Prolyl oligopeptidase inhibitor suppresses the upregulation of ACSDKP in patients with acute myeloid leukemia

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Received August 18, 2017; Accepted February 2, 2018

DOI: 10.3892/etm.2018.6111

Abstract. The aim of the current study was to measure the expression of acetyl-N-Ser-Asp-Lys-Pro (ACSDKP) in patients with acute myeloid leukemia (AML) and the effect of prolyl oligopeptidase inhibitor (POPi) on the bone marrow stromal cells of these patients. Serum and bone marrow stromal cell samples were collected from 33 patients with AML admitted to Wuxi Second People's Hospital, Nanjing Medical University between September 2011 and August 2016. ACSDKP levels were measured using a highly specific competitive enzyme immunoassay (EIA). Bone marrow stromal cells were treated with synthetic ACSDKP (10 μM/ml) or different concentrations of POPi S17092 (25, 50 and 100 μg/ml). Cells that received no treatment were used as control. An MTT assay was conducted to measure the proliferation of bone marrow stromal cells. The results demonstrated that serum levels of ACSDKP in patients with AML were significantly higher than those of controls ($P<0.05$). Following treatment with ACSDKP, cell proliferation was significantly increased compared with untreated cells ($P<0.05$). However, following treatment with different concentrations of POPi, the expression of ACSDKP was significantly decreased in a dose-dependent manner ($P<0.05$). Furthermore, the proliferation of bone marrow stromal cells was also decreased in a dose-dependent manner. Therefore, the present study demonstrated that ACSDKP levels were increased in the serum and bone marrow stromal cells of patients with AML and that ACSDKP promoted the proliferation of bone marrow stromal cells of these patients, which was inhibited by POPi. These results may identify a novel target for the treatment of AML.

Introduction

Acute myeloid leukemia (AML), is the most common type of leukemia in adults, is a disease that leads to impaired hematopoiesis and bone marrow failure induced by clonal expansion of undifferentiated myeloid precursors (1). Epidemiological studies demonstrate that in 2017 there were 19,950 cases of AML in the USA, (11,130 males and 8,820 females) and 10,430 individuals succumbed (2). In China, the incidence and mortality rates of all types of leukemia have markedly increased between 2011 and 2015 (3). There were ~18,000 new cases in 2011 and ~75,300 new cases in 2015, while 24,526 people succumbed to the disease in 2011 compared with 53,400 in 2015 (3). As the population of China is ageing rapidly, the incidence of AML is expected to increase by ~50% by 2030 (4).

The etiology of most cases of AML is unknown. Environmental and genetic factors are considered to be the most possible risks for AML (5,6). It is likely that many different mutations, epigenetic aberrations and downstream abnormalities are involved in development of AML (7). The results of whole-genome sequencing have indicated that AML is a complex, dynamic disease (8,9). Despite the improvement of chemotherapy for AML, refractory disease is common and relapse is the primary cause of treatment failure (10,11). The exact etiology of AML remains unknown and current treatments of AML are not as effective as they could be; therefore, it is important to determine the molecular biological mechanisms of AML and identify novel potential treatment targets for AML in order to develop novel potential treatments for AML.

The natural tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (ACSDKP) is generated from the N-terminus of thymosin-β4 via enzymatic cleavage by prolyl oligopeptidase (POP) (12). It has been demonstrated that ACSDKP is associated with endothelial cell proliferation (13), the promotion of angiogenesis (14) and the inhibition of myofibroblast differentiation (15). ACSDKP is also considered to be abnormally expressed in some human malignant tumors, including tumors of the thyroid gland (16), breast, colon, head and neck, kidney, lung, skin, ovary and prostate (17,18). It has been demonstrated that ACSDKP expression varies during chemotherapy to treat patients with AML, in certain patients the ACSDKP level increased sharply during treatment, whereas in others it did not change or decreased (19); however, few studies investigating ACSDKP have been conducted since. It has been demonstrated that ADSCKP is upregulated in human leukemia cells and may enhance the proliferation of U87-MG glioblastoma cells via the phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit α (PI3KCA)/protein kinase B (Akt) signaling

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Key words: acute myeloid leukemia, acetyl-N-Ser-Asp-Lys-Pro, prolyl oligopeptidase inhibitor

pathway (17). Furthermore, it was indicated that prolyl oligopeptidase inhibitor (POPi) may inhibit the stimulation of U87-MG glioblastoma cell proliferation by ADSCKP (20). Therefore, the present study focused on the expression of ADSCKP and its effects on AML.

In present study, the serum level of ADSCKP in patients with AML was determined and the effect of POPi on ADSCKP expression and the proliferation of bone marrow stromal cells in patients with AML was assessed. The aim of the present study may identify a novel target for the treatment of patients with AML.

Materials and methods

Patients. Serum and bone marrow stromal cell samples were collected from 33 patients with AML admitted to the Wuxi Second People's Hospital, Nanjing Medical University (Wuxi, China) between September 2011 and August 2016. All patients were diagnosed with AML according to the World Health Organization criteria and no patients received chemotherapy prior to the study (21). The mean age of patients was 57.33±9.32 (range, 18-77) years, and patients consisted of 23 males and 10 females. Patients were classified according to the French, American and British (FAB) classification system for AML (22). Sera samples were obtained from 25 healthy individuals who underwent a physical examination at the Wuxi Second People's Hospital over the same time period. The control patients had a mean age of 53.48±12.50 (range, 18-75) years and consisted of 17 males and 8 females. A total of 5 ml blood was obtained from the ulnar vein of each participant. To obtain the sera the blood samples were centrifuged for 10 min at room temperature at 2,795 x g and stored at -20˚C. Written informed consent was obtained from all participants and the present study was approved by the Research Committee of Wuxi Second People's Hospital (Wuxi, China). Baseline clinical data from the patients and healthy volunteers is presented in Table I.

ACSDKP measurement. ACSDKP concentration was measured using a highly specific ACSDKP ELISA kit (cat. no. 69-99161; MSKBio, Ltd.; Merck KGaA, Darmstadt, Germany) with acetylcholinoesterase conjugate as a tracer (Bertin Pharma, Montigney le Brettoneux, France), as described previously (19,20). ACSDKF levels were measured using ELISA on the serum fraction following extraction by methanol, evaporation and reconstitution in saline buffer. The extracts were then added to 0.5 ml buffer and used to determine ACSDKP concentration, according to the manufacturer's protocol.

Bone marrow stromal cell culture and treatment. Bone marrow stromal cell samples were collected from all patients with AML. Briefly, 3-5 ml marrow fluid was extracted using a sterile injector with heparin sodium (20 IU/M1) anticoagulant (Sigma Aldrich; Merck KGaA). Bone marrow fluid was diluted with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and centrifuged for 10 min at room temperature at 2,795 x g and the cell supernatant was extracted. Subsequently, the cell fluid was cultured with RPMI 1620 medium in a humidified incubator with 5% CO_2 at 37˚C. Following 72 h culture, all supernatant and suspension cells were discarded and the complete culture medium of the aforementioned system was added. When the adherent cells were spread across >90% of the bottom of the culture flask, cells were collected and stored at -20˚C prior to further investigation.

The cells were subsequently treated with 10 μM/ml synthetic ACSDKP (Genepep, Saint-Jean de Védas, France) or different concentrations of POPi S17092 (25, 50 and 100 μg/ml; Institut de Recherche Servier, Croissy, France) and cultured for 72 h. Untreated cells were used as control.

MTT assay. An MTT assay was then conducted to measure the proliferation of bone marrow stromal cells, following a previously described protocol in which the formazan crystals were dissolved in 0.2 ml dimethyl sulfoxide for 30 min at 37˚C (23). The optical density (OD) of each well was measured at 590 nm using a BioTek microplate reader (BioTek Instruments, Winooski, VT, USA). Values are expressed as the percentage of the OD of the control cells.

Statistical analysis. All data are expressed as the mean ± standard deviation, apart from the particular ADSCKP values of each patient. All analyses were conducted using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). A χ^2 test was performed to analyze characteristics, such as the gender ratio. A Student's t-test was used to compare differences between continuous variables, including the ages of patients and controls. A Kruskal-Wallis test followed by Turkey's post hoc test was used to compare levels of ADSCKP in patients with different FAB stages of AML. The Mann-Whitney U test was used in the *in vitro* study to compare differences between the groups. $P<0.05$ was considered to indicate a significant difference.

Results

Serum levels of ACSDKP are upregulated in patients with AML. The baseline clinical characteristics of all participants are presented in Table I. Serum levels of ACSDKP in patients with AML and healthy controls were measured and compared. The results demonstrated that serum levels of ACSDKP in patients with AML were significantly higher than those of control group ($P<0.05$; Fig. 1A). This indicates that serum ACSDKP levels are upregulated in patients with AML. Subsequently, serum levels of ACSDKP were analyzed in patients with different FAB stages of AML; however, no significant differences were observed among these groups (Fig. 1B).

ACSDKP promotes the proliferation of bone marrow stromal cells of patients with AML. To determine the effect of ACSDKP on bone marrow stromal cell proliferation in patients with AML, 10 μM/ml ACSDKP was used to treat cells and proliferation was measured using MTT. The results demonstrated that following treatment with ACSDKP, proliferation was significantly promoted compared with untreated cells ($P<0.05$; Fig. 2), indicating that ACSDKP stimulates the progression of AML.

POPi reverses the effects of ACSDKP on bone marrow stromal cells taken from patients with AML. Finally the

Table I. Basic clinical data of all participants.

Characteristic	Patients (n=33)	Healthy controls (n=25)	P-value
Mean age ± SD, years	57.30±15.1	55.36±12.57	0.198
Age range, years	(18-77)	(18-73)	
Sex (male:female)	23:10	18:7	0.720
FAB group			
0	1	N/A	N/A
1	8	N/A	N/A
2	6	N/A	N/A
3	4	N/A	N/A
4	4	N/A	N/A
5	3	N/A	N/A
6	2	N/A	N/A
7	1	N/A	N/A
Unc	4	N/A	N/A

Unc, unclassified; N/A, not applicable; SD, standard deviation; FAB, French, American and British classification system.

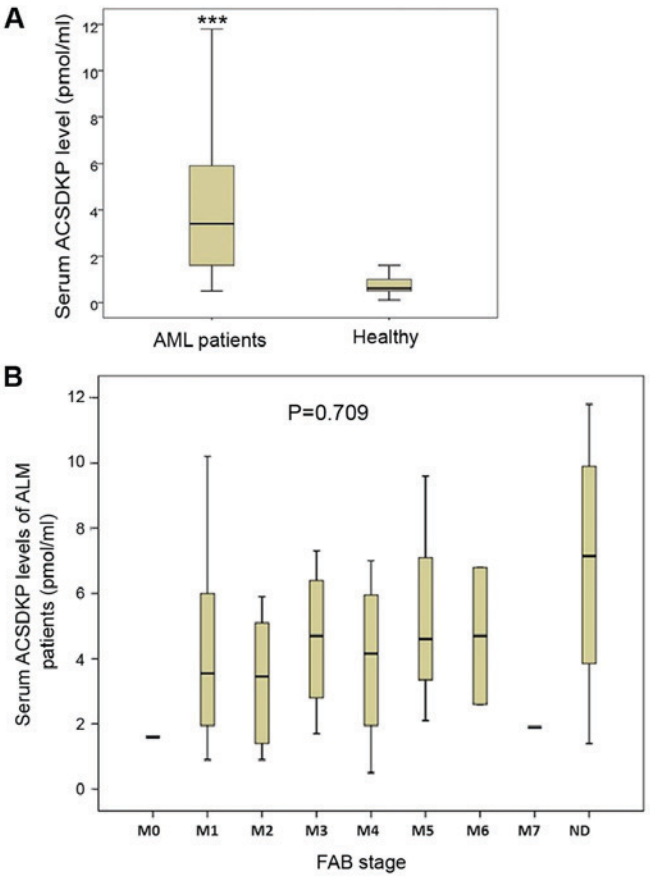

Figure 1. (A) Serum ACSDKP levels of patients with ALM and healthy controls. (B) Serum ACSDKP levels of patients with ALM with different FAB stages. ***P<0.05 vs. Healthy controls. ALM, acute myeloid leukemia; ACSDKP, acetyl-N-Ser-Asp-Lys-Pro; FAB, French, American and British classification system.

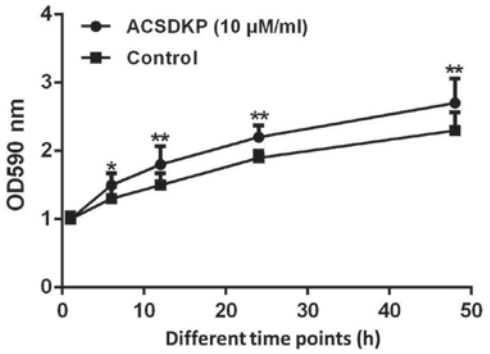

Figure 2. The proliferation of bone marrow stromal cells taken from patients with acute myeloid leukemia following treatment with ACSDKP. *P<0.05 and **P<0.01 vs. control. OD, optical density; ACSDKP, acetyl-N-Ser-Asp-Lys-Pro.

effects of POPi on ACSDKP expression and the proliferation of bone marrow stromal cells of patients with AML were determined. The results indicated that cell proliferation was significantly inhibited following POPi treatment (P<0.05; Fig. 3A). Furthermore, treatment of bone marrow stromal cells with different concentrations of POPi (25, 50 and 100 μg/ml) decreased the expression of ACSDKP in a dose-dependent manner (P<0.05; Fig. 3B).

Discussion

High-risk AML is characterized primarily by cytogenetic features of the blast population and less often by immunophenotypic abnormalities (24). Patients with AML usually respond to induction therapy but may also experience secondary disease manifestation following myelodysplastic syndrome or cytotoxic treatment for another malignant disease (25). Numerous studies have focused on the pathogenesis of AML; however, the mechanisms by which AML develops remain unknown.

The function of ACSDKP in angiogenesis and its association with leukemia has been characterized (19,26). However the precise role that ACSDKP serves in leukemia has not yet been elucidated. It was identified that ACSDKP expression is upregulated in human leukemia (17) and it was demonstrated

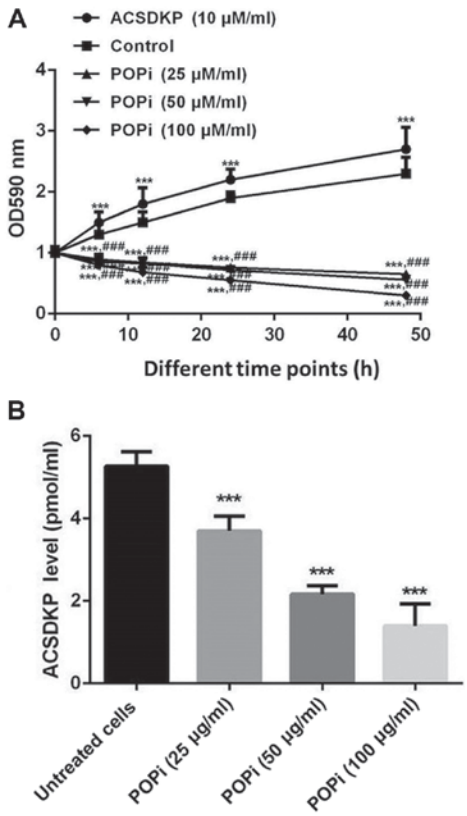

Figure 3. (A) The proliferation of bone marrow stromal cells taken from patients with acute myeloid leukemia following treatment with ACSDKP or different concentrations of POPi. (B) ACSDKP levels of bone marrow stromal cells of patients with AML following treatment with ACSDKP or different concentrations of POPi. ***P<0.001 vs. the control. $^{\#\#\#}$P<0.001 vs. the ACSDKP group. OD, optical density; ACSDKP, acetyl-N-Ser-Asp-Lys-Pro; POPi, prolyl oligopeptidase inhibitor.

that ACSDKP could enhance the proliferation of U87-MG glioblastoma cells via the PI3KCA/Akt signaling pathway (20). Therefore, the current study aimed to determine the role of ACSDKP in AML.

In the present study, the serum level of ACSDKP in patients with AML and healthy controls were assessed and compared and it was demonstrated that ACSDKP expression was significantly increased in patients with AML. Subsequently, the proliferation of bone marrow stromal cells of patients with AML was determined and the results indicated that ACSDKP promoted bone marrow stromal cell proliferation. Finally it was determined that POPi inhibits ACSDKP expression and the proliferation of bone marrow stromal cells from patients with AML in a dose-dependent manner.

Hu *et al* (20) identified that ACSDKP expression was significantly upregulated in U87-MG glioblastoma cells; furthermore, the expression of ACSDKP in K562 human leukemia cells was not particularly high. Other studies obtained different results; for example, Liozon *et al* (19) indicated that serum levels of ACSDKP varied between 0.6 and 13.0 pmol/ml in patients with AML. Liu *et al* (17) demonstrated that serum levels of ACSDKP were significantly increased in a mouse model of leukemia and in patients with leukemia. In the present study, it was demonstrated that ACSDKP levels were enhanced in the serum and bone marrow stromal cells of patients with AML.

There were certain limitations of the current study. The molecular mechanisms underlying the ACSDKP-mediated increase in bone marrow stromal cell proliferation remain unclear. Furthermore, the mechanism by which POPi suppresses the ADSCKP-induced increase in bone marrow stromal cell proliferation remains unknown. Further studies are required to elucidate these mechanisms of action.

In conclusion, the current study measured the serum levels of ADSCKP in patients with AML and determined the effect of POPi on ADSCKP expression and the proliferation of bone marrow stromal cells of patients with AML. The results indicated that ACSDKP levels were enhanced in the serum and bone marrow stromal cells of patients with AML and indicated that ACSDKP may promote the proliferation of bone marrow stromal cells. Treatment with POPi reversed this increase in bone marrow stromal cell proliferation. Therefore, the current study indicated that POPi treatment may present a novel therapeutic strategy for the treatment of AML.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HC wrote the manuscript and conducted the experiments. XZ and SL collected and analyzed the data. ZW designed the study and approved the final version of the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants and the present study was approved by the Research Committee of Wuxi Second People's Hospital (Wuxi, China).

Consent for publication

Written informed consent was obtained from all patients for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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