

Clinical significance of androgen receptor expression in triple negative breast cancer-an immunohistochemistry study

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Abstract. Androgen receptor (AR) is closely associated with the occurrence and progression of breast cancer; however, the clinical significance of it in triple negative breast cancer (TNBC) has been controversial. There is a limited amount of research regarding the effect of neoadjuvant chemotherapy on AR expression. By examining the expression of AR in patients with TNBC, the aim of the present study is to explore the clinical significance of AR and provide evidence for AR-directed treatment in TNBC. A total of 188 patients with primary TNBC with complete medical records were included in this retrospective study. Tumor sections from 41 patients (21.8%) were positive for AR, which was more often detected in small tumors ($P=0.042$) and cases with no lymph node involvement ($P=0.032$). Among them, 102 were treated with neoadjuvant chemotherapy (NAC). A total of 17 patients (16.7%) exhibited pathological complete response. However, the patient response was irrelevant to AR expression. Matched pathological tissues before and after NAC were collected for 49 cases, suggesting an enrichment of AR-expressing tumors following chemotherapy ($P=0.008$). Further analysis indicated that AR expression had no correlation with the disease-free and overall survival of patients with general TNBC; rather, it predicted a poor survival of the patients with stage III TNBC in comparison with those at earlier stages ($P=0.035$). AR expression occurs more often in small TNBC tumors or in cases with no lymph node metastasis. It is associated with a poor prognosis of the patients with advanced stages of tumors.

Introduction

Breast cancer is the most prevalent and lethal malignancy among females worldwide (1). In 2018, 1,735,350 incident breast cancer cases are estimated to be diagnosed in the United States of America and 609,640 associated mortalities are anticipated (2). Breast cancer is highly heterogeneous in biological and clinical features, and multimodality measures, including surgery, endocrinotherapy, chemotherapy and radiotherapy have been developed for treatment, in the past few decades. Precision medicine arising in recent years has been significant in prolonging the survival of patients with specific genetic backgrounds and improving their quality of life (3).

Molecular diagnosis allows the stratification of breast cancer into four major subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (4). Targeted therapies blocking the functions of ER or HER2 have exhibited prominent clinical benefits in patients with tumors positive for the ER, or HER2 receptors (5,6). However, the clinical outcome of a large number of patients remains poor due to 30-40% of breast cancer cases being ER-negative and 70-80% being HER2-negative. Furthermore, 15-20% of patients with triple negative breast cancer (TNBC) are negative for ER, PR and HER2 (7). TNBC is a distinct subtype of breast cancer that is characterized by frequent recurrence and metastasis (8), and chemotherapy is currently the only available systemic treatment approach. Chemotherapy has been effective; however, it results in strong side effects and high costs (9). In general, patients who achieve pathological complete responses (pCR) following neoadjuvant chemotherapy (NAC) typically have a favorable prognosis (10). However, at present, it is unclear what clinicopathological and molecular features may be used to identify this subpopulation of patients.

Androgen receptor (AR) is a nuclear receptor, which, upon the binding of androgen, forms a hormone-receptor complex that acts on the androgen response elements of target genes to mediate gene transcription (11). AR is widely expressed in human tissues, including testis, ovary and breast (12). Deletion of the AR-encoding gene in mice leads to abnormal mammary gland development and growth retardation (13). AR has drawn increasing attention in the management of breast

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cancer in recent years, as AR is expressed in ~80% of primary breast cancers and often at a higher level in comparison with ER (14,15). This AR alteration explains the clinical benefit rate of 20-25% in patients with breast cancer treated by testosterone, as demonstrated in the 1970s (16). Testosterone was later replaced with tamoxifen and aromatase inhibitors, due to its masculinizing effects (17). These ER-modulating drugs have been widely used; however, their efficacy can be limited by patient intolerance (18,19). The observation that aromatase inhibitors elevate androgen levels highlights the potential significance of AR-modulating agents (20).

AR is upregulated in up to 53% of TNBC tumors (14,21,22). There are six subcategories of TNBC classified by gene expression profiles: Basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like and luminal androgen receptor (LAR) (23). The LAR-type tumors are usually abundant with AR upregulation (7). Unsurprisingly, a preclinical study demonstrated that LAR-type breast cancer cell lines are sensitive to AR antagonists (24). These findings suggest AR may be a valuable prognostic marker in TNBC.

In order to explore the clinical significance of AR in TNBC, the expression of AR in 188 TNBC patients was examined and its association with the outcome of 102 patients who were treated with NAC was assessed. Using a cohort of 49 patients with tissue samples collected prior to and following NAC, the effect of NAC on AR expression in TNBC was also studied, and the prognosis function of AR in correlation with survival rates was evaluated.

Materials and methods

Ethical approval. The present study was approved by the Research Ethics Committee of Xiangya Hospital Central South University (Changsha, China; approval no. 201303083). Written informed consent was obtained from all patients to include their data in this retrospective study.

Patient selection. A total of 188 patients, aged 49.42±9.73 years old (mean ± standard deviation) with primary TNBC who underwent treatment at Xiangya Hospital, Central South University, between July 2011 and July 2014 were included. The patients were chosen based on the pathological features, therapeutic approaches, metastatic status, and availability of a complete medical record, which included age, menstrual status, body mass index (BMI), relevant family history, tumor grade and size, lymph node involvement, clinical stage, Ki-67 expression, and clinical follow-up information. All patients were diagnosed to have invasive ductal carcinoma with no systemic metastases. Expression of HER2 was re-evaluated due to the positive threshold of the HER-2 testing was 10%, reduced from 30% in 2009, and in fluorescent *in situ* hybridization for positivity, the HER2/CEP17 ratio is ≥2, or HER2 copy number is >6 signals per cell (25). HER2-positive patients were excluded from the present study. Of the total 188 patients, 102 were treated with NAC, which included 3-4 cycles (3 weeks/cycle) of docetaxel (75 mg/m²), pirarubicine (50 mg/m²), or cyclophosphamide (500 mg/m²). Matched pre- and post-chemotherapy tissues were available for 49 patients. The pre- and post-chemotherapy tissues were collected by needle core biopsy and surgical excision, respectively.

Immunohistochemistry. Immunohistochemical analysis was performed following a commonly used protocol outlined by the study of Shi *et al* (26) with minor modifications. Briefly, tissues were fixed, paraffin-embedded, and dissected into 4-μm thick sections. Serial sections were dewaxed in xylene, rehydrated by a series of decreasing percentages of ethanol in water, and rinsed with PBS. Antigen retrieval was performed by heating the sections in a 95°C water bath in the presence of EDTA in a microwave for 20 min. The slides were additionally treated with 3% hydrogen peroxide (reagent 1; catalog no. PV-9000; ZSGB-BIO; OriGene Technologies, Beijing, China) and blocked with normal goat serum (ZSGB-BIO; OriGene Technologies) for 40 min in room temperature. The tissue sections were then incubated overnight at 4°C with a monoclonal mouse anti-AR (dilution 1:50; catalog no. ab9474; Abcam, Cambridge, UK). The next day, the slides were incubated with an undiluted polymer helper (reagent 2; catalog no. PV-9000; ZSGB-BIO; OriGene Technologies) for 20 min at 37°C, followed by staining with appropriate undiluted secondary antibodies (reagent 3-mouse, catalog no. PV-9000, ZSGB-BIO; OriGene Technologies) conjugated with poly-peroxidase for 20 min at 37°C. Color was developed using diaminobenzidine as a chromogen. All slides were assessed and scored by pathologists (light microscope; Leica Microsystems GmbH, Wetzlar, Germany; magnifications, x100 and x400). By using the double-blind reading, pathologists selected 10 high magnification fields of view (x400) randomly, and counted >100 cells in each field. Staining of AR was considered positive when ≥1% of the tumor cell nuclei were stained.

Prognostic analysis. When accessible, patients were followed up monthly until mortality or July 2016, the cutoff date for data collection. Complete follow-up information was obtained for 188 patients by outpatient review and phone communication. The patient data, including dates of treatment and first recurrence, metastatic status, and the TNBC-associated mortality were used to assess the overall (OS) and disease-free survivals (DFS). OS was defined as the period of time from the date of surgery to the date of mortality associated with breast cancer or the last follow-up time. DFS was defined as the period of time from the date of surgery to the date of first recurrence, metastasis, or mortality associated with breast cancer.

Statistical analysis. The data were analyzed by Statistical Package for Social Sciences software version 22.0 (IBM Corp., Armonk, NY, USA). Associations between AR expression, and clinicopathological features and the outcome of NAC were assessed using χ^2 or Fisher's exact tests. A Kaplan-Meier estimator and log-rank test were used to assess the patient survival rate. A multivariate analysis using the Cox proportional hazard regression model was performed to assess prognosis. P<0.05 was considered to indicate a statistically significant difference.

Results

AR is expressed in TNBC. AR expression in TNBC was assessed by immunohistochemistry (Table I). Among the

Table I. Association between AR expression and clinicopathological characteristics in 188 patients with TNBC.

Characteristics	AR		χ^2	P-value
	Positive (%)	Negative (%)		
Age (years)				
≤50	24 (21.6)	87 (78.4)	0.006	0.941
>50	17 (22.1)	60 (77.9)		
Menstrual status				
Pre-menopause	27 (21.8)	97 (78.2)	<0.001	0.987
Post-menopause	14 (21.9)	50 (78.1)		
BMI				
<24	28 (23.5)	91 (76.5)	0.563	0.453
≥24	13 (18.8)	56 (81.2)		
Family history				
No	36 (23.4)	118 (76.6)	1.228	0.268
Yes	5 (14.7)	29 (85.3)		
Tumor grade				
I-II	29 (21.5)	106 (78.5)	0.030	0.862
III	12 (22.6)	41 (77.4)		
Tumor size (cm)				
≤5	37 (25)	111 (75)	4.155	0.042
>5	4 (10)	36 (90)		
Lymph node metastasis				
No	27 (28.1)	69 (71.9)	4.590	0.032
Yes	14 (15.2)	78 (84.8)		
Clinical stage				
I-II	31 (24.4)	96 (75.6)	1.553	0.213
III	10 (16.4)	51 (83.6)		
Ki-67				
<14	10 (23.3)	33 (76.7)	0.068	0.794
≥14	31 (21.4)	114 (78.6)		

AR, androgen receptor; TNBC, triple negative breast cancer; BMI, body mass index.

188 patients diagnosed with TNBC, tumor sections from 41 patients (21.8%) stained positively for AR (AR⁺; Fig. 1A), while others exhibited no evident AR expression (AR⁻; Fig. 1B). Statistical analysis indicated a significant association between AR expression and smaller tumors (P=0.042; Table I), suggesting AR was likely expressed during the early stage of cancer progression. Consistently, the AR protein expression was significantly associated with the localization of the tumors; TNBC with no lymph node metastases more likely expressed AR (P=0.032, Table I). No significant association between AR expression with age, menstrual status, BMI, family history, tumor grade, clinical stage or Ki-67 expression was identified (Table I).

AR expression has no significant effect on the outcome of chemotherapy. Of the 188 patients, 102 were treated with NAC (Table II). Their responses were assessed according to the guideline of the response evaluation criteria in solid tumors (27) and are summarized in Table II, together with the

clinicopathological features of the patients, in order to identify the factors that affect response to NAC. The results indicated that a higher BMI was the only parameter predicting pCR. Among 102 patients treated by NAC, 20 (19.6%) were positive for AR prior to treatment whereas the other 82 were negative (Table II). Following chemotherapy, 5/20 AR-positive patients (25%) exhibited pCR, while 12/82 AR-negative patients (14.6%) exhibited pCR. However, no statistically differences were identified between the two cohorts (P=0.316).

A total of 49/102 patients underwent post-chemotherapy surgery to remove residual tumors, which were sampled *ex vivo* for immunohistochemistry analysis. The results indicated that tumors from 21/49 patients (42.9%) expressed AR (Table III). Which was significantly higher than the pre-chemotherapy data (19.6%) (Table III), suggesting an enrichment of AR-expressing tumors following chemotherapy (P=0.008). As shown in Table III, 12 patients with AR-negative tumors were identified to have AR-positive nodules following chemotherapy.

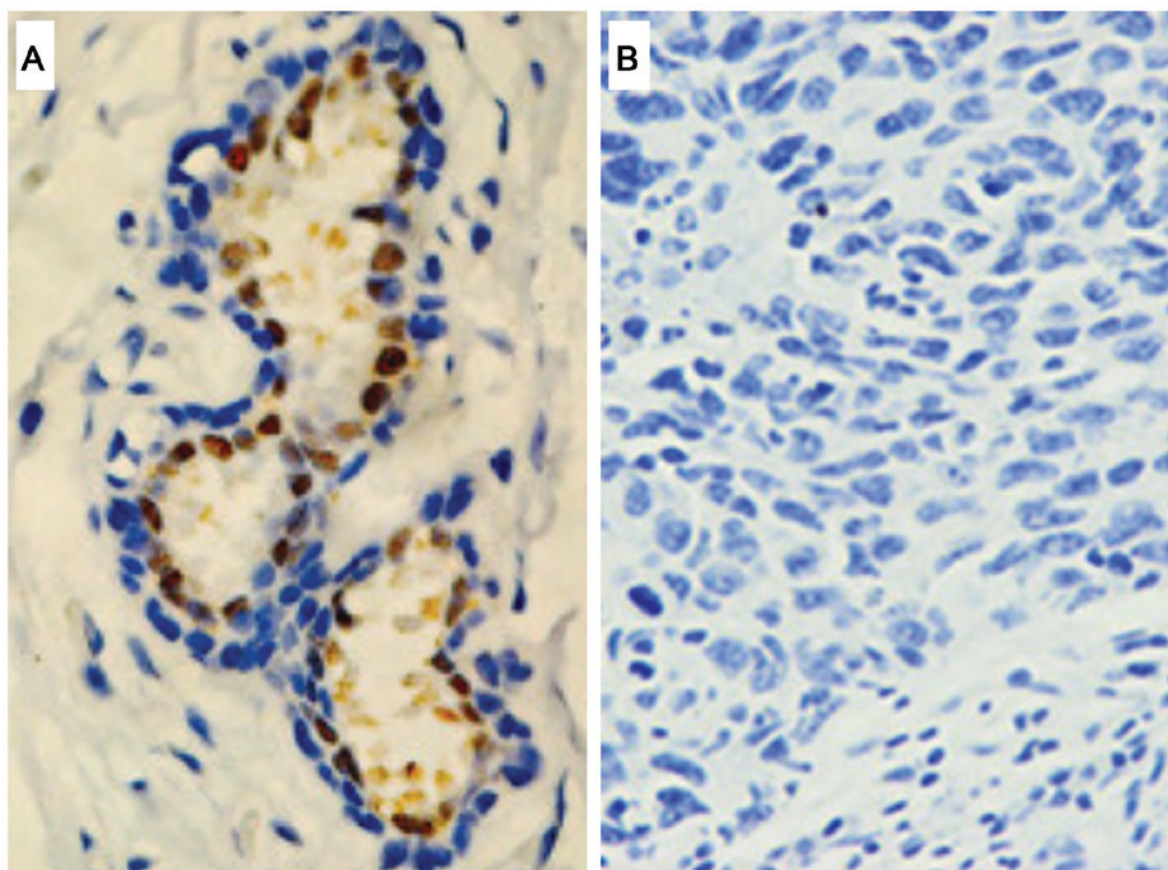


Figure 1. Representative immunohistochemistry images of (A) AR-positive and (B) AR-negative tissue sections (magnification, x400) of patients with triple negative breast cancer. AR, androgen receptor.

AR expression predicts a poor prognosis for stage III TNBC. Whether AR expression was associated with patient prognosis was then investigated. A total of 188 patients were followed-up for up to 60 months, with 37 developing recurrent diseases and 10 succumbing to breast cancer-associated mortality. Kaplan-Meier survival analysis with a log-rank test was performed to assess the association between AR expression and patient survival. In AR-positive patients, the recurrence rate was 19.5% (8/41), which was similar to 19.7% (29/147) in AR-negative patients. During this period, the mortality in AR⁺ TNBC was 9.76% (4/41) and the mortality in AR-TNBC was 4.08% (6/147). The results indicated no significant correlation of AR expression with the disease-free and overall survivals of patients (Fig. 2A and B). However, AR expression in stage III tumors (10/61 stage III cases) predicted a poor survival of the patients (Fig. 2C; $P=0.035$) compared with those with no AR positivity (51 patients). In stage III tumors, the mortality in AR⁺ TNBC was 30% (3/10) and the mortality in AR-TNBC was 7.8% (4/51). Among 127 stage I-II patients AR expression was not associated with the survival of patients with early stage of cancer (data not shown).

DFS is associated with age of patients and clinical stage of disease. Univariate and multivariate analyses were performed using 188 patients to identify crucial factors for DFS. Age >50 and clinical stage III were identified to be major risk factors for reduced DFS compared with a younger age and early

stages, respectively, by univariate and multivariate analyses (Table IV). Lymph node metastasis was also a risk factor for shorter DFS based on univariate analysis (Table IV). Advanced clinical stage was also significantly associated with reduced OS based on univariate and multivariate analyses.

Discussion

Androgen receptor mediates key processes in mammary gland development, including ductal branching, formation of the milk-producing alveoli and lobuloalveolar development (13). Accumulating evidence highlights its crucial functions in cancer progression (7,28-30). In the present study, the expression of AR in 188 patients with TNBC was determined using immunohistochemistry and its potential value in predicting the prognosis of patients with TNBC treated with NAC was assessed. The results of the present study demonstrated that AR expression was induced by NAC treatment and that AR expression in advanced-stage tumors predicts a poor prognosis in patients with TNBC.

The immunohistochemistry data indicated that 21.8% of the 188 TNBC cases are positive for AR. This is consistent with previous findings that 10-53% TNBC tumors express AR (21,31-34). The significant variations between different studies are attributable to a lack of commonly accepted standards and analytical protocols to determine the expression of AR by immunohistochemistry. Since AR is not recognized as

Table II. Association between chemotherapeutic effect and clinicopathologic characteristics in 102 patients with TNBC.

Characteristics	Chemotherapeutic effect		χ^2	P-value
	pCR (%)	Non-pCR (%)		
Age (years)				
≤50	11 (18.3)	49 (81.7)	0.291	0.589
>50	6 (14.3)	36 (85.7)		
Menstrual status				
Pre-menopause	13 (18.6)	57 (81.4)	0.583	0.445
Post-menopause	4 (12.5)	28 (87.5)		
BMI				
<24	5 (7.8)	59 (92.2)	9.697	0.002
≥24	12 (31.6)	26 (68.4)		
Family history				
No	15 (17.9)	69 (82.1)	0.486	0.730
Yes	2 (11.1)	16 (88.9)		
Tumor grade				
I-II	15 (19.7)	61 (80.3)	2.023	0.226
III	2 (7.7)	24 (92.3)		
Tumor size (cm)				
≤5	10 (14.5)	59 (85.5)	0.726	0.394
>5	7 (21.2)	26 (78.8)		
Lymph node metastasis				
No	6 (16.2)	31 (83.8)	0.008	0.927
Yes	11 (16.9)	54 (83.1)		
Clinical stage				
I-II	10 (18.9)	43 (81.1)	0.385	0.535
III	7 (14.3)	42 (85.7)		
AR				
Negative	12 (14.6)	70 (85.4)	1.244	0.316
Positive	5 (25.0)	15 (75.0)		
Ki-67				
<14	3 (13.0)	20 (87.0)	0.281	0.756
≥14	14 (17.7)	65 (82.3)		

TNBC, triple negative breast cancer; AR, androgen receptor; pCR, pathological complete responses.

a prognostic molecule marker in breast cancer, ER is usually assessed instead (35). Furthermore, the thresholds for ER and HER2 expressions in the American Society of Clinical Oncology/College of American Pathologists guideline have been changed (25,35), which resulted in significant decreases in the number of TNBC diagnoses. In the present study, all TNBC cases were diagnosed following the most recent guideline recommendations for the evaluation of ER, PR, AR and HER-2 (25,35). Furthermore, patients with TNBC of various ethnic backgrounds may express AR at different levels, with previous meta-analysis demonstrating that AR expression was slightly increased in Asians when compared with Caucasians (36).

The results of the present study suggested that AR is detected more often in smaller tumors or in cases with no lymph node metastases. This is consistent with previous findings that AR⁺

carcinomas were highly differentiated and had a low Ki-67 labeling index (33,34,37,38). In preclinical experiments, AR had an anti-proliferative effect through stimulating the expression of ER β , which inhibited cell growth (39), and AR has been demonstrated to mediate signaling pathways, including Janus kinase/signal transducer and activator of transcription 3, microtubule affinity regulating kinase, NOTCH and phosphatidylinositol 3-kinase (PI3K)/mechanistic target of rapamycin kinase (mTOR)/AKT serine/threonine kinase (40). The multifaceted roles of AR in TNBC implicate that it may be a useful clinical marker.

No significant association was identified between AR expression and the response to NAC in the present study although a lower pCR rate has previously been demonstrated in AR⁺ compared with AR⁻ patients (41). This may be due to the limited number of AR⁺ cases treated with NAC in the present

Table III. Association between AR status and NAC in 49 patients with TNBC.

AR status following NAC	AR status prior to NAC		χ^2	P-value
	Positive (%)	Negative (%)		
Positive	6 (28.6)	15 (71.4)	6.772	0.008
Negative	3 (10.7)	25 (89.3)		

AR, androgen receptor; TNBC, triple negative breast cancer; NAC, neoadjuvant chemotherapy.

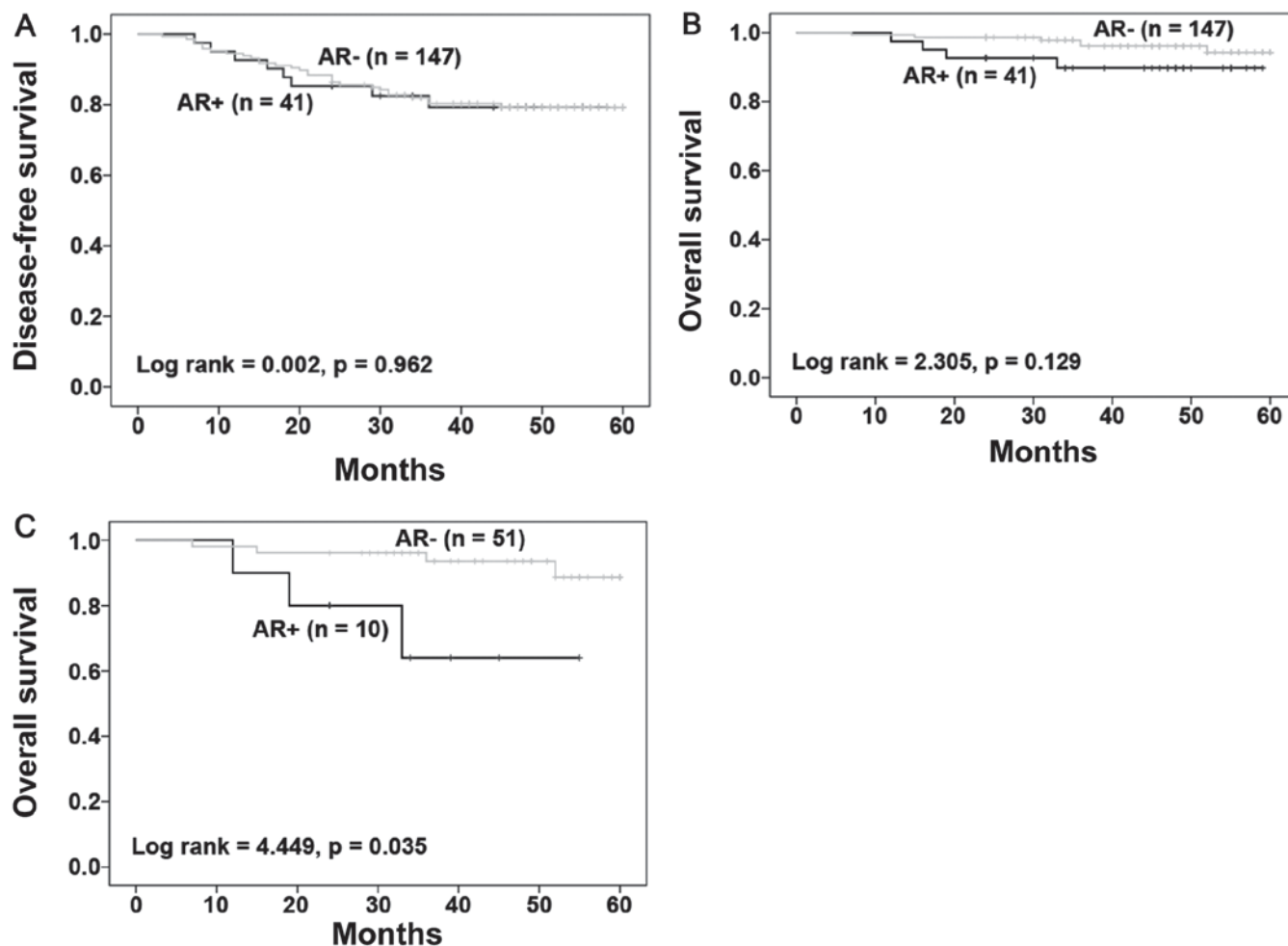


Figure 2. Kaplan-Meier analysis of (A) disease-free and (B) overall survival of 188 patients with triple negative breast cancer stratified by AR expression. (C) Overall survival of 61 patients with stage III tumors subgrouped based on AR positivity. AR, androgen receptor.

study. Also, NAC induced AR expression in certain patients with TNBC, which is likely due to a lower susceptibility of AR⁺ cells to NAC when compared with AR⁻ cells in the present study. A hypothesis is that chemotherapy drugs kill more AR⁻ cells than AR⁺ cells, resulting in the upregulation of AR gene expression and AR⁺ cells exhibiting chemotherapeutics resistance (42), thus hormone receptor negative breast cancer are more likely to benefit from chemotherapy. Chemotherapy insensitive or resistant triple-negative breast cancer may have high levels of AR expression; therefore, AR-directed

therapy may be used in AR⁺ TNBC, which poorly responds to chemotherapy.

No consistent findings have been reported regarding the association between AR expression and patient survival. While AR expression predicts better OS and DFS in general breast cancer or patients with TNBC (41,43-45), there are also reports that AR positivity is associated with poor prognosis (34,46-48) or is irrelevant to patient survival (37). The results of the present study demonstrated no significant association between AR expression with the survival of 188 patients with TNBC

Table IV. Univariate and multivariate analysis of disease-free survival in 188 patients with TNBC.

Parameters	Univariate			Multivariate		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (>50 years vs. ≤50 years)	2.007	1.041-3.870	0.038	2.003	1.030-3.896	0.041
Tumor grade (III vs. I-II)	1.490	0.759-2.926	0.247	1.538	0.781-3.029	0.213
Tumor size (>5 cm vs. ≤5 cm)	1.258	0.594-2.666	0.549	0.700	0.291-1.687	0.427
Lymph node metastasis (Yes vs. no)	2.350	1.181-4.678	0.015	1.783	0.808-3.935	0.152
Clinical stage (III vs. I-II)	2.502	1.312-4.770	0.005	2.378	1.029-5.497	0.043
AR (Positive vs. negative)	1.019	0.466-2.229	0.962	1.272	0.572-2.828	0.555
Ki-67 (≥14 vs. <14)	1.677	0.699-4.023	0.247	1.488	0.614-3.603	0.379

TNBC, triple negative breast cancer; HR, hazard ratio; CI, confidence interval; AR, androgen receptor.

without stratification. The contradictory conclusions warrant future multi-institutional studies, in which universal standards should be used in the examination of AR expression and the definition of TNBC. However, AR⁺ status was significantly associated with poor overall survival of stage III patients, suggesting the prognostic value of AR for patients with advanced stage TNBC. This result is consistent with previous findings that AR⁺ TNBC cells are chemoresistant (42). In ER-negative TNBC, AR stimulates tumor growth by activating the ER signaling pathway (49). As with the molecular apocrine profile (ER⁻, AR⁺), it exhibits a high invasive ability and poor prognosis (50). A total of 90% of patients with TNBC have gene mutations, deletions or amplifications (23), consequently the mechanism of AR in TNBC is not clearly understood.

Prostate cancer is the second most prevalent cancer among males worldwide and it is also a hormone dependent cancer (2). Androgens stimulate the occurrence and development of TNBC molecular by binding AR, and the modulation of androgen levels can be effective in the treatment of prostate cancer (51). Therefore, AR-directed therapy may be effective in a specific group of patients, those with AR⁺ TNBC, which may increase survival rates. As bicalutamide treatment gains great success in prostate cancer (52), numerous preclinical or clinical studies are committed to the application of AR antagonists in TNBC (53-57). LAR breast cancer cell lines are sensitive to AR antagonists. Furthermore, the study of Cuenca-Lopez *et al* (53) reported that AR⁺ TNBC cell line, which did not belong to the LAR subtype, also had a sensitivity to AR inhibition. In early clinical trials, patients with advanced AR⁺ TNBC were treated with bicalutamide with a clinical benefit rate of 20% (54). In a phase II clinical trial of enzalutamide, which has a six-fold higher affinity to AR than previous bicalutamide, 42% patients with advanced AR⁺ TNBC attained a clinical benefit time of 16 weeks in preliminary data (55). Subsequently, cytochrome P450 enzyme inhibitors, including abiraterone acetate, act on microsomal enzyme to suppress androgen production (56). The study of O'Shaughnessy *et al* (57), identified that in post-menopausal women with letrozole-pretreated metastatic ER⁺ breast cancer, combining abiraterone acetate with exemestane did not improve progression free survival compared with treatment with single exemestane.

Selective androgen receptor modulators (SARMs) are novel AR-directed therapies, which have high specificity

for AR without masculinizing side effects. Additionally, SARMs improve the side effects of advanced breast cancer by increasing muscle mass and restoring bone mineral density (58). GTx-024 is the one of the precedent SARMs (59). At present, there are a number of drugs about TNBC currently undergoing clinical trials. Nevertheless, an absence of adequate evidence has resulted in these drugs requiring approval. The combination therapy of TNBC may be considered due to the involvement of AR-mediation in numerous signaling pathways. The study of Lehmann *et al* (24), discovered that in AR⁺ TNBC cells, PI3K/mTOR inhibitors in combination with an AR antagonist had an additive growth inhibitory effect. The present study merely discussed AR expression and its relation to survival time in TNBC. Whether AR will function as a therapeutic target is subject to the outcome of clinical trials.

In the 188 patients with TNBC evaluated in this study, AR was expressed in ~21% of them, most often in small nodules or tumors with no lymph node metastases. AR expression does not determine the outcome of NAC; however, NAP may be enriched during chemotherapeutic treatment. The results of the present study suggest that AR expression has potential prognostic value in the prognosis of TNBC, but is limited to patients in the advanced stage of disease.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author contributions

LT and KZ conceived and designed the experiments. YL performed the experiments and analyzed data. LT, KZ and YL provided final approval of the manuscript.

Ethical approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent was obtained from all patients to publish their data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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