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Original Work

Role of endogenous hormones in premenopausal females with breast carcinoma - a pilot study in north Indian population

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ABSTRACT: The objective of this study is to evaluate hormone levels in premenopausal females with breast cancer at the time of diagnosis and to predict their role as a risk factor of breast cancer in females. Circulating hormone levels were measured in 345 previously untreated premenopausal breast cancer patients during luteal phase (19th-21st day). Their results were compared with a group of 345 age-matched healthy controls. Serum prolactin, testosterone, estrogen and progesterone levels were higher in patients as compared to controls. Regression analysis showed that changes in hormone levels have significant impact on breast cancer. Female patients with breast cancer have abnormalities in hormone levels. These abnormalities may be considered in the pathogenesis of the disease and should be taken into account for the treatment of patients of breast cancer. Reduction of hormone levels might prove to be helpful in preventing breast cancer but further studies are required to prove the benefit of analyzing hormone levels at an early stage.

KEY WORDS: ***Breast cancer; Prolactin; Testosterone; Estrogen; Progesterone***

INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled cell division leading to growth of abnormal tissue. Breast carcinoma is the most common malignancy and most common cause of cancer deaths among women worldwide. It originates from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. It occurs more commonly in developed countries, accounting for 3-5% of deaths while it is 1-3% in the developing countries[1].

Various etiological factors implicated are Caucasian race, family history of first degree relative with breast carcinoma, breast cancer in family members before 40 yrs of age, prior breast disease and mutation of BRCA1 and BRCA2 gene[1]. Post menopausal females on hormone replacement therapy (HRT), radiation exposure, increased fat intake, moderate to heavy alcohol intake, obesity and less breast feeding are also risk factors for breast cancer[2].

Prolactin, estrogen, progesterone, growth hormone and corticosteroids are the main hormones responsible in the development of breast cancer[3].

Prolactin affects cellular growth, angiogenesis, proliferation and differentiation of breast tissue and initiation and maintenance of milk production after priming of breast tissue by estrogen and progesterone. In conjunction with estrogens and progesterone, prolactin also leads to full lobulo-alveolar development of the breasts. Further, prolactin may act as an autocrine/paracrine factor within mammary tissue[4].

Several studies have demonstrated that prolactin mRNA is produced in normal human breast epithelium and that breast cancer cells can

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synthesize appreciable quantities of prolactin *in vitro*. Little is known about the relationship between blood and breast tissue prolactin concentrations; however prolactin staining in breast tumors has been significantly correlated with plasma prolactin concentrations at the time of diagnosis. It has also been suggested that prolactin is associated with higher cellular motility and angiogenesis[4].

The breast cancer cells also produce prolactin locally. Clevenger *et al* studied the role of prolactin and prolactin-receptors in mammary carcinoma and found positive correlation between elevated levels of serum prolactin and carcinoma breast. *In vitro* studies have also indicated that antiprolactin agents can block human breast cell lines[5]. Significant association of serum prolactin levels and breast cancer risk was also observed by Tworoger *et al*[6].

The prolactin receptor is detectable by immunohistochemical staining in both normal and malignant breast tissues. A study by Manjer and associates observed no association between prolactin levels and risk of breast cancer in premenopausal and postmenopausal women[7].

Estrogens produce duct growth in the breasts and are largely responsible for breast enlargement at puberty in girls; they have been called the growth hormones of the breast. They are responsible for the pigmentation of the areolas, although pigmentation usually becomes more intense during the first pregnancy than it does at puberty[8-9].

A number of prospective studies in postmenopausal women have shown that increased probability (two to five times) of breast cancer diagnosis later in life is associated with slightly higher levels of estrogens. Prolonged exposure to higher endogenous estrogens (eg. as in women with early menarche and late menopause) is known to be associated with increased probability of breast cancer and oophorectomy at an early age decreases this risk. It has been suggested that breast tumor stromal cells may indeed contribute to estrogen production. These small increases in estrogen levels may, therefore, be more likely a consequence of the 'spill-over' of these locally-produced estrogens into the circulation. Not much literature is available for premenopausal women because of discrepancy of serum estrogen levels during different phases of menstrual cycle[8].

Progesterone stimulates the development of lobules and alveoli in the breast. It acts through progesterone receptors (PR) and induces the differentiation of estrogen-prepared ductal tissue and supports the secretory function of the breast during lactation[8,10,11]. Effect of exogenous progesterones on the breast can be proliferative or anti-proliferative depending on the type, dosage and duration of exposure. Basically, short bursts of progesterone or progestin tend to stimulate breast cell proliferation, whereas long duration, high dose progesterone or progestins tend to down-regulate the breast[10,11].

The effects of progestins on cell proliferation in *in vivo* studies in patients with breast cancer are very limited. Most of the data were observed after a combined treatment of estrogens plus progestins. In one study progesterone alone was administered in patients with breast cancer, where a decrease in growth was found in four of six tumors; however, in the other two there was stimulation of growth. The same authors reported that in a combined treatment with estradiol and progesterone, growth increased in four of seven cases at low doses, but treatment with 10–100-fold higher concentrations of both hormones invariably led to a decrease in proliferation[11]. Immaculata and coworkers observed positive correlation between progesterone receptors (PR) and risk of breast carcinoma[12].

The literature available for the impact of progesterone on breast cancer is contradictory; some reports suggest it inhibits the breast cancer cells others have reported the stimulation of breast cancer cells and a few studies have reported no effect of progesterone on the proliferation of breast cancer cell lines[10,11].

Testosterone levels have been found to be low in females. Studies have shown that elevated androgen levels may be related to increased incidence of breast cancer and the risk of cancer is three times greater in females with higher concentration of testosterone[13].

A prospective study reported an association between breast cancer and premenopausal levels of testosterone, androstenedione, and DHEAS. For premenopausal women, the association with circulating androgens appears to be stronger than the data for circulating estrogens, but it is not known how much of the observed differences are due to measurement issues, since androgens can be more reliably measured and have less variation according to the menstrual cycle than estrogens. It is also not known whether androgens independently influence breast cancer risk or simply provide additional substrate at the tissue level for conversion into estrogens; androstenedione can be converted directly by aromatization into estradiol[9,13].

Hormones play a key role in the normal physiological development of breast tissue and breast cancer is usually present for many years (as long as 5–10 years) before it is clinically diagnosed (theory of the 'dormant malignant cell'). This implies that breast cancer cells, during their subclinical period, are likely to have been exposed for a considerable time to endogenous and exogenous sex hormones[3,14]. Breast cancer is generally more aggressive in the premenopausal females with higher production of angiogenic growth factors, a higher proliferation rate, and a

higher degree of lymph node involvement and lymphovascular invasion[15].

Endogenous hormonal milieu predicts the chances of development of breast cancer in females. The hormonal environment is markedly different in premenopausal and postmenopausal women. Studies are available suggesting the role of serum hormones in postmenopausal females in Indian population but not much data is available regarding the role of hormones in premenopausal females in Indian population. The problem in premenopausal women is that their hormone levels fluctuate due to physiological variations during the menstrual cycle whereas in the postmenopausal patients, levels are rather constant, and tend only to vary over long time spans[14]. So, we planned to evaluate the role of endogenous hormones in premenopausal females with breast cancer during luteal phase (19th-21st day) of menstrual cycle.

METHODOLOGY

The present study was conducted in the Department of Biochemistry in collaboration with Department of Radiotherapy, Pt. B. D. Sharma University of Health Sciences, Rohtak. The patients were randomly divided into two groups; three hundred and forty five females with breast carcinoma at the time of diagnosis were taken as the study group. These patients were subjected to various routine investigations along with serum prolactin, testosterone, estrogen and progesterone levels during the luteal phase of the menstrual cycle (19th-21st day). Their results were compared with a group of three hundred and forty five age matched healthy controls (Group B). Informed consent was taken from all patients and volunteers. Females on oral contraceptive pills / hormone therapy or drugs affecting hormone levels were excluded from the study, females with BMI ≥ 30 Kg/m2 were also excluded. Staging of breast carcinoma was done according to TNM staging.

Five ml of venous blood was collected aseptically from antecubital vein. Serum was separated by centrifugation (2000 rpm for 15 minutes) and subjected to analysis of hormone levels measured using direct chemiluminescent technology by ADVIA Centaur CP.

Prolactin (PRL)[16]

The ADVIA Centaur CP Prolactin assay is a two-site sandwich immunoassay using direct chemiluminometric technology, which uses constant amounts of two antibodies. The first antibody, in the Lite Reagent, is a polyclonal goat anti-prolactin antibody labeled with acridinium ester. The second antibody, in the Solid Phase, is a monoclonal mouse anti-prolactin antibody, which is covalently coupled to paramagnetic particles. Normal range: Premenopausal females = 59.36-619.04 µIU/mL

Testosterone (TSTO)[16]

Testosterone assay is a competitive immunoassay using direct chemiluminescent technology. Testosterone in the patient sample competes with acridinium ester-labeled testosterone in the Lite Reagent for a limited amount of polyclonal rabbit antitestosterone antibody bound to monoclonal mouse anti-rabbit antibody, which is coupled to paramagnetic particles in the Solid Phase. The assay uses Testosterone Releasing Agent to release bound testosterone from the endogenous binding proteins in the sample. Normal range: Testosterone in females = 0.49-2.64 nmol/L

Estradiol-6III (E2-6III)[16]

Estradiol-6 III assay is a competitive immunoassay using direct chemiluminescent technology that derives its name from the coupling of the estradiol immunogen at the specificity-enhancing sixth position, allowing for the production of a highly specific antibody. This 17β-estradiol-6-antibody allows the ADVIA Centaur CP Estradiol-6 III assay to be used across a wide range of applications. Estradiol in the patient sample competes with acridinium ester-labeled estradiol in the Lite Reagent for a limited amount of rabbit antiestradiol antibody in the Antibody Reagent. Rabbit anti-estradiol is captured by mouse antirabbit IgG, which is coupled to paramagnetic particles in the Solid Phase. Normal range: Estradiol during luteal Phase = 82.21–939.59 pmol/L

Progesterone (PRGE)[16]

The ADVIA Centaur CP Progesterone assay is a competitive immunoassay using direct chemiluminescent technology. Progesterone in the patient sample binds to an acridinium ester-labeled mouse monoclonal anti-progesterone antibody in the Lite Reagent. Unbound antibody binds to a progesterone derivative, covalently coupled to paramagnetic particles in the Solid Phase. Normal range: Progesterone levels during luteal phase= 3.34−10.62 nmol/L

RESULT

Values are expressed as Mean ± S.E. Statistical analysis was carried out using SPSS for Windows 15.0 software (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard error of mean. The p value was calculated using unpaired ‘t’ test with equal variance. The mean age of patients was 42.56 ± 4.46 years while

the mean age of controls was 41.57 ± 2.49 years (**Table 1**).

Regression analysis was done and unstandardized coefficient (B) was calculated to predict the dependent variable from the independent variable. On individual calculation of standardized beta coefficient by linear regression analysis, serum prolactin had beta coefficient of .725 which suggests for every unit increase in breast cancer lesion a .725 unit increase in prolactin occurs holding all other variables constant. Regression analysis showed that all the values had high significance (0.000) except for serum progesterone which is significant (0.016) which suggests that change in serum hormone levels has significant impact on occurrence of breast cancer (**Table 2**).

Sensitivity and specificity of each individual hormone at a particular cut off point has been shown in **Table 3**. Prolactin was found to be most sensitive as screening test.

Table 1: Serum hormone levels in patients with breast carcinoma (Values are expressed as mean ± S.E)

Parameter	Breast cancer patients (n=345)	Healthy controls (n=345)	p Value
Estrogen (pmol/L)	585.48 ± 27.23	247.29 ± 9.84	<0.000*
Progesterone (nmol/L)	79.19 ± 6.13	40.48 ± 4.56	<0.000*
Testosterone (nmol/L)	1.82 ± 0.09	0.88 ± 0.07	<0.000*
Prolactin (μIU/mL)	767.92 ± 18.46	312.38 ± 17.18	<0.000*

*Very highly significant

Table 2: Regression analysis

	Unstandardized coefficients		Standardized coefficients	Significance
	B	S. Error	Beta	
Estrogen	.023	.000	.497	.000*
Progesterone	.004	.002	.075	.016**
Testosterone	.014	.001	.386	.000*
Prolactin	.037	.001	.725	.000*

*Very highly significant; **Significant

Table 3: Sensitivity and specificity at a particular cut-off point

Parameter	Sensitivity	Specificity	Cut-off point
Testosterone	0.717	0.705	1.15 nmol/L
Estrogen	0.891	0.876	436.06 pmol/L
Progesterone	0.719	0.773	64.46 nmol/L
Prolactin	.923	.914	435.49 μIU/mL

DISCUSSION

Of all breast cancer cases, family history of breast cancer was present in 16%. 28% of females with breast cancer belonged to the high-income group. Increased incidence in females of high-income group is probably because of lower birth rate (parity <3), possible over-detection and over-treatment because of better access to breast cancer screening and the postulation of as yet unexplained lifestyle and dietary factors correlated with higher income[1].

Females with sedentary lifestyles are more prone to breast cancer. In our study we found that 72% of females had a sedentary lifestyle. The effect of physical activity on incidence of breast cancer is mediated by an increased capacity for glucose transport into the muscle and adipose tissue in response to insulin stimulation. Lack of exercise results in decreased insulin sensitivity and increased insulin concentration. The overabundance of insulin, called hyperinsulemia, amplifies the bioavailability of IGF-I. IGF-I and insulin together have been shown to stimulate

motility in human breast cancer cell lines, an effect that could enhance migration and invasion. IGF-I also promotes angiogenesis in breast tissue[17].

The association between breast cancer and hormone replacement therapy has been well documented but the role of endogenous sex steroids in causing breast cancer is not well known and could be an important avenue of investigation in the general population to determine the risk of breast cancer and in treatment, once cancer has been detected. Therefore, the present study was planned to determine hormone levels in premenopausal patients at the time of diagnosis and these levels were compared with controls.

In the present study we found that serum estrogen levels in breast cancer patients were significantly high as compared to healthy controls. Our study supports the common opinion that estrogens are detrimental in breast cancer[4].

Obesity has been associated with increased risk of breast cancer in postmenopausal females because of peripheral aromatization of testosterone to estrogen in fat tissue[4].

Serum estrogen enters the cell by free diffusion and its intracellular concentration is enhanced in those organs and tissues that express estrogen receptors (ERs). Breast tissue expresses estrogen receptors so increase in serum estrogen leads to increase in estrogen levels in breast tissue and enhances breast tumor development[10]. Estrogen is also produced locally in breast tissue by enzyme aromatase cytochrome 450[18]. Estrogen formed in breast tissue and after entering the cell through estrogen receptor increases the chances of cancer by promoting tumor proliferation, by influencing the expression and transcription of growth factors, by activation of proto-oncogenes and oncogenes (e.g. c-fos, c-myc), nuclear proteins and other factors involved in the physiological cell cycle. Estrogens and some of their metabolites may also be directly genotoxic[12,18].

In our study we found serum progesterone levels in patients were raised as compared to controls. Exogenous progestins have been shown to induce growth factors (EGF, TGF-β), to stimulate the expression of the growth factors and growth factor receptor (EGFR) protein and its mRNA but there is paucity of literature regarding the relationship of breast cancer and serum progesterone levels in female patients[12].

Exogenous progestins also increase the expression of proto-oncogenes, such as c-myc, c-fos, and c-jun. A sequence sharing a great homology with the progesterone response element (PRE) has recently been identified in the 5′ flanking region of the human c-myc gene. This positive progesterone regulatory region confers progestin responsiveness in PR-rich T-47D cells, but not in PR-negative MDA-MB-231 cells. The expression of the tumor-suppressor protein p53 is also decreased by progestins in T-47D cells which can contribute to the stimulatory activity. Endogenous progesterone might lead to breast cancer through the same mechanism but exact pathogenesis for this is yet to be identified[12,18].

Clinical studies have provided conflicting results when looking for a clear correlation between testosterone blood levels and breast cancer in postmenopausal women[19,20]. In one study testosterone levels were shown to decrease the risk of breast cancer when conventional hormone therapy (i.e. estrogen and progesterone) included testosterone[21]. Another study looked at androgen receptor antagonist in primates and concluded that endogenous androgens (such as testosterone) inhibit mammary proliferation, thus potentially decreasing its impact on breast cancer[21].

In our study we found serum testosterone levels in breast cancer patients were high as compared to controls. Normal mammary tissue contains aromatase cytochrome P450 enzyme that catalyzes the conversion of androgens into estrogens thus increasing the estrogen level, which would increase the risk of breast cancer as explained earlier[18].

Prolactin levels were significantly higher in patients as compared to controls and serum prolactin had highest sensitivity and specificity in comparison to other hormones. The prolactin receptor may be associated with the estrogen (ER) and progesterone (PR) receptor expression. Several *in vitro* studies have reported that long-term prolactin or estrogen exposure can increase both prolactin receptor and ER expression. Increased prolactin positivity has been significantly associated with increased tumor size, higher stage, nodal involvement, and a worse overall survival in univariate analyses[22-24].

Prolactin may lead to breast cancer by promoting cell proliferation and growth by altering the expression of cyclin D1, an important cell cycle regulator, increasing cell motility, and supporting tumor vascularization. It has a mitogenic action in breast cells[4]. Gutzman and colleagues studied breast cancer cell line which was induced to produce high levels of prolactin, proliferation of breast cancer cells was increased 1.5 times as compared to non-induced cells; this effect was magnified by the addition of estradiol, suggesting that prolactin could increase the responsiveness of cells to estrogenic effects[13].

Prolactin also appears to inhibit apoptosis of breast cancer cell lines. One study reported that endogenous prolactin expression in breast cancer cell lines was negatively correlated with C2-ceramide induced apoptosis. Introduction of a prolactin antibody was associated with a three to sevenfold increase in cell death in the same model system[23].

Rose and coworkers studied the role of prolactin in mice mammary tissue. They found that 65 to 80%

of transgenic mice with constant prolactin expression in the mammary epithelium developed mammary carcinomas compared to 5% of nontransgenic controls. Transgene expression was associated with an increased percentage of cells in the S-phase at age 6 months and the end of life. Similarly, transgenic mice over-expressing prolactin had all developed mammary carcinomas by age 11-15 months, while controls had normal mammary tissue only. Mice injected with high prolactin expression cell cultures had a 2–4 times faster tumor growth rate and an increased number of metastases compared to controls[24].

Prolactin has also been reported to stimulate capillary formation in a chick chorioallantoic membrane assay system and up-regulate vascular endothelial growth factor, suggesting that prolactin may be involved in tumor vascularization[23].

A better understanding of the autocrine/paracrine actions of prolactin in the breast may help to characterize pathways through which prolactin influences breast cancer cells. Recent evidence of crosstalk between estradiol and prolactin in activating AP-1 activity, a factor that promotes many carcinogenic processes, suggests that additional research concerning the potential inter-relationship between the prolactin and estrogen is warranted[23].

Premenopausal females with breast cancer have abnormalities in hormone levels. These abnormalities may be important in the pathogenesis of the disease. Reduction of hormone levels might prove to be helpful in preventing breast cancer. Evaluating hormone levels might also be helpful to screen for risk of breast cancer in the general population. This might also be helpful to delay the onset of cancer by normalizing the levels of these hormones and in deciding the treatment modality for patients once breast cancer has been diagnosed, but the sample size in our study was small so further long-term prospective research is necessary to confirm our findings.

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