biosciences).

Cytogenetic analysis

All samples were submitted to karyotype determination by standard G banding. Accordingly, PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 20% fetal calf serum in the presence of IL-2 and CpG-oligonucleotide DSP30 (TIBMolBiol) for 72 h, following which metaphase was induced by colcemid. At least 20 metaphases were analyzed per patient. Standard procedures were followed for chromosome preparation, and cytogenetic analysis was performed according to the International System for Human Cytogenetic Nomenclature (ISCN).

Real-time PCR (RT-PCR)

All collected samples were submitted to RNA extraction using TRIzol reagent (Invitrogen), according to the manufacturer's instructions. cDNA was generated from 1 µg total RNA with reverse transcriptase and random primers using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). RT-PCR reactions were performed using TaqMan® probes (Applied Biosystems) for *KDM4A* (Hs00206360_m1), *KDM4B* (Hs00392119_m1), and *KDM4C* (Hs00909577_m1). All reactions were carried out in duplicate and relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with the mean of the control samples used as a calibrator. As an endogenous control, a *GAPDH* (Hs99999905_m1) probe was used. The assays were performed using a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

Statistical analysis

The Mann–Whitney test was used for numerical comparisons between two groups. The influence of KDM expression on cytogenetic findings was determined by Kruskal–Wallis followed by the multiple comparison test (Dunn's). All analyses were performed using the Prism 7 software (GraphPad Software, San Diego, CA, USA) with a significance level of $p < 0.05$.

Results

The clinicopathological characteristics of our CLL cohort were described previously [11]. In brief, the patients were aged between 43 and 85 years old (mean, 63 years old); 25 were women and 34 were men; and there were 38 cases of Binet A, 12 cases of B, and 9 cases of C. Regarding ZAP-70 protein expression, 19 patients were negative and 39 were positive (cut-off for positivity, 20%). Among all patients, 17 were classified in the favorable karyotype group, 18 in the intermediate, and 24 in the unfavorable. Yet, 15 patients showed a normal karyotype, while 29 patients had 1 or 2 alterations and 15 patients had a complex karyotype [3, 4, 12].

Interestingly, *KDM4A* gene expression was higher in CLL patients as compared with control samples ($p = 0.01$). In contrast, CLL samples showed a decreased level of the *KDM4B* transcript in relation to control cases ($p = 0.001$). Finally, no difference in *KDM4C* expression was detected between CLL and control cases (Fig. 1a–c). To investigate whether these histone demethylases had any association with the number of leukemic cells, we adopted the median value of *KDM4A, KDM4B*, and *KDM4C* to dichotomize CLL patients into "low" and "high" *KDM4* expression groups. For *KDM4A*, the median expression was 2.63, while for *KDM4B* and *KDM4C*, the median expression was 0.31 and 3.60, respectively. Importantly, the low expression levels of *KDM4A, KDM4B*, and *KDM4C* were associated with a higher number of white blood cells (WBC) ($p = 0.004$, $p = 0.0004$, and $p = 0.01$, respectively) (Fig. 1d–f). Considering these data, we investigated whether there was differential expression of these genes relative to ZAP-70 protein expression. Interestingly, although no association could be identified between *KDM4A* and ZAP-70, patients with positive expression of ZAP-70 had lower expression of *KDM4B* ($p = 0.01$) and *KDM4C* ($p = 0.03$) as compared with ZAP-70-negative patients (Fig. 2a, b).

Notably, investigation into the impact of *KDM4B* and *KDM4C* on cytogenetic risk revealed that there was a continuous decrease in the levels of *KDM4B* in patients with

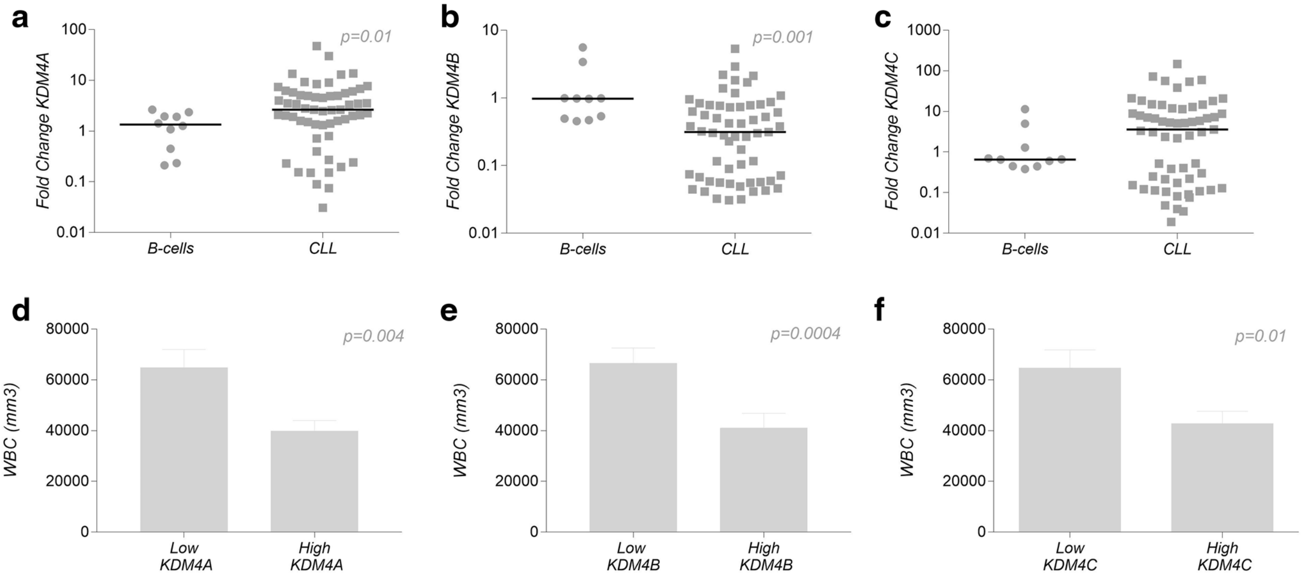

Fig. 1 *KDM4* expression in CLL patients and normal B-cells and its association with WBC count. **a** *KDM4A*, **b** *KDM4B*, and **c** *KDM4C* relative fold change values were calculated by the formula $2^{-\Delta\Delta C_t}$ using the median C_t value from the control samples as a reference. Patients with low expression of **d** *KDM4A*, **e** *KDM4B*, and **f** *KDM4C* presented with a higher number of white blood cells (WBC). The Mann–Whitney test was employed to compare differences between the groups

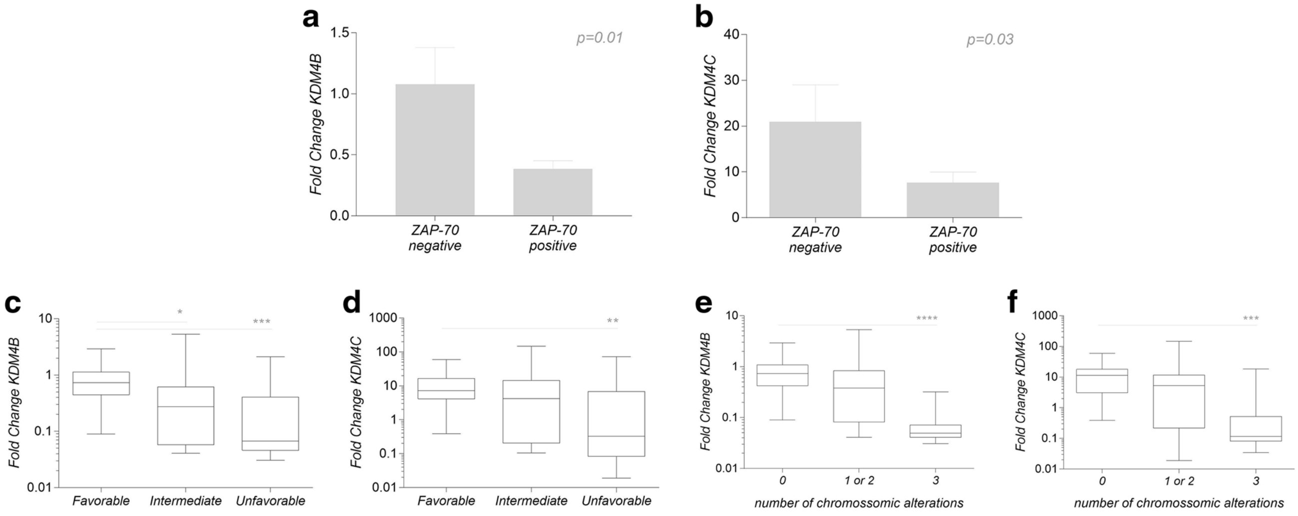

Fig. 2 Association of *KDM4* levels with ZAP-70 protein expression and with cytogenetic findings in CLL patients. CLL patients with positive expression of ZAP-70 showed lower expression of **a** *KDM4B* and **b** *KDM4C* as compared with ZAP-70-negative patients. The Mann–Whitney test was employed to compare differences between the groups. **c** *KDM4B* and **d** *KDM4C* expression in patients with favorable, intermediate, and unfavorable risk. $*p=0.02$; $**p=0.002$; $***p=0.0006$. **e** *KDM4B* and **f** *KDM4C* expression according to the number of chromosomal alterations. $***p=0.0002$; $****p<0.0001$. Kruskal–Wallis followed by the multiple comparison test (Dunn's) was used to compare differences between the groups

intermediate and unfavorable karyotypes ($p=0.001$) as compared with patients with favorable cytogenetic results. Similarly, expression of *KDM4C* was significantly reduced in patients with an unfavorable karyotype ($p=0.005$). No association between *KDM4A* and cytogenetic findings was noticed. We subsequently investigated the impact of the differential expression of these histone demethylases on the number of chromosomal alterations. Strikingly, decreased levels of *KDM4B* and *KDM4C* were associated with the acquisition of a complex karyotype in CLL ($p<0.0001$ and $p<0.0003$, respectively) (Fig. 2c–f).

Discussion

In the present study, we characterized the transcriptional levels of *KDM4A, KDM4B*, and *KDM4C* in CLL and demonstrated that the expression of these histone demethylases was associated with classical prognostic markers, such as the accumulation of leukemic cells, ZAP-70 protein expression, and adverse cytogenetic findings. Interestingly, CLL samples showed high expression of *KDM4A* as compared with healthy donors, and cases with low expression of this gene presented a high WBC count. *KDM4A* has previously been shown to be overexpressed in bladder and lung cancer, and in these tumors, *KDM4A* inhibition induces significant suppression of tumor progression [13]. Importantly, it has been demonstrated that dysregulated expression of *KDM4A* promotes localized copy gain of 1q12, 1q21, and Xq13.1 without indices global chromosome instability [14]. However, we did not detect any association between the expression of *KDMA4A* and the acquisition of cytogenetic abnormalities in the present study.

KDM4A and *KDM4B* are overexpressed in several cancer types including breast, lung, prostate, and melanoma [15]. Interestingly, our CLL samples showed low transcript levels of *KDM4B* as compared with control B-cells, but no difference was detected in *KDM4C* expression. Notably, our data reveal that CLL patients with positive expression of the prognostic marker, ZAP-70, have low expression of *KDM4B* and *KDM4C*. In accordance, beyond showing an increased number of leukemic cells, these patients also presented with adverse cytogenetic findings and the acquisition of a complex karyotype, which are useful markers for the identification of patients with aggressive CLL and a short overall survival time [3, 4, 16]. Importantly, beyond *KDM4A*, the overexpression of *KDM4B* and *KDM4C* also promotes chromosomal instability; once aberrant expression of these histone demethylases impairs the integrity of DNA mismatch repair, microsatellite instability (MSI) is promoted in addition to an increase in the frequency of spontaneous mutations [17]. More specifically, the association between *KDM4* members and genomic instability has been reinforced by the demonstration that *KDM4C* controls the expression of MDM2, which can destabilize the p53 protein and induce genomic instability [18]. It has been speculated that, due to the dysregulated expression of *KDM* members in the same cancer and their similar physiological functions, KDM4 proteins may perform overlapping functions [7]. Our data are in agreement with this speculation, considering the different associations of both *KDM4B* and *KDM4C* with indicators of poor prognosis in CLL.

Conclusion

To the best of our knowledge, this study is the first to compressively investigate the gene expression pattern of *KDM4* members in CLL and to demonstrate their impact on the prognosis of this leukemia, mainly with the acquisition of adverse cytogenetic findings. These findings are useful for a better understanding of the impact of epigenetics on CLL progression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval and informed consent The study was approved by the Ethical Committee of the Medical School Hospital of Ribeirão Preto, University of São Paulo, Brazil, and samples were collected after informed consent was obtained from patients and health donors.

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