Published in final edited form as:

Cancer. 2013 July 15; 119(14): . doi:10.1002/cncr.28092.

Expression of Androgen Receptor and its Phosphorylated Forms in Breast Cancer Progression

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Abstract

Background—Androgen receptor (AR) expression in breast cancers may serve as a prognostic and predictive marker. We examined the expression pattern of AR and its phosphorylated forms, Ser-213 (AR-Ser(P)-213) and Ser-650 (AR-Ser(P)-650), in breast cancer and evaluated their association with clinicopathological parameters.

Methods—Immunohistochemistry was performed on primary and distant metastatic breast cancers and benign breast tissue using antibodies against AR, AR-Ser(P)-213, and AR-Ser(P)-650. The levels of cytoplasmic and nuclear expression were scored semiquantitatively using a histoscore.

Results—Nuclear staining of AR was observed in all benign breast tissue and 67% of cancer cases. Nuclear and cytoplasmic AR-Ser(P)-213 was increased in breast cancers 2-fold ($p=0.0014$) and 1.7-fold ($p=0.05$), respectively, compared to benign controls, whereas nuclear and cytoplasmic AR-Ser(P)-650 expression was decreased in tumors by 1.9-fold and 1.7-fold (both $p<0.0001$), respectively. Increased expression of nuclear or cytoplasmic AR-Ser(P)-213 was observed in metastatic breast cancers (1.3-fold, $p=0.05$), ER-negative (2.6-fold, $p=0.001$) and invasive ductal carcinoma (6.8-fold, $p=0.04$). AR-Ser(P)-650 expression is downregulated in

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CONFLICTS OF INTEREST DISCLOSURES

The authors declare no financial disclosures or conflicts of interest.

lymph node-positive (1.4-fold, $p=0.02$) breast cancers, but is upregulated in invasive ductal carcinomas (3.2-fold, $p<0.0001$) and metastases (1.5-fold, $p=0.003$). Moreover, in ER-negative breast cancers nuclear AR-Ser(P)-650 was decreased (1.4-fold, $p=0.005$) and cytoplasmic ARSer(P)-650 was increased (1.4-fold, $p=0.003$).

Conclusion—AR and its phosphorylation at serines 213 and 650 are differentially expressed in breast cancer tumorigenesis and progression. Phosphorylation of AR at serines 213 and 650 is increased in ER-negative, ductal carcinomas, and metastases and may have predictive value in breast cancer prognosis.

Keywords

breast; breast neoplasms; androgen; androgen receptor; phosphorylation

INTRODUCTION

The study of steroid receptor (SR) function in breast cancer biology has been largely focused on estrogen and progesterone receptors. However, recent studies have revealed the presence and importance of the androgen receptor (AR) in the biology of breast cancer (reviewed in ^{1, 2}). In fact, it may be possible to target the AR as a therapeutic strategy in estrogen receptor negative breast cancer.^{3, 4} Thus, in addition to the estrogen receptor, it is predicted that modulating the activity of AR will provide novel prevention and treatment approaches for breast cancer patients.

Androgens act on target cells by binding to the androgen receptor, a ligand-dependent transcription factor, and are important in the development of male reproductive organs and prostate cancer. AR has also been shown to have an inhibitory effect on ER- α activity and to be a critical player in the growth and malignancy of breast cancer cells.⁵ AR expression has been examined in several subtypes of breast cancer from patients with clinical follow-up. A study by Agoff et al. shows high levels of AR expression in 49% of ER-negative and 89% of ER-positive cases.⁶ AR expression is associated with a good prognosis in ER/PR -negative cancers. Conversely, loss of AR is associated with a poor prognosis in lymph node positive ER/PR/HER2-negative breast cancers.⁷ These findings are consistent with *in vitro* studies, where AR activation with the agonist 5- α -dihydrotestosterone ⁸ or dehydroepiandrosterone sulfate (DHEAS) ⁹ inhibits cell growth in AR-positive breast cancer cell lines, suggesting that AR initiates a growth inhibitory effect in breast cancer. In evaluating the metastatic potential of breast cancer cells, ligand activated AR has also been reported to induce cell motility by changing both cellular morphology and E-cadherin expression.¹⁰ Immunohistochemical studies on large cohorts of breast tumor samples have shown the potential for AR to be an effective biomarker for breast cancer survival.¹¹ Studies looking at different subsets and classes of breast cancer tumors based on immunohistochemical staining and gene expression microarray analysis have determined that AR plays an inherent role in cell signaling in breast cancer, and that targeting AR could be a potential therapeutic strategy for treatment of some ER-negative cancers.^{3, 4}

The expression and activity of steroid receptors, including ER, PR and AR can be regulated by posttranslational modifications including phosphorylation, ubiquitination, acetylation, and sumoylation.¹² A large number of phosphorylation sites have been identified in these receptors, with the majority being located within the N-terminus. Site-specific phosphorylation of SRs has been shown to be modulated by a wide variety of kinases depending on the cellular context; affecting hormone sensitivity, receptor stability and localization, DNA binding and cofactor interaction.¹² The expression and function of

phosphorylated forms of AR has been reported in prostate cancer but the biological implications of phosphorylated AR, particularly in breast cancer, remain largely unknown.

The androgen receptor can be phosphorylated at multiple serine residues. AR phosphorylation is increased in response to androgen at serines 16, 81, 256, 308, 424, and 650. In addition, Ser-94 is constitutively phosphorylated.^{13, 14} Studies in prostate cancer cell lines show AR phosphorylation at Ser-650 (AR-Ser(P)-650) is enhanced by treatment with forskolin, epidermal growth factor, and phorbol-12-myristate-13-acetate, suggesting that AR phosphorylation may be intricately linked to signal transduction processes regulating tumor promotion and cell growth.¹³ AR-Ser(P)-650 phosphorylation also plays an important role in nuclear export of AR in response to stress kinase signaling through p38 and JNK kinases.¹⁵ In the prostate, expression of AR phosphorylated at Ser-213 (AR-Ser(P)-213) has been shown to be restricted to epithelial cells and specific developmental and cellular contexts (high levels of androgens and differentiated luminal cells).¹⁶ It has been reported that Akt phosphorylates AR at serines 213 and 791 *in vitro*^{17, 18} and we have recently observed that serine 213 can also be phosphorylated by the PIM1 kinase. In addition, detection of AR-Ser(P)-213 was more prevalent in recurring compared to non-recurring prostate cancers.¹⁹ Interestingly, a recent study found that PIM1 kinase expression was higher in breast cancers compared to benign breast tissues and is associated with increased invasiveness, higher tumor grade, ER/PR/HER2 negative status, and poorer survival rates.²⁰

In this study, immunohistochemistry was performed using antibodies against the androgen receptor (recognizing phosphorylated and non-phosphorylated (total) AR), and AR phosphorylated at serine residues 213 and 650. We characterized the expression of AR, ARSer(P)-213 and AR-Ser(P)-650 in breast cancer epithelial cells on a tissue microarray (TMA) and correlated AR expression and phosphorylation with clinicopathological parameters.

MATERIALS AND METHODS

Breast Cancer Specimens

Tissue microarrays (TMA) of 156 formalin-fixed, paraffin-embedded breast cancer cases were obtained from therapeutic or diagnostic procedures performed as part of routine clinical management between 1999 and 2004 at the Memorial Sloan-Kettering Cancer Center (MSKCC), New York, NY. The TMA includes primary (n=104) and distant metastatic (n=52) breast cancers with three tissue cores representing each case. The predominant site of disease and metastatic deposits was used for the TMA. The primary cases were not matched to the distant metastases cases. The clinicopathological parameters include age, tumor size and type, hormone receptor status, clinical stage, grade, lymph node status and clinical follow-up. Tumors were graded according to the modified Scarff-Bloom-Richardson system. The H&E stain of the TMA was reviewed by two pathologists at MSKCC to confirm the tumor histology, grade and stage based on the most recent American Joint Commission on Cancer (AJCC) cancer staging criteria.²¹ The ER, PR and HER2 status were obtained from the original pathology reports (greater than or equal to 10% immunohistochemical staining for ER or PR was considered positive). Lymph node positivity was defined as nodes with macrometastases (>2.0mm). The benign controls (n=34) include 17 cases from mastectomy and 17 cases from the benign breast tissue of cancer cases from therapeutic or diagnostic procedures performed as part of routine clinical management between 2009 and 2010 at the New York University Langone Medical Center, New York, NY. Tissues were acquired according to each institution's institutional review board's policies.

Immunohistochemistry

Immunohistochemistry was performed using affinity-purified antibodies against AR, ARSer(P)-213 and AR-Ser(P)-650. Paraffin-embedded tissue sections were dewaxed in xylene, rehydrated, and washed in phosphate -buffered saline, pH 7.4. For antigen retrieval, paraffin sections were heated in a microwave oven (900 watts) in 10 mM citrate buffer followed by treatment with 3% H₂O₂ and blocked with 20% normal goat serum. Sections were then incubated with antibody against AR (1:250 dilution; N-20, Santa Cruz Biotechnology, Santa Cruz, CA), AR-Ser(P)-213 (1:100)¹⁶, and AR-Ser(P)-650 (1:250) (unpublished results, S. Logan), followed by incubation with a biotinylated rabbit secondary (1:1000, Vector Labs, Burlingame, CA). An avidin-biotin complex was formed and developed using diaminobenzidine chromagen, followed by a counter-stain with hematoxylin. Microscopy was performed on a Zeiss Axio Imager A2 microscope and representative images were acquired using QCapture Pro Software.

Evaluation of Immunostaining

Immunohistochemical staining was examined and scored independently, in a blinded manner, by two observers using a semiquantitative weighted histoscore. The weighted histoscore represents staining intensity (negative (0), weak (1), moderate (2) and strong (3)) and the percentage of positive cells within each intensity category, providing a score of 0–300.²² The nuclear and cytoplasmic staining was scored separately. The final histoscore was determined for each case by taking the mean histoscore of all the present cores and was used for statistical analysis.

Statistical Analysis

The cases were divided into different groups according to clinicopathological parameters. Statistical analyses of the histoscores were performed by unpaired Student's t-test with Prism 4 software (GraphPad Software, Inc, La Jolla, CA). The standard deviations were calculated to estimate the degree of variation in each group. All testing was two-tailed with 0.05 as the level of significance. Cases with missing data were excluded from analyses. Based on the results of the univariate analysis, variables with significant p values were selected and evaluated with a multivariate linear regression analysis to estimate their potential as independent predictors for clinical parameters. The multivariate linear-regression analysis was performed with SPSS software package (IBM Inc., Armonk, NY).

RESULTS

Patient and Tumor Characteristics

The breast cancer patients ranged in age from 30 to 87 years old (median=54). Of the 156 cancer cases, 104 cases were localized cancers (67%) and 52 cases were distant metastatic breast cancers (33%). The sites of metastatic breast cancer included: bone (n=17), lung (n=9), brain (n=8), ovary (n=8), liver (n=5), soft tissue (n=3), and chest wall (n=2). The majority of tumors were ductal carcinomas (n = 84), with a few lobular carcinomas (n = 15). 59% of tumors (n=92) were high stage (T 2). 78 cases (50%) were ER-positive cancers, while 65 were ER-negative (42%). 83 cases (53%) were recurrent cancer, either locally (n=31) or at a distant metastatic site (n=52). Immunohistochemical staining was performed and the mean histoscores for AR, ARSer(P)-213, and AR-Ser(P)-650 are presented in Table 1.

Androgen Receptor, AR-Ser(P)-213, and AR-Ser(P)-650 Expression in Benign and Malignant Breast Cells

We first examined the expression profiles of nuclear and cytoplasmic AR in benign and malignant breast epithelial cells, including primary and metastatic cancers. We observed nuclear staining of AR in all the benign breast tissue (100% cases), while 105 of 156 cancers (67%) show nuclear AR staining (Figure 1A). The mean expression of nuclear AR is decreased in breast cancers by 1.8-fold compared to benign tissues ($p<0.0001$). Cytoplasmic detection of total AR under the staining conditions of the tissue microarray was negative or weak and therefore not scored.

Nuclear and cytoplasmic expression of AR-Ser(P)-213 and AR-Ser(P)-650 were observed in both malignant and benign breast tissue (Figure 1B and C). However, the patterns of phosphorylated AR expression were distinct. Nuclear and cytoplasmic staining of AR-Ser(P)-213 were both increased in breast cancers compared to benign controls by 2-fold ($p=0.0014$) and 1.7-fold ($p=0.05$), respectively. Conversely, nuclear and cytoplasmic AR-Ser(P)-650 expression were both significantly decreased in tumors compared to benign by 1.9-fold and 1.7-fold ($p<0.0001$), respectively (Table 1).

AR, AR-Ser(P)-213, and AR-Ser(P)-650 Expression in ER-positive vs -negative Breast Cancers

The breast cancer cases were grouped according to the available clinicopathological parameters and detection of AR, AR-Ser(P)-213 and AR-Ser(P)-650 was analyzed. Among the ER-negative cancers, AR nuclear staining was detected in 34 cases (52%), compared to 64 cases (82%) of ER-positive cancers. ER-negative cancers, which in general have a more aggressive clinical course, showed an increase in cytoplasmic AR-Ser(P)-213 (2.6-fold, $p=0.001$) and ARSer(P)-650 (1.4-fold, $p=0.003$) expression compared to the ER-positive breast cancers (Figure 2). ER-negative breast cancers also showed decreased nuclear AR (2.1-fold, $p<0.0001$) and nuclear AR-Ser(P)-650 expression (1.4-fold, $p=0.005$). There was no significant difference in the nuclear expression of AR-Ser(P)-213 between ER-negative and -positive cancers. These data indicate that the phosphorylation of AR at Ser-213 and Ser-650 may play a role in aggressive ER-negative tumors. We also observed a similar expression pattern in PR-negative breast cancers (Table 1).

AR-Ser(P)-213 and AR-Ser(P)-650 Expression in Histologic Subtypes of Breast Cancer

Compared to invasive lobular carcinoma ($n=15$), invasive ductal carcinoma ($n=84$) exhibited increased cytoplasmic AR-Ser(P)-213 (6.8-fold, $p=0.04$) and AR-Ser(P)-650 expression (3.2-fold, $p<0.0001$) (Figure 3, panels B, E, C, and F). This data is consistent with the findings from ER-negative breast cancers, indicating that cytoplasmic AR phosphorylation may correlate with more aggressive breast cancers. There were no significant differences in the expression levels of nuclear AR, AR-Ser(P)-213 or AR-Ser(P)-650 between ductal and lobular type cancers (Table 1).

AR-Ser(P)-213 and AR-Ser(P)-650 Expression in High Stage Tumors

According to the AJCC Cancer Staging Handbook (7th Ed.) criteria, 92 cases (59%) were high stage (T 2). We observed decreased cytoplasmic AR-Ser(P)-650 expression in T 2 cancers by 1.7-fold ($p=0.02$) compared to T 1. The nuclear expression of AR-Ser(P)-213 was decreased in high-stage breast cancers (1.4-fold) but the difference was not statistically significant ($p=0.06$). The expression of nuclear AR, nuclear AR-Ser(P)-650 and cytoplasmic AR-Ser(P)-213 did not reach statistical significance comparing T 1 and T 2 (Table 1).

AR-Ser(P)-213 and AR-Ser(P)-650 Expression in Tumor Progression

A total of 81 breast cancer cases (52%) metastasized to the lymph nodes at the time of diagnosis. We observed decreased cytoplasmic AR-Ser(P)-650 expression (1.4-fold, $p=0.02$) in breast cancers with positive metastatic lymph nodes compared to cancers with negative lymph node status. There was no significant difference in the expression of nuclear AR, nuclear ARSer(P)-650, as well as nuclear and cytoplasmic AR-Ser(P)-213 between lymph node -positive vs. -negative breast cancers.

Distant metastases to other organs are represented in 52 breast cancer cases (33%). Breast cancers with distant metastasis had higher expression of nuclear AR-Ser(P)-213 (1.3-fold, $p=0.05$) and nuclear AR-Ser(P)-650 expression (1.5-fold, $p=0.0003$) (Figure 3H and I). There was no significant difference in the expression of nuclear AR, cytoplasmic AR-Ser(P)-213, and cytoplasmic AR-Ser(P)-650 between primary breast cancer and those with distant metastases. These data suggest that phosphorylation of AR (Ser-213 and Ser-650) in the nucleus may play a role in metastasis.

97 of the 104 primary tumor cases with and without lymph node involvement were followed for disease recurrence. 96 of the primary cancers and 23 metastatic cases were followed for tumor progression. The follow-up time interval ranged from 1 to 171 months, with a mean of 43 months. During this time, 33 patients (34%) with primary tumor developed recurrent cancer, locally or at a distant site. As for tumor progression, 58 patients (49%) remained disease free (NED), 19 patients (16%) are alive with recurrent disease (AWD), and 22 patients (18%) were deceased from recurrent cancer (DOD). We did not observe any significant difference in the expression pattern of AR, AR-Ser(P)-213, or AR-Ser(P)-650 in primary versus recurrent breast cancers or in DOD versus NED/AWD groups (Table 1).

Multivariate analysis of AR Expression and Phosphorylation

Clinical parameters found to be statistically significant via univariate analysis (Table 1: tumor histologic type, ER status, pathologic stage, lymph node status and distant metastasis) were evaluated using multivariate linear regression analysis for AR and phosphorylated AR expression in the breast cancer cases (Table 2). The fold change in the mean histoscores of AR or phosphorylated AR expression within each clinical parameter was used to examine if AR or phosphorylated AR are independent predictors associated with the clinical parameter. Increased expression of AR-Ser(P)-213 correlated with ductal carcinoma, ER-negative status, and distant metastases using univariate analysis, but multivariate analysis of AR-Ser(P)-213 and these parameters did not maintain statistical significance. However, decreased nuclear AR-Ser(P)-650 expression was an independent predictor of ER status ($p=0.006$) and increased expression was predictive of distant metastasis ($p=0.007$). In addition, increased cytoplasmic AR-Ser(P)-650 expression was associated with histological subtype (ductal carcinoma, $p=0.007$).

DISCUSSION

Steroid hormone receptors and their coactivators play an important role in breast cancer development and progression. Although the functions of AR and androgens in breast cancer are still unclear, multiple studies in epidemiology and human breast cancer implicate androgens in breast carcinogenesis. High levels of androgen in postmenopausal women have been correlated with an increased incidence of breast cancer.^{23, 24} A significant correlation between AR status and tumor grade has been reported.²⁵ Also, increased levels of AR expression in ER-negative breast cancer correlated to increased age, postmenopausal status, tumor grade, tumor size, and HER-2/neu overexpression has been shown.⁶ However, increased AR expression has also been reported in ER/PR-positive breast cancers with low

histological grades.^{26, 27} Our results are consistent with the studies by Secretò et al. and Park et al.: 64 of 77 (83%) of ER-positive breast cancers and 34 of 62 (55%) of ER-negative breast cancer cases expressed AR. The ER-positive cancers also show significantly higher expression of AR than the ER-negative cancers.

Compared to other studies where AR expression was dichotomized as positive or negative, we utilized a histoscore which combines the intensity score with the percentage of positive staining, which we believe more accurately reflects the expression level of AR and its phosphorylation status in breast cancer. We also used a collection of benign breast tissue as controls. Our analysis of the expression of AR and phosphorylated AR in breast cancer and benign controls suggests that AR plays an active role in breast cancer tumorigenesis and progression.

We observed decreased total AR expression in breast cancer compared to benign controls, and in ER-negative cancers compared to ER-positive ones. Importantly, our data indicate that there are increased levels of phosphorylated AR in the cytoplasm compared to the nucleus in breast cancer, especially in ER-negative cancer and invasive ductal type carcinoma. Lastly, phosphorylated AR expression is increased, especially AR-Ser(P)-650, in nuclei of breast cancer with distant metastases. All these data support the role of the androgen receptor and its phosphorylation in the tumorigenesis and progression of breast cancer.

Our results show that both nuclear and cytoplasmic AR phosphorylation at Ser-213 are increased in breast cancer, suggesting that phosphorylation could alter gene expression in the nucleus and may also play a non-genomic role in the cytoplasm to promote breast cancer progression. This idea is consistent with the increased nuclear expression of AR-Ser(P)-213 observed in breast cancer with distant metastases. We also observe increased cytoplasmic expression of AR-Ser(P)-213 in the ER-negative cancers and invasive ductal carcinomas, which generally have a more aggressive clinical course. Invasive ductal carcinomas are traditionally considered more aggressive than invasive lobular carcinomas but recent evidence shows that survival rates, stage for stage, may be similar.^{28, 29} Altogether, our data suggests a role of AR phosphorylation at Ser213 in tumor progression. However, it should be noted that under multivariate analysis these parameters did not maintain statistical significance, likely due to sample size and the relationship between the clinical parameters and aggressive breast cancers.

Our data show that both nuclear and cytoplasmic AR phosphorylation at Ser-650 is generally decreased in breast cancer cells, suggesting that regulation of the receptor plays a role in breast cancer progression. There are specific instances where AR-Ser(P)-650 expression is increased. AR phosphorylation at Ser-650 is increased in the cytoplasm of ER-negative breast cancer cells and ductal carcinoma, while the nuclear AR phosphorylation at Ser-650 is decreased, supporting the idea that phosphorylation at Ser-650 may be important in nuclear export of AR in more aggressive breast cancers. In addition, nuclear expression of AR-Ser(P)-650 is increased in cancers with distant metastases, suggesting that phosphorylation in aggressive disease may direct the AR to activate transcription of genes that promote metastases. Of note, it is possible that results from the recurrences or metastases could be complicated by prior systemic and/or local therapy.

Recently, De Amicis et al. reported the role of AR in tamoxifen-resistant breast cancers. Gene expression profiling showed that AR mRNA was increased while ER- α mRNA was reduced in tamoxifen-resistant tumors and overexpression of AR in the MCF-7 cell line (ER α -positive) caused them to become tamoxifen-resistant, which could be reversed with an AR antagonist.³⁰ Although the expression of AR and ER were inversely correlated with

histopathological grade, AR expression still remained significantly higher than ER. The prevalence of AR expression in breast cancer, particularly in triple negative or hormone resistant disease makes it an attractive therapeutic target.³¹

In summary, we report that the androgen receptor and its phosphorylation at serines 213 and 650 may play a role in the development and progression of breast cancer. To our knowledge, this is the first study examining the phosphorylation status of the androgen receptor in breast cancers. Future studies will focus on the mechanism of AR phosphorylation in the tumorigenesis and progression of breast cancer. It may also be of great interest to study the pattern of other serine residues and their significance in breast versus prostate cancer progression.

Acknowledgments

FUNDING SOURCES

This study was supported by the NYU Clinical and Translational Science Institute (1UL1RR029893) a seed fund to P.L. and NIH R01CA112226 (S.L.).

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Figure 1. AR and Phosphorylated AR Expression in Benign Breast Tissue

Immunohistochemical staining showing the representative expression patterns of AR (A), ARSer(P)-213 (B), and AR-Ser(P)-650 (C) in benign breast tissue. Images are at 20x magnification and inserts are at 40x magnification.

Figure 2. AR and Phosphorylated AR Expression in ER-positive versus ER-negative breast cancer

Immunohistochemical staining showing the expression patterns of AR (A and D), ARSer(P)-213 (B and E), and AR-Ser(P)-650 (C and F) in ER-positive and ER-negative breast cancers. AR-Ser(P)-213 and AR-Ser(P)-650 cytoplasmic staining is increased in ER-negative compared to ER-positive cancers. Images are at 20× magnification and inserts are at 40x magnification.

Figure 3. AR and Phosphorylated AR Expression in Invasive Lobular Carcinoma, Invasive Ductal Carcinoma, and Distant Metastasis

Immunohistochemical staining showing the staining patterns of AR (A,D, and G), AR-Ser(P)-213 (B,E, and H), and AR-Ser(P)-650 (C,F, and I) in invasive lobular carcinoma, invasive ductal carcinoma, and distant metastasis. There is increased cytoplasmic AR-Ser(P)-213 and AR-Ser(P)-650 staining in ductal carcinomas, and there is also increased nuclear AR-Ser(P)-213 and AR-Ser(P)-650 in breast metastases. Images are at 20× magnification and inserts are at 40× magnification.

Table 1

AR, AR-Ser(P)–213, and AR-Ser(P)–650 expression in clinical subgroups. Mean histoscores, standard deviations and p-values are shown. Bold indicates statistical significance using univariate analysis. The level of significance is $p = 0.05$. (N=nuclear staining, C=cytoplasmic staining)

Clinical Subgroup	AR-N	AR-Ser(P) 213-N	AR-Ser(P) 213-C	AR-Ser(P) 650-N	AR-Ser(P) 650-C
Tumor/ Benign					
Tumor (n=152)	67.7±5.7	88.9±5.3	29.6±4.2	91.2±5.2	74.5±4.5
Benign (n=34)	12.4±9.6	44.9±11.8	17.1±4.7	170.5±11.3	125.4±8.7
p-value	<0.0001	0.0014	0.05	<0.0001	<0.0001
Histologic Subtype					
Ductal (n=84)	63.9±7.8	85.0±6.5	34.7±6.0	77.9±6.6	82.1±5.3
Lobular (n=15)	100.6±19.3	74.6±11.3	5.1±4.1	94.6±13.5	25.8±9.3
p-value	0.0757	0.5197	0.0413	0.3147	<0.0001
ER Status					
Negative (n=65)	42.2±7.1	86.7±8.2	45.7±7.6	74.5±7.4	91.0±7.0
Positive (n=78)	90.7±8.3	91.8±7.2	17.5±4.5	104.1±7.3	63.1±5.7
p-value	<0.0001	0.6459	0.0012	0.0054	0.0025
PR Status					
Negative (n=85)	53.0±6.8	93.0±7.5	41.0±6.7	83.0±6.7	85.0±6.0
Positive (n=56)	96.0±10.0	87.0±7.6	15.0±3.9	110.0±8.6	63.0±7.1
p-value	0.0005	0.5947	0.0012	0.0461	0.02
HER2 Status					
Negative (n=107)	69.0±7.1	87.8±6.4	25.9±4.7	86.6±6.0	69.7±5.3
Positive (n=13)	73.5±20.1	101.9±18.0	52.2±16.2	113.8±18.1	97.3±10.4
p-value	0.8369	0.4731	0.1415	0.1759	0.029
Tumor Stage					
1(n=13)	76.4±19.4	115.9±16.2	36.0±12.8	94.2±12.3	103.5±17.7
2(n=89)	97.4±22.8	84.4±5.9	29.0±5.8	77.8±6.7	67.4±5.3
p-value	0.4936	0.0627	0.6603	0.3546	0.0187
Lymph Nodes Involved					
0 (n=25)	56.7±14.8	100.4±12.2	42.2±13.3	85.7±12.2	95.9±10.0
1 (n=81)	75.7±8.1	81.8±6.5	24.6±4.9	82.5±6.9	66.6±5.9
p-value	0.2511	0.1632	0.1282	0.8241	0.0162
Distant Metastases					
Yes (n=52)	75.6±9.8	102.6±10.8	29.9±7.0	117.5±9.3	76.9±8.2

Clinical Subgroup	AR-N	AR-Ser(P) 213-N	AR-Ser(P) 213-C	AR-Ser(P) 650-N	AR-Ser(P) 650-C
No (n=101)	65.6±7.0	81.2±5.5	28.9±5.2	78.0±5.9	73.2±5.3
p-value	0.4091	0.05	0.9077	0.0003	0.7007
Recurrence					
No (n=64)	65.4±9.1	89.5±7.4	31.0±6.9	85.5±7.7	77.7±7.1
Yes(n=33)	70.7±12.66	67.3±8.3	28.2±8.8	65.0±8.5	62.5±7.9
p-value	0.7378	0.0686	0.8081	0.0972	0.1838
Disease-Specific Survival					
NED/AWD (n=77)	70.1±8.6	86.9±6.519	27.77±6.0	84.7±7.2	73.6±6.5
DOD (n=38)	58.5±9.5	88.0±10.8	32.9±8.1	85.4±9.4	77.3±7.5
p-value	0.4038	0.923	0.6174	0.9516	0.9428

Table 2

Fold change in expression levels of AR, AR-Ser(P)–213, and AR-Ser(P)–650 in clinical subgroups. P-values from univariate analysis (U) and multivariate analysis (M) are shown. Bold indicates statistical significance using multivariate analysis. (N=nuclear staining, C=cytoplasmic staining). $p \leq 0.05$ were considered statistically significant.

Subgroup Ratio	AR-N	AR-Ser(P)–213-N	AR-Ser(P)–213-C	AR-Ser(P)–650-N	AR-Ser(P)–650-C
Tumor/Benign	0.5 p-value: <0.0001 (U)	1.9 p-value: 0.0025 (U)	1.8 p-value: 0.05 (U)	0.5 p-value: <0.0001(U)	0.6 p-value: <0.0001(U)
ER -/ER +	0.5 p-value : <0.0001 (U)	0.9 p-value: 0.65 (U)	2.61 p-value: 0.0012(U) 0.71 (M)	0.72 p-value: 0.0054(U) 0.006(M)	1.44 p-value: 0.0025 (U) 0.295 (M)
Her2 -/Her2+	0.9 p-value: 0.84 (U)	0.9 p-value: 0.47 (U)	0.5 p-value: 0.14 (U)	0.8 p-value: 0.18 (U)	0.72 p-value: 0.029 (U) 0.78 (M)
Ductal/Lobular	0.6 p-value: 0.076 (U)	1.1 p-value: 0.52 (U)	6.79 p-value : 0.0413 (U) 0.338(M)	0.8 p-value: 0.31 (U)	3.18 p-value: <0.001 (U) 0.007(M)
T-Stage 2-4/T-Stage 1	1.3 p-value: 0.49 (U)	0.73 p-value: 0.0627 (U) 0.267 (M)	0.8 p-value: 0.66 (U)	0.8 p-value: 0.35(U)	0.65 p-value: 0.0187 (U) 0.101 (M)
Lymph Node+/ Lymph Node -	1.3 p-value: 0.25 (U)	0.8 p-value: 0.16 (U)	0.6 p-value: 0.13(U)	1.0 p-value: 0.82(U)	0.69 p-value: 0.0162 (U) 0.076 (M)
Distant Met+/ Distant Met -	1.2 p-value: 0.41 (U)	1.26 p-value: 0.05 (U) 0.074 (M)	1.0 p-value: 0.9 (U)	1.51 p-value: 0.003 (U) 0.007 (M)	1.0 p-value: 0.7 (U)