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CLINICAL STUDIES

Correlation of serum toll like receptor 9 and trace elements with lipid peroxidation in the patients of breast diseases

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ABSTRACT

Toll-like receptors are recognized as redox sensitive receptor proteins and have been implicated in cellular response to oxidative stress. Altered pro-oxidant-antioxidant balance leads to an increased oxidative damage and consequently play an important role in breast diseases. The study was designed to access the oxidative stress status by quantification of byproducts generated during lipid peroxidation and inadequate trace elements during oxidative damage and its effects on the toll like receptor (TLR) activity in patients of breast diseases. Decreased levels of selenium, copper, zinc, magnesium and iron with elevated levels of malondialdehyde (marker of lipid peroxidation) were accompanied by decreased TLR activity in patients of benign breast diseases as well as breast carcinoma. A similar pattern was observed with the advancement of disease and its subsequent progression in breast carcinoma patients. Results of multinomial regression analysis suggest benign breast disease patients are at higher risk of developing breast cancer with high odds ratio of lipid damage.

Introduction

Breast cancer is the most common cancer diagnosed in women worldwide with nearly 1.7 million new cases diagnosed in 2012 which represents about 12% of all new cancer cases and 25% of all cancers in women. Breast cancer is the leading cause of mortality in women worldwide [1]. Benign breast diseases are at least ten times more common than breast carcinoma and hence their recognition, management and delineation from malignancy are as important as their malignant counterpart. Women with previous benign breast disease such as fibroadenoma are at a higher risk of developing breast cancer [2]. The etiology of breast cancer is multifactorial. Hormonal, genetic and environmental factors appear to interplay in the pathogenesis of breast cancer, however the specific factors are not yet known [2,3].

When excessive production of reactive oxygen species (ROS) overwhelms the counter-regulatory antioxidant defense system cells undergo oxidative stress. It results from diminished antioxidant protection as well as increased free radical production [4,5]. This production of unstable free radicals is directly involved in the peroxidation of cellular components such as lipids which can initiate a chain of events resulting in the onset of variety of diseases. Malondialdehyde (MDA), a carbonyl group produced during lipid peroxidation, has been recognized to be prominent feature of various diseases and their progression therefore widely used in determining oxidative stress.

A growing body of evidence has indicated that many trace elements play an important role in a number of biological processes by activating or inhibiting enzymes, by competing with other elements and metalloproteins for binding sites or by affecting the permeability of cell membranes. Thus trace elements may exert action, directly or indirectly, on the carcinogenic process [6]. Trace elements are major components of antioxidant enzymes such as selenium is an essential trace element and a co-factor for glutathione peroxidase [7]. Zinc and copper are also essential trace elements, being a co-factor for about 200 human enzymes, including the cytoplasmic antioxidant Cu–Zn SOD [8]. Iron is an essential metal involved in the activity of many enzymes such as catalase and cytochrome [9]. Magnesium (Mg) is an essential mineral rich in wheat germ, green vegetables, legumes, algae, nuts and seeds, which acts as a cofactor in enzymatic reactions in the human body. Altered levels of magnesium increases genomic instability and Mg intake has been reported to be inversely associated with a risk of colorectal cancer [10]. Therefore it is reasonable

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to investigate the role of these trace elements in the process of carcinogenesis.

Infections caused by different pathogens are often associated with systemic symptoms and may compromise the functional integrity. In the mediation of the systemic effect of pathogens toll-like receptors (TLRs) play a significant role. TLRs are a type of pattern recognition receptors and recognize molecules that are broadly shared by pathogens but distinguishable from host molecules. TLRs are broadly distributed on cells of the immune system and function as primary sensors of invading pathogens. Oxidative stress significantly up regulates the expression of these receptors [11]. TLRs are mostly associated with initiation of the innate response and inflammation, and inhibition of TLR activity, which may help combat an overactive innate response characteristic of numerous inflammatory disorders such as breast cancer [12].

Although a number of studies have unraveled the role of numerous well-established risk factors like advancing age, early menarche, late menopause, late age at first birth and family history of breast cancer as well as the imbalance in oncogenes and tumor suppressor genes in breast cancer [13,14] but there are only few reports on the oxidant–antioxidant and trace elements profile in the serum of breast carcinoma and benign breast patients. The present work is an attempt to evaluate the relationship between oxidative state by quantification of levels of MDA and some trace elements associated with antioxidant defense system along with TLR activity in patients of breast diseases so as to have a better understanding of the contributions of the reactive oxygen species to breast diseases.

Materials and methods

Selection of patients and control cases

For the case control study, histo-pathologically confirmed patients of breast carcinoma and benign breast disease admitted in the Department of General Surgery, University Hospital, Banaras Hindu University, Varanasi were selected. The clinical and pathological staging of breast cancer was done according to the tumor-node-metastasis (TNM-UICC) classification [15]. Age and sex matched healthy volunteers having socio-economic status similar to that of patients were taken as controls. Specific exclusion criteria considered for the present study were the healthy controls having no acute or chronic diseases such as diabetes, parasitosis, immune dysfunction or any other malignancy and they were not under any pharmacological therapy. None of the study subjects were under oral contraceptives, hormonal therapy or antioxidant supplementation. The venous blood without using anticoagulant was collected from patients and healthy volunteers in sterile tubes. Blood was then centrifuged at 5000 rpm for 10 min at 4 °C. The top serum layer was removed without disturbing the white buffy layer. The serum was stored at −20 °C for various biochemical investigations. The study protocol was approved by the ethical committee of the Institute of Medical Sciences, Banaras Hindu University. Informed consent of each patient and healthy volunteers was obtained purely for research purpose.

Analytical methods

All indices were determined in serum obtained from blood of the studied groups. Collected sera were stored at −20 °C for further examination.

Reagents

All reagents used in this study were of analytical grade and obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Malondialdehyde – marker of lipid peroxidation

Assay of oxidative damage in the serum of the patients as well as healthy control samples was assessed by measurement of products of lipid peroxidation in serum by thiobarbituric acid reactive substances (TBARS) technique of Philpot [16]. 1 ml of sample was mixed thoroughly with 2 ml of TCA-TBA-HCl (15% w/v TCA and 0.375% w/v TBA in 0.25 N HCl). At the time of assay 0.01% butyl hydroxytolvene (BHT) was added to stock reagent to abolish the metal catalyzed auto-oxidation of lipids, which could result in falsely elevated TBA reactivity. The mixture was heated in a boiling water bath for 15 min. The samples were centrifuged at 1000 rpm for 10 min. The absorbance of each sample was determined at 530 nm in a spectrophotometer against a suitable blank. The malondialdehyde concentration of each sample was calculated by using extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Estimation of selenium

An aliquot of sample was transferred into 40 μL of 4 M HCl and 30 μL of 0.5% phenylhydrazine-p-sulphonic acid and the mixture was allowed to stand for 30 min at room temperature. There after 20 μL of 0.5% acetylacetone and 100 μL of 6 M NaOH solution were added. The total volume was made to 250 μL and allowed to stand for 10 min at room temperature. The absorbance of the azo dye was measured at 490 nm against the reagent blank [17,18].

Copper

Serum copper was determined in all the cases and controls by using colorimetric kit obtained from Crest Biosystems, Goa, India by using 3,5-dibromo-2-pyrodylazo-N-ethyl-N-sulphopropylaniline color reagent in acid solution [19]. Copper is released from ceruloplasmin and reduced. The cuprous ion forms a colored complex with colored reagent. The absorbance was measured by spectrophotometer at wave length 580 nm.

Zinc

Serum zinc concentration was determined in all the cases and controls by using colorimetric kit obtained from Crest Biosystems, Goa, India by using colorimetric method with Nitro-PAPS color reagent [20]. Nitro-PAPS react with zinc in alkaline solution to form a purple color complex. The absorbance was measured at 570 nm and is proportional to the amount of zinc present in the sample.

Magnesium

Serum magnesium (Mg^{2+}) concentration was determined in all the cases and controls by using colorimetric kit obtained from Crest Biosystems, Goa, India. The assay was based on Calmagite method [21]. Magnesium combines with calmagite in alkaline medium to form red colored complex. The absorbance was measured at 510 nm. Intensity of the color formed is directly proportional to the amount of Mg^{2+} present in sample.

Estimation of iron

The iron levels in the serum of all the cases and controls were assayed by using colorimetric kit obtained from Crest Biosystems, Goa, India. Iron, bound to transferrin, is released in an acidic medium and the ferric ions are reduced to ferrous ions. The Fe (II) ions react with ferrozine to form a violet colored complex [22]. Intensity of the complex formed is directly proportional to the amount of iron present in the sample. The absorbance was measured at 570 nm.

Estimation of serum toll like receptor-9

TLR-9 concentrations was measured in the serum of all the cases and controls using competitive *in vitro* enzyme linked immunosorbent assay (ELISA) kits obtained from USCN Life Science & Technology Company, China [23]. The test principle applied in