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Review

The Role of the Androgen Receptor Signaling in Breast Malignancies

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Abstract. *Breast cancer (BrCa) is the most common malignancy among women worldwide, and one of the leading causes of cancer-related deaths in females. Despite the development of novel therapeutic modalities, triple-negative breast cancer (TNBC) remains an incurable disease. Androgen receptor (AR) is widely expressed in BrCa and its role in the disease may differ depending on the molecular subtype and the stage. Interestingly, AR has been suggested as a potential target candidate in TNBC, while sex hormone levels may regulate the role of AR in BrCa subtypes. In the presence of estrogen receptor α (ERa), AR may antagonize the ERα-induced effects, whereas in the absence of estrogens, AR may act as an ERα-mimic, promoting tumor. Thus, depending on the BrCa micro-environment, both agonists and antagonists of the AR have been suggested as therapeutic approaches. Herein, we review the role of AR signaling in BrCa and the molecular cross-talk mechanisms with other molecules/pathways, as well as its therapeutic implications in the different subtypes of the disease.*

Breast cancer (BrCa) is the most common solid tumor among women with an annual incidence of 123 new cases/100,000 females according to the United States Cancer Statistics and 252.710 estimated new cases in the U.S. in 2017. Despite significant progress made in therapeutics during the last 2 decades, BrCa still has a poor prognosis with 5-year survival rates of metastatic disease reaching to 26% only. BrCa is the second leading cause of death among female cancers with 40,610 estimated deaths in the U.S. expected in 2017 (1).

Breast cancer comprises a heterogeneous group of diseases with variable course and outcome. Currently, BrCa is sub-classified into distinct molecular subtypes named: normal breast like, luminal A/B, HER-2 related, basal-like and claudin-low (2, 3). Estrogen receptor (ER), progesterone receptor (PR) and HER2 have long been established as useful prognostic and predictive biomarkers. Hormonal therapy in ER and PR positive tumors (4), as well as the use of monoclonal antibodies in HER2 over-expressing tumors (5) have shown promising results, however overall survival of metastatic disease remains relatively low (1). To date, androgen receptor (AR) has been suggested to play a key role in breast cancer biology in certain disease subgroups (6, 7).

AR is expressed in all stages of breast cancer (in-situ, primary and metastatic disease) and its contribution to the progression of disease may differ depending on the stage (8). Overall, AR expression among patients with breast cancer has been estimated at approximately 77% and varies significantly among molecular subtypes of BrCa (9). Human observational studies have associated AR with better outcome in ER^+ tumors, but this positive effect may be lost in ER- tumors (10, 11). Of interest, AR expression correlates with better clinicopathological features in the most aggressive form of BrCa, triple-negative breast cancer (TNBC) (12). AR seems to have distinct roles in disease development and progression depending on the tumor's hormonal environment and specifically upon relative levels of androgens and estrogens.

The historically used androgens together with anti-androgens, Selective AR Modulators (SARMs) and Androgen Receptor antagonists constitute valuable options for the treatment of specific disease subpopulations. In the past decade, a wealth body of studies have focused on AR targeting along with hampering major signaling pathways

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implicated in BrCa biology in combinational therapeutic approaches. Herein we review the experimental and clinical evidence investigating the role of AR in BrCa development, progression and metastasis, as well as exploring its therapeutic efficacies in the different subgroups of the disease. Special emphasis has been given on the AR-induced signaling in BrCa and the molecular cross talk mechanisms with other molecules/pathways that may hold promising therapeutic implications.

The Androgen–AR Axis in Normal and Cancerous Breast Tissue

While the estrogen-ERα axis constitutes the predominant regulator of female breast development, androgens also play a significant role through their balanced antagonism with estrogens (13). The main circulating active androgen in females is testosterone, which is synthesized both in the ovaries and the adrenal glands, as well as by peripheral conversion of the inactive androgen precursors such as dehydroepiandrosterone (DHEA) (14). In the breast tissue, testosterone is converted either to dihydrotestostone (DHT) by 5α-reductase or to 17β-estradiol (E2) by aromatase and can function as an AR or ERα agonist respectively, thus having a dichotomous effect on breast development (8, 15). Studies have shown that testosterone is converted to E2 under estrogen deprivation, but is preferentially metabolized into DHT when both hormones are present at physiological levels, thus hampering the E2-induced effects (16, 17). Maintenance of this balance ensures the physiological response of the mammary gland depending on the hormonal needs and the menopausal status.

Although data remain conflicting, AR is indispensable for normal breast growth. AR-knock-out mice showed reluctant ductal extension and branching, as well as markedly reduced epithelial cell proliferation in the breast tissue (18), whereas transcriptional inactivation of AR in another *in vivo* model led to accelerated mammary growth and increased number of terminal end buds, an effect that was reversible with DHT treatment (19). Abnormal cell proliferation could be partially attributed to reduced insulin-like growth factor-1 receptor (IGF-1R) signaling *via* cross-talk mechanisms (impaired MAPK and cyclin D1 activation), underlining the importance of a functional AR for the physiological regulation of breast development (18, 20) .

The addressed effects of exogenous androgen treatment on breast tissue proliferation are also controversial (21). Androgens have been shown to inhibit growth of breast tissue probably *via* interfering with both basal and estrogen-induced proliferation of AR-expressing breast cancer cell lines and epithelial breast cells (21, 22). However, the molecular pathways underlining this inhibitory effect are rather unclear due to the ability of testosterone to act both as AR and ERα agonist. As discussed above, the effect of androgens on breast growth is dependent upon relative levels of estrogens. In the vein to shedding more light on this balance, studies have investigated breast growth under extreme states of androgen signaling. Congenital adrenal hyperplasia, which is characterized by extremely high levels of androgens, associates with inhibition of normal breast growth (15), whereas AR knock-out *in vivo* shows incomplete mammary gland development (18). Moreover, during the menstrual cycle, breast cell proliferation is maximal during the luteal phase, when testosterone levels are reduced and estrogen levels are highest, whereas during the follicular phase, where testosterone levels are stable and estrogens are significantly reduced, breast cell apoptosis is increased (8, 23). In general, evidence shows that although androgens have an inhibitory effect on breast tissue development in the presence of estrogens, AR-induced signaling is compulsory for normal breast tissue growth.

Polymorphisms and Post-translational Modifications of the AR in Breast Cancer

Nowadays cancer is considered as a genetic disease characterized by both inter-tumoral and intra-tumoral (spatial and temporal) genetic heterogeneity. As such, it is conceivable that in the vein of personalized therapeutics, focus has been turned into studying the genetic background of the distinct breast cancer subtypes in selective population subgroups (24).

The gene encoding for the *AR* is located on the X chromosome, having a length of more than 90 kb and is subjected to alternative splicing modifications giving rise to various isoforms. The role of the most common splice variant, V7, has recently been explored in breast cancer tissues. AR V7 was associated with androgen deprivation resistance and was expressed in more than 50% of BrCa cases. Moreover, treatment with enzalutamide, a non-steroidal anti-androgen, led to AR V7 up-regulation suggesting a potential mechanism of acquired resistance to anti-androgen therapy (25).

Variable length of a CAG repeat in exon 1 of the *AR* gene constitutes a functional polymorphism with potential prognostic significance. However, the association between number of triplet-repeats and risk of BrCa development is rather unclear (26). The initial study by Giguere *et al*. showed that smaller numbers of CAG repeats were protective against BrCa development (27). In contrast, the majority of studies that followed showed either positive or no correlation between the CAG repeat length and BrCa risk (28), whereas, a meta-analysis incorporating 6,788 cases of breast cancer patients and 7,648 controls showed that increased number of repeats is protective against BrCa (29). The controversies regarding a clear correlation between CAG repeats and risk of BrCa may be attributed to the lack of an established threshold clearly defining the length of high or low CAG repeats (*i.e*. 21 repeats). Furthermore, population-specific diversity may lead

to distinct frequency patterns (28). Recently, Gottlieb *et al*. described an alternative approach analyzing laser capture micro-dissected tissue specimens from BrCa patients using next generation sequencing in contrast to previous studies that stratified peripheral blood samples. As expected, high intra-tumoral genetic heterogeneity regarding the CAG repeats was revealed, while the preferential selection of 18-25 CAG repeat length was associated with breast cancer. In the same study, it was shown that shorter CAG repeats may be protective against breast cancer risk (26).

The role of AR genotypes in response to endocrine therapy has also been explored. In this vein it has been suggested that certain AR diplotype variants may be associated with longer breast-cancer free survival in response to tamoxifen (30).

AR is also subjected to post-translational processing, predominantly phosphorylation at various sites, serine, threonine and tyrosine residues, which are of major importance for its functions (31, 32). The existence of a series of possible phosphorylation sites of the AR has also been proven *in vivo*, the majority of which are located in the N-terminal of the AR protein. Their role, however, remains unclear (31). In a recent paper, Ren *et al*. explored the value of two specific phosphorylation sites at Ser(P)-213 and Ser(P)-650, as prognostic factors for BrCa using immunohistochemistry. AR-Ser(P)-213 was found significantly increased both in the nucleus and the cytoplasm of breast cancer specimens compared to benign ones, whereas, AR-Ser(P)-650 was significantly decreased (33). It seems that different phosphorylation patterns for AR may apply in benign and cancerous breast tissue. More studies are warranted to shed more light into the role of the distinct post-translational modifications of AR in the pathophysiology of the disease.

AR Signaling in Anti-estrogen-resistant Breast Cancer

Hormonal therapy using Selective Estrogen Receptor Modulators (SERMs) -like Tamoxifen (Tam) and Raloxifen – are the first choice of targeted therapeutics in pre-menopausal women with both early or metastatic disease (34). However, up to 30% of patients show resistance to tamoxifen either from the beginning (*de novo* resistance) or during the course of treatment (acquired resistance) (35). In the vein of understanding the mechanisms of Tam-resistance, focus has turned into the investigation of potential cross talks with other molecules and signaling pathways such as HER-2 (36), IGF-1R (37) and MAPK (38). Currently and despite its central role in BrCa pathogenesis, data exploring the role of AR signaling in Tam-resistant breast tumors are relatively limited.

Expression micro-arrays and qRT-PCR analysis of cancerous breast tissues have shown increased mRNA levels of AR in Tam-resistant tumors compared to Tam-responsive ones (39). Furthermore, *in vitro* studies of AR-overexpressing MCF-7 cells showed increased resistance to Tam, with Tam having AR-agonistic effects. Treatment with the anti-androgen bicalutamide restored Tam-sensitivity, alluding to the underlying interaction between AR and ERα signaling as a key mechanism regulating the response to Tam (39). Of note, aromatase overexpression, leading to increased androgen conversion to estrogens, also restored tam-sensitivity (40).

Aromatase inhibitors (AIs), like anastrozole, letrozole and exemestane, constitute first-line treatment for ER-positive breast tumors in post-menopausal women (41). However, as with tamoxifen, an increasing number of patients do not respond to recurrent treatment with AIs making the development of novel biomarkers that may predict responsiveness a necessity. In this vein, AR-induced signaling may serve as a legitimate resistance mechanism due to its frequent expression in BrCa cells (9), strong co-expression with ER (42), as well as cross-talk with both ERα and IGF-1R signaling (18, 20).

Results from engineered estrogen-responsive MCF-7 cells overexpressing both AR and aromatase showed resistance to the inhibitory effect of anastrazol in contrast to the non-AR overexpressing cells (40). Anastrozole was unable to fully inhibit ERα signaling in AR-overexpressing cells, a notice that might be of clinical importance since tamoxifen- and/or AI-resistant BrCa cells endogenously overexpress AR (40). Immunohistochemical analysis of primary versus recurrent BrCa lesions showed decreased ER and PR, but increased AR expression, in AI-treated recurrent lesions, suggesting an AR dependent growth of AI-resistant lesions (43). Furthermore, *in vitro* models simulating the BrCa microenvironment during AI-treatment (steroids-free, testosterone supplemented media), confirmed an AR-dependent, ER-independent pattern of growth. These results suggested that a subset of patients with AI resistance may benefit from treatment with AR-inhibitors (43). Of interest, Hole *et al*. showed that both letrozole and exemestane were able to inhibit testosterone-stimulated growth of AI-resistant cell lines. In addition, fulvestrant, an ERα antagonist, was able to fully abrogate testosterone-stimulated as well as the basal growth of the same cell lines (44), suggesting that testosterone acted primarily *via* ERα pathway *via* its conversion to E2, underscoring the importance of local sex hormone conversion.

Cross-talk of AR with other Molecules/ Pathways in Breast Cancer

AR signaling has also cross-talks with other molecules that already have been tested as potential biological targets in clinical trials. AR activation induces ERK phosphorylation through an Erbb2 (HER2)-dependent pathway (45). In contrast to androgen treatments which cause transient phosphorylation of ERK, addition of Erbb2 inhibition to these, lead to

permanent ERK phosphorylation, which has been suggested to negatively impact tumor growth (46), probably *via* negative feedback-loop mechanisms. Consequently, therapeutic regimens leading to persistent ERK phosphorylation in combination with AR inhibitors were highlighted as potential therapeutic options in molecular apocrine BrCa, a subcategory of ER^- breast tumors which express AR. Simultaneous treatment of BrCa cell line MDA-MB-453 with flutamide, a non-steroidal anti-androgen, and PM-20, a Cdc25A phosphatase inhibitor, revealed synergy between these drugs as shown by growth inhibition and down-regulation of steroid response genes (47). Furthermore, xenograft tumors in NOD/SCID mice using MDA-MB-453, as an *in vivo* molecular apocrine BrCa model, also showed that combinational therapy with flutamide and PM-20 led to decreased tumor growth, lower tumor cellularity and proliferation index (47).

In addition to ERK, correlation with AKT pathway has also been suggested for AR signaling. AR expression has been associated with *PI3K* activating mutations, while increased mTOR activity and *PIK3CA* mutant TNBC tumors are more likely to have higher AR expression (48, 49). Moreover, combinational therapies targeting both PI3k and AR have shown additive anti-tumor effects in *in vitro* and *in vivo* models of AR^+ TNBC tumors alluding to the potential clinical significance of this regimen in that disease subgroup (50).

As described above, the balance between androgens and estrogens in the tumor microenvironment plays a key role in the progression of the disease. A growing body of experimental studies point to the cross-talk between AR and estrogens. Among others it has been suggested that AR can directly bind to ERα opposing its action (51). In accordance to studies showing that AR overexpression may be associated with down-regulation of the prot-oncogene Myc (52), simultaneous administration of E2 and testosterone *in vivo* resulted in inhibition of the MYC which is usually activated by estrogens (17). In addition to ERα, AR may inhibit growth of BrCa cell lines *via* ER-β up-regulation (53). One of the most widely studied genes in BrCa biology is the breast cancer 1 (*BRCA1*), mutations of which have been correlated with the development of the disease. It has been shown that BRCA1 may act both as an inhibitor of estrogen signaling (54, 55), as well as a co-activator of AR signaling (18, 56).

AR as a Potential Biomarker in Breast Tumors

AR is expressed in more than 70% of primary BrCa, more frequently compared to ER or PR (42, 57) , although its expression is usually correlated with these two receptors (42, 58) and has been suggested as a prognostic factor of the disease with dichotomous outcomes depending on the current tumor microenvironment (10, 11, 59). AR expression is higher in ER^+ tumors (60), with a mean expression of 74.8% in contrast to ER^- tumors where it is estimated at approximately 31.8% of cases (11).

A wealth body of evidence has suggested AR as an independent prognostic factor in ER^+ BrCa, positively associated with favorable outcomes (time to relapse, disease free survival as well as overall survival) (61-63). AR expression has also been associated with favorable clinico-pathological features of the disease, such as lower tumor grade, smaller tumor size and negative lymph-node metastasis (42, 58), as well as, older age at diagnosis (63). Mechanistically, these observations may be explained by the AR-induced inhibitory effects of androgens on estrogen-dependent tumor growth (8).

Estrogen status seems to play a major role in determining the prognostic significance of AR, since the same studies are not supportive for any association between AR positivity and better outcomes in ER- tumors (62, 63). The high histological and molecular diversity of the heterogeneous diseases comprising ER^- BrCa may partially explain the inconclusive results regarding the prognostic value of AR in this molecular subtype of the disease (49). In addition, in the absence of ER (ER- tumors), testosterone may preferentially be metabolized into DHT rather than E2, leading to more potent AR-stimulation (8, 16, 17). On the other hand, AR has been associated with HER-2 overexpression in ER^- tumors (63). Further studies are warranted to elucidate if this might be of translational significance in anti-androgen/anti-HER2 combinational approaches and whether AR may serve as a legitimate biomarker predicting response to anti-HER2 therapeutics in ER^- tumors.

More importantly, AR may serve as a marker predicting response to standard chemotherapeutic regimens in the most difficult to treat and frequently relapsing subgroup of the disease, TNBCs. Of note, ATP-based chemotherapy response assay of tumor cells from 47 patients diagnosed with TNBC showed increased susceptibility of AR-expressing tumors to methotrexate and 5-Fluorouracil (64).

AR as a Therapeutic Target: Experimental and Clinical Evidence

Androgens have been widely used as the main hormonal therapy in the treatment of BrCa (65, 66), but their use has faded after the introduction of tamoxifen and other anti-estrogens, primarily due to their masculinizing side effects. Despite the major progress in controlling the breast malignancies, TNBC remains an incurable disease. As discussed above, depending on the hormonal status in the tumor microenvironment, AR may play a key regulatory role in BrCa progression. As such, in the absence of estrogens, AR may replace the tumor promoting effects of ERα. Consequently, research efforts have focused in targeting the AR in the distinct molecular subtypes of BrCa.

More than 20 years ago, Birrell *et al*. described the effect of DHT and mibolerone, an orally active anabolic-androgenic steroid, on both AR expressing and non-expressing BrCa cell lines and described an AR-dependent inhibitory effect on breast cancer cell growth, reversible by treatments with anti-androgens (67).

SARMs are another class of AR targeting drugs recently being tested for their therapeutic efficacies in TNBC. SARMs show agonistic effects upon AR-binding acting locally with less side-effects compared to systemic androgen therapy. SARMs have been suggested to strongly inhibit breast tumor growth both *in vitro* and *in vivo* (68). DHT and SARMs (GTx-027 and Gtx-024), both acting as agonists of AR, were able to inhibit proliferation of MDA-MB-231 BrCa cells engineered to express AR in a molecular apocrine BrCa model (68). *In vivo* evidence from the same study showed that SARMs in addition to reducing the tumor weight by more than 90% were also able to inhibit the tumor-induced cachexia with the mice gaining 3-5gr of weight in 5 weeks (68). These effects of SARMS in muscle mass have also been tested in human studies including patients with cancer-related cachexia (69, 70).

To date, patients with TNBC are exclusively treated with chemotherapeutic agents, as their response rates to standard endocrine therapy are rather poor (71-73). Propelled by studies using stratified analysis to show that in the TNBC subgroup, but not in the other subgroups (defined by ER, PR and HER2), AR expression may predict a better disease-free survival (74), recent focus has turned towards inhibiting the AR signaling in this disease subgroup. Data however remain conflicting since AR expression among the TNBC patients varies considerably (from 0 and 53%) as addressed by the different studies (75). A phase II clinical trial evaluating the use of bicalutamide in $ER^-/PR^-/AR^+$ advanced breast tumors, showed a clinical benefit rate; complete or partial response or stable disease for more than 6 months, in 19% of patients (76). Two more phase II clinical trials evaluating the efficacy of bicalutamide in AR^+ TNBC are currently ongoing (NCT02353988, NCT00468715), whereas another one was terminated due to slow enrollment of patients (NCT02348281). In the same molecular subgroup of the disease, results from a phase II clinical trial of enzalutamide, are also supportive for a 35% clinical benefit rate at 16 weeks, 29% at 24 weeks and a mean progression free survival of 14 weeks (77). Two more phase II trials of enzalutamide in $TNBC/AR^+$ subjects (NCT01889238) or in HER^+/AR^+ locally advanced or metastatic breast cancer patients in combination with trastuzumab (NCT02091960) are currently ongoing.

Following the promising experimental evidence, the efficacy of AR targeting in ER+ tumors, primary or metastatic, resistant to conventional endocrine therapy (SERMs, aromatase inhibitors) has also been tested (78). In the presence of a malfunctioning ERα, AR becomes the primary mediator of cell growth (39), rendering blockade of the androgen-AR axis an intriguing target for the anti-hormonal resistant ER^+/AR^+ tumors. In this vein, a phase I/II trial of abiraterone acetate, an inhibitor of adrenal androgen synthesis through CYP17 blockade, in postmenopausal women with advanced or metastatic BrCa has recently been completed (NCT00755885) with results pending publication. In another ongoing trial (NCT01381874), abiraterone is being evaluated as combinational therapy with prednisone with or without exemestane in postmenopausal women with ER^+ metastatic breast tumors who have already been treated with aromatase inhibitors. Interestingly, the same compound is currently evaluated in molecular apocrine breast cancer subjects (NCT01842321). More advanced phase clinical trials, in selected population subgroups, may identify AR as a legitimate target in endocrine resistant and triple negative breast tumors.

Conclusion

The significance of AR expression as a predictive biomarker in breast tumors remains unclear. Several reasons may account for the contradictory results addressed, including different methodological approaches, disease heterogeneity, lack of common cut off points on what is considered positive or negative AR expression, distinct population subgroups analysis *etc*. Although several issues need to be elucidated, the era of AR signaling in BrCa research remains imperishable.

The perspective of targeting a commonly expressed hormone receptor, in otherwise treatment unresponsive breast tumors is certainly attractive. The interplay between androgens and estrogens in the breast cancer micro-environment is of major importance in the development and progression of the disease. In the presence of ERα, AR may act as a tumor suppressor by inhibiting the ERα induced effects, whereas in the absence of estrogens, AR may act as an ERα mimic and thus serve as an oncogene (79). In this vein, both AR agonists and antagonists could serve as therapeutic regimens in the different molecular subtypes of the disease. With the limited to no treatment modalities available for the treatment of TNBC, a growing era of developing novel anti-androgens has emerged as potential therapeutic agents. To date, experimental and clinical evidence has shown some early promising results. However, more studies are needed to shed more light in the complex interactions of AR with other molecules in BrCa, and identify novel biomarkers that will predict population subgroups that may benefit from anti-androgen approaches.

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