Escalated Oxidative Stress and Estrogen Level Stimulate Invasion of Tumor Cells in Infiltrating Ductal Carcinoma

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ABSTRACT

Breast cancer (CaBr) accredits one of the highest numbers of deaths due to cancer in women on the planet. Severity of the breast cancer is dreaded, mostly due to its invasiveness and expedition of cancer progression. Since, infiltrating ductal carcinoma (IDC) is the commonest type of breast cancer and alone accounts for more than 80% occurrence as compared to other types of breast cancer which requires more focus. Oxidative stress has been implicated to play a pivotal role in cancer development. MDA is a by-product of lipid peroxidation, a chain of reaction initiated by reactive oxygen species (ROS) leading to death of cells. Estrogen has been reported to be causative agent of cancers of epithelial origin including IDC, mullerian epithelial ovarian cancer etc. Thus, MDA and estrogen levels have been selected as biomarker for IDC. MDA assessment in serum and tissue was performed according to the standard protocol. RBC count and haemoglobin level were measured by standard protocol. Estimation of estrogen was performed by ELISA. MDA level in serum of CaBr patients was found to be higher than normal persons with postmenopausal CaBr patients having highest level (p<0.0001) and similar fashioned graph was established while analyzing MDA in tissue with p=0.0493. RBC count was observed to be lower in both premenopausal and postmenopausal CaBr patients than normal person, Haemoglobin level also shows similar level as RBC. It can be concluded that oxidative stress devastate the cells structure and estrogen enhances proliferation which induces the invasion of epithelial cells into stromal cells of breast and begins the formation of IDC.

Keywords: CaBr, Estrogen, IDC, LPO, MDA, Menopausal.

INTRODUCTION

Cancer is a class of diseases also known as malignant tumor, in which a group of cells develop capacity of uncontrolled growth, invasion and sometimes metastasis.1 The etiologies of cancer are diverse, intricate, and only partially understood. Many things are known to intensify the risk of cancer, including tobacco use, dietary factors, certain infections, exposure to radiation, lack of physical activity, obesity, and environmental pollutants. Approximately, 5–10% of cancers are due to genetic mutations inherited from the parents.2 A number of ways can be employed to characterize cancer, including the presence of certain signs and symptoms and screening tests. Breast cancer is one of the commonest causes of death due to cancer, worldwide.3 Some of the risk factors associated with breast cancer are: Menarche and menstrual cycle: Risk of breast cancer increases when a woman starts menstruating early.4 The risk of breast cancer declines by around 5% after every 1 year procrastination in menarche.5 It has been reported that, although menarche is associated with breast cancer risk at all ages, the effect may be stronger in younger woman. Childbearing: When compared with nulliparous women, it was found that there was 25% reduction in risk of breast cancer in women having one full pregnancy at least once in their life time.6,7 Menopause: Woman who experiences menopause at late age are at a higher risk of breast cancer than those who cease menstruating earlier, with risk increasing by about 3% for each year older at menopause.9 Lifestyle: A lack of physical activity has been linked to ~10% of cases.10 There is a relationship between diet and breast cancer, including an increased risk with a high fat diet,11 alcohol intake12 and obesity13 related to higher cholesterol levels.14 Dietary iodine deficiency may also play a role.15 Lipid peroxidation (LPO) is an oxidative disintegration of lipid containing a number of carbon-carbon double bonds (PUFA- polyunsaturated fatty acid).16 A various toxic byproducts are generated during LPO. Reactive oxygen species (ROS) example of byproducts of normal cellular metabolism, primarily in the mitochondria17, which include free radicals such as superoxide anion (O₂⁻), perhydroxyl radical (HO₂⁺), hydroxyl radical (OH•). The breakdown products formed during LPO, mostly aldehydes, such as malondialdehyde (MDA), hexanal, 4-hydroxynonenal, or acrolein have attracted many scientists because they are the most reactive compounds.18 The lipid peroxidation can lead to changes in the permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity.19 Sex hormones are implicated in the development of a variety of human cancer.20 Estrogen acts as a chemical messenger in the body, essential for normal sexual development and functioning of female organs important for childbearing. It is also necessary for normal
development of the breast. Biosynthesis of estrogen is catalyzed by aromatase enzyme by aromatization of circulating androgen like testosterone to form estradiol and estrogen. The chief site for biosynthesis of estrogen is adipose tissue in postmenopausal women and aromatase activity increases with age and body weight. It has been affirmed that estrogen production in breast adipose tissue locally induces breast cancer development in postmenopausal women. Aromatase expression in tumor cells and surrounding breast tissue has been observed to be escalated due to higher production of factors that stimulate the aromatase expression.

Estrogen may be implicated in breast cancer risk because of: (i) its role in stimulating breast cell division (ii) its work during the critical periods of breast cancer growth and development and (iii) its effect on other hormones that stimulate breast cell division.

In current paper, we have attempted to analyze MDA level, RBC count and haemoglobin level in premenopausal and postmenopausal breast cancer patients. Moreover, estrogen level was also evaluated in premenopausal and postmenopausal CaBr patients. Examination of tissue architecture through microscopic study of infiltrating duct carcinoma tissue provides perspicuous information.

**MATERIALS AND METHODS**

Blood samples were collected from 110 breast cancer patients and 65 normal women at pathology department and tissues of 24 non-cancerous persons and 43 breast cancer patients were collected from operation theatre of Mahavir cancer Institute and research centre, Patna with the consent of patients. Part of blood was used for RBC count and haemoglobin level in premenopausal and postmenopausal breast cancer patients. Moreover, estrogen level was also evaluated in premenopausal and postmenopausal CaBr patients. Examination of tissue architecture through microscopic study of infiltrating duct carcinoma tissue provides perspicuous information.

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**Haematological Parameter**

RBC count and Haemoglobin level were estimated by standard procedures using Cell Counter (Medonic M-Series) in the Department of Haematology, Mahavir Cancer Institute, Patna.

**Assessment of MDA**

Serum and tissue- The blood was centrifuged at 3000RPM for 10 minutes and serum was collected and stored at -80°C. Tissues were homogenized in 1M Tris HCl and its extract was used for LPO. TBARS (ThioBarbituric Acid Reactive Substance) level in each breast cancer patient was estimated by standard procedure with slight modifications.

**Estrogens level**

Estimation of estrogen in blood of 39 out of 110 breast cancer patients was conducted with ELISA. 25µl of test serum was added in the wells, followed by 100µl of enzyme conjugate solution and was incubated for 1hr. The wells were washed 3 times with washing buffer and soaked on absorbent paper. 100µl of TMB solution was dispensed in wells and kept for incubation for 30 minutes after shaking gently for 20 seconds. 50µl of stop solution was added to stop the reaction, followed by shaking gently. Yellow colour reaction was taken for optical density reading at 450nm and concentration was analyzed from the standard prepared.

**Histopathological procedure**

Histological parameters were studied by collecting tissues of breast cancer patients from operation theatre. Tissues were fixed in 10% formalin and dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin wax and blocks were prepared. Sections (6µm) were cut and fixed on slide with the help of Mayer’s albumin. Double staining was performed in haematoxyline and eosin and the sections were dehydrated in ascending concentrations of ethanol, cleared in xylene, mounted with DPX and examined under light microscope.

**Statistical analysis**

The data obtained in this paper were represented as mean ± S.D and P value was calculated using one way analysis of variance (ANOVA) and SPSS software package 11.5. P<0.05 was considered statistically significant.

**RESULTS**

Levels of MDA in serum and tissue were usually found to be higher in CaBr patients than normal person. In text fig. 1A, mean±S.D of MDA (nMol/ml) in serum of normal persons (27.34±3.599) was significantly lower as compared to mean MDA in serum of premenopausal (42.39±9.30) and postmenopausal (52.73±13.42) conditions of CaBr patients. Similarly, mean MDA concentration in tissues of normal persons (36.53±5.15) was analyzed to be quite lower than mean MDA in tissues of premenopausal (52.095±5.66) and postmenopausal (125±73.89) conditions of CaBr patients in text figure 1B.

RBC count (thousand/µl) of breast cancer patients seemed to be lower than that of normal. Mean ± S.D of RBC count in normal persons (4.71±0.599) which was higher than premenopausal (4.43±0.50) and postmenopausal (3.60±0.66) conditions of breast cancer patients (Figure 1 C). It is interesting to note that postmenopausal CaBr patients tend to produce more number of RBC when compared to their premenopausal counterpart. It is agreeable to find mean values of haemoglobin (Figure 1D) depicting similar fashioned graph as in mean RBC count (Figure 1C). Mean ± S.D value of haemoglobin (g/dl) of healthy persons was 13.2±1.39 and premenopausal CaBr patients 9.888±1.44 whereas postmenopausal CaBr patients had 10.82±1.78 (Figure 1D).

Estrogen levels were estimated for 39 out of 110 patients of breast cancer, out of which 21 patients belonged to clique of postmenopausal CaBr patients and 18 from...
premenopausal CaBr patients (Figure 1E). Mean estrogen level for 21 postmenopausal patients was calculated to be 12.96pg/ml, out of which 13 patients didn’t produce any estrogen and with few patients producing exceptionally high. On the other hand, mean estrogen for 18 premenopausal CaBr patients were calculated to be 29pg/ml, only six patients produced no estrogen and rest had significant level of estrogen as shown in Figure 1E.

Table 1: Mean ± S.D of MDA levels in serum as well as tissue, RBC count and Haemoglobin level in healthy persons and premenopausal and postmenopausal CaBr patients.

<table>
<thead>
<tr>
<th></th>
<th>Normal persons (Mean ± S.D)</th>
<th>Premenopausal CaBr patients (Mean ± S.D)</th>
<th>Postmenopausal CaBr patients (Mean ± S.D)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA in serum (nMol/ml)</td>
<td>27.34±3.599</td>
<td>42.39±9.30</td>
<td>52.73±13.42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MDA in tissue (nMol/ml)</td>
<td>36.53±5.15</td>
<td>52.095±5.66</td>
<td>125±73.89</td>
<td>&lt;0.0493</td>
</tr>
<tr>
<td>RBC count (million/µl)</td>
<td>4.71±0.599</td>
<td>4.43±0.50</td>
<td>3.60±0.66</td>
<td>&lt;0.0003</td>
</tr>
<tr>
<td>Hemoglobin Level (g/dl)</td>
<td>13.2±1.39</td>
<td>9.888±1.44</td>
<td>10.82±1.78</td>
<td>&lt;0.0002</td>
</tr>
</tbody>
</table>

Figure 1: (A) mean MDA in blood serum, (B) mean MDA levels in tissues, (C) mean RBC count, (D) haemoglobin levels of normal and breast cancer patients of premenopausal and postmenopausal condition and (E) estrogen levels of different cabr patients in premenopausal and postmenopausal condition.

Figure 2: Microphotographs of (A) normal breast tissue at X400 magnification, (B) pleomorphic cells with no tubules formation in infiltrating ductal carcinoma (IDC) at 100X magnification, showing intrusion of stroma, (C) infiltrating ductal carcinoma at 1000X magnification, enlarged and hyperchromatic nuclei (straight black arrow) and pervading cytoplasm with no definite structure of cells (elbow arrow connector), (D) IDC at 1000X magnification, nuclei with apparent nucleoli (NL) and diffusion of cytoplasm (straight arrow).
Histopathological studies done for normal tissue showed normal architecture of the tissue as depicted by the Figure 2A. Section illustrates lobules lined by uniform ductal cells with bland nuclei lined by myoepithelial cells. on the other hand, section of infiltrating ductal carcinoma tissue (Figure 2B) exhibits pleomorphic tumor cells and stroma is prominent and seems to obscure tumor cells, mitotic number of cells is plethora, and no myoepithelial cell lining is seen. When same slide was observed at even higher magnification (X1000), curved arrow connector shows fat tissue evaded by tumor cells in Figure 2C. Moreover, loss of definite structure of cells, pervading of cytoplasm and enlarged nucle are trivial (Figure 2 C & D).

DISCUSSION

There are several etiological factors assigned to be responsible for causing cancer. Healthy lifestyle and nutritional supplements have been promulgated to prevent the onset of cancer. Furthermore, modulation in oxidative stress status can help to mitigate the effect of cancer. The best method to measure the oxidative stress is by estimating the concentration of by-products of free radicals in serum or tissue.\textsuperscript{26} According to a report, oxidative stress causes progression of cancer.\textsuperscript{27} Moreover, oxidative stress has been reported to be elevated in both blood serum and tissue of malignant breast cancer.\textsuperscript{28-30} Our current study correlates the results of MDA obtained from serum and tissue with RBC count, haemoglobin level and estrogen level. Higher MDA level in breast cancer patients signifies higher oxidative stress in serum and tissue (figure 1 A & B). Postmenopausal patients of CaBr tend to generate more MDA level in both blood serum and tissue when compared with premenopausal counterpart. Similar data were obtained in blood serum and meat of turkey.\textsuperscript{31} As it can be inferred from text Figure 2 A & B that MDA level is higher in tissues as compared to MDA in serum of normal, premenopausal and post menopausal CaBr patients. The reason can be attributed to degradation of PUFA from plasma membrane which leads to generation of MDA that gets accumulated mostly in the cellular matrix and some of it is transferred to circulatory blood. It has been proposed that aging is provoked by increase in oxidative damage to biomolecules like lipid, protein or DNA including hassle in developmental process governed by gene regulation.\textsuperscript{32-34} Sex-dependent differences in TBARS level in tissue due to the effect of aging has been reported in liver and brain of Winstar rats.\textsuperscript{35,36}

Committed stem cells that differentiate and multiply through different erythroblast stages are responsible for production of RBCs. Nephrn senses hypoxia, and the kidney responds to it by producing erythropoietin. A tumour oxygenation or erythropoietin dose of regular schedule which is associated with improved survival in patients with various malignancies has been used to treat anaemia in cancer.\textsuperscript{37-40} A report claims, reoxygenation damages cells due to increased oxygen free radicals generation from endothelial cells, parenchymal cells and in filtrating leukocytes\textsuperscript{41,42} and according to Figure 1 C & D, RBC count and haemoglobin level are higher in postmenopausal condition than premenopausal, which means more oxygen is supplied to tissues than in premenopausal condition, this possible reason why postmenopausal breast cancer patients exhibit higher MDA level than premenopausal women. Furthermore, superoxides are produced in reperfused (artificial restoration of blood supply in ischemia or in cancer) tissues due to incomplete electron transfer or reduction of oxygen by damaged mitochondria\textsuperscript{43} and cellular antioxidant defence mechanism is also vitiated. According to a report, Breast cancer erythropoietin survival Trial (BEST) has been devised to determine the effect of Haemoglobin.\textsuperscript{44} RBC count and haemoglobin levels show similar pattern in text Figure 2 C&D, which explicit that RBC count and haemoglobin are directly correlated. Estrogen is synthesized by aromatase which is a member of cytochrome P450 super family, a product of cyp19 gene.\textsuperscript{45} Aromatase is expressed at various sites, including granulosa cells and corpus luteum of ovary in females,\textsuperscript{46,47} adipose tissue of the breast, abdomen, thighs, and buttocks;\textsuperscript{22,48} and various sites in the brain including hypothalamus and hippocampus.\textsuperscript{49,50} The activity of aromatase is believed to show 10 times more in preadipocytes than mature adipocytes and has site specificity with greater activity in preadipocytes. It is well established that estradiol helps in growth and proliferation of mature adipose tissue in postmenopausal women and older men.\textsuperscript{16} Estrogen levels of postmenopausal patients in CaBr patients show lower mean value than premenopausal patients.\textsuperscript{51} Moreover, estrogen effect acts as carcinogen and reinforces severity of CaBr and has direct relationship with menopausal conditions.\textsuperscript{32,53}

As depicted in Figure 2B, microphotograph of Infiltrating Ductal Carcinoma (IDC) contains increased number of nuclei with clearly visible nucleolus. The growth and development of IDC are critically dependent on their stroma. An adequate stromal blood supply is required as stromal connective tissue seems to provide framework for epithelial tissue. Cancer related anaemia signifies low oxygen supply to stromal cells. Oxidative stress along with low oxygenation stimulates disruption of stromal connective tissue through which proliferating parenchyma evade. Mitotic count can be heeded to be significantly elevated in number and hence outnumbering normal cell mitotic count. IDC pertains to uncontrolled proliferation of epithelial cells of breast tissue which is induced by the effect of estrogen surge. Estrogen has been indicated to be responsible for growth of epithelial cells of breast tissue and ovarian surface in women.\textsuperscript{54,55} Moreover, microphotographs of IDC at X1000 magnification (Figure 2D) discloses structure of cells in IDC tissue unambiguously. Due to higher oxidative stress, tumor cells have lost fluidity of plasma membrane and hence, their structure has become irregular and deformed. Tumor cells are seen to be evading into fat
tissue as shown in figure 1C, and cytosol is observed to be pervading throughout tumor area.

CONCLUSION

Despite of innumerable work on etiology of breast cancer, oxidative stress and estrogen still proves to play a key role in breast cancer development. Oxidative stress stimulates loosening of stromal cells through which proliferating epithelial cells induced by estrogen infiltrate. Hence, MDA and estrogen synergistically enhance the growth of Infiltrating Ductal carcinoma.

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