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EXPRESSION OF DEUBIQUITINASE GENES AND INFLAMMATORY RESPONSE IN MYELOID LEUKEMIA

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SUMMARY

Myeloid leukemia (ML) is a cancer of the blood that begins when cells of the myeloid lineage uncontrollably change and grow. Acute myeloid leukemia (AML) is a disorder of rapid, uncontrolled growth of immature myeloid cells in the blood and bone marrow. Chronic myeloid leukemia (CML) is characterized by the aberrant proliferation of myeloid cells and driven by the translocation of regions of the BCR and ABL genes to form the Philadelphia (Ph) chromosome. The deubiquitinase enzymes (DUBs) including A20, OTUB1, OTUB2, and Cezanne play important roles in inhibiting NF-κB activation in response to various stimuli. Cytokines including tumor necrosis factor-alpha (TNF-α), IL-6, and IL-1β are released from immune cell activation triggered by antigenic stimulation. To this end, blood samples of 20 AML and 62 CML patients and the control group consisting of 37 healthy individuals were used to examine the mRNA expression of *A20*, *OTUB1*, *OTUB2* and *Cezanne* genes by using quantitative RT-PCR and determine IL-6, TNF-α and IL-1β concentrations by using ELISA. As a result, the mRNA level of *OTUB1* was significantly decreased in both AML and CML patients compared to that in healthy individuals, however, no difference in the transcriptional expression of *OTUB2* among AML and CML patients and control group was detected. Unlike the levels of OTUB1 and OTUB2, the expressions of *A20* and *Cezanne* in CML, but not in AML patients were significantly lower than healthy individuals. For serum cytokine analysis of the study groups, in AML and CML samples, IL-6 and TNF-α concentrations significantly increased in comparison with the control group, however, IL-1β level was similar among CML, AML patients and healthy individuals. In conclusion, this study revealed the different DUB involvement in the pathogenesis of ML, suggesting further investigations on gene polymorphisms and their functions linked to biological properties of leukemia cells.

Keywords: A20, AML, Cytokine, CML, Otubain.

INTRODUCTION

Myeloid leukemia is a cancer of the blood that begins when cells of the myeloid lineage change and grow uncontrollably and can spread quickly in the acute stage of the disease. ML starts in the bone marrow at the soft inner part of certain bones, in which large numbers of abnormal white blood cells are produced. There are two main types of ML: Acute (AML) and chronic myeloid leukemia (CML). AML is a disorder of rapid, uncontrolled growth of

immature myeloid cells in circulatory system. Most AML patients have presented with a combination of leukocytosis and signs of bone marrow failure such as anemia and thrombocytopenia (De Kouchkovsky *et al.*, 2016). CML is characterized by the aberrant proliferation of myeloid cells and driven by the translocation of regions of the BCR and ABL genes, leading to the fusion gene BCR-ABL, which forms the Philadelphia (Ph) chromosome.

Investigations on activations of the deubiquitinases (DUBs) including A20, otubain (OTUB) and Cezanne in ML patients are not fully understood yet, although their inhibitory effects on inflammatory response, cell proliferation and apoptosis are extensively documented. The DUBs are mostly considered as negative regulators of the NF-κB and STAT signalling pathways in response to different stimuli. Inhibition of the signaling pathways may significantly increase immunogenicity and anti-leukemic activity in AML (Habbel *et al.*, 2020). Recently, A20 is inactivated in multiple leukemia and lymphomas and role of A20 is indicated as an inducer of apoptotic cell death by inhibiting cell proliferation and activation of leukemia and lymphoma cells (Jia *et al.*, 2017). Unlike A20, the effects of OTUB1, OTUB2 and Cezanne are little known in regulating functional activation of leukemia and lymphoma cells. The deubiquitinating role of OTUB1 involves in cleaving ubiquitin chains from tumor necrosis factor receptor-associated factor (TRAF)s to inhibit virus-induced IFN-β expression and overexpression of OTUB1 facilitates solid tumor growth and metastasis in colorectal cancer (Zhou *et al.*, 2014). The inhibitory role of OTUB2 on TRAF6/NF-κB signaling is shown in pancreatic beta cells (Beck *et al.*, 2013). Cezanne participates in suppressing inflammatory response by removing polyubiquitin chains from TRAF3 and receptor-interacting protein (RIP)3-signaling intermediaries (Abe *et al.*, 2013).

Roles of inflammatory cytokines in modulating the immune response in ML patients is well documented. The secretion of cytokines including tumor necrosis factor-alpha (TNF-α), IL-6 and IL-1β are released from activated cells by antigenic stimulation. TNF-α level is elevated in AML and chronic myeloid leukemia (CLL) patients and changes in its concentration provides potential value as a prognostic marker of these patients (Bolkun *et al.*, 2015; Dürr *et al.*, 2018). IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory cytokine. Several recent studies have shown that IL-6 concentration is higher in leukemia patients including CLL and AML (Reittie *et al.*, 1996; Zhang *et al.*, 2020). IL-1β is a potent pro-inflammatory cytokine for host defense responses to infection and injury, and high level of IL-1β is associated with poor prognosis in CML (Matti *et al.*, 2014).

Little is known about effects of the DUBs on the pathogenesis of ML, in this study, mRNA expressions of these genes and inflammatory reactions in Vietnamese patients with ML were determined. The results indicated that the expression levels of *A20, OTUB1, OTUB2* and *Cezanne* were different among control individual, AML and CML groups, which suggests further studies on genetic alteration and their effects on the development of ML cells.

MATERIALS AND METHODS

Patients and control subjects

Fresh peripheral blood samples were collected from naïve adult patients who were diagnosed with AML and CML based on cytomorphology and cytochemistry according to the French-America-British (Bennett *et al.*, 1985) and the WHO (Harris *et al.*, 2000) classifications, at the 103 Hospital, Military Medical University, Hanoi, Vietnam. The patient group consisted of 62 CML and 20 AML patients. The control group comprised 37 healthy subjects. No individuals in the control population took any medication or suffered from any known acute or chronic disease. All patients and volunteers gave written consent to participate in the study. Person care and

experimental procedures were performed according to the Vietnamese law for the welfare of humans and were approved by the Ethical Committee of the Institute of Genome Research, Vietnam Academy of Science and Technology.

RNA extraction and real-time PCR

Total mRNA was isolated using the Qiashredder and RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. For cDNA first-strand synthesis, 1 µg of total RNA in 12.5 mL DEPC-H2O was mixed with 1 mL of oligo-dT primer (500 mg/mL, Invitrogen) and heated for 2 min at 70ºC. To determine transcript levels of *A20*, *OTUB1*, *OTUB2*, and *Cezanne*, the quantitative real-time PCR with the LightCycler® 96 (Roche Diagnostics) was applied. The following primers were used: *A20* primers: 5'-TCCTCAGGCTTTGTATTTGA-3' (forward) and 5'-TGTGTATCGGTGCA-TGGTTTT-3' (reverse); *OTUB1* primers: 5'-ACAGAAGATCAAGGACCTCCA-3' (forward) and 5'-CAACTCCTTGCTGTCAT-CCA-3' (reverse); *OTUB2* primers: 5'-CTCACGTCGGCCTTCATCA-3' (forward) and 5'- GCCATGGGCTCTACTTCGT-3' (reverse); *Cezanne* primers: 5'-ACAATGTCCGATTGGCCAGT-3' (forward) and 5'-ACAGTGGGATCCACTTCACATTC-3' (reverse) and *GAPDH* primers: 5'-GGAGCGAGATCCCTCCAAA-3' (forward) and 5'- GGCTGTTGTCATACTTCTCAT-3' (reverse). PCR reactions were performed in a final volume of 20 µL containing 2 µL cDNA, 2.4 µL $MgCl_2$ (3 µM), 1 µL primer mix (0.5 µM of both primers), 2 µL cDNA Master SybrGreen I mix (Roche Molecular Biochemicals), and 12.6 µL DEPC-treated water. The target DNA was amplified during 40 cycles of 95ºC for 10 s, 62ºC for 10 s, and 72ºC for 16 s, each with a temperature transition rate of 20ºC/s, a secondary target temperature of 50ºC, and a step size of 0.5ºC. Melting curve analysis was performed at 95ºC, 0 s; 60ºC, 10 s; 95ºC, 0 s to determine the melting temperature of primer dimers and the specific PCR products. The ratio between the respective gene and corresponding GAPDH was calculated per sample according to the ΔΔ cycle threshold method of qPCR data analysis (Livak *et al.*, 2001).

Determination of cytokines

Sera were isolated from the blood samples of AML and CML patients and healthy subjects and stored at −20ºC until used for ELISA. TNF-α, IL-6, and IL-1β concentrations were determined by using ELISA kits (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol.

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. Correlation between cytokine concentrations in ML patients was tested for significant relevance using a non-parametric test, Spearman's rank correlation test. Differences were checked for significance using Student's unpaired two-tailed t-test or ANOVA. All of the statistical tests were two-sided. $p < 0.05$ was considered statistically significant.

RESULTS

Analysis of gene expression profile in AML and CML cells

Comparative analysis of DUB genes regulated in AML and CML, we indicated that the mRNA level of *OTUB1* was significantly decreased in AML cells compared to that of the healthy group ($p = 0.032$), however, no difference in the transcript expression of other DUB genes including *A20, Cezanne,* and *OTUB2* between AML patient and control groups was detected (Figure 1). The mean mRNA expression of *OTUB1* in AML cells was about 1.9-fold lower than in control cells (Fig. 1C). Differently, the results in Figure 1A-C revealed that the mRNA expressions of *A20, Cezanne* and *OTUB1,* but not *OTUB2* were significantly down-regulated in CML patients in comparison to healthy individuals ($p < 0.001$, $p < 0.01$ and $p < 0.001$, respectively). The results revealed the difference in the expression level of the DUB genes between AML and CML patients.

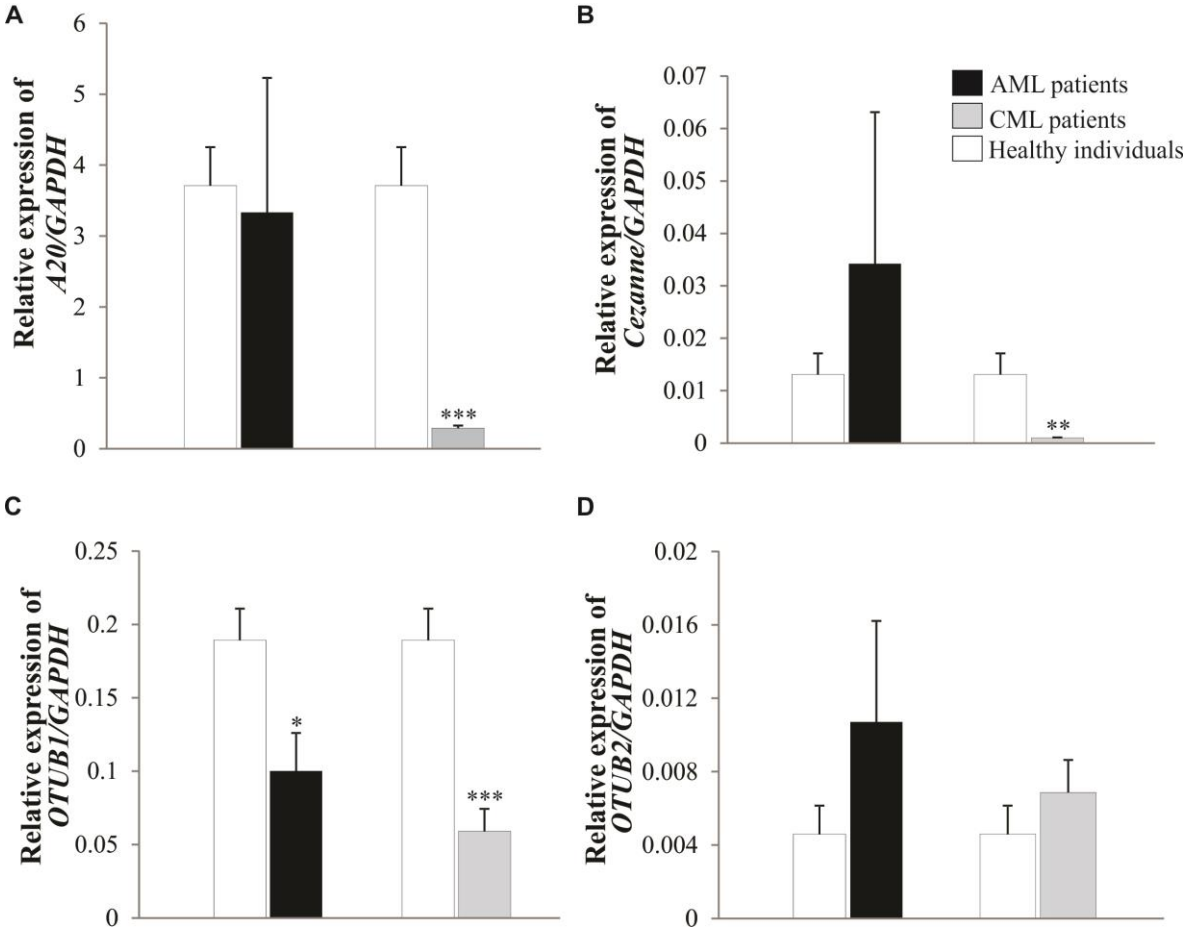

Figure 1. Gene expression profile in AML and CML patients. **A-D.** Transcript levels of *A20*, *Cezanne*, *OTUB1,* and *OTUB2* are shown in healthy donors (white bars), patients with AML (black bar) and CML (grey bar). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) indicate significant differences from healthy donors (ANOVA).

Serum profile in AML and CML patients

For cytokine analysis in serum, the levels of IL-6, TNF-α and IL-1β were measured by ELISA. The results showed that IL-6, TNF-α and IL-1β concentrations in AML patients and healthy individuals were 206.8 pg/mL, 0.1 pg/mL; 11.52 pg/mL, 0.05 pg/mL; and 0.1 pg/mL, 0.05 pg/mL, respectively. Therefore, levels of IL-6 and TNF-α in AML group showed significant increases as compared to control group (p = 0.024 and p = 0.036), however, IL-1β concentration did not change in AML patients (Fig. 1A-C).

The results shown in Figure 1A-C also revealed that concentrations of IL-6, TNF-α and IL-1β in CML group were 47.06 pg/mL, 22.2 pg/mL and 0.26 pg/mL, respectively, while levels of cytokines including IL-6, TNF-α and IL-1β were 0.027 pg/mL, 0.057 pg/mL, and 0.1 pg/mL, respectively. Thus, similar to AML group, mean concentrations of IL-6 and TNF-α in CML patients demonstrated significantly higher in comparison with control group (p = 0.012 and p = 0.00067), however, IL-1β concentration was not different between the two groups.

Association of inflammatory cytokine responses in ML patients

For determination of the relationship among

the cytokines and the DUB gene expression in ML patients, we indicated that there was a positive correlation between concentrations of IL-6 and TNF-α in CML patients (Fig. 3). In addition, no correlation among concentrations of IL-6 and TNF-α in AML patients as well as the DUB gene expression in ML patients were found in this study (data not shown). The evidences suggested that the regulating effect of inflammatory cytokines IL-6 and TNF-α in the development of CML.

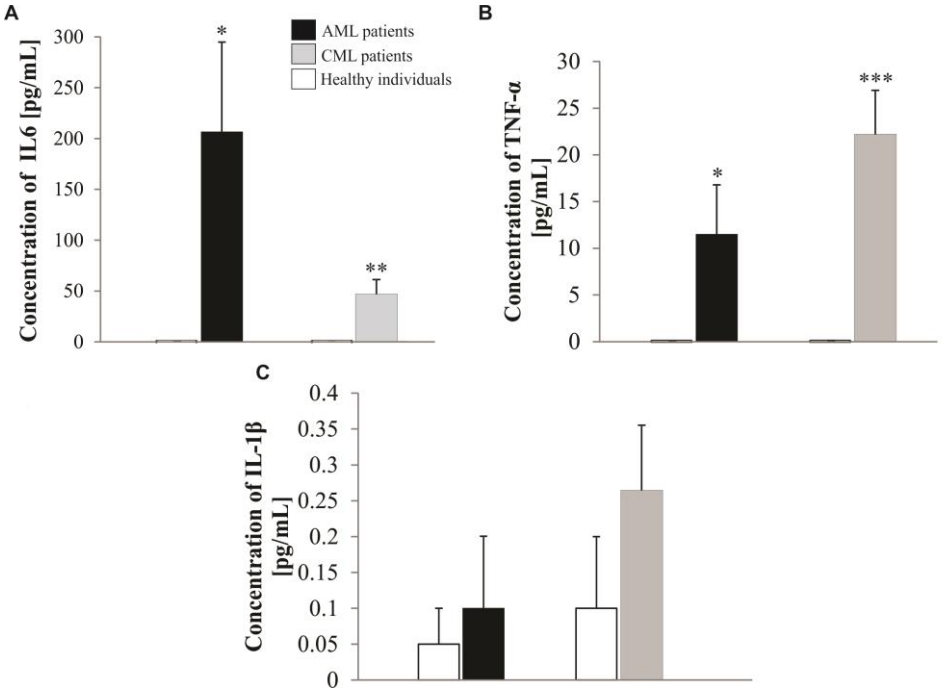

Figure 2. Serum profile of AML and CML patients. **A-C.** IL-6, TNF-α, and IL-1β concentrations are measured from serum of healthy donors (white bars), patients with AML (black bar) and CML (grey bar). *($p < 0.05$) and *** ($p < 0.001$) indicate significant differences from healthy donors (ANOVA).

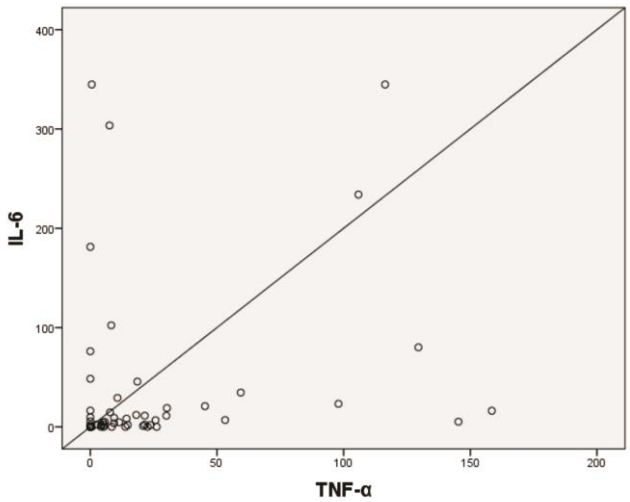

Figure 3. Positive correlation between concentrations of IL-6 and TNF-α in CML patients. P= 0.004** indicates significant relevance (Spearman's rank non-parametric correlation test).

DISCUSSIONS

ML primarily occurs in adults and is very rare in people under the age of 20. Among ML, AML differs from CML by activated expression of myeloid cells. Most myeloid cells present in AML cells are immature, while in CML cells are partly mature but not completely. In this study, we observed that the profile of DUB gene expression in these patients was distinct each other. Expression level of *A20* was significantly lower in CML patients compared to healthy individuals, while there was no difference in *A20* expression between AML patient and the control groups. In consistent, A20 is reported as a tumor suppressor, and its inactivation is closely associated with leukemia and lymphomas. A20 dysregulation has been also implicated in different autoimmune diseases and cancers (Lee *et al.*, 2000).

Next, expression level of *Cezanne* in the two patient groups was examined. The results revealed that *Cezanne* was lower expressed in CML group in comparison with the control group, while the expression of *Cezanne* gene in AML patients was similar to that in the control group. Recent investigations indicated that Cezanne deficiency leads to an increased B-cell response to antigens, and an elevated intestinal immune response against the intestinal bacterial pathogens. Differently, *Cezanne* expression was usually higher in several solid cancers including squamous cell lung cancer and adenocarcinoma (Lin *et al.*, 2019).

Unlike A20 and Cezanne, level of *OTUB1*, but not *OTUB2* expression was decreased in both AML and CML patients compared to healthy individuals, suggesting an important role of OTUB1 in regulation of immune response in ML patients. In contrast, *OTUB1* overexpression is associated with metastasis of cancer cells and a poor prognosis in colorectal cancer (Zhou *et al*., 2014). According to a study by Jing Li et al., *OTUB2* decreased its expression and is negatively associated with poor prognosis in lung cancer (Li *et al.*, 2019). Therefore, activation of *OTUB1* and *OTUB2* in leukemia is distinct from several solid cancers.

Cytokines are normally secreted by activated immune cells to trigger inflammation. The inflammatory cytokines such as IL-6, TNF-α, and IL-1β are known to be involved in controlling the growth of human bone marrow progenitor cells (von Palffy *et al.*, 2020). TNF-α concentration is enhanced in AML patients and the higher level of TNF-α is related to an adverse prognostic factor for survival in AML patients (Bolkun *et al*., 2015). Another study indicated the enhanced level of TNF-α in chronic leukemia and acute lymphoblastic leukemia (ALL), but not in AML (Aguayo *et al.*, 2000). IL-6 concentration is also found higher in CLL patients than healthy individuals (Reittie *et al.*, 1996) and the high level of IL-1β is associated with poor prognosis in CML (Matti *et al.*, 2014). The enhanced serum levels of the cytokines IL-6, TNF-α and IL-1β was exhibited in myeloid compartment of mice lacking A20 (Matmati *et al.*, 2011). In this study, we observed that the levels of cytokines IL-6, TNF-α were increased in both AML and CML patients compared to the healthy group, while IL-1β levels were similar in all study groups. Importantly, we indicated the positive correlation between levels of IL-6 and TNF-α in CML, but not AML patients, suggesting a crucial role of inflammatory cytokines in regulating the pathogenesis of CML disease.

In this study, expression levels of the DUBs in patients with AML and CML were different from each other, although levels of inflammatory cytokines IL-6, TNF-α and IL-1β in the two patient groups were relatively similar. Among the DUBs investigated, level of OTUB1 only was significantly lower in AML group, whereas CML group had inactivation of all *A20*, *OTUB1* and *Cezanne*. The evidences revealed different roles of the DUBs in their involvement in the pathogenesis of ML. Based on the expression levels of the DUBs, further investigations on gene polymorphisms and their functions linked to biological properties of leukemia cells should be performed.

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