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Antioxidant Enzyme Levels as Surrogate Markers for Genomic Instability in Leukemia Patients

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Abstract

Oxidative stress due to increased generation of reactive oxygen species (ROS) leads to above than genomic instability and damages proteins, lipids and DNA, leading to development of pathological conditions such as inflammation, cardiovascular diseases and cancer. In the present study, we investigated the total antioxidant status and levels of reduced glutathione, catalase and lipid peroxidation in leukemia patients. The patients were divided into two groups one with normal karyotype and another group of patients with abnormal karyotype. The study included 35 untreated leukemia patients and 23 age and sex matched healthy controls. We report elevated plasma lipid peroxidation (MDA levels), catalase, and GSH levels, and reduced FRAP activity in patients as compared to control subjects. Increased activity of antioxidative enzymes in leukemia patients indicates the generation of free radicals causing the undesired pathological changes in leukemia patients. In summary, levels of specific antioxidant enymes may serve as surrogate markers of genomic instability in leukemia patients.

Key Words: *Antioxidative Enzymes, Leukemia, catalase (CAT) and glutathione reductase (GSH), lipid peroxidation, FRAP, Karyotype*

1. Introduction

Reactive oxygen species (ROS) has been established in the etiology and development of leukemia and have been implicated in the development of leukemogenesis[1]. The ROS/ free radicals are metabolites produced during abnormal cellular process. The excessive production of ROS with abnormal response of antioxidant system disturbs the homeostasis eventually leading to oxidative stress. ROS plays a dual role in tumorigenicity, particularly during generation of neoplasms, induces cell death, including apoptosis, and protects the cells from apoptosis thereby promoting cell survival, leading to proliferation, migration, metastasis and drug-resistance[1-6]. ROS are involved in the pathogenesis of malignancies incluidng major types of leukemia e.g., acute myeloid leukaemia, chronic lymphocytic leukemia, chronic myeloid leukaemia, and acute lymphoblastic leukaemia[7-11].

The oxidative stress markers have been implicated in the progression and pathogenesis of leukaemia by several mechanisms. The products of lipid peroxidation and ROS leads to genomic instability and aberrant DNA by producing 7,8 dihydro-8-oxo-2′deoxyguanosine (8-oxoG) and other oxo-base derivatives, which may lead to point mutations subsequently leading to tumorigenesis. Studies have reported that 8-OHG and its nucleoside form 8-OHdG are the indicators of oxidative DNA damage in vivo and in vitro[11, 12]. The 8-OHG in the DNA causes a G-T and a C-A transversion, as 8-OHG allows the incorporation of cytosine and adenine nucleotides opposite the lesion during DNA replication and has been reported to be involved in carcinogenesis and pathogenesis of aging associated disease and cancer[13-18].

The BCR-ABL, induced ROS production results in malignant transformation, resistance to apoptosis, and increased DNA damage[19-21]. The

FLT3-ITD mutation induces increased production of free radicals leading to DNA double strands breaks and repair errors[1, 22]. Also the mutant NRAS and HRAS induces the production of superoxide and peroxide ions leading to accelerated cell differentiation[23].

ROS participate in several cell growth pathways by interfering with the regulation of certain genes and signal transduction pathways, including tumor protein p53 mutation, activator protein-1 (AP-1) activation, vascular endothelial growth factor (VEGF) leading to several haematological malignancies including leukemia[1, 24-26].

Additionally, it has been observed that high-dose chemotherapy, used as mainstay in leukemia treatment, is often accompanied with generation of reactive oxygen species resulting in cytotoxicity[1]. Thus, the utilization of chemotherapy in combination with antioxidants may attenuate leukemia progression, particularly for cases of refractory or relapsed neoplasms.

Understanding the mechanisms of ROS generation in leukaemia patients may be helpful in disease susceptibility and designing better therapeutic strategies with less toxicity. Therefore, the present study focuses on the roles of OS in leukaemia patients characterizing the associations between ROS and disease pathology.

2. Materials and methods

The mean age was 35 years. Informed consent was taken from all the subjects. Peripheral blood samples were taken from leukemia patients (n=35) along with age and sex matched healthy control (n=23). Conventional cytogenetic using bone marrow karyotyping was used and the patients were divided into subgroups: a) Leukemia Patients with normal karyotype (n=17), and b) Leukemia patients with abnormal karyotype (n=18). The blood sample was separated by centrifugation at 3000 rpm per 15 minutes and plasma was separated for further biochemical estimation of total antioxidant status, reduced glutathione (GSH) levels, catalase enzyme activity and lipid peroxidation levels. Protein estimation was done by Lowry's method using bovine serum albumin (BSA) as standard[27].

Cytogenetic studies: Karyotyping

Chromosome analysis was performed for karyotyping, routine cytogenetic procedures were followed[28] (as described in Rooney and Czepulkowski). Heparinised bone marrow samples obtained at the time of diagnosis, were processed and cultured for 24 h/48 h in RPMI 1640 medium (Caission labs, Cat. no RPMI-012P) + 20% fetal bovine serum (GIBCO Cat. no. 10270), COLCEMID (0.05µg/ml) (Biological Industries 10µg/ml Cat. no 12004-1D) was added for the last 60 min of culture, followed by hypotonic treatment with a 0.075-KCl solution and a final fixation in methanol/acetic acid (3:1). Chromosomes were G-banded for identification. Whenever possible, at least, 20 metaphase were analysed according to the International System for Cytogenetic Human Nomenclature, 2009).

FRAP assay- to measue total antioxidant power

The reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous fromcan be measured by this method of Iris et al., 1996 taking absorbance at 593nm[29].This non-specific reaction drive the ferric ions to ferrous ions. The absorbance level is directly related to the combined or total reducing power of the electrondonating antioxidants present in the reaction mixture. Ferrous sulphate (1mM) was used as standard.

Glutathione Reductase (GSH)

Glutathione involves oxidation of GSH by sulfahydryl reagent 5'-dithio-bis (2 –nitrobenzoic acid) DTNB to form TNB, which is measured at 412nm[30]. The GSSG formed can be recycled to GSH by glutathione reductase in presence of NADPH.

Catalase

Catalase activity is essentially measured by the method of Beers and Sizer, (1952)[31]. Hydrogen peroxide (H_2O_2) was used as the substrate and disappearance of peroxide is measured spectrophotometrically. The absorbance at 240nm is measured directly to calculate the reaction rate. Results were expressed as H_2O_2 decomposed per minute per mg.

Lipid Peroxidation

The Lipid peroxidation involves the reaction of chromogenic reagent , N methyl-2-phenyl-indole with MDA and 4hydoxyalkenals at 45^0C[32]. The TBARS was evaluated by using spectrophotometric method based on the reaction between MDA and thiobarbitturic Acid (TBA). Absorbance was measured at a wavelength 532 nm with molar extinction coefficient ε532=1.56×105 M-1cm-1.22 Plasma level of TBARS were expressed as nmole MDA/mL.

Statistical Analysis

All biochemical analyses were performed in triplicate. The data is represented as Mean ±

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standard deviation. The levels between various groups and subgroups were compared using Student's t-test using the graphical software SPSS, and differences with $p<0.01$ were considered to be significant.

3. Results

The leukemia patients were categorized into two groups based on karyograms: patients with normal karyotype (n=17), and patients with abnormal karyotype (n=18). The total antioxidant level as measured by FRAP levels was found to be significantly lower in leukemia patients compared to healthy controls (Fig. 1A). Total antioxidant levels were signicantly lower for both groups of leukemia patients compared to control; no significant differences for total antioxidant status was found within the two groups of leukemia patients the leukemia pateints, i.e., normal. *vs.* abnormal karyotype (Fig. 1B).

The GSH levels were significantly higher in leukemia patients compared to the control group (Fig. 2A); it was observed to be higher in both subgroups of patients as compared to control group. Further, subgroup analysis among the patients showed that GSH levels were significantly higher in patients with abnormal karyotype compared to those in patients with normal karyotype (Fig. 2B).

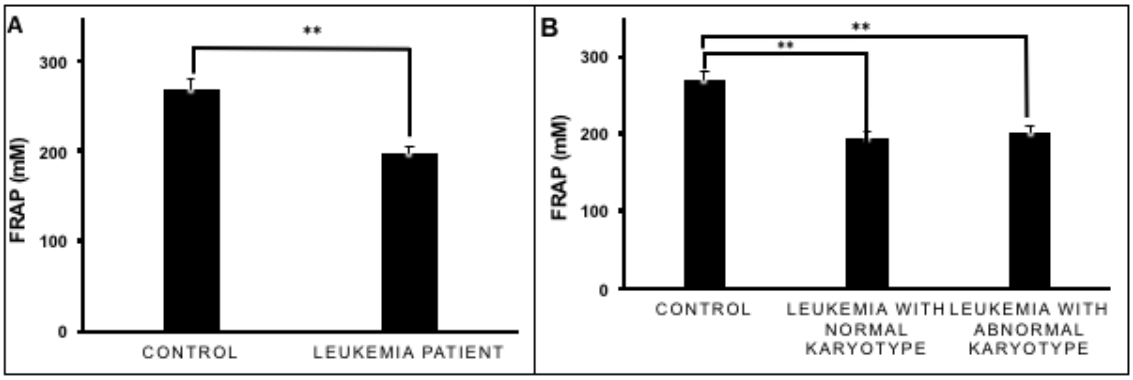

Figure 1: Comparison of total antioxidant status measured by FRAP assay between **(A)** control and leukemia patients, and **(B)** control, leukemia patients with normal karyotype and leukemia patients with abnormal karyotype. Values represent Mean ± SD. ** Indicates statistical difference with $p < 0.01$.

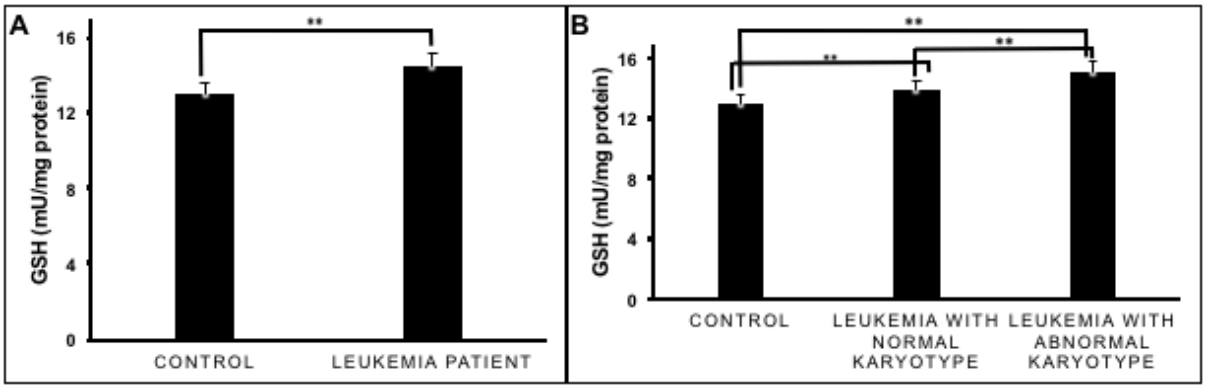

Figure 2: Comparison of reduced glutathione (GSH) levels between **(A)** control and leukemia patients, and **(B)** control, leukemia patients with normal karyotype and leukemia patients with abnormal karyotype. Values represent Mean ± SD. ** Indicates statistical difference with $p < 0.01$.

Significant increase in total catalase activity levels in leukemia patients compared to the healthy control individuals (Fig. 3A). As observed for GSH levels, significantly elevated catalase activity was also observed in both the subgroups of patients as compared to control group (Fig. 3B). Among leukemia patients, catalase activity was significantly higher in patients with abnormal karyotype compared to those in patients with normal karyotype (Fig. 3B).

For lipid peroxidation activity, no significant differences were found between controls and leukemia patients (Fig. 4A). Intrestingly, the lipid peroxidation levels for the leukemia patients with abnormal karyotype was significantly higher than control healthy individuals (Fig. 4B). Further,

leukemia patients with abnormal karyotype also had significantly higher lipid peroxidation compared to patients with normal karyotype (Fig. 4B). No significant differences for lipid peroxidation levels were observed between controls and leukemia patients with normal karyotype.

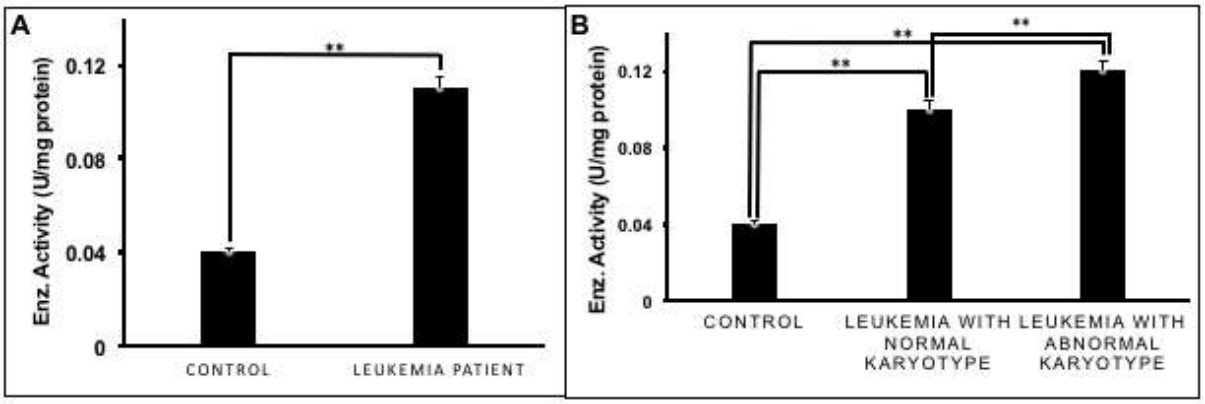

Figure 3: Comparison of Catalase activity levels between **(A)** control and leukemia patients, and **(B)** control, leukemia patients with normal karyotype and leukemia patients with abnormal karyotype. Values represent Mean ± SD. ** Indicates statistical difference with $p < 0.01$.

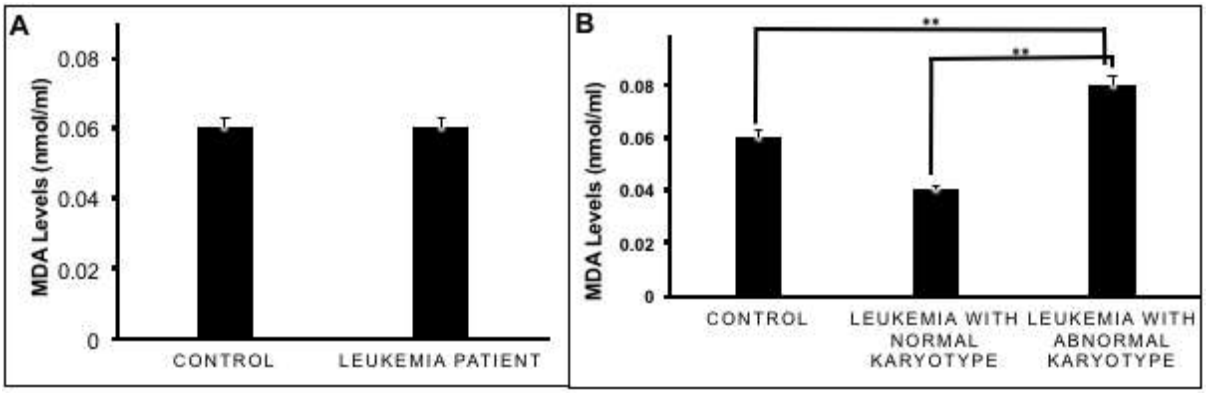

Figure 4: Comparison of lipid peroxidation status as measured by MDA levels between **(A)** control and leukemia patients, and **(B)** control, leukemia patients with normal karyotype and leukemia patients with abnormal karyotype. Values represent Mean ± SD. ** Indicates statistical difference with $p < 0.01$.

Discussion

Oxidative stress is the result of imbalance between free radicals and antioxidant defense system and serve an important role in pathogenesis of leukemia. Conventional cytogenetic analysis using Giemsa banding of chromosomes detects the numerical and structural abnormalities. The Metaphase cytogenetic analysis of is of gold standard to identify the translocations and related changes common in AML, ALL and CML diseases. In the present study, we studied the antioxidant enzyme status in two groups of patients categorized by using conventional cytogenetics method of karyotyping, one group of patients was with normal karyotype and another was with abnormal karyotype.

We observed a significant decrease in total antioxidant level in FRAP in both the subgroups of patients as compared to control group. Our results are in agreement with report from Mazor *et al.*, (2008)[33] who reported significantly decreased total FRAP activity in pediatric patiients with acute lymphocytic leukemia (ALL) and solid tumors.

In our study, GSH activity was found to be elevated significantly in both the subgroups of patients as compared to control group Our results are in parallel with results reported in previous studies[33, 34]. Several researchers have reported that the elevated leves of GSH are directly proportional to disease duration as a consequence of increased tripeptide synthesis depending on the increased lymphocytes glutathione peroxidase activity further leading to increase in GSH activity in CLL[34].

The antioxidant enzyme catalse catalyzes the conversion of hydrogen peroxide to water and molecular oxygen.Variations in catalase level has

been observed in many disease conditions including acute myeloid leukemia[35]. A significant increase in total catalase levels was found in both the subgroups of patients as compared to control group [control *vs.* normal karyotype ($p<0.0001$) and control *vs.* abnormal karyotype ($p<0.04$)] between both the subgroups of patients ($p<0.005$). Our results are in concordance with previous studies [34, 36]. However, a few studies have also reported the low levels of catalse in leukemia patients suggesting various mechanisms[35].

Lipid peroxidation causes damage to cell membranes and lipid-containing structures. One of the intermediate products of this reaction are hydroperoxides and secondary products [malondialdehyde (MDA) and 4-hydroxynonenal/4-hydroxy-2-nonenal (HNE)] interacts with the membranes and endanger cells[37, 38]. The products of lipid peroxidation can cause irreversible damage to proteins and nucleic acid via modifying the amino acid residues to form stable adducts or covalent adducts with nucleic acids and membrane[39, 40]. The MDA levels are an important biomarker in leukemia having diagnostic and prognostic role and helps in predicting the disease progression[35]. We observed a significant increase in lipid peroxidation levels in two subgroups of patients, with normal karyotyotype and with abnormal karyotype in comparison with control group ($p<0.002$). However, the increase was insignificant when compared between control group and normal karyotype leukemia patients group.

In summary, this pilot study suggests that assessment of differences in the specific antioxidants such as GSH and catalase may be used as surrogate markers of genomic instability in leukemia patients, and may be beneficial in deciding treatment decisions for better patient outcomes.

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