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Original Paper

Androgen Receptor Expression and Bicalutamide Antagonize Androgen Receptor Inhibit β-Catenin Transcription Complex in Estrogen Receptor-Negative Breast Cancer

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Key Words
Androgen receptor-positive • Estrogen receptor-negative • Breast cancer • Prognosis • Bicalutamide • Antagonize

Abstract
Background/Aims: Little is known about the potential mechanism of action for androgen receptor (AR) targeting treatment in estrogen receptor (ER)-negative breast cancer. This study aimed to evaluate AR status and its prognosis in four breast cancer subtypes. Bicalutamide has been identified as an AR antagonist and used for treating AR+/ER- breast cancer in a phase II trial. Our studies will clarify its mechanism in breast cancer treatment. ***Methods:*** A total of 510 consecutive cases of invasive ductal cancer (IDC) were evaluated in this study. The expression of AR was analyzed by immunohistochemistry and compared with patient survival, and its implications were evaluated in four subtypes of IDC. We examined bicalutamide as an AR antagonist to inhibit proliferation and increased apoptosis in AR+/ER- breast cancer cell lines. We explored the tumor suppressive functions of bicalutamide *in vitro* and vivo and its related mechanisms in AR+/ER- breast cancer. ***Results:*** AR expression was related to that of ER (P<0.001), PR (P<0.001), Her2 (P=0.017), Ki-67(P=0.020) and to four subtypes (P<0.001).

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AR retained independent prognostic significance (*P*=0.007, ER- cases; *P*=0.001, ER+ cases; *P*=0.001, total cases). We found that bicalutamide significantly decreased viability and increased apoptosis *in vitro* and vivo. The mechanistic analysis revealed that bicalutamide blocked androgen-stimulated oncogenic AR and Wnt/β-catenin signaling and inhibited the growth of AR+/ER- breast cancer. ***Conclusion:*** Our studies provide novel insights into bicalutamide as an antagonist of AR function in AR+/ER- breast cancer and reveal the mechanistic basis for targeting AR as a therapeutic opportunity for patients with AR+/ER- breast cancer.

Introduction

Androgen receptor (AR) is expressed frequently and is widespread in breast cancer, and breast cancer tissue has the third highest expression of AR among various tissues [1](Fig. 1). Currently, it is indicated that AR and AR inhibition play an important role in patients with breast cancer [2, 3]. AR is typically expressed in a greater proportion of breast cancers (80-90%) than the estrogen receptor (ER) (50-80%) [4], and previous studies have suggested the potential for AR to predict disease progression [5, 6] and provide a target for therapy [3, 7]. Studies of estrogen and ER have led to significant progress in the development of endocrine therapies targeting estrogen production or ER, contributing to both breast cancer prevention and treatment [8, 9]. Despite androgen and AR being widely recognized for their expression in breast cancer, little is known concerning a potential mechanism of action for the targeted treatment of this disease. Molecular expression profiling has been used to classify breast cancer into four subtypes, luminal A, luminal B, triple negative breast cancer (TNBC, ER-/PR-/Her2-) and human epidermal growth factor receptor 2 (Her2) over expressing(ER-/PR-/Her2+), according to the St. Gallen International Expert Consensus 2013 [10, 11]. ER and Her2 serve as prognostic markers and direct therapeutic targets (hormonotherapy and trastuzumab, respectively) for the luminal A, luminal B and Her2-overexpressing subtypes. Patients with breast cancer that lacks expression of the ER and progesterone receptor (PR) have not traditionally derived any benefit from conventional endocrine therapies such as selective ER modulators or aromatase inhibitors. AR expression varies across the clinical subtypes: approximately 80-95% in ER+ tumors [12], 30-65% in ER−/Her2+ tumors [13, 14], and 10-50% in TNBC [10, 12, 14, 15]. However, despite the high frequency of AR expression in breast cancer, it is still not standard clinical practice to use AR antagonists as therapy, though it has been used in clinical trials (NCT00468715 and NCT01889238) [16, 17].

AR is a ligand-dependent transcription factor that controls the expression of specific genes involved in the onset of breast cancer [18]. For targeting AR, bicalutamide has been used clinically as an anti-androgen. Bicalutamide, an oral, non-steroidal anti-androgen that competitively inhibits the binding of androgens to AR, has provided a proof of principle for the efficacy of minimally toxic androgen inhibition in a select group of patients with AR+/ER-/PR- breast cancer. AR antagonists may have a particularly in AR+/ER- (AR positive and ER negative) breast cancers, including the TNBC and Her2-overexpressing subtypes. In the absence of ER, the role of AR and anti-androgen signaling in regulating cell proliferation has been a little clearer.

Khramtsov and colleagues [19] reported that Wnt/β-catenin activation is an important feature of TNBC and predictive of worse overall survival. Moreover, these strong data suggested that Wnt/β-catenin pathway activation is implicated in mouse models of breast cancer [20]. Chesire [21] studied the interaction between β-catenin and AR in several cell lines, including kidney epithelial cells and human breast cancer cells and demonstrated that β-catenin enhanced AR-dependent transcription by directly interacting with this receptor. A recently concluded clinical trial (NCT00468715) of bicalutamide in patients with metastatic AR+/ER- breast cancer reported a modest clinical benefit rate of 20%. The underlying mechanisms and signaling pathways are poorly understood. We hypothesized that AR+/ER- breast cancer might also critically depend on the AR and β-catenin transcription complex and may benefit from treatment with bicalutamide. Our study detected the expression of

AR by immunohistochemistry (IHC) in four breast cancer subtypes. In this study, we show that AR level is significantly associated with disease outcome and indicate the biological functions of bicalutamide in AR+/ER- subtype breast cancer cells *in vivo* and vitro.

Materials and Methods

Patient characteristics and analysis of clinicopathological parameters

A total of 510 consecutive cases of invasive ductal (IDC) breast cancer were collected in this study, with a mean age of 50.5 years, who underwent mastectomy at Harbin Medical University Cancer Hospital (HMUCH) from January 2006 to December 2007. The patient's medical and follow-up data were retrieved from the hospital records and interviews. The following information was extracted from them: histopathology and treatment characteristics, such as tumor size, histological grade, hormone receptor status and lymph node involvement. The tumor grade and stage were classified according to the 7th edition of the AJCC staging system [22]. All patients were treated according to modern guidelines, including the use of adjuvant chemotherapy for IDC breast cancer and endocrine therapy for ER+/PR+ subtypes. No patient received any therapy before surgery. Ethical approval for the study was obtained through the Heilongjiang Regional Ethics Committee. Informed consent was obtained from all patients before surgery and examination of the specimens.

Immunohistochemical assay and evaluation of staining

Breast cancer tissue microarrays (TMA) were used as described previously [23, 24] and immunohistochemically stained for AR, ER, PR, Her2, Ki67 and β-catenin expression levels. Briefly, after deparaffinization, sections were hydrated and underwent Ethylene Diamine Tetraacetic Acid (EDTA) buffer (pH 8.0) or sodium citrate buffer (pH 6.0) retrieval. The slides were treated with methanol containing 0.3% hydrogen peroxide to block any endogenous peroxidase activity. Heat-mediated antigen retrieval with a pressure cooker method was used for all staining. A rabbit polyclonal antibody to the AR antibody (ab74272; 1:100dilution; Abcam) was used to evaluate AR protein expression level. The primary antibodies used in this study included ER (ab180900; 1:200dilution; Abcam), PR (ab63605; 1:300dilution; Abcam), Her2 (ab16901; 1:200dilution; Abcam), Ki67 (ab66155; 1:150dilution; Abcam), and β-catenin (ab32572; 1:400dilution; Abcam). For each antibody, the location of immunoreactivity, percentage of stained cells and intensity were determined. The evaluation of each protein's expression was determined from the mean of the individual cases. AR, ER, PR and Ki67 stains were assessed using Allred scores [25]. Her2+ was defined as strong membrane staining in >10% of the tumor cells. Tumors with a >2.2-fold increase in Her2 gene amplification, as determined by fluorescence in situ hybridization (FISH), were considered to be positive for Her2 overexpression [26]. Immunostaining was scored by two pathologists who were blinded to the patient's clinicopathological characteristics and outcomes. The analysis of breast cancer subtypes has been described previously [10, 11] and was based on the gene expression patterns. The subtype definitions were as follows: luminal A (ER+ and/or PR+ and Her2- and Ki67<14%), luminal B (ER+ and/or PR+ and Her2- and Ki67>14%; ER+ and/or PR+ and Her2+ and Ki67 anyway), Her2 overexpression (ER-, PR- and Her2+), TNBC (ER-, PR- and Her2-). To eliminate nonspecific staining, a negative control was performed with PBS.

Cell culture

MDA-MB-231 (MB-231) and MDA-MB-453 (MB-453) cells were purchased from the American Tissue Culture Collection (ATCC). MB-231 (TNBC) and MB-453 (Her2 overexpression) were chosen because two represents cell lines express moderate and high levels of AR in the absence of ER and PR breast cancer [7, 13, 27-29]. LnCap cell lysates, used as a positive control for AR, were a present from Dr Yongsheng Chen (Harbin Medical University). All cell lines were maintained at 37°C in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen, CA, USA), 1% penicillin/streptomycin, L-glutamine, nonessential amino acids, and insulin supplementation. Cell line identity was confirmed by analysis of short-term repeat loci and cells were routinely tested for mycoplasma infection. MB-231 and MB-453 cell with either AR or β-catenin knockdown cells were generated by lentiviral transduction of shRNAs targeting AR and β-catenin (Invitrogen, CA, USA)

Cellular assays and reagents

MB-231 and MB-453 cells were treated with 10 nmol/L Dihydrotestosterone (DHT) (Sigma-Aldrich), 10 mmol/L bicalutamide (AstraZeneca, Germany). A concentration of 10 mmol/L bicalutamide approximates the IC_{50} for the two cell lines studied [3, 13] and is a clinically achievable concentration. Androgen concentrations have been previously examined in breast cancer [13, 30]. The DHT levels in the FBS used during routine tissue culture propagation were monitored [31]. Colony formation assays were performed using monolayer culture. Cells (MB-231 and MB-453) were plated in six-well plates and treated with DHT and bicalutamide or transfected with shRNA-AR or shRNA-β-catenin plasmids (Invitrogen, CA, USA). Plating efficiency was calculated by dividing the number of colonies by the original seeding density. The surviving fraction was determined by comparing the plating efficiency of the treated wells to that of the control wells. Surviving colonies were counted after staining with Gentian Violet. All experiments were performed three times.

In situ hybridization

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining for apoptosis was performed using the ApopTag Plus Peroxidase In Situ Apoptosis Detection kit (Invitrogen, CA, USA), per the manufacturer's instructions.

Real-time quantitative PCR

RNA was isolated from cell lines using TRIzol Reagent (Invitrogen, Beijing, China), RNA quality and concentration were measured using a GeneQuant pro (GE Healthcare, Piscataway, NJ, USA), and cDNA was synthesized from 1 μg total RNA, using M-Mulv reverse transcriptase enzyme (Takara Bio, Otsu, Japan). qPCR was performed using the ABI 7500 Fast Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR green quantitative gene expression analysis was performed using the following primers: AR forward, 5′-GAACCACAAATACCTGGCTA-3′and AR reverse, 5′-TCCATTTTTGCCTCCCTTTT-3′; β-catenin forward, 5′-GAGGAGAA-GAGGAAAGAGA-3′and β-catenin reverse, 5′-TTGAGGACCTC-TGTGTATTTG-3′; β-ACTIN forward, 5′-CCTGTGGCATCCACGAAACT-3′ and β-ACTIN reverse, 5′- GAAGCATTTGCGGTGGACGAT-3′. Relative gene expression was calculated using the comparative cycle threshold method and values were normalized to β-actin, AR and β-catenin.

Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Sigma Aldrich) as per the manufacturer's instructions.

Western blot

Total proteins were quantified using the Pierce BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA), and 50μg of protein per sample was separated onto a denaturing sodium dodecyl sulfate polyacrylamide gel containing SDS-PAGE, and then transferred to a methanol-activated PVDF filter membrane (Thermo Scientific, Rockford, IL, USA). The lysates were then transferred to PVDF membranes for antibody incubation. After blocking with 5% nonfat milk and 0.1% Tween 20 in TBS, the membranes were incubated with anti-AR (ab74272; 1:1000 dilution; ABcam) or β-catenin antibody (ab180900; 1:1200 dilution; ABcam), CMYC (ABcam, Burlingame, CA) at 4°C overnight.. After subsequent washing with TBST, membranes were incubated with secondary antibody for 1 h at room temperature. GAPDH was used as a control. The bands were detected by enhanced chemiluminescence detection reagents (Applygen Technologies, Beijing, China).

Xenografted tumor model

Xenograft experiments were approved by the Animal Feeding Center of Harbin Medical University Cancer Hospital and Use Committee (HMUCH protocol). All animal experiments were conducted in accordance with the NIH Guidelines of Care and Use of Laboratory Animals. A total of 5×10^5 MB-231 or 1×10^6 MB-453 cells were mixed with Matrigel (BD Biosciences) and injected into the mammary fat pads of female BALB/c nude mice (Weitong Lihua Experimental Animal Technology Co., Ltd, Beijing). Tumor burden was assessed by caliper measurements [tumor volume was calculated as volume = (length × width^2)/2]. Once tumors were established, the mice were randomized into groups based on the total tumor burden as measured by Vernier calipers. Mice were administered bicalutamide in their chow (10 mg/kg daily dose) [13]. The feed was irradiated and stored at 4°C before use. Bicalutamide was mixed with ground mouse

chow (Research Diets Inc.) at 0.2 mg/g chow. Mice in the control group received the same ground mouse chow but without bicalutamide. All mice were given free access to bicalutamide formulated chow or control chow during the study period. Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation, and the tumors and mammary glands were harvested.

Statistical analysis

Statistical analyses were performed with SPSS version 16.0 statistical software. Statistical analysis of the data was performed with paired Student's t-test or one-way ANOVA for differences between experimental group mean values. Overall survival (OS) time was calculated from the date of the first definite operation to the date of the last follow-up, or death from any cause. OS curves were generated according to the Kaplan–Meier method. Multivariate analysis was carried out with Cox regression analysis. For all the tests, $P<0.05$ was considered as statistical significance.

Results

AR expression in breast cancer is related to OS in different subtypes

To assess the expression and prognostic value of AR expression in four molecular subtypes of breast cancer, we conducted immunohistochemical analysis of AR in a cohort of 510 IDC lesions. Among the 510 patients with follow-up data, a large percentage (79.0%) (403/510) of patients displayed nuclear immunoreactivity for AR, and AR expression was found in 76% (60/79) of Her2 overexpression patients and 39% (36/93) of the triple-negative (TN) subtype patients. AR expression was related to pathological node stage ($P=0.040$), ER ($P<0.001$), PR ($P<0.001$), Her2 ($P=0.017$), Ki-67 ($P=0.020$), subtype ($P<0.001$) and hormone therapy ($P<0.001$) but showed no relation to other parameters, such as age, menopause status, tumor size, clinical stage, histological grade, operation or chemotherapy in the treated cohort (Table 1). IHC staining of each protein marker and AR expression in the four subtypes of breast cancer tissues is illustrated in Fig. 2. The expression of AR was significantly associated with improved survival. Fig. 3A shows the 5 Year-OS ($P=0.007$) curve of 236 ER-negative patients with AR-positive and AR-negative breast cancer. Fig. 3B shows the 5 Year-OS ($P=0.001$) curve of 274 ER-positive patients with AR-positive and AR-negative breast cancer. Fig. 3C shows the 5 Year-OS ($P=0.001$) curve of 510 patients with AR-positive and AR-negative breast cancer. In 510 breast cancer patients, multivariate Cox regression analysis indicated a 2.36-fold increase in the risk of cancer-related death (5 Year-OS) ($P=0.023$) for AR-negative patients compared with AR-positive patients (Fig. 3D).

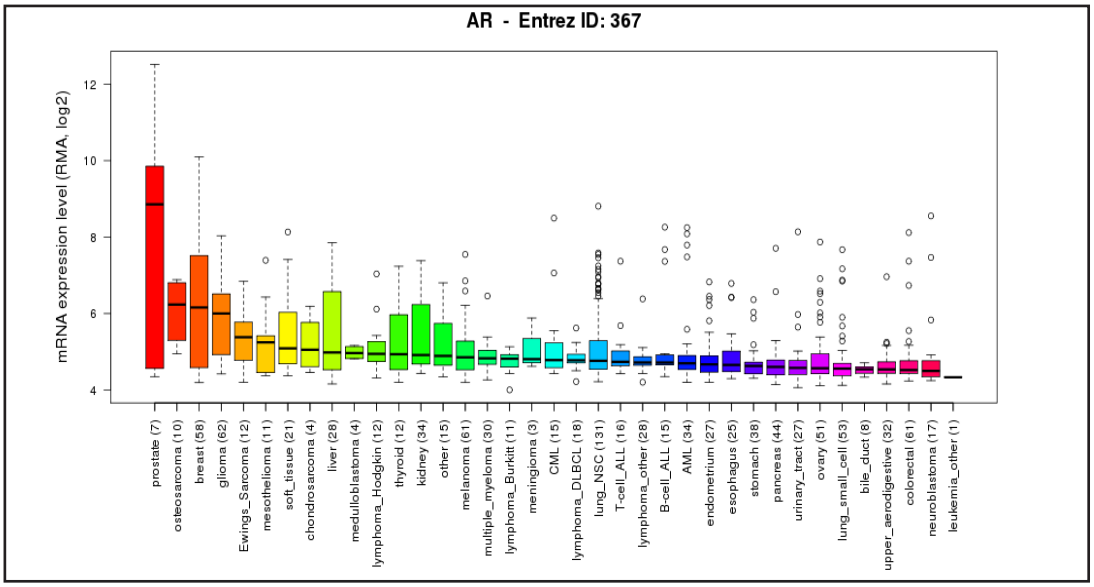

Fig. 1. Gene Expression data for AR extracted from Cancer Cell Line Encyclopedia (CCLE).The breast cancer tissue has the third highest expression of AR among various tissues.

Bicalutamide decreases proliferation and increases apoptosis in ER-negative breast cancer cells

AR inhibition was studied in MB-453 andMB-231 cell lines, which both represent ER-negative breast cancer cells. Using crystal violet staining, DHT was observed to increase the proliferation of the MB-453 and MB-231 cell lines, and bicalutamide significantly decreased ligand-mediated and baseline proliferation inserum (Fig. 4A, ***P*<0.001). The MTT assay was used to examine the effect of combination treatment with DHT and bicalutamide, which resulted in a more significant suppressive effect on cell proliferation at 72 hours (Fig. 4B, ***P*<0.001). Colony formation assays were performed to assess the effect of bicalutamide on cell proliferation in breast cancer. An approximate 70-80% reduction in colony numbers was observed in MB-453 and MB-231 cells compared with the vehicle control (Veh) (Fig. 4C, ***P*<0.001), suggesting that bicalutamide decreases growth and may decrease tumorigenicity *in vitro*. Bicalutamide also increased apoptosis and caspase-3/7 activity compared with the vehicle control in MB-453 and MB-231 cells (Fig. 4D, ***P*<0.001).

Bicalutamide inhibits ER-negative breast tumor growth in vivo

To determine the antitumor effect of targeting AR *in vivo*, we established MB-453 and MB-231 xenograft models. The MB-453 and MB-231 cells were implanted in the mammary gland of BALB/c nude mice, and one group of the mice received the concomitant implantation of a DHT slow-release pellet. DHT dramatically promoted the

Table 1. AR expression of breast cancer in relation to clinical and pathological features; BCS: breast conservative surgery. MRM: modified radical mastectomy

Factor	N	No. of AR (n=510) AR (–)	AR (+)	P
Age				.062
<50	279	51 (18%)	228 (82%)	
≥50	231	56 (24%)	175 (76%)	
Menopause status				.129
Premenopausal	275	52 (19%)	223 (81%)	
Postmenopausa	235	55 (23%)	180 (77%)	
Pathological T stage				.192
T1 (≤2cm)	173	32 (18%)	141 (82%)	
T2-4 (>2cm)	337	75 (22%)	262 (78%)	
Pathological N stage				.040
N0	231	57 (25%)	174 (75%)	
N1-3	279	50 (18%)	229 (82%)	
Clinical stage				.217
Stage I	102	18 (18%)	84 (82%)	
Stage II-III	408	89 (22%)	319 (78%)	
Histological grade				.083
G1	54	7 (13%)	47 (87%)	
G2-3	456	100 (22%)	356 (78%)	
ER				<.001
ER (–)	274	94 (34%)	180 (66%)	
ER (+)	236	13 (6%)	223 (94%)	
PR				<.001
PR (–)	204	79 (39%)	125 (61%)	
PR (+)	306	28 (9%)	278 (91%)	
Her2				.017
Her2 (–)	399	92 (23%)	307 (77%)	
Her2 (+)	111	15 (14%)	96 (86%)	
Ki-67				.020
Ki-67<14%	276	48 (17%)	228 (83%)	
Ki-67≥14%	234	59 (25%)	175 (75%)	
Subtype				<.001
Luminal A	123	14 (8%)	169 (92%)	
Luminal B	155	17 (11%)	138 (89%)	
Her2 (+)	79	19 (24%)	60 (76%)	
Tripple negative	93	57 (61%)	36 (39%)	
Operation				.148
BCS	127	22 (17%)	105 (83%)	
MRM	383	85 (22%)	298 (78%)	
Chemotherapy				.519
Yes	373	78 (21%)	295 (79%)	
No	137	29 (21%)	108 (79%)	
Hormone therapy				<.001
Yes	271	30 (11%)	241 (89%)	
No	239	77 (32%)	162 (68%)	

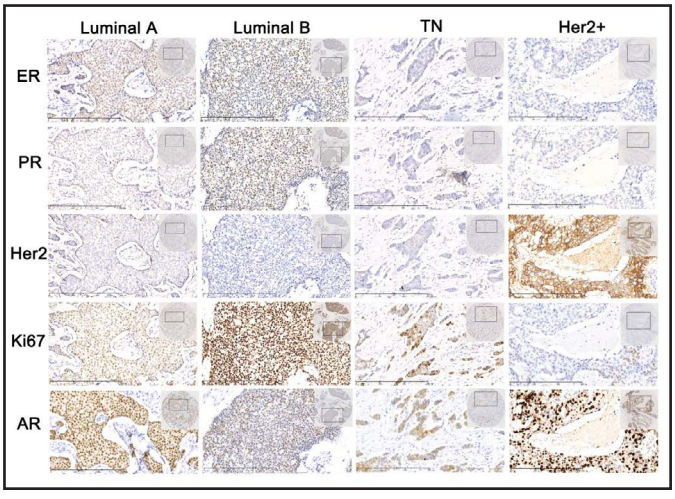

Fig. 2. Expression of ER, PR, Her2, Ki67 and AR by Immunohistochemical staining in Luminal A, Luminal B, TN and Her2+ breast cancer (the same patient with the same lesion site of each type). Positive expression of ER, PR, Ki67 and AR revealed nuclear staining, original magnification ×50 and ×200. Positive expression of Her2 revealed membrane staining, original magnification×50 and ×200.

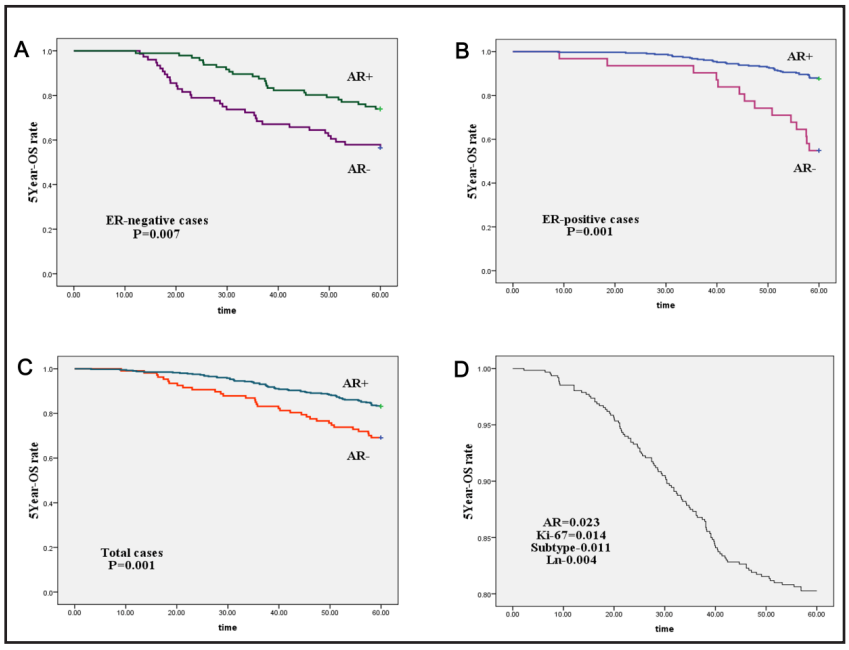

Fig. 3. Survival curves according to androgen receptor (AR) expression stratified by estrogen receptor (ER) status. (A) Kaplan-Meier analysis for 5-Year overall survival (OS) in patients with ER-negative tumor. (B) Kaplan-Meier analysis for 5-Year OS in patients with ER-positive tumor. (C) Kaplan-Meier analysis for 5-Year OS in total patients. (D) Cox regression model multivariate analysis for 5-Year OS in total patients.

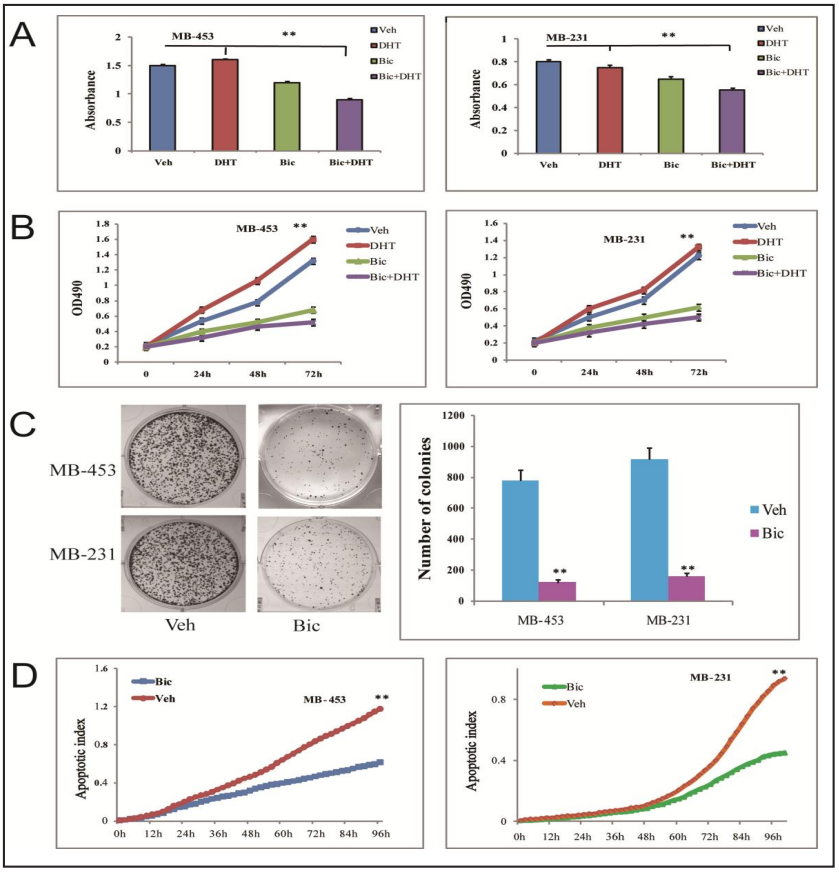

Fig. 4. Bicalutamide decreases proliferation and increases apoptosis in Her2 overexpression and TNBC cell lines. (A) Crystal violet assay of MB-453 and MB-231 treated with vehicle control (Veh), bicalutamide (Bic), and/or DHT. (B) MTT assay was used to measure MB-453 and MB-231 cell proliferation. (C) Soft agar assays of MB-453 and MB-231 cell lines treated with Veh or Bic in full serum. (D) Apoptotic index of nuclear red MB-453 and MB-231 cell lines treated with Veh (solid circle) or Bic (solid square). **, P<0.01.

growth of MB-453 tumors. After tumors reached a volume of 500 mm^3, daily oral administration of bicalutamide at 10 mg/kg was delivered to 50% of the mice carrying DHT pellets (n=3). As expected bicalutamide treatment resulted in a significant inhibition of the DHT-stimulated growth of MB-453 and MB-231 xenograft tumors (**P<0.001), and tumors re-

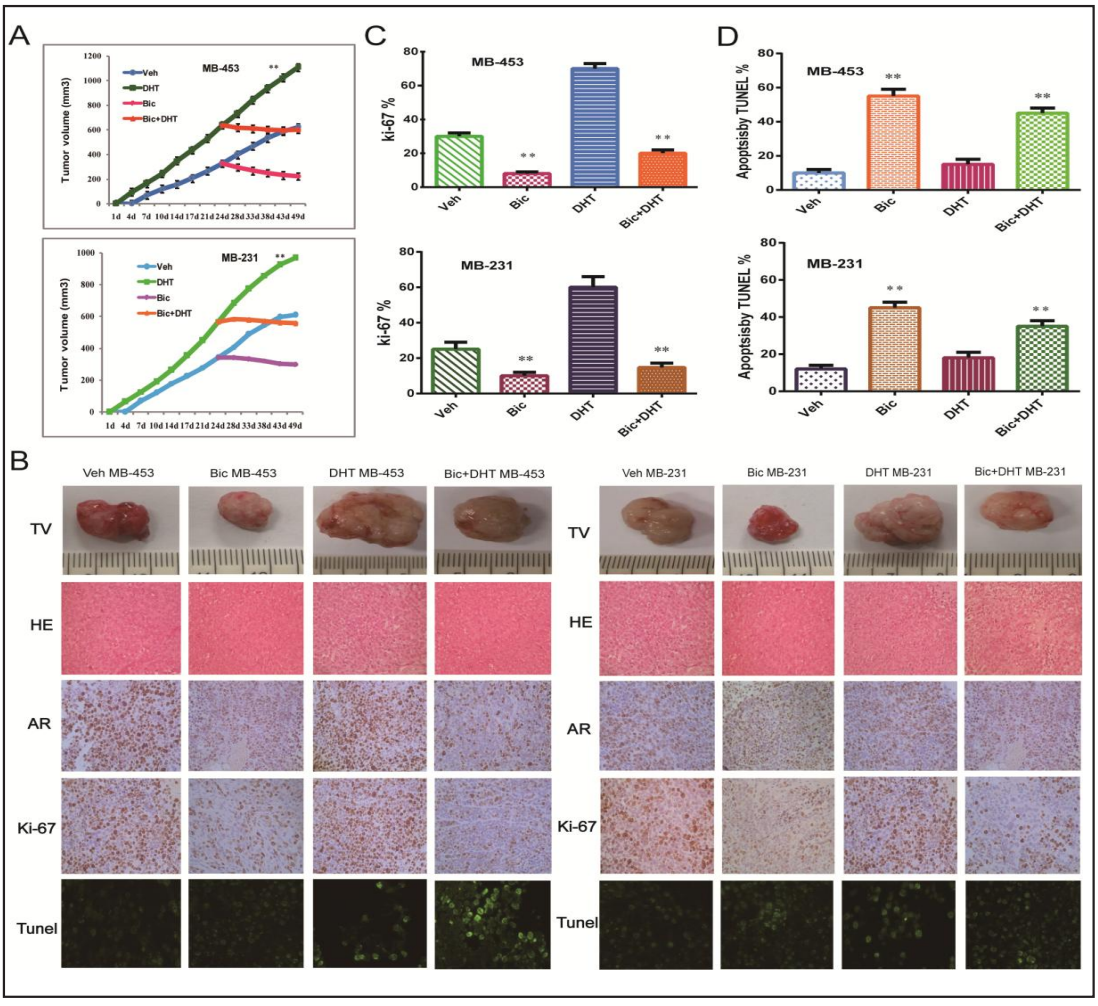

Fig. 5. Bicalutamide (Bic) decreases cellular viability and increases necrosis and apoptosis in MB-453 and MB-231 xenografts. (A) Effects of vehicle control (Veh), Bic, and/or DHT on the growth of xenograft tumors. MB-453 and MB-231 cells were orthotopically implanted in the fourth inguinal gland of SCID mice. (B) Solid tumor, immunohistochemisty (IHC) and Tunel staining of MDA-MB-453 xenograft tumors from different treatment groups to examine the expression. (C) Ki67 proteins expression of Veh, Bic, and/or DHT by IHC. (D) Tunel analysis for apoptosis from different treatment groups.

gressed to control levels after 4 weeks of treatment (Fig. 5A). Representative images from the analyzed tumor sections and the subcutaneous tumors are shown in Fig. 5B. IHC was performed to analyze the expression of the cell proliferation marker Ki-67 in xenograft tumors. In hematoxylin and eosin (H&E)-stained sections from xenograft tumors of MB-453 and MB-231 cells without bicalutamide treatment, numerous tumor cells with positive Ki-67 expression were observed compared to cells with bicalutamide treatment (Fig. 5C, $^{**}P<0.001$). These results indicate that bicalutamide acts as a tumor suppressor in breast tumorigenesis. Bicalutamide treatment enhanced cell death compared to xenograft tumors without treatment, as detected by the Tunel assay (Fig. 5D, $^{**}P<0.001$).

Bicalutamide inhibits AR cooperates with β-catenin in transcriptional downregulation of CMYC

The molecular mechanism of how bicalutamide influences the development of AR is unclear. As shown in Fig. 6A, we explored the different interaction partners of AR using Ingenuity Pathway Analysis (IPA) software (String Database). The analysis speculated

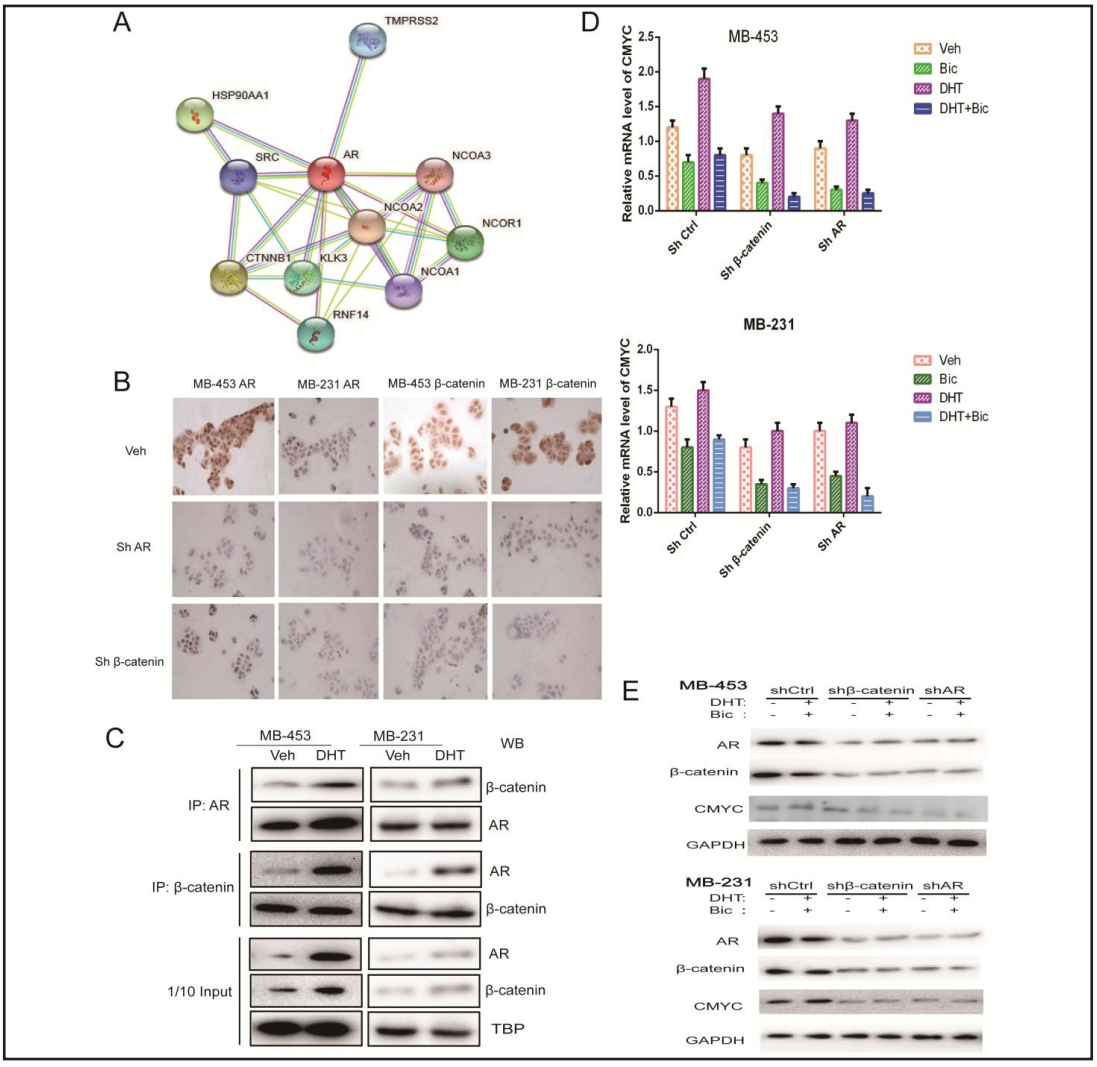

Fig. 6. Bicalutamide (Bic) inhibits AR cooperates with β-catenin in MB-453 and MB-231 cell lines. (A) Identification of molecular interactions of AR from Ingenuity Pathway Analysis. AR can interact and regulate the function of CTNNB1 (β-catenin). (B) AR and β-catenin knockdown inhibits reciprocal expression by cell histochemistry in MB-453 and MB-231 cell lines. (C) Coimmunoprecipitation of endogenous AR and β-catenin from the extracts of MB-453 and MB-231 cell lines. (D) MB-453 and MB-231 cells transduced with the indicated shRNA lentivirus were treated with vehicle, Bic, and/or DHT, and the total RNA was subjected to RT-PCR of CMYC. mRNA levels are presented as means with SD. (E) Immunoblotting to determine CMYC expression in MB-453 and MB-231 cells after transduction of the indicated lentiviral shRNA followed by vehicle (–) or DHT and Bic (+) treatment.

that AR can interact with and may regulate the function of many genes, such as CTNNB1 (β-catenin), NCOA1-3, SRC, and KLK3. We verified our hypothesis that AR+/ER- breast cancer might depend on the AR and β-catenin transcription complex and benefits from treatment with bicalutamide. AR silencing caused a decrease in β-catenin at the protein expression level. Interestingly, β-catenin knockdown also diminished AR expression, as determined by immunocytochemical analysis of MB-453 and MB-231 cells (Fig. 6B). We detected the interaction between AR and β-catenin in the extracts of MB-453 and MB-231 breast cancer cells by coimmunoprecipitation (Fig. 6C). We confirmed by RT-PCR that bicalutamide treatment substantially reduced the mRNA expression level of CMYC and that the silencing of β-catenin and AR effectively impeded the expression of CMYC (Fig. 6D). Bicalutamide

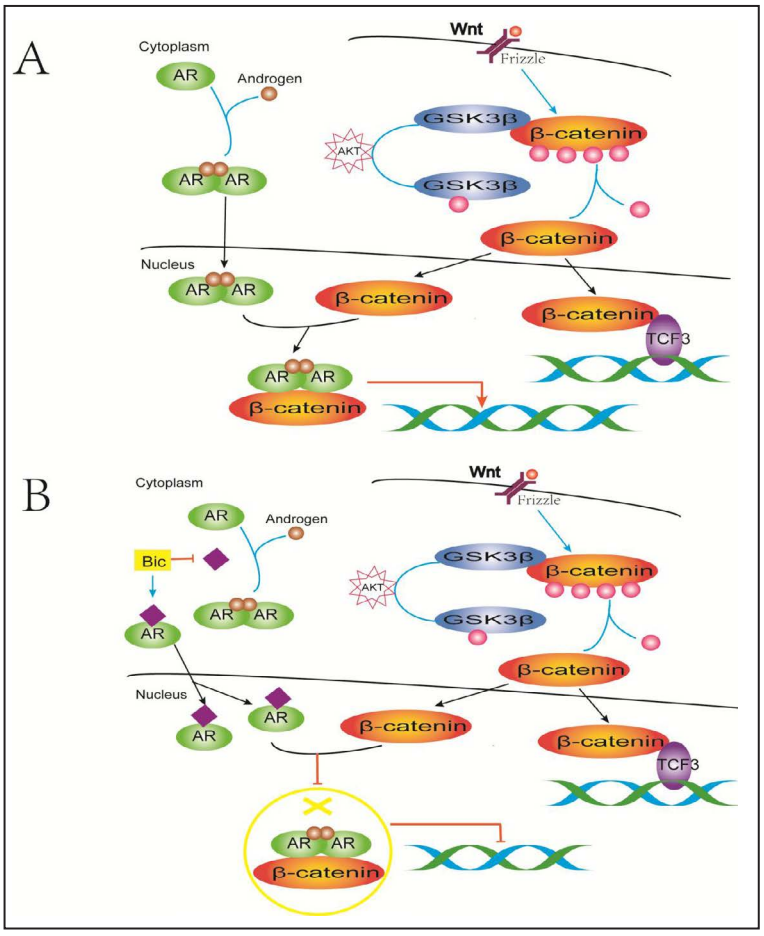

Fig. 7. Model of AR antagonism role of bicalutamide (Bic) in inhibiting Wnt/β-catenin in ER-negative breast cancer. Bic target AR and indirect suppression transcription complex AR and β-catenin by Wnt/β-catenin pathway. (A) Before Bic. (B) After applying Bic.

inhibition led to a reduced expression of CMYC, which was also observed with AR or β-catenin silencing. As expected, the Western blot assay suggested that knockdown of AR diminished β-catenin expression; interestingly, β-catenin silencing caused a decrease in the AR protein expression level, and knockdown of AR or β-catenin inhibited CMYC expression in MB-453 and MB-231 cells (Fig. 6E). In short, these results demonstrate that inhibiting AR with bicalutamide blocks androgen-stimulated oncogenic Wnt/β-catenin signaling and inhibits the growth of ER–/AR+ breast tumors.

Discussion

Endocrine therapies that target ER and estrogen signaling pathways play a crucial role in the treatment of the majority of breast cancer patients. However, over a quarter of breast cancers fail to express ER, and ER-negative patients are not suitable for these endocrine therapies. Our findings show that a considerable number of ER-negative breast cancers express AR and that their growth is stimulated by androgens. We have suggested that AR+/ER- breast cancer might critically depend on the AR and β-catenin transcription complex and may benefit from treatment with bicalutamide.Our study found that bicalutamide antagonizes AR to inhibit oncogenic Wnt/β-catenin signaling pathways through the transcriptional down regulation of CMYC in AR+/ER- breast cancer (as summarized in Fig. 7). This regulatory network indicates a reciprocal link between AR and Wnt/β-catenin pathways in ER-negative breast cancer. Although the existence of AR in breast cancer has been studied for decades, it has not been widely investigated as a potential therapeutic target in malignant breast neoplasms [28].

TNBC and Her2 overexpression breast cancers are biologically more aggressive, are associated with a higher recurrence rate during the first 1–3 years and have a significantly worse overall survival [7, 32, 33].The poor prognosis of patients with TNBC is due to a lack of effective endocrine and targeted therapy. However, AR is expressed in up to 30-50% of patients with TNBC and represents an opportunity for endocrine and targeted therapy [27, 34-36]. Our studies showed that the expression levels of AR in TNBC and Her2 overexpression similar to the results of several other studies [5-7, 12, 15, 32-36]. This range concurs with the rate of AR expression of 39% and 76% achieved in our triple-negative and Her2 overexpression series [4, 15, 33-35]. Our results reported that ER-negative tumors with AR expression has are associated with significantly better disease-free survival than AR-negative tumors [12, 14, 15, 32-36]. It was postulated by Doane [37] that AR functions as an antiproliferative effector in ER-positive breast cancer by antagonizing ER, whereas it facilitates tumor cell proliferation and growth in an androgen-dependent manner in ER-/AR+ breast cancer. Identifying the underlying mechanisms of AR is crucial for designing therapies for estrogen-insensitive neoplasms.

Steroid hormone receptors are crucial components of signaling pathways and act as transcription factors to regulate gene expression [38]. Along with their coactivators, these transcription factors play an important role in breast cancer oncogenesis and progression. AR has been implicated in breast tumorigenesis. However, delineating its precise function has proven difficult. Our study demonstrated that bicalutamide decreases proliferation and increases apoptosis *in vitro*. Moreover, the effects of bicalutamide on viability and apoptosis through AR inhibition *in vivo* were recapitulated in MB-453 and MB-231 xenografts in nude mice. Bicalutamide inhibits cell proliferation and induces apoptosis in AR-positive mesenchymal stem-like TNBC cell lines increasing the expression of p73 and p21 and negatively regulating p53 and Cyclin D1 [39]. Several studies have focused on the role of the high AR-expressing molecular subtype in TNBC and have found that this subtype is responsive to bicalutamide, whereas this subtype is less responsive or nonresponsive to enzalutamide [7, 40].

Our work indicates that targeting AR with antagonists such as bicalutamide may be another way to target the Wnt/β-catenin signaling pathway. A role for Wnt signaling in primary breast cancers of the ER-negative subtype has been described [19, 41]. A phase II clinical trial of bicalutamide in AR+/ER- metastatic breast cancer reported a 19% clinical benefit rate and a median increase of 12 weeks in progression-free survival [3]. In addition, a phase II clinical trial is currently testing the efficacy of enzalutamide in AR+ TNBC (NCT01889238). Bartonreported that MB-231 cells were less sensitive to enzalutamide, as determined by soft agar and caspase-3/7 assays. In our studies, we have shown that the AR level is significantly associated with disease outcome, and our results indicate that the biological functions of bicalutamide decrease proliferation and increase apoptosis in AR+/ER- subtype of breast cancer cells *in vitro* and vivo. The discrepancy in results may be due to differences in the mechanisms of action of the two AR antagonists. Bicalutamide permits AR nuclear localization and binding to chromatin, recruiting corepressors rather than coactivators, whereas enzalutamide inhibits nuclear localization and DNA binding [42].

The direct effect of AR on the Wnt/β-catenin expression level was previously unknown in normal mammary glands and breast tumors. We found that the interaction between β-catenin and AR formed a transcription complex to regulate the downstream gene of the Wnt/β-catenin pathway: CMYC. This regulatory network indicated an intrinsic link between AR and the Wnt/β-catenin pathway in AR+/ER- breast cancer. These results demonstrated that bicalutamide blocks androgen-stimulated oncogenic AR and Wnt/β-catenin signaling and inhibits the growth of AR+/ER- breast cancer.

Although our study generates some important findings, it also has some limitations. Our population is too small and limited to completely determine the prognostic outcomes of different regions and races. Therefore, more multi-ethnic cases and longer follow-up periods may be necessary for this type of analysis. In addition, more studies examining the

mechanism of other cell lines and pathways are needed. Much remains to be done, and more accurate data need to be generated in order to yield more valuable findings in the future.

In conclusion, our study not only provides novel insights into the antagonism of bicalutamide on AR function in AR+/ER- breast cancer but also reveals the mechanistic basis for targeting AR as a therapeutic opportunity for patients with AR+/ER- breast tumors.

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Disclosure Statement

The authors declare no conflicts of interests.

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