

A Survey of the Relationship between Serum Testosterone Level and Expressions of Androgen, Progesterone and Estrogen Receptors and HER2 in Iranian Women with Breast Cancer

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Abstract

Background: Breast cancer is the most common cause of cancer in women. This tumor is hormone dependent tumor and oncologists use estrogen receptor (ER), progesterone receptor (PR) and HER2 for treatment of this malignancy. Androgens like testosterone and their receptors (AR) have a role in the pathophysiology of breast cancer but their clinical values are not clear.

Method: AR expression was evaluated in 49 patients with breast cancers using immunohistochemistry. Testosterone was evaluated with ELISA. The relation of clinical characteristics and biomarkers with AR and testosterone were analyzed. According to the percentage of stained cells AR categorized to: AR-absent (0%), AR-poorly (1%-10%), AR-moderately (>10%-50%), and AR-highly (>50%) positive.

Results: Among 49 patients with breast cancer, 34% were AR-positive and 44% of ER-positive and 22% of ER negative patients were AR-positive. There was no significant association between mean of testosterone and AR, ER, PR and HER2. AR was positive more frequently but not significantly statistically in older patients and patients less than 45 years of age. Testosterone level was higher in ER positive patients than ER negative and lower in AR positive patients than AR negative patients, but these findings were not statistically significant. ten percent of breast cancers were triple negative and AR was negative in all of them.

Conclusion: Androgens and AR have role in pathophysiology of breast cancer and in the future one can use the potency of this pathway for the treatment of breast cancer.

Keywords: Testosterone, AR, ER, PR, Triple negative, Breast cancer

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Introduction

Breast cancer is the clonal proliferation of epithelial cells, lining ducts and lobules of the breast. This cancer is the most common cancer in women with the exception of skin cancer. Approximately 230,000 new cases of breast cancer and 40,000 breast cancer deaths have occurred in 2013 among women in the US.¹ Breast cancer is a hormone dependent neoplasm. Hormones and their receptors have been used to treat breast cancer for many years.² The effect of ovarian function and its main hormone, estrogen, on breast cancer development has been considered after regression of cancer following oophorectomy. A higher dose and prolonged exposure of estrogen is a major risk factor for breast cancer. Women with increased numbers of estrogen receptors (ER) and higher levels of estrogen will develop more ER positive breast cancer in the future. The most known carcinogenic mechanism of estrogen is via its ER and its direct and indirect effects on cell proliferation by stimulation of growth hormone production. In addition, estrogen can play a role in non-ER related and genotoxic mechanisms.³ Androgens are the dominant sex steroid hormones in women because they are secreted from both the adrenals and ovaries. The concentration of androgens is higher than estrogen during the menstrual cycle and similar to estrogen in the pre-ovulatory peak. Although secretion of both androgens and estrogens decrease after menopause, however secretion of androgens is proportionally higher than estrogens in this period. Hence, the importance of androgens is clear. In post-menopausal women the aromatization of androgen to estrogen in peripheral tissue, especially adipose tissue, is the main provider of circulating estrogen. There is a strong association between circulatory testosterone (as a main, powerful androgen) and estradiol in developing breast cancer, particularly ER positive tumors. Testosterone level is also associated with a higher recurrence of breast cancer.⁴ The androgen receptor (AR) is a member of a nuclear hormone family receptor which is expressed by 70% to 90% of breast cancers. This rate of expression is higher than ER (70% to 80%) and PR (50% to 60%). The effect and function of AR in breast cancer in several studies is controversial but it seems that AR expression is associated with smaller tumor size, negative lymph node metastasis and

low histological grade. Thus the effect of AR in breast cancer development is inhibitory.⁵ Androgens can inhibit the growth of breast cancer via an AR-mediated genomic action. In addition they can decrease mRNA and protein of ER in breast cancer cells. In another way AR can inhibit ER α activity by direct binding to it.⁶ Androgens may be controllers of breast proliferation. Because of the high rate of expression and protective effect of AR in breast cancer cells, it seems this receptor can be a target for therapy in the future.

HER2, an epidermal growth factor receptor 2 belongs to the epidermal growth family receptor (EGFR). It plays a role in growth and differentiation of epithelial cells and probably angiogenesis. As a prognostic factor, HER2 expression is association with disease recurrence and death in the absence of treatment. Androgens can increase the expression of EGFR and they are markers of poor prognosis.

Approximately 20% of human breast cancers are triple negative. These tumors are associated with larger size, higher grade, pushing margins, increased rate of recurrence, distant metastasis and decreased survival.⁷ About 40% of triple negative breast cancers (TNBC) are AR positive. Expression of AR is associated with lower histologic grade, recurrence and distant metastasis.^{7,8} AR can be a unique choice for treatment of AR positive TNBC. Triple negative breast cancers are an obscure point in treatment of breast cancer.

Because the high rate of AR expression and the effect of this receptor and its ligand in development of breast cancer, the association of AR with good prognosis makes this receptor an attractive choice for treatment of breast cancer in the future. However testosterone, as a potent androgen and ligand of AR, is associated with poor prognosis. Hence there is controversy in the function of AR and its ligand testosterone. This adverse effect may be due to the peripheral alteration of androgens to estrogens by aromatase.

Due to the importance of androgens and the possible effect of the androgen/AR pathway in TNBC, we have designed a study to determine if there is a relationship between AR, testosterone (as a ligand of AR and potent androgen), ER, progesterone receptor (PR) and HER2.

The results of this study can be helpful in designing the best way to use the potency of the androgen system as a treatment for breast cancer.

Materials and Methods

This was a cross-sectional study that included 49 pathologically confirmed breast cancers in the Department of Pathology at Shiraz University of Medical Sciences during one year (Aban, 1391 until Aban, 1392). All patients underwent mastectomy versus lumpectomy. Age and menopausal status were evaluated as clinical parameters. Hormonal therapy and previous chemotherapy were considered exclusion criteria.

Histopathological examination and subsequent immunohistochemical (IHC) studies were performed for biomarkers AR (Biogenex; Netherlands, F39.4.1), ER (Dako; Denmark, 1:50), PR (Dako; Denmark, 1:50), and HER2 (Dako; Denmark, 1:50). Cases were labelled as positive for ER and PR when >10% of tumor cells expressed the marker. In order to further analyze the association between AR and testosterone levels, we classified AR expression into four categories: AR-absent (0%; 0), AR-poor ($\geq 1\%$ to 10%; 1+), AR-moderate (10% to 50%; 2+), and AR-high (>50%; 3+) positive.

The strength of HER2 staining was classified as 0 to 3+ where 0 indicated no or fewer than 10% of cells that stained; 1+: faint/barely perceptible membrane staining detected in >10% of tumor cells; 2+: weak-moderate complete membrane staining in >10% of the malignant cells; and 3+: strong complete membrane staining in >10% of tumor cells. We defined 1+, 2+ and 3+ as HER2-positive.

We used 4 μ m paraffin sections for IHC. Citrate buffer was used for antigen retrieval. Primary mouse monoclonal antibody was incubated overnight at room temperature with diaminobenzidine (DAB) as the chromogen. We considered tumor cells to be positive when they expressed nuclear ER, AR and PR receptors and cytoplasmic HER2.

The association of various factors such as age (<45 and >45 years); menopause (pre/post); IHC expressions of three steroid receptors (SRs) AR, ER and PR (-/+) and growth factor (GF) as HER2 in tumor cells was assessed by the chi-square test. The associations between testosterone and receptors were analyzed by the chi-square and Mann-Whitney tests. Co-expression patterns between SRs and GFs receptors were analyzed by chi-square.

Results

We analyzed clinical characteristics and cell markers in a group of 49 Iranian women with an age range of 30-75 years. The mean age was 52 years. The majority of patients were above 45 (70%) years of age ($P < 0.05$).

Most patients were in the postmenopausal group (54%); the others were not in menopause (46%; $P > 0.05$).

The mean testosterone level was 0.44 ± 0.61 ng/ml and median level was 0.26 ng/ml with a normal range of 0.26 to 1.22 ng/ml. Mean testosterone level in most (91%) cases was below 0.75 ng/ml ($P < 0.05$). Over 50% of patients had testosterone levels lower than 0.26, the lower limit of normal. The minimum testosterone level was 0.1 ng/ml and maximum level was 3.76 ng/ml.

AR was expressed in 34% of tumor cells; 66% were AR-absent ($P > 0.05$). There were 10% of patients with poor AR expression, 10% who had moderate expression and 14% with highly positive AR expression.

A total of 73% of patients were ER positive and 26% were ER negative ($P < 0.05$).

PR was positive in 59% of patients and negative in 41% ($P > 0.05$).

HER2 was negative in 46% of patients and the numbers of positive cases were as follows: 1+ (20%), 2+ (18%) and 3+ (16%). Thus only 16% of cases were suitable for clinical use ($P < 0.05$).

Analysis of data showed that in the over 45 year age group, 58% ($P > 0.05$) of patients were AR negative and only 22% were highly positive (Table 1). In patients less than 45 years, 76% ($P < 0.05$) were AR negative and there were no highly positive patients (Table 1). Most tumors in the younger patients were AR negative. We observed no significant association between age and AR status because in both groups (>45 and <45 years) the majority of patients were AR negative ($P > 0.05$).

In patients less than 45 years, 78% were ER positive ($P < 0.05$) and over age 45, 69% were ER positive ($P < 0.05$). There was no significant association between age and ER status because most patients in both age groups were ER positive ($P > 0.05$).

There were 57% of cases in both age groups who were PR positive, so there was no significant association between age and PR status ($P > 0.05$).

There were 50% HER2 negative cases in the under 45 year old age group and 14% were 3+. A total of 43%

Table 1. Androgen receptor (AR) expression and characteristics of breast cancer in Iranian women.

	AR expression							
	Absent		Poor		Moderate		High	
	No	%	No	%	No	%	No	%
Age (years)								
<45	13	26	2	4	2	4	0	0
>45	19	38	3	6	3	6	7	14
Menopause								
Pre	15	30	3	6	2	4	3	6
Post	16	32	3	6	2	4	5	10
Estrogen receptor (ER)								
Negative	10	20	0	0	2	4	1	2
Positive	20	40	5	10	4	8	7	14
Progesterone receptor (PR)								
Negative	14	28	0	0	3	6	3	6
Positive	16	32	4	8	3	6	6	12
HER2								
Negative	12	24	3	6	0	0	5	10
Positive	17	34	2	4	4	8	5	10

of cases over the age of 45 years were HER2 negative with 18% that were 3+. We observed no significant association between age and HER2 status ($P>0.05$). In both age groups, there were a low number of 3+ HER2 patients for which pharmacological treatment was available ($P<0.05$).

In the postmenopausal group, 61% of patients were AR negative; in non-menopausal patients, 65% were AR negative. There was no significant association between menopause and AR status ($P>0.05$).

There were 72% of postmenopausal patients who were ER positive ($P<0.05$); in non-menopausal patients 71% were ER positive ($P<0.05$). There was no significant association between menopause and ER status ($P>0.05$).

A total of 56% of postmenopausal patients were PR positive ($P>0.05$) whereas 57% of non-menopausal patients were PR positive ($P>0.05$). There was no significant association between menopause and PR status ($P>0.05$).

In postmenopausal patients, 37% were HER2 negative ($P>0.05$) and in non-menopausal patients 52% were HER2 negative ($P>0.05$). We observed that 25% of postmenopausal patients were 3+ positive ($P<0.05$) whereas only 9% of non-menopausal patients were 3+ positive for HER2 ($P<0.05$). Only this group (3+) benefits from treatment via HER2 by trastuzumab. There was no significant association between

menopause and HER2 status ($P>0.05$).

Testosterone level in 48% of patients with age more than 45 y/o was normal and high, and also testosterone level in 42% of patients less than 45y/o was normal and high but these differences were not significant. There was no significant association between testosterone level and menopause ($P>0.05$).

The mean testosterone level in AR negative patients was 0.44 and the median was 0.26 ng/ml. AR positive patients had a mean testosterone level of 0.36 and a median level of 0.26 ng/ml. Hence there was no significant association between AR and testosterone level ($P>0.05$; Table 2).

The mean testosterone level in ER negative patients was 0.26 ng/ml; in ER positive patients it was 0.50 ng/ml, thus there was no significant association between ER and testosterone level ($P>0.05$).

In the ER positive group, women in the AR negative subset showed higher testosterone levels than women in the AR positive subset but this difference was not significant ($P>0.05$; Table 2).

AR positive women in the ER negative group had insignificantly higher testosterone levels compared to women in the AR negative subset ($P>0.05$; Table 2).

Mean testosterone level in PR negative patients was 0.36 ng/ml and 0.49 ng/ml in PR positive patients. There was no significant association between PR and testosterone level ($P>0.05$).

There were 44% of ER positive patients who were also AR positive, which was not significant ($P>0.05$). In addition, 46% of PR positive patients were AR positive, which was also not significant ($P>0.05$; Table 1). In the ER negative group, 22% of patients were AR positive.

Of HER2 positive patients 40% were AR positive, which was not significant ($P>0.05$; Table 1).

Amongst ER negative patients, age was not significantly associated with AR expression ($P>0.05$), nor was there a significant association in HER2 negative in terms of age and AR expression ($P>0.05$).

In this study 10% of patients were triple negative. Both mean and median of testosterone level in triple negative patients were 0.28 ng/ml. There was no significant difference between mean testosterone level in triple negative patients and mean of testosterone in the others patients ($P>0.05$). All triple negative patients were negative for AR.

The mean age in triple negative patients was 46 years whereas for others it was 52 years. Non-menopausal women comprised 80% of triple negative cases.

Table 1 shows the association between tumor characteristics, age, menopause and AR expression. Table 2 shows the association between serum testosterone according to AR and ER expression in women with breast cancer.

Discussion

Breast cancer is a hormone dependent malignancy where the roles of estrogen, progesterone and their receptors in its pathophysiology and treatment have previously been described. Androgens and their receptors (AR) also have an important role in development of breast cancers. Androgen receptors present in most malignant breast cell lines but the pathophysiology is not well documented. Using the potency of androgens in treatment of breast cancer, especially in triple negative cancer is a goal of numerous studies.

In this pilot study, for the first time, we evaluated patient (age, menopause) and tumor (ER, PR, HER2, AR) characteristics in 49 Iranian women with breast cancer. We also evaluated the relationship between AR, testosterone and other receptors to determine the role of AR in the pathophysiology of breast cancer.

Table 2. Association of serum testosterone, androgen (AR) and estrogen receptor (ER) expression in women with breast cancer.

Patients	No.	Testosterone	
		%	mean(ng/ml)
All patients			
AR expression			
Absent	30	61	0.434
Poor	5	10	0.280
Moderate	5	10	0.470
High	9	18	0.156
ER-positive			
AR expression			
Negative	20	40	0.523
Positive	16	32	0.380
ER-negative			
AR expression			
Negative	10	20	0.255
Positive	3	6	0.270

Ogawa et al. evaluated AR in 227 primary breast cancer cases. The AR was positive in 62% of patients.⁸ Secreto et al. evaluated 592 post-menopausal patients and observed that AR was positive in 85%. They also evaluated ER and PR and they reported that 90% of ER-positive tumors were AR positive and also 55% of ER-negative tumors were AR positive.⁹ Agoff et al. evaluated 88 patients with breast cancers and observed that approximately 60% were AR positive. They divided the patients into two groups, ER positive and ER negative. A total of 89% of ER positive breast cancers were AR positive whereas only 49% of ER negative cancers were AR positive. The researchers described an association between ER and AR expression.¹⁰ In our study AR was positive in 34%. After we divided the patients into two groups, ER positive and ER negative, we noted that 44% of ER positive patients and 22% of ER negative patients expressed AR. As with other studies we concluded that ER positive tumors expressed AR more than ER negative tumors. We believe there is a type of association between AR and ER expressions. This co-expression may play a role in cell proliferation. However further studies are necessary to find the exact molecular basis of this relationship and modulating that, as a target of treatment. The lower expression of AR in the current study was probably race dependent.

We observed higher testosterone levels in ER positive patients compared to ER negative cases. There were also higher testosterone levels in AR negative

patients compared to AR positive. The lowest level of testosterone was seen in highly positive AR cases, which was not statistically significant. When we divided the patients into ER positive and ER negative and compared AR and testosterone, we found that in the ER positive category, women in the AR negative subset had higher testosterone levels compared to women in the AR positive subset, but this difference was not significant. In the ER negative category, women who were AR positive had insignificantly higher testosterone levels than women in the AR negative subset. Secreto et al. reported a significant positive association between testosterone level, highly positive AR and ER.⁹ The most important mechanism that describes the role of androgens in proliferation of ER positive tumors is the aromatization of androgens, particularly testosterone to estradiol. When ER is not present androgens act via AR. This mechanism is supported by data that has shown higher concentrations of estradiol in tumor tissue than blood¹¹ and also a higher concentration of estrogen producing enzymes in tumor tissue.¹²

This discrepancy between the current results and those by Secreto et al. might be attributed to the race effect and the fact that their research only included post-menopausal women. In addition, testosterone levels in most of our patients were at the lower limit of normal, thus the effect on receptors was less.

Finding a positive or negative relation between testosterone and AR would help to determine if the main effect of the testosterone/AR pathway is inhibitory or stimulatory in development of breast cancer. Modulating this pathway can be effective in treatment of this malignancy.

We observed no association between AR, PR, HER2 and menopause status. In agreement, Ogawa et al. did not find any relation between AR and menopause status however they stated that most breast cancer cases with high AR expression were postmenopausal.⁸ Agoff et al. showed a significant relationship between AR and menopausal status in ER negative cancers but not in all patients.¹⁰ In postmenopause, changes in levels of different hormones, may change the expression of receptors, so different receptors in premenopause and postmenopause patients may describe different modalities of treatment. We did not see significant change in presentation of receptors before and after

menopause.

Although there was no association between AR and age, in patients less than 45 years most were AR negative. In our study AR was positive more frequently but not significantly in older patients which supported the results from previous studies.^{9,10,13} Previous studies have shown that androgen secretion increased in older patients.¹⁴ In our study testosterone was higher in patients over the age of 45 years but this was not statistically significant. On the other hand the risk of developing breast cancer is higher with increasing age.¹ This association has suggested an AR/androgen pathway in older individuals for developing breast cancer. In patients less than 45 years, because most are AR-absent therefore development of breast cancer is estrogen-dependent.

Because the higher expression of AR in ER positive tumors and higher level of testosterone in ER positive patients, we suggest that androgens should be considered important factors in the development of breast cancer via an ER mediated pathway.

In this study we divided tumor cells into two groups, AR negative and AR positive. The AR positive group was divided into three groups - poor, moderate, and highly positive. Testosterone level was higher in AR negative and AR moderately positive than the poor and highly positive group. A higher level of testosterone in the AR negative group was reported in a previous study.⁹ This supported the role of androgens in the development of breast cancer via a pathway other than the AR receptor. In ER negative tumors, 80% were AR negative and the testosterone level in this group was not significantly lower than the ER negative, AR positive group so androgens might act via pathways other than the AR/ER pathways. Secreto et al.⁹ suggested that androgens might induce production of epidermal growth factor (EGF) and this growth hormone can stimulate development of breast cancer.

HER2 was positive in approximately 60% of tumors. There were 57% of AR-positive cancers that were HER2 positive and 58% of AR negative cancers were HER2 positive. There was no significant association between AR and HER2. Secreto et al. reported the same results⁹ but other studies reported an association between AR and HER2. Naderi et al. described a cross-action between AR and HER2 in androgen sensitive

tumors.¹⁵ Further studies are necessary to describe the clinical significance of this pathway.

Triple negative breast cancer is an aggressive form of breast cancer that has an initial response to chemotherapy but a higher rate of relapse and poor prognosis. In this study, 10% of breast cancers were TNBC. In this group, 80% were not in menopause. These patients were less than 45 years of age, therefore TNBC is more prevalent in younger patients. Testosterone levels in TNBCs are low. In previous reports AR was positive in approximately 40% of TNBCs^{7,8} and Ogawa et al. reported this positivity up to 25%.¹⁶ However in the current study all TNBCs were AR-negative thus these results were evidence of lower dependency of this group of tumors to hormones. We believe that the BRCA1 mutation and an evaluation of EGFR expression might be of more assistance to be used in adjuvant therapy of TNBCs.

The various ways to use the AR/Androgen pathway as a target in treatment of breast cancer include the following. Androgen production inhibitors such as 5 α -reductase, inhibit conversion of testosterone to Dihydrotestosterone (DHT). Concentration of DHT in tissue is three time higher than plasma,⁷ so treatment with a 5 α -reductase inhibitor is a treatment choice for AR positive tumors. Direct AR blocking involves non-steroidal anti-androgens like bicalutamide that bind to cytosolic AR, then inhibit its action. Activation of membrane AR (mAR) has a different biological effect compared to cytosolic AR. This AR decreases cell motility and controls apoptosis in breast cancer cells and potentiates the action of paclitaxel. Ligands of mAR are natural androgens and albumin-conjugated androgens. Androgen receptor co-regulating factor inhibits ARA70 β , the AR co-activator that induces proliferation of breast cancer cells. High-dose medroxyprogesterone acetate (MPA) - the presence of AR can predict response to this anti-proliferating agent.¹⁶ These agents can develop new strategies for future treatment of breast cancer.

Conclusion

In this study we evaluated a number of breast cancer indexes in Iranian women diagnosed with breast cancer for the first time. We have shown that: (a) as with previous studies, AR expression and testosterone levels

were proportionally higher in older patients. This fact was in favor of the AR/androgen pathway responsible in the --pathophysiology of breast cancer in older patients. (b) There was a group of AR negative patients who had high levels of testosterone. Most were ER positive, however a group of ER negative, AR negative patients had insignificantly low androgen levels. These results might suggest that androgens act via different ways in breast cancer. (c) A substantial number of our results were markedly, but not statistically significant, thus other studies with larger groups of participating patients would be necessary to evaluate the role of the AR/androgen pathway in pathophysiology of breast cancer and defining the best manner to use this system as treatment.

Conflict of Interest

No conflict of interest is declared.

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