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Serum Chymotrypsin-Like Activity on Digestion of Ala-Ala-AMC as a Substrate, Among CML and AML Patients at TASH, Addis Ababa Ethiopia: A Comparative Cross-Sectional Study

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Abstract: Proteosome, composed of 19S and 20S subunits, is vital for AML and CML cell cycling, proliferation, adhesion and as a means to by-pass apoptosis through three distinguishable proteolytic activities. Chymotrypsin-like (CT-L) activity is rate limiting and a well-established therapeutic strategy for AML and CML cells, by proteosome inhibitors than the other two proteolytic activities: trypsin-like activities and caspase-like activities. As the main objective of the study, the serum levels of Chymotrypsin-like activity was assessed among chronic and acute myeloid leukemia patients, and compared among each other and with those of healthy controls. A hospital-based comparative cross-sectional study was conducted among CML and AML patients from February 2016 up to December 2016. Serum samples were obtained from 24 AML, 60 CML and 35 presumed healthy controls. Fluorogenic assays for serum chymotrypsin-like activity using aminomethylcoumarin (AMC) peptide derivatives were carried out. Statistical analysis was done by using SPSS version 20. Descriptive statistics, Paired Samples T-test, Wilcoxon Signed Rank test and Spearman's rho test were used to investigate any correlation among different parameters. The minimum level of statistical significance was set at p-value <0.05. The mean and median serum levels of Chymotrypsin-like activity were significantly higher in patients with CML and AML than in the healthy controls (P-value < 0.05). CML patients in chronic phase (CP) and secondary AML patients had significantly higher mean and median serum levels of Chymotrypsin-like activity than CML patients in accelerated/blast phase (AP/BP) and *de novo* AML patients (p-value < 0.05). As a conclusion, the serum Chymotrypsin-like activity level might be a useful diagnostic test, and may be used as prognostic test particularly in a subset of CML patientsin chronic phase (CP) and Secondary AML patients. However, further studies that incorporate other protocols such as chymotrypsin-like activity enzyme-immunoassay with large scaled study population are warranted to decide on prognostic and diagnostic role of the enzyme more accurately.

Keywords: CML, AML, Serum Chymotrypsin-Like Activity

1. Introduction

Leukemia cells, either AML or CML cells, bypasses growth arrest, terminal differentiation or apoptosis in response to appropriate environmental stimuli. Acute myeloid leukemia (AML) is heterogeneous hematopoietic cancer characterized by the clonal expansion and accumulation of immature myeloid precursors (blast cells) in the marrow and blood [1, 3]. Chronic myeloid leukemia (CML) pathogenesis originates from a reciprocal

translocation that occurs between the long arms of chromosomes 9 and 22 generating the structure called a *Philadelphiachromosome* (Ph). Such translocation transforms the entire cell to have BCR/ABL (breakpoint-cluster region/Abelson leukemia gene) oncogene which inhibits apoptosis and induces cell growth and proliferation, and transforming the cell into malignancy known as CML [7].

Proteasome, an intracellular organelle providing a targeted mechanism for protein degradation, composed of 19S and 20 S subunits in eukaryotes including human beings, and is vital for cancer cell (such as AML and CML cells), cycling, adhesion, proliferation, and apoptosis via chymotrypsin-like (CT-L) catalytic specificities [1]. Other two distinguishable proteolytic activities localized to the 20S proteasome subunits are trypsin-like activity and caspase-like activity [6].

Proteasome-mediated protein degradation via chymotrypsin-like (CT-L) activity is a key regulator of protein homeostasis or is rate limiting and a well-established therapeutic strategy for cancer cell (such as AML and CML cells), by proteosome inhibitors [2, 4, 6]. Currently, literatures on natural compounds indicate that flavonoids and isoflavones down regulate the expression of anti-apoptotic proteins and inhibit proteasome mediated protein degradation via chymotrypsin-like (CT-L) activity and bring their anti-tumeric activity [3, 5].

Recent findings demonstrated the novel proteasome inhibitors (NPI) that could have a potential to inhibit all three proteolytic activities (chymotrypsin-like activity, trypsin-like activity and caspase - like) of proteosome more effectively and efficiently [6]. Under NPI inhibition of proteosome system, the variation exists among the degree of inhibition between three enzyme activities. Thus, NPI inhibits chymotrypsin-like activity, and caspase - like activity more efficiently than that of trypsin-like activity [6, 7].

Indeed, inhibition of proteasome mediated protein degradation via chymotrypsin-like (CT-L) activity has the direct consequence, to induce cell cycle arrest and apoptosis, and, more significantly in CML and AML cells at the level of normal cells [4, 5]. One study conducted on Morrocan hematological malignant patients indicated that serum and intracellular chymotrypsin-like activity in patients with hematological malignancies is significantly higher than that of healthy control groups [4].

Moreover in Ethiopia no study has been performed on the proteasome, even more on the relationship between the proteasome and the onset or progression of a disease given in the Ethiopian population. In this work, we proposed to study the variations of serum chymotrypsin-like activity in Ethiopian patients with CML and AML as well as in between AML and CML patients and healthy controls. The study and analysis of the proteolyticpotential of chymotrypsin-like activity was conducted in parallel with the measurement of concentration of chymotrypsin-like activity in the serum of Both patients and healthy controls.

The objective of our work can be summarized in these points:

1) To determine whether there were differences in serum chymotrypsin-like activity levels between samples from patients with CML and AML.
2) To determine whether there were differences in serum chymotrypsin-like activity levels between normal samples from healthy controls and samples from patients with CML and AML.
3) To determine whether there were differences in serum chymotrypsin-like activity levels between samples from patients with CML and AML at different stages and classes.

2. Materials and Methods

2.1. Study Area

The study was conducted in the Department of Biochemistry, College of Health Sciences, Addis Ababa University in collaboration with Hematology-Oncology clinic, Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia.

Tikur Anbessa Specialized Hospital, located in the nation's capital Addis Ababa, is Ethiopia's largest referral hospital in the country. The hospital was given to Addis Ababa University by the Ministry of Health as a main teaching hospital in school of medicine. TASH offers diagnosis and treatment for approximately 370,000 to 400,000 patients a year. The hospital has 600 beds (18 beds dedicated for cancer care), with 16 specialists, 50 non-teaching physicians.

2.2. Study Design and Period

A hospital based comparative cross-sectional study was conducted from February up to December, 2016 to achieve the objective of the study.

2.3. Population

2.3.1. Source Population

All adult CML and AML patients visiting Hematology-Oncology Clinic at TASH, Addis Ababa, Ethiopia and all presumed healthy staff members of Kolfe Keraniyo Sub-city Lome Meda Health Center.

2.3.2. Study Population

During the study period 119 individuals between 18 and 75 years old, including 65 males and 54 females, were studied. AML and CML patients were recruited from the Hematology-Oncology Clinic at Tikur Anbessa Hospital. The study participants were organized into the following study groups: one group consisted of 24 AML patients, ranging from 19 to 75 years old; another group consisted of 60 CML patients, ranging from 18 to 70 years old; the third group consisted of 35 healthy controls, ranging from 26 to 66 years old. Presumed healthy controls were randomly selected individuals from the staff of Kolfe Keranyo sub-city Woreda-13 Lomi Meda Health Center who were presumed healthy. They had no known chronic diseases and were not taking any chronic medications.

2.4. Sampling Procedure

CML and AML patients who were able to comply with this study and volunteered to be involved in the study were recruited during the specified study period. Due to the restrictions on time and cost as well as availability of patients, only 84 morphologically confirmed leukemia (60 CML and 24 AML) patients and 35 healthy control individuals were recruited.

2.5. Study Variables

2.5.1. Dependent Variables

a) Serum levels of chymotrypsin-like activity

2.5.2. Independent Variables

a) Age
b) Sex
c) Classes of AML: *de novo* (primary) *vs* secondary
d) Stages of CML: chronic *vs* accelerated/blast stage

2.6. Exclusion and Inclusion Criteria

AML/CML patients with a previous history of allergic airway diseases (e.g. asthma), rheumatoid arthritis, mastocytosis, hyper-eosinophilia and anaphylactoid reactions (e.g. skin rashes, blushing and urticaria) were excluded from the study (as the serum chymotrypsin-like activity levels are elevated abnormally under these conditions).

2.7. Ethical Considerations

After being approved by the Research and Ethics Committee of the Department of Biochemistry, School of Medicine, College of Health Sciences, Addis Ababa University, a formal letter was written to Hematology-Oncology clinic at TASH. Similarly, the objectives of the study were explained to all concerned bodies assigned in the hospital to get permission and support. Also the aim of the study was clearly stated for the participants (both patients and controls) under the study. Moreover, confidentiality was strictly maintained throughout the course of the study and the study outcome was submitted to the hospital as well.

2.8. Data Collection and Techniques

All necessary information was collected from patients' medical records (charts) using structured formats consisting of collection of data on demographic characteristics, and laboratory investigations. All the data collection formats were filled in the Hematology-Oncology center of TASH.

2.8.1. Serum Sample Preparation

About 5 mL of blood was drawn from the antecubital vein of patients and healthy controls and then left to clot at room temperature for one hour. The blood was centrifuged at 3000 rpm for 10 minutes and the serum removed and stored at minus 70 degrees centigrade until use.

2.8.2. Assays for Chymotrypsin-Like Activity

Principles: Serum Chymotrypsin-like activity cleaves a scissile peptide bond in a protein or peptide or an amide bond in a synthetic peptidyl chromogenic or fluorigenic substrate. Substrate amino acids that are on the amino (N) side of the scissile bond are numbered P1, P2, and P3, and so on (called P sites) with the one closest to the scissile bond numbered P1, and those that are on the carboxyl (C) side of the scissile bond are numbered as P1', P2', P3' and so on (called P' sites). Their corresponding binding sites on the protease (chymotrpsin-like activity enzyme) are labeled with S (S1, S2, and S3…) and S' (S1', S2' and S3'…), respectively.

In this study a fluorogenic assay protocol was used in which a fluorescent dye, 7-amino-4-methylcoumarin (AMC) that contains a reactive amine group covalently attached to the carboxyl end of a flourogenic substrate (Ala-Ala-Phe-AMC) via an amide bond. The sequence of the peptide moiety provides chymotrypsin-like activity enzyme specificity and the fluorescent AMC dye moiety functions as a fluorogenic reporter for chymotrypsin-like activity enzyme activity. Chymotrypsin-like activity enzyme cleaves the amide bond, which mimics a peptide bond, on the carboxyl side of Phenylalanine. At the excitation and emission wavelengths used, the fluorescence of the attached AMC is quenched when it is covalently attached to the peptide via an amide bond. At these wavelengths, the fluorescence increases when AMC is released by chymotrypsin-like activity enzyme cleavage of the amide bond that joins the AMC to the peptide moiety.

Chymotrypsin-like activity enzyme within the serum samples cleaves the substrate; Ala-Ala-Phe-AMC specifically on the C-terminal side of the phenylalnine residue and aminomethylcoumarin (AMC) is released as a product. AMC released was detected using an excitation wavelength of 360 nm and emission wavelength of 460nm. The fluorescence intensity of the AMC released is linearly proportional to the enzyme activity in the original serum sample.

Procedure: First, 70ul of assay buffer (composed of 1mM EDTA, 50ug/ml heparin, 150mM NaCl, tris buffer at pH 7.4 and 1.5% dimethylsulphoxide (DMSO, used as solvent for substrate solution), and 5ul of stock substrate (Ala-Ala-Phe-AMC, obtained from Sigma Aldrich, plc) were added to each well of 96-well microtiter plates [11] and mixed. Then, 25 ul of serum sample was added and the whole 100 ul sample mixtures in each well were mixed and incubated at room temperature for 10 minutes. Fluorescence was then measured using a microtiter plate fluorimeter (excitation wavelength 360 nm, emission wavelength 460 nm). The process was automated and the fluorescence converted to enzyme activities measured as pM/sec of AMC released, which is equivalent to pM/sec of substrate digested.

2.9. Statistical Analysis

All the data collected were coded and entered into Excel data sheet on computer. Double entry method was applied to preserve data quality. Analysis was done using the Statistical Package for Social Sciences (SPSS) version 20. Results were analyzed statistically using descriptive statistics, Paired

Samples T-test, Wilcoxon Signed Rank test and Spearman's Rho test. All the analyzed data were expressed as Median and Mean ± SD. The minimum level of statistical significance was set at p-value < 0.05.

3. Results

3.1. Age-Sex Distribution of the Study Participants

Table 1 shows the age/sex distribution of study participants. Sixty morphologically confirmed adult CML patients between 18 and 70 years old; 24 morphologically confirmed AML patients between 19 and 75 years old; and 35 presumed healthy individuals between 26 and 66 years old participated in this study. The mean age for CML patients was 39.1 ± 15.2 years; for AML patients was 35.7 ± 14.1 years old; and for healthy individuals was 38.5 ± 10.1. Therefore, healthy and leukemia patients were age-matched (Paired Samples T-test, % CI = 95, p-value = 0.00). However, there were more females (68.6%) than males (31.4%) in the healthy group, but more males than females in both AML and CML patients, so leukemic patients were not well sex-matched with the healthy controls (Paired Samples T-test,% CI = 95, p-value = 0.38).

Table 1. *Age-sex distribution of study participants at Hematology-Oncology Clinic of TASH, Addis Ababa, Ethiopia, 2016.*

Participants	Sex			Age (Mean ± SD) years
	Males N (%)	Females N (%)	Total N (%)	
CML patients	39 (65%)	21 (35%)	60 (100%)	39.1± 15.2
AML patients	15 (62.5%)	9 (37.5%)	24 (100%)	35.7± 14.1
Healthy controls	11 (31.4%)	24 (68.6%)	35 (100%)	38.5± 10.1
Total (%)	65 (54.6%)	54 (45.4%)	119 (100%)	
P- Value	P = 0.38 (P > 0.05)			P = 0.00 (P < 0.05)

3.2. Results of Serum Levels of Chymotrypsin-Like Activity

The mean ± SD of serum levels of chymotrypsin-like activity of study participants (assayed as Ala-Ala-Phe-AMC hydrolysis) was 54.7 ± 18.6 pM/sec for CML patients, 49 ± 20.2 pM/sec for AML patients and 58.8 ± 21.7 pM/sec for healthy controls. Paired Samples T-test was performed to see if there was significant difference in the mean serum levels chymotrypsin-like activity between AML patients, CML patients and healthy controls. It was found that the mean level of serum chymotrypsin-like activity (in pM/sec) was significantly lower in healthy controls than in CML patients and AML patients (Paired Samples T-test,% CI = 95, p-value = 0.00) (Table 1).

Similarly, Wilcoxan Signed Rank test was performed to see if there was significant difference in the median serum level of chymotrypsin-like activity between AML patients, CML patients and healthy controls. It was found that the median level of serum chymotrypsin-like activity (in pM/sec) was significantly lower in healthy controls (50pM/sec) than in AML patients (55.5 pM/sec) and CML patients (64pM/sec) (Wilcoxan Signed Rank test,% CI = 95, p-value = 0.00) (Table 1).

Table 2. *Levels of serum Chymotrypsin-like activity (assessed by Ala-Ala-Phe-AMC hydrolysis) of study participants at Hematology-Oncology Clinic of TASH, Addis Ababa, Ethiopia, 2016.*

Statistical parameters	Serum Levels of chymotrypsin-like activity (pM/sec.)			P-Value
	Healthy Controls	CML-patients	AML-patents	
	Ala-Ala-Phe-AMC	Ala-Ala-Phe-AMC	Ala-Ala-Phe-AMC	
Mean ± SD	49 ± 20.2	58.8 ±21.7	54.7 ±18.6	P<0.05
Median	50	64	55.5	P<0.05
Range	10-88	10-96	19-90	

3.3. Serum Levels of chymotrypsin-Like Activity and Classes of AML

In the present study, the serum levels of chymotrypsin-like activity of AML patients with respect to two etiological classes: *de novo* AML and secondary AML, were assessed and compared to each other. The mean ± SD and median level of serumchymotrypsin-like activity (in pM/sec) (assessed by Ala-Ala-Phe-AMC hydrolysis) was determined for each class of AML. The mean ± SD and median level of serum chymotrypsin-like activity was higher in secondary AML patients than in patients with *de novo* AML (mean ± SD = 49.9 ± 17.8, median = 54 *vs* 47.7 ± 24.9, 50) ((Paired samples T-tests and Wilcoxan Rank Test,% CI = 95, p-value = 0.04).

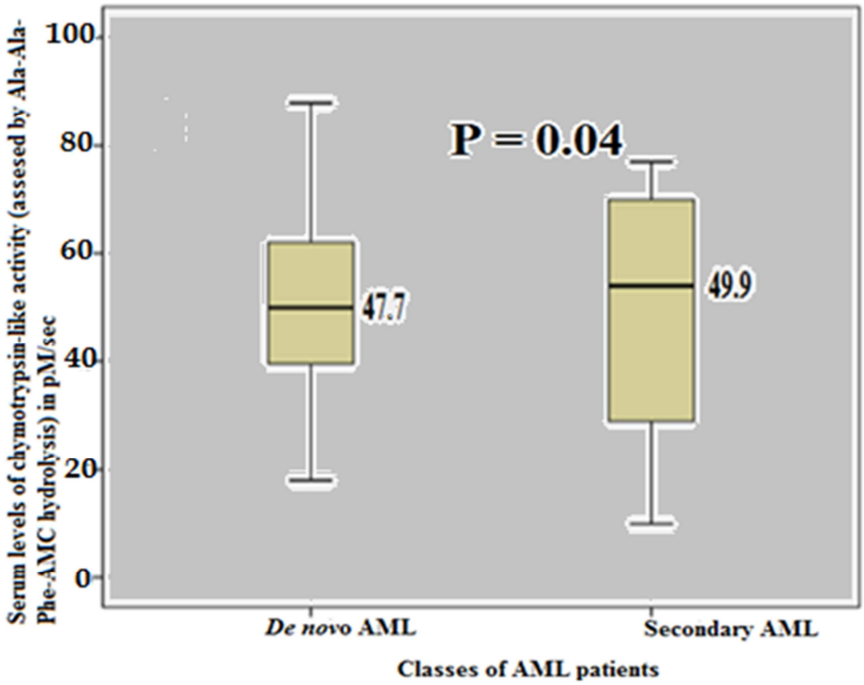

Figure 1. *Serum levels of chymotrypsin-like activity (assayed as Ala-Ala-Phe-AMC hydrolysis) of de novo and secondary AML patients at Hematology-Oncology Clinic of TASH, Addis Ababa, Ethiopia, 2016.*

3.4. Serum Levels of Chymotrypsin-Like Activity and Stage of CML

Comparison of the serum levels of chymotrypsin-like activity in different stages of CML revealed higher mean ± SD and median difference in CML patients in the chronic (CP) phase than CML patients in the accelerated/blast (AP/BP) phase. The mean ± SD and median level of serum chymotrypsin-like activity (in pM/sec) (assessed by Ala-Ala-Phe-AMC hydrolysis) was determined for each phases of CML and it was found that the mean ± SD and median level of serum chymotrypsin-like activity for CML patients in the chronic phase (CP) was higher than that of those in the accelerated /blast (AP/BP) phase (mean ± SD = 55.9 ± 19.3, median = 57 *vs* 52.3 ± 17.4, 52 (Paired samples T-tests and Wilcoxan Rank Test,% CI = 95, p-value = 0.04)).

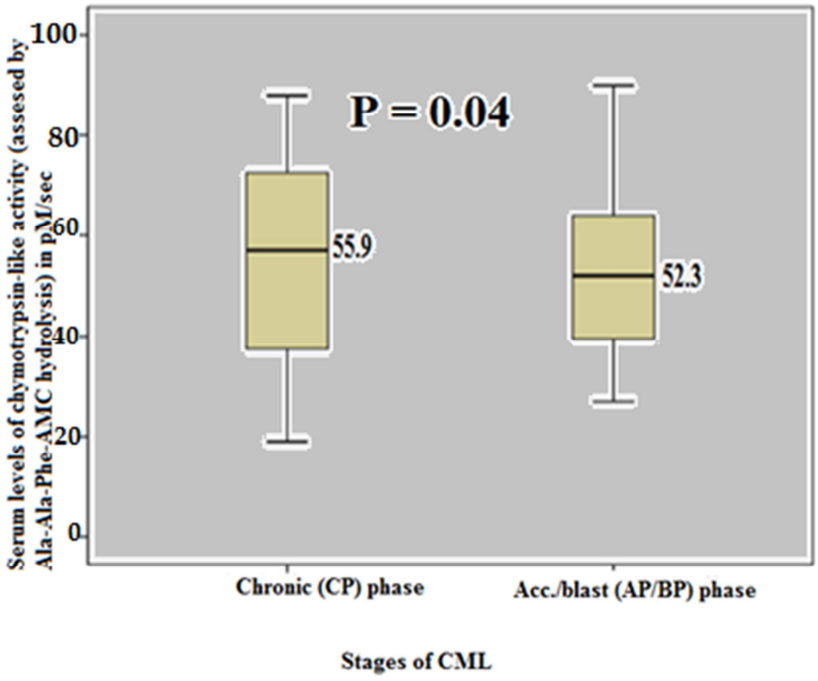

Figure 2. *Serum levels of chymotrypsin-like activity (assayed as Ala-Ala-Phe-AMC hydrolysis) of CML patients in CP and AP/BP stages at Hematology-Oncology Clinic of TASH, Addis Ababa, Ethiopia, 2016.*

4. Discussion

In the present study it was found that the median and mean ± SD values of serum levels of chymotrypsin- like activity assessed by hydrolysis of Ala-Ala-Phe-AMC were higher in patients with AML and CML when compared with values of serum chymotrypsin- like activity levels in healthy controls with statistical significance of P-value < 0.05. In agreement with this finding, other studies also showed higher serum levels of chymotrypsin- like activity among AML and CML patients [4], acute pancreatitisin rat models [8], Lymphoma and myloma [4] and breast cancer [9] patients, than in healthy controls.

In the present study, it was found that the mean ± SD and median value of serum levels of chymotrypsin- like activity assessed in terms of digestion of Ala-Ala-Phe-AMC was higher in secondary AML patients than in patients with *de novo* AML with statistical significance of P-value < 0.05. In agreement with this finding, other studies [12, 15] also showed higher serum levels of chymotrypsin- like activity in patients with secondary AML than *de novo* AML. This may be due to the reason that secondary AML patients with the serum levels of chymotrypsin- like activity simply had a genetic predisposition for having such elevated serum levels of chymotrypsin- like activity with having other pathological condition. Another possibility might be that secondary AML patients with the serum levels of chymotrypsin- like activity had an allergic condition or reaction that was asymptomatic [13] or other conditions namely, rheumatoid arthritis [14] that might raise the secondary AML patients with the serum levels of chymotrypsin- like activity.

Studies [15] showed that patients with advanced (accelerated and blast) phase CML more often possess serum levels of chymotrypsin- like activity than patients with chronic phase CML. In the present study, however, it was found that the mean ± SD and median value of serum levels of chymotrypsin- like activity assessed in terms of digestion of Ala-Ala-Phe-AMC for CML patients in CP was higher than that of those in AP/BP. The reason behind this disagreement is unclear, but it might be due to the difference in chymotrypsin- like activity method used this study.

5. Conclusions

In this study, elevated mean ± SD and median serum levels of chymotrypsin- like activity were seen in patients with AML and CML when compared with healthy individuals. Among leukemic patients included in this study, mean and median serum levels of chymotrypsin- like activity was higher in CML patients than AML patients; in patients with secondary AML than *de novo* AML and in CML patients in the chronic phase (CP) than in the accelerated/ blast phase (AP/BP).

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