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Original Article

Biochemistry Section

Evaluation of Nitric Oxide, Endothelial Nitric Oxide Synthase and Lipid Profile in Breast Cancer: A Case-control Study

SHRIKANT SHANKARRAO SHINGARWAD[1], SWATI DIGAMBAR SAWANT[2], AMIT AMRUT BANSODE[3], RAVI RAMKISHAN YADAV[4]

ABSTRACT

Introduction: Breast cancer accounts for 25% of all female cancer cases in India. Although, lipids and free radicals like Nitric Oxide (NO) have been related to breast cancer, but its pathogenesis remains controversial.

Aim: To estimate NO, endothelial NO Synthase (eNOS) and lipid profile in serum of breast cancer patients and also to determine their possible role in patients of breast cancer in Indian women.

Materials and Methods: This was a hospital-based case-control study conducted for a period of 24 months from July 2012 to June 2014 in Dr. V.M. Government Medical College, Solapur, with a total of 100 women (50 breast cancer cases and 50 healthy age-matched controls) of age group 35-65 years. Fasting venous blood samples were collected. Biochemical parameters analysed in the study were serum NO, serum eNOS, serum Total Cholesterol (TC), serum Triglycerides (TG), serum High Density Lipoprotein (HDL) and serum Low Density Lipoprotein (LDL). Results were expressed as mean±Standard Deviation (SD) and Student's unpaired t-test was used to compare the pairs of means.

Results: There was no significant difference in mean age between cases (49.73±8.16 years) and controls (48.23±8 years). Serum levels of NO, eNOS, TC, TG and LDL were significantly higher in cases than in controls (55.92±6.54 µmol/L vs 49.58±6.74 µmol/L for NO; 175.4±22.92 U/mL vs 148.6±9.77 U/mL for eNOS; 271±9.16 mg/dL vs 177.58±6.41 mg/dL for TC; 180.70±8.48 mg/dL vs 107.57±8.98 mg/dL for TG; 177.39±6.21 mg/dL vs 98.16±8.90 mg/dL for LDL). However, serum HDL levels did not showed significant difference in case group (57.55±6.40 mg/dL) when compared with control group (57.15±5.35 mg/dL).

Conclusion: The present study demonstrated that high serum levels of NO, eNOS and lipids are associated with breast cancer and thereby, suggests that increased NO, eNOS and abnormal lipid profile may have a role in pathogenesis of breast cancer.

Keywords: Cholesterol, Diagnostic biomarkers, Indian women, Lipoprotein, Pathogenesis, Triglycerides

INTRODUCTION

Breast cancer is the most common cancer in Indian women and is the second most common cancer in the world [1]. About 1.2 million cases are detected every year, affecting 10-12% of women and is responsible for approximately 500,000 deaths per year [2]. In India, approximately 25% of the female cancer cases are of breast cancer. Overall, 1 in 28 Indian women is likely to develop breast cancer during her lifetime [3].

Regarding the causative factors contributing towards the development of breast cancer, researchers have postulated that neoplastic transformation of a normal cell is the result of various factors such as demographic factor, personal factor, altered hormonal milieu and exposure to chemical carcinogens, radiation and oncogenic viruses [4]. Free radicals have also been implicated in the pathogenesis of breast cancer [5]. There are various free radicals out of which NO is an important free radical. It is the only endogenous molecule that acts as a paracrine messenger, a hormone, a Reactive Oxygen Species (ROS), neurotransmitter, cytoprotective and cytotoxic molecule [6]. The role of NO in tumour biology is not properly understood. Interactions of endothelial cells of tumour vasculature, tumour infiltrating immune cells, macrophages and tumour cells themselves regulate growth of solid tumours. These cellular components have been shown to produce NO [7]. NOS converts L-arginine to NO [6]. Various tumour cell lines express NOS and it has been reported that NOS activity is present in human ovarian and uterine tumours [8]. Out of various forms of NOS, nNOS (neuronal NOS) and eNOS are two important forms of NOS existing in nature; eNOS being solely responsible for endothelial synthesis of NO [6].

Lipids are essential to carry out several vital physiological functions. However, lipids might be associated with cancers because they play a key role in the maintenance of cell integrity. In malignancies, blood cholesterol undergoes early and significant changes. Cholesterol serves as a precursor for the synthesis of many sex hormones linked to increased risk of various cancers. To add to this, the pathway for cholesterol synthesis also produces various tumourigenic compounds. However, the mechanism for the link of cancer and cholesterol remains controversial. Both hypolipidaemia and hypercholesterolemia have been connected with cancers in various studies leading to genesis of undistinguished and conflicting relationship between cancer and cholesterol [9].

To the best of authors' knowledge, only one documented study conducted by Shelgikar PJ and Abhang SA, had measured eNOS levels in the serum of breast cancer patients in Indian women [10]. Also, several previous studies have investigated the association between lipid levels and breast cancer [11-15]. However, it is noteworthy that only few studies reported by Shah FD et al., and Hasija K and Bagga HK, have included the investigation of Indian women specifically [16,17]. Consequently, very less is known about the lipid levels in breast cancer among Indian women. Therefore, the present study was aimed to estimate NO, eNOS and lipid profile in serum of breast cancer patients among Indian women, and to determine their possible role in patients of breast cancer.

MATERIALS AND METHODS

The present study was a hospital-based case-control study and was reviewed and approved by the Dr. V.M. Government Medical College Institutional Ethical Review Board (IRB file no. 2012-12-028). The study was conducted for a period of 24 months from July 2012 to June 2014 in Dr. V.M. Government Medical College, Solapur, Maharashtra, India. Informed written consent was taken from all the study participants.

Inclusion criteria: Fifty female patients diagnosed as having breast cancer (diagnosis confirmed by biopsy) were selected as cases for this study. Control group consisted of 50 healthy age matched women attending the routine health check-up in the hospital. None of the patients had received any therapy including radiation, surgery, or chemotherapy but were waiting for surgical intervention.

Exclusion criteria: Patients suffering from myocardial infarction, other malignancies, smokers, diabetes mellitus, hypothyroidism, liver disease, kidney disease, Cushing's syndrome and a history of familial dyslipidaemia were excluded. In addition, patients receiving medications affecting lipid metabolism, such as lipid lowering drugs, oral contraceptives, thyroxine were excluded from the study.

Sample size calculation: Statistical calculation for measuring sample size was done by the statistical formula- sample size, $n=\{k^2\times4\,(SD)^2\}\div d^2$, where SD is Standard Deviation obtained from pilot study [18]; d is the desired width of the Confidence Interval (CI); k is a constant depending on the two-sided CI. For this, two-sided CI was taken as 95%, k as 1.96, d as 10, alpha as 5%, power as 90%. Here, SD was taken as 17.2 that was actually calculated from the pilot study conducted at the start of the study. It is to be noted that the sample size for the pilot study was taken as 12 as per the recommendations by Julious SA and Van Belle G [19,20]. The calculated sample size came out to be 46 for case and control groups implying that the total sample size would be 92. Therefore, on approximation, a total of 100 women (50 cases and 50 controls) of age group 35-65 years, willing to give informed consent were enrolled in the study.

Study Procedure

Fasting venous blood samples (5 mL) were collected from antecubital veins from both the groups (in the morning immediately after the diagnosis before giving any medication). The blood was allowed to clot at room temperature in plain blood collection tube for one hour. Serum was collected by centrifugation at 1500xg for 10 minutes which was then used for estimation. Analysis was performed within 24 hours of sample collection. The biochemical parameters analysed in the study were serum NO, serum eNOS, serum TC, serum TG, serum HDL and serum LDL [Table/Fig-1] [21-25].

S. No.	Biochemical parameters	Methods of estimation	Reference interval
1.	Serum Nitric Oxide (NO)	Miranda Katrina method [21]	Reference interval not established
2.	Serum endothelial Nitric Oxide Synthase (eNOS)	Sandwich ELISA.	Reference interval not established
3.	Serum Total Cholesterol (TC)	Cholesterol oxidase-peroxidase (CHOD-POD) method [22]	120-200 mg/dL
4.	Serum Triglycerides (TG)	Glycerophosphate oxidase peroxidase (GPO-POD) method [23]	50-150 mg/dL
5.	Serum HDL Cholesterol (HDL)	Phosphotungstic acid method [24]	40-60 mg/dL
6.	Serum LDL Cholesterol (LDL)	Friedwald's formula [25]	50-130 mg/dL

[Table/Fig-1]: Serum parameters along with their methods of estimation [21-25].

Lipid profile parameters were analysed in ERBA EM-200 fully automatic clinical chemistry analyser while serum NO and serum eNOS were analysed in semi-automated clinical chemistry analyser and ELISA respectively. The eNOS levels were measured in the serum samples using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit according to the manufacturer's instructions (Human eNOS 3 kit made by USC Life Science Inc., Wuhan, China) [21-25].

STATISTICAL ANALYSIS

The results obtained in the study were evaluated using Mystat statistical software (version 12.0) at 95% CI. Values of $p<0.05$ was considered to be statistically significant and $p<0.01$ as statistically highly significant, whereas $p>0.05$ was considered to be statistically non significant. Results were expressed as mean±SD for all continuous variables. Student's unpaired t-test was used to compare the pairs of means.

RESULTS

There was no significant difference in mean age between cases (49.73±8.16 years) and controls (48.23±8 years) (p=0.31). Also, there were no significant differences in maternal characteristics between the two groups, with regard to the number of pregnancies (2.0±0.54 in cases and 2.1±0.61 in controls) (p=0.49).

Serum NO showed a significant increase in cases (as compared to controls (p=0.009). Serum eNOS also showed a significant increase in cases as compared to control group (p=0.007) [Table/Fig-2].

Parameters	Cases (50)	Controls (50)	p-value
NO (µmol/L)	55.92±6.54	49.58±6.74	0.009*
eNOS (U/mL)	175.4±22.92	148.6±9.77	0.007*
TC (mg/dL)	271.0±9.16	177.58±6.41	0.001*
TG (mg/dL)	180.70±8.48	107.57±8.98	0.001*
HDL (mg/dL)	57.55±6.40	57.15±5.35	0.790
LDL (mg/dL)	177.39±6.21	98.16±8.90	0.005*

[Table/Fig-2]: Serum parameters in case and control groups.
*p<0.05 is statistically significant; *p<0.01 is statistically highly significant; p>0.05 is statistically non significant; Student's unpaired t-test

Serum level of TC was significantly higher in cases than in controls (p=0.001). Serum level of TG was also significantly higher in cases than in controls (p=0.001). Similarly, serum level of LDL was significantly higher in cases than in controls (p=0.005). However, serum HDL levels have not shown significant difference in cases when compared with control group (57.55±6.40 mg/dL vs 57.15±5.35 mg/dL, p=0.790) [Table/Fig-2].

DISCUSSION

In the present study, the authors have investigated the possible role of NO, eNOS and lipid profile in breast cancer patients among Indian women. Serum NO levels showed a statistically significant increase in patients with breast cancer (55.92±6.54 µmol/L in cases vs 49.58±6.74 µmol/L in controls). In support of the present study, Gunel N et al., and Kadam C and Abhang SA have found elevated level of NO at operable serum in samples of patients with breast cancer [26,27]. Favourable results were provided by Guler E et al., who demonstrated decreased plasma total NO level after chemotherapy of breast cancer patients [28]. Elevated levels of NO production increase the tumour vascularity and facilitate tumour metastasis in breast cancer patients [29]. The NO production is a part of the angiogenic switch in tumour development [30]. NO may promote tumour growth by modulating the production of prostaglandins. The NO can activate Cyclooxygenase-2 (COX-2) which generates prostaglandins that promote angiogenesis and suppress the apoptosis of cancer cells [31,32].

In the present study, there was statistically significant increase in eNOS levels in breast cancer patients in comparison to control group (175.4±22.92 U/mL in cases vs 148.6±9.77 U/mL in controls). In agreement with the present study, studies conducted by Shelgikar PJ and Abhang SA; and Thomsen LL et al., showed a significant increase in serum eNOS in the breast cancer patients [10,33]. Therefore, the present study demonstrates that high eNOS levels are associated with breast cancer and thus, provides an important biological link between the eNOS system and human breast cancer. Moreover, Papapetropoulos A et al., showed that in cancer cells, on long term exposure to VEGF proteins, there is Vascular Endothelial Growth Factor (VEGF)-induced eNOS production and consequently increased NO synthesis that stimulates angiogenesis

and vascular permeability [34]. Thus, in the present study, serum NO and eNOS levels showed a significant increase in breast cancer patients compared with control subjects. So, it can be elucidated that elevated NO levels in the patients might be a result of increased eNOS activity and further, NO and eNOS might play a possible role in pathogenesis of breast cancer.

In the present study, when compared with control groups, serum TC, TG, LDL levels were found to be increased in breast cancer cases (for TC, 271±9.16 mg/dL in cases vs 177.58±6.41 mg/dL in controls; for TG, 180.70±8.48 mg/dL in cases vs 107.57±8.98 mg/dL in controls; for LDL, 177.39±6.21 mg/dL in cases vs 98.16±8.90 mg/dL in controls). These differences among case and control groups were statistically highly significant as $p<0.01$ for these parameters. These findings were in accordance with various previous studies. Bani IA et al., found a higher level of serum TC in breast cancer patients prior to surgery [11]. Goodwin PJ et al., examined fasting lipids in females who underwent breast biopsies and discovered that plasma TG concentrations were significantly higher in breast cancer patients compared to patients whose biopsies were negative for proliferative disease [12]. Several studies such as Shah FD et al., and Michalaki V et al., have also shown a significant increase in TG levels of breast cancer patients [16,35]. Capasso I et al., reported that prevalence of increased serum TC and TG levels are increasing in parallel with increasing breast cancer incidence worldwide [13]. Similarly, a previous study by Owiredu WK et al., showed that increased TC and TG levels may be related to increased risk of breast cancer [36]. In accordance with the current findings related to serum LDL, Llaverias G et al., reported that there is greater tumour growth in the high LDL groups in breast of genetic mice models [14]. Also, it is noteworthy that the role of lipids in maintaining cell integrity of cancer cells has been documented by Qadir MI and Malik SA [37]. The present study thereby confirms the occurrence of abnormal lipid profile among women with breast cancer and further, abnormally high lipids might play a possible role in pathogenesis of breast cancer.

In the present study, HDL level did not differ between the breast cancer patients and control group (57.55±6.40 mg/dL in cases vs 57.15±5.35 mg/dL in controls). Also, HDL level did not show any statistically significant difference between the two groups ($p>0.05$). This was in accordance with the studies conducted by Moorman PG et al., Agurs-Collins T et al., and Gaard M et al., [15,38,39]. In the nested case-control study of breast cancer patients conducted by Moorman PG et al., HDL levels were not significantly different between the cases and controls [15]. Also, a study by Agurs-Collins T et al., showed that there was no statistically significant difference in the HDL levels between the breast cancer patients and controls [38]. Similarly, Gaard M et al., showed no association between breast cancer and serum HDL in the Norwegian female population [39].

Thus, in the present study, the authors have demonstrated statistically significant increased serum levels of NO, eNOS and lipid profile parameters such as TC, TG and LDL in breast cancer among Indian women.

Limitation(s)

The present study was based on a small sample size. Yet, the authors have shown some interesting and significant findings that warrant further investigation in a larger study population of Indian women specifically.

CONCLUSION(S)

The present study provides a possible insight into the understudied relationships of biochemical parameters such as NO, eNOS and lipid profile with Indian breast cancer women. It suggests that high serum levels of NO, eNOS, lipid profile parameters such as TC, TG and LDL are associated with breast cancer in Indian women. This adds to the current literature, and expands information available on such an understudied population. Hereby, the authors suggest that increased NO, eNOS and high lipid levels and thereby cholesterol may have a role in pathogenesis of breast cancer. Future epidemiological studies are needed to investigate the relationship between breast cancer and cholesterol so as to have an in-depth understanding of cancer disparities. Further, the estimation of these biochemical parameters can add up to the diagnostic predictability of breast cancer. However, more research is warranted to establish these parameters as a routine diagnostic marker in breast cancer patients.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Biochemistry, Dr. S.C. Government Medical College, Nanded, Maharashtra, India.
2. Assistant Professor, Department of Biochemistry, Dr. V.M. Government Medical College, Solapur, Maharashtra, India.
3. Assistant Professor, Department of Pharmacology, Dr. V.M. Government Medical College, Solapur, Maharashtra, India.
4. Associate Professor, Department of Biochemistry, Saraswati Medical College, Unnao, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Ravi Ramkishan Yadav,
Saraswati Medical College, LIDA, Madhu Vihar, P.O. Asha Khera, NH-27,
Lucknow-Kanpur Highway, Near Nawabganj Toll Plaza,
Unnao-209859, Uttar Pradesh, India.
E-mail: drraviaugust@gmail.com

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