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Studies on biomarkers for oxidative stress in patients with chronic myeloid leukemia

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BACKGROUND: Chronic myeloid leukemia (CML) is a myeloproliferative disorder with a unique genetic rearrangement, the Philadelphia chromosome. High reactive oxygen species (ROS) levels favor oxidative stress, which could play a vital role in normal processes and various pathophysiologies including neoplasm. Biomarkers of oxidative stress are measured as products of oxidized proteins and lipids. Plasma levels of protein carbonyl (PC), thiobarbituric acid reactive substances (TBARS) and total lipid hydroperoxide (LOOH) were used as biomarkers of oxidative stress in the past. The aim of this study was to evaluate the products of protein oxidation and lipid peroxidation in plasma as biomarkers of oxidative stress in CML patients.

PATIENTS AND METHODS: The study included 40 CML patients and 20 age- and sex-matched healthy volunteers. Of 40 CML patients, 28 were in chronic phase (CML-CP) and 12 in accelerated phase (CML-AP). Plasma levels of PC, TBARS and LOOH as biomarkers of oxidative stress were evaluated by spectrophotometric methods.

RESULTS: There were significant differences ($P<.05$) in plasma levels of PC, TBARS and LOOH in CML, CML-CP and CML-AP patients as compared to controls.

CONCLUSION: PC, TBARS and LOOH might reflect oxidative stress in CML patients and might be used as biomarkers in such patients.

Chronic myelogenous leukemia (CML) is characterized by the increased and unregulated growth of predominantly myeloid cells in the bone marrow and the accumulation of these cells in the blood. This myeloproliferative disease is associated with a characteristic chromosomal translocation called the Philadelphia chromosome.[1] In this translocation, parts of two chromosomes (9th and 22nd) switch places. As a result, part of the BCR gene from chromosome 22 is fused with the ABL gene on chromosome 9.[1] This abnormal "fusion" gene generates a protein of p210 kDa or sometimes p185 kDa weight and also known as tyrosine kinase.[1] The bcr-abl transcript is continuously active and does not require activation by other cellular messaging proteins.[1] In turn, bcr-abl activates a cascade of proteins that control the cell cycle, speeding up cell division.[1]

The annual incidence of CML is 1-2 per 100 000 people in Western countries.[1] Its incidence is not well known in India. It occurs slightly more in men than women and the etiology of CML is not well known. CML is often divided into three phases and in the absence of intervention, CML typically begins in the chronic phase, and over the course of several years progresses to an accelerated phase and ultimately to a blast crisis. Blast crisis is the terminal phase of CML and clinically behaves like an acute leukemia.

Historically, CML has been treated with busulfan, hydroxyurea, interferon and bone marrow transplantation, although targeted therapies such as tyrosine kinase inhibitor (imatinib mesylate) introduced at the beginning of the 21st century have radically changed the management of CML.[1]

The oxidative stress can be defined as an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage.[2] These ROS can be generated within the cell not only by external sources of radiation such as x-ray or atomic bombing, but also within the body as a consequence of normal metabolic processes, which include drugs, electron transport chain and chemicals including toxins and dyes collectively termed as xenobiotics.[3] ROS are responsible for DNA, lipid and

protein damage and play an important role in the development and progression of many human diseases including cancer.[4-7]

Thus, there is a need for biomarkers to quantify as the intensity of oxidative stress. Direct measurements of ROS are not reliable due to the very short half-life of ROS and none of the techniques is sensitive for quantification of ROS.[8] The indirect methods for quantification of ROS include measurement of damage products of protein carbonylation and lipid peroxidation.[8] Protein carbonylation was measured as protein carbonyl (PC) is a product of irreversible non-enzymatic oxidation of protein whereas lipid peroxidation is evaluated in term of thiobarbituric acid reactive substances (TBARS) and total lipid hydroperoxides (LOOH).[8,9] Oxidative stress is now recognized to be a prominent feature of many acute and chronic diseases, and even cancer and leukemias.[4-13] The levels of these parameters have been shown to have value as indicators or biomarkers for oxidative stress and disease progression in a number of pathophysiologies.[4-13] Carbonylation of protein often leads to a loss of protein function, which is considered a widespread marker of severe oxidative stress, damage and disease-derived protein dysfunction in various pathophysiologies.[8,14] Increased level of lipid peroxidation products were found in plasma of patients with gynecological malignancies, breast cancer, squamous cell carcinoma, colon cancer and various types of leukemia.[6,9,15-19] We therefore, prospectively studied the products of protein carbonylation and lipid peroxidation in plasma as biomarkers of oxidative stress in chronic myeloid leukemia patients.

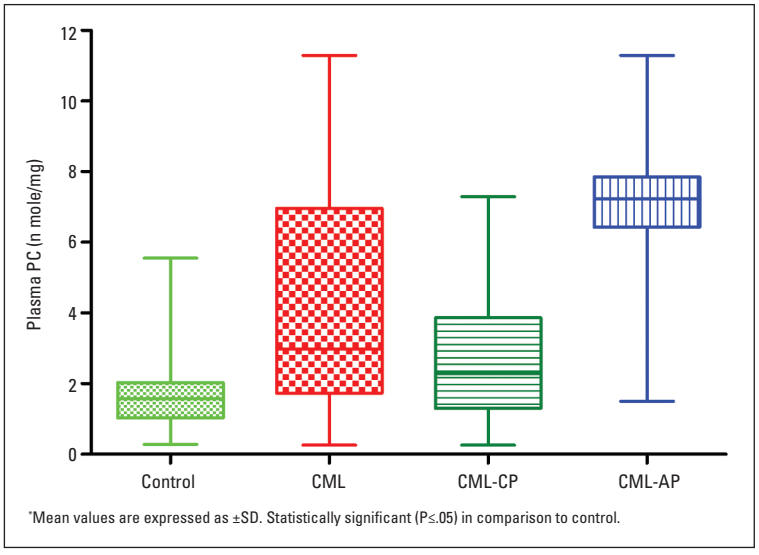

Figure 1. Plasma protein carbonyl (PC) levels in CML patients and controls. (CP: chronic phase, AP: accelerated phase)

PATIENTS AND METHODS

Forty patients were diagnosed as having chronic myeloid leukemia on the basis of standard clinico-hematological and cytogenetic criteria. Twenty age- and sex-matched healthy subjects were included in this prospective study. Of 40 CML patients, 28 were in chronic phase (CML-CP) and 12 in accelerated phase (CML-AP). The mean age of CML patients was 35.40 (11.19) years, the median age 33 years and the range 20-60 years. The mean age of healthy volunteers was 31.70 (10.53) years, the median age 34 years and the range 18-55 years. Patients were given hydroxyurea or imatinib mesylate and the dose was adjusted on each follow-up to maintain total leukocyte counts between 5000/mm^3 and 10000 mm^3. There were no nutritional or supportive antioxidant treatment given to patients and no control subjects have taken any nutritional antioxidant as well.

Informed consent was obtained from all the individuals included in the study. The present investigations were approved by the institutional ethical committees for biomedical research. At the time of blood collection, there was no evidence of tissues injuries, infection or any inflammatory manifestation in the patients or controls. Blood samples were taken from patients and control following an overnight fasting period into K_2 EDTA vials to avoid the probable influence of nutritional factors on the ROS level.

The plasma was separated by centrifugation at 3000 rpm per 15 minutes. Plasma PC content was measured by spectrophotometric detection of protein hydrazone, which is formed by the reaction between 2,4-dinitrophenyl hydrazine (DNPH) and PC.[20] The results were expressed as n moles of PC content per milligram of protein by using wavelength 370 nm with molar extinction coefficient ε_{370}=22 000 $M^{-1}cm^{-1}$. The protein content was determined by the Lowry method using bovine serum albumin as a standard.[21] The TBARS in the plasma was evaluated by slightly modified spectrophotometric method based on the reaction between MDA and thiobarbituric Acid (TBA).[20] Absorbance was measured by spectrophotometerically at a wavelength 532 nm with molar extinction coefficient $\varepsilon_{532}=1.56\times10^5$ $M^{-1}cm^{-1}$.[22] Total hydroperoxide (LOOH) level in plasma was estimated using the ferrous oxidation in xylenol orange Fox-2 assay by spectrophotometerically.[23] Plasma level of TBARS and LOOH were expressed as n mole MDA/mL and mM/mL respectively.

The findings were expressed as the mean and standard deviation. The differences were compared with the use of unpaired *t* test. A value of $P<.05$ was considered statistically significant.

RESULTS

Present study was conducted to assess the PC, TBARS and LOOH as biomarkers of oxidative stress in chronic myeloid leukemia patients. Plasma protein carbonylation or oxidation was assessed as PC, which was significantly (P<.05) higher in CML patients as compared with controls (Figure 1). The mean (SD) plasma PC levels in CML, CML-CP, CML-AP patients and controls were 3.94 (2.83), 2.72 (1.87), 6.78 (2.69) and 1.88 (1.43) n mole/mg respectively. Plasma lipid peroxidation levels were measured in term of TBARS and LOOH and were found significantly increased (P<.05) in CML patients as compared with controls (Figures 2, 3). The mean plasma levels TBARS in CML, CML-CP, CML-AP patients and controls were 3.91 (1.64), 3.47 (1.05), 4.94 (2.28) and 2.47 (0.80) n mole MDA/mL, respectively. We found significance variation between levels of mean plasma TBARS in control and clinical phases of CML. The mean plasma level of LOOH in CML, CML-CP and CML-AP were 0.64 (0.24), 0.55 (0.21) and 0.85 (0.15) mM/mL whereas in control was 0.41 (0.16) mM/mL, respectively. We also found significantly different levels of mean plasma LOOH between clinical phases of CML and control.

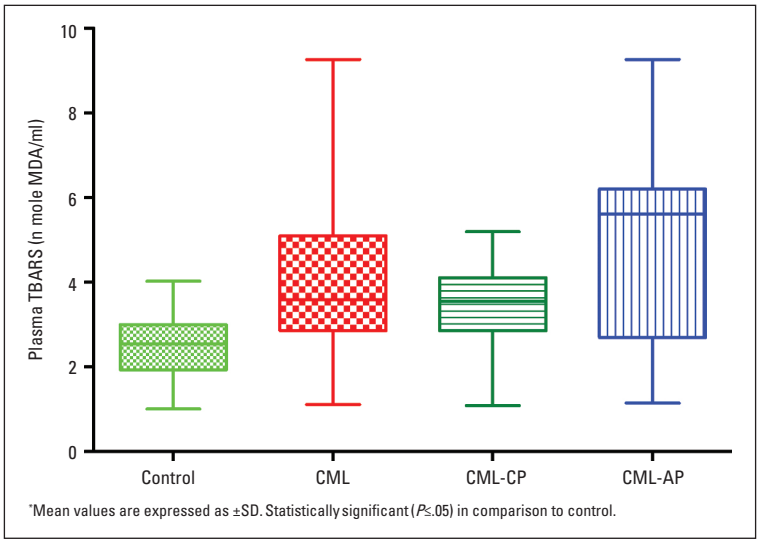

Figure 2. Plasma thiobarbituric acid reactive substances (TBARS) levels in CML patients and controls. (CP: chronic phase, AP: accelerated phase)

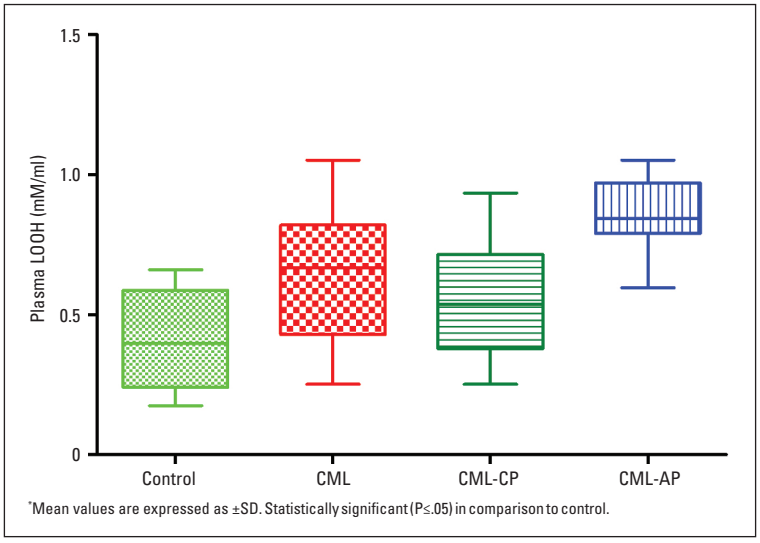

Figure 3. Plasma lipid hydroperoxide (LOOH) levels in CML patients and controls. (CP: chronic phase, AP: accelerated phase)

DISCUSSION

Generally speaking, oxidative stress is defined as an imbalance between the level of ROS and the antioxidant defense system, in favor of the former.[2] Oxidative stress plays a variety of roles in normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention.[4-19] Biomarkers are defined as characteristics that can be autonomously measured and analyzed as indices of oxidative stress.[8] Various *in vitro* markers of oxidative stress are available, including ROS themselves, but most have imperfect value *in vivo* because they lack sensitivity, specificity, or require invasive methods.[8] Although some ROS have been directly detected *in vitro* by chemiluminescence or by electron spin resonance with or without spin-trapping reagents, these direct detection methods are not yet appropriate for clinical research because of the instability of ROS and the need for costly equipment.[8] Furthermore, ROS are generally extremely reactive and have a half-life too short to measure in cells, tissues, or body fluids.[8]

Molecular products or metabolic products formed from the reaction between ROS and bio-molecules are generally considered more stable than ROS themselves.[8] These products are too stable to evaluate more easily than direct evaluation of ROS.[8] In previous studies, various metabolic products have been described as biomarkers for oxidative stress in a numbers of pathologies.[8] Most commonly metabolic products include products of oxidation of proteins and lipids. PC is an irreversible product of protein oxidation whereas TBARS and LOOH is the product of lipid oxidation.[8,9,14] A study on breast cancer provided evidence that protein oxidation might be associated with cancer risk.[24] A significant level of plasma PC was observed in patients with Hodgkin lymphoma, colon cancer, bladder cancer and lung cancer and in children with various malignancies.[16,25-27] TBARS and LOOH are well-defined lipid peroxidation or oxidation end products. The TBARS is considered to be mutagenic and carcinogenic.[4] It can

also modulate the expression of genes related to initiation and progression of cancer.[4] Increased level of TBARS was found in patients with breast cancer, gynecological malignancies, squamous cell carcinoma, colon cancer, cervical cancer and various types of leukemia.[6,9,13,15-19] A significant increased level of LOOH was found in patients with obstructive jaundice and colon cancer.[16,28]

Elevated level of PC, TBARS and LOOH support the hypothesis that high ROS generation occurs in neoplastic or cancer cells. It was demonstrated that bcr-abl fusion protein is associated with increased levels of ROS in hematopoietic cell lines compared with their non-transformed parental cell lines.[29] High ROS and increased activity of tyrosine kinase protein play an important physiological role in signal transduction and induced proliferative pathway in the cell.[29] A current report states that bcr-abl kinase stimulates ROS, which causes oxidative DNA damage, resulting in mutations in the kinase domain.[30] It was suggested that ROS might play a major role in resistance to given therapy, which could contribute to progression of CML.[30]

Our suggestion for the mechanism of higher PC, TBARS and LOOH levels in plasma is that mature and immature myeloid cells as well as other unknown factors are the major source of ROS, which leads to oxidative stress in the cells, if antioxidant system is not powerful. There may be comparability between numbers of mature/immature myeloid cells and bcr-abl fusion protein with disease progression. The bcr-abl fusion protein has also been linked with genomic instability, progression of the disease, increased ROS production and additional karyotypic abnormalities.[30] These circumstances build an intracellular milieu more favorable for macromolecules damage, mutations and disease progression. Hence, it can be implied that in CML, plasma PC, TBARS and LOOH levels may precisely reflect the degree of oxidative stress with disease phenotype. We suggest that plasma PC, TBARS and LOOH may serve as biomarkers for oxidative stress in patients with CML. A highly structured study with a larger sample size is required to establish the precise role of oxidative stress in pathobiology of CML.

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