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An androgen receptor regulated gene score is associated with epithelial to mesenchymal transition features in triple negative breast cancers

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ABSTRACT

Background: Androgen receptor (AR) is considered a marker of better prognosis in hormone receptor positive breast cancers (BC), however, its role in triple negative breast cancer (TNBC) is controversial. This may be attributed to intrinsic molecular differences or scoring methods for AR positivity. We derived AR regulated gene score and examined its utility in BC subtypes.

Methods: AR regulated genes were derived by applying a bioinformatic pipeline on publicly available microarray data sets of AR+ BC cell lines and gene score was calculated as average expression of six AR regulated genes. Tumors were divided into AR high and low based on gene score and associations with clinical parameters, circulating androgens, survival and epithelial to mesenchymal transition (EMT) markers were examined, further evaluated in *invitro* models and public datasets.

Results: 53% (133/249) tumors were classified as AR gene score high and were associated with significantly better clinical parameters, disease-free survival (86.13 vs 72.69 months, log rank $p = 0.032$) when compared to AR low tumors. 36% of TNBC ($N = 66$) were AR gene score high with higher expression of EMT markers ($p = 0.024$) and had high intratumoral levels of 5α-reductase, enzyme involved in intracrine androgen metabolism. In MDA-MB-453 treated with dihydrotestosterone, *SLUG* expression increased, E-cadherin decreased with increase in migration and these changes were reversed with bicalutamide. Similar results were obtained in public datasets.

Conclusion: Deciphering the role of AR in BC is difficult based on AR protein levels alone. Our results support the context dependent function of AR in driving better prognosis in ER positive tumors and EMT features in TNBC tumors.

Introduction

Breast cancer (BC) is the most common cancer in women and is associated with significantly high mortality rates [1]. It is a heterogeneous disease with multiple subtypes, and this has implications on varied response to treatment. Molecular profiling has been used to classify BC into subtypes that has led to identification of targets for treatment and has significantly improved the prognosis for hormone receptor positive (HR+HER2-) and HER2 amplified (HER2+) tumors [2]. On the other hand, triple negative breast cancers (TNBC) are mainly treated with cytotoxic chemotherapy due to lack of actionable therapeutic targets. Hence, new therapeutic targets that can overcome drug

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resistance and improve clinical outcomes remain a clinical unmet need for TNBC.

The Androgen receptor (AR) plays an important role in the biology of BC and is considered a useful marker for prognosis [3,4]. Although AR is commonly expressed by all subtypes of BC, the proportion of AR expressing cells and the activity of AR regulated pathways, differ among the various subtypes [5,6]. Evidence from published literature supports the role of AR as a potential tumor suppressor in HR+HER2- BC and its expression has been associated with better prognosis [3,7,8]. The role of AR in TNBC is debatable and has been reported to be associated with either better or worse prognosis, based on the ethnic population and different methods employed for identifying the AR driven tumors [3, 9–12]. Moreover, the prognostic significance of AR in TNBC is unclear due to usage of different antibodies for detection and varying cut-off values used for assessment of protein expression [13,14].

The role of AR and androgens in carcinogenesis and in promoting metastasis through epithelial to mesenchymal transition (EMT) is well established in prostate cancer [15]. Similar reports of AR promoting EMT in BC has been recently reported using in vitro models [16–19]. Though EMT is commonly thought to be associated with tumor progression, emerging insights of this complex cellular process over the past few years, has shown that the association of EMT with poor prognosis is not consistent across tumors of all solid organs. A recent report by Tan et al. showed that the estimation of EMT through a gene expression based score was not associated with poor disease-free survival (DFS) in BC [19]. DHT treatment of MCF7 cells showed an upregulation of the mesenchymal-associated features suggesting that AR activation may be involved in promoting EMT [20], though mechanistic perspective of this process lacks clarity.

AR is considered as a potential drug target in BC and AR targeted therapies have been evaluated in multiple clinical trials [21–24]. Both AR agonists such as enobosarm [25] and antagonists which are employed as therapeutic agents in prostate cancer are under evaluation in various clinical trials. Treatment of advanced and metastatic AR positive TNBC using AR antagonists like bicalutamide/enzalutamide in combination with other therapeutic agents have shown variable clinical benefit rate (CBR), ranging from 19 to 33% at 16 weeks [26–28]. Similar results were observed in the PREDICT AR trial, where a gene signature was used to identify AR driven disease and to assess any correlation with patient outcome in advanced AR positive TNBC tumors. PREDICT AR+ TNBC tumors showed a better CBR of 39% at 16 weeks when treated with enzalutamide [29]. These results suggest that preselection of patients based on AR activity could improve the clinical response to AR targeted therapies. Moreover, these studies emphasize the need for assessment of the pathways regulated by AR, to gain a better understanding of the molecular mechanisms involved in AR mediated signaling in the various BC subtypes.

A recent multi-institutional study [30] concluded that assessment of AR protein level by IHC alone did not emerge as a reliable marker for predicting prognosis, and variations in the levels of AR protein was observed across populations despite using well standardized consistent methodologies for detection. Conflicting results could also be due to intrinsic molecular differences in AR based signaling among BC subtypes. The use of multiple markers to derive AR regulated gene signature instead of AR protein alone, may help in identifying breast tumors driven by AR signaling or those tumors where AR plays a functional role and hence may be more responsive to AR targeted therapies. Here, we have attempted to develop an AR gene score using AR regulated genes and assessed the association of this gene score with the clinicopathological features and commonly altered pathways in BC.

Material and methods

We applied bioinformatics based methods to identify AR regulated genes using publicly available datasets from both estrogen receptor positive (ER+) and negative (ER-) BC cell lines as explained below.

Bioinformatic based methods to identify the AR regulated genes

Collection of datasets

The gene expression profiles of BC cell lines with series identifier GSE61368 (ZR-75-1 cell line (ER+/AR+) treated with dihydrotestosterone (DHT), estradiol and both vs control) was examined and differentially expressed genes (DEGs) between these conditions were identified to derive the AR regulated genes in ER+ BC.

Similarly, data from series identifier GSE28305 (MDA-MB-453 cell line (ER-/AR+) treated with 10 nM DHT for 16 h) was used to identify DEGs in comparison to the untreated control to identify the AR regulated genes in ER- BC.

Next, AR regulated genes obtained from both the analysis (GSE61368 and GSE28305) were compared. The non-overlapping DEGs in GSE61368 (ER+/AR+) in comparison to GSE28305 (ER-/AR+) were classified as AR regulated genes “in presence of ER” and likewise the non-overlapping DEGs in the GSE28305 (ER-/AR+) were classified as AR regulated genes “in absence of ER”. The DEGs which were overlapping in both datasets were considered as AR regulated genes “independent of ER”.

The detailed sample information is given in the supplementary data S1. The curated genes from various studies related to androgen or estrogen in BC were collected and termed as ‘base genes’.

Data analysis and prioritization of candidate genes

Analysis of the expression data was performed using R package *limma* [31]. To standardize and reduce the technical noise in the probe level data, the raw signal values of each probe sets were normalized using Robust Multiarray Average (RMA)[32] algorithm. DEGs between the control and treatment groups were filtered based on the significant p-value ($p<0.05$).

Protein interaction map and network analysis

The list of DEGs and ‘base genes’ were mapped to the Human Integrated Protein-Protein Interaction Reference (HIPPIE) database [33,34] for constructing Protein Interaction Map (PIM). All the protein interactions of DEGs and ‘base genes’ obtained from the gene expression analysis were extracted with an association score of ≥ 0.4 to create PIM. Visualization and calculation of topological parameters of PIM were performed using Cytoscape (version 3.8.2) [35]. We adopted an approach, which has been formerly applied by Rakshit et al.[36] to identify the hubs. The degree centrality (DC) cut-off threshold formula for choosing the hub protein is defined as:

$$\text{Hubs} = \text{M} + (2 \times \text{SD}), \text{where M} = \text{Mean degree across the genes and SD} = \text{Standard deviation of the degree across genes.}$$

From the PIM, genes and their primary partners that belong to hubs and base genes were extracted to decompose the complex interactome PIM to a significant Protein Interaction Map (sPIM). Figures on protein interaction map and network analysis are provided in supplementary files (supplementary figure 1, supplementary data S2).

Calculation of semantic similarity

Using encoded evidence in the gene ontology (GO) hierarchy, the functional similarity between the gene pairs in the subnetwork sPIM was assessed using R package *GoSemSim* [37]. In this study, we used Wang’s similarity metric [38] to compare the biological process (BP) hierarchy. Next, we filtered the gene pairs in which one gene among the pair has at least absolute fold change of 1.4. To retain genes which have differential expression but were removed due to lack of connectivity, absolute fold change with a specific threshold was screened from the initial list of significant genes and those which fall above top 75 quantile were screened and included in the final list.

Cohort details

Tumor samples were chosen from a retrospective cohort of 244 women with primary BC including 5 women with bilateral tumors. These samples were collected as part of an observational longitudinal study from two tertiary cancer care hospitals in Bangalore, India between 2008 and 2013 and these women were followed-up for up to 9 years, with a total loss to follow up of less than 5%, and a median follow-up duration of more than 72 months. Informed consent was obtained from all the patients to use their tissue and blood sample for research and the study was approved by the ethical committee of both institutions. Information on clinical variables like age, grade, tumor size, lymph node status, stage of the disease with ER, Progesterone receptor (PgR) and HER2 were obtained from their clinical records (supplementary table S3). Formalin fixed paraffin embedded (FFPE) blocks from tumor tissue having more than 50% of representative tumor were selected for the study.

Immunohistochemistry of AR

Immunohistochemistry for AR was done on each of the tumor sections as per standard protocol using the Ventana Benchmark XT staining system (Ventana Medical Systems, Tucson, AZ, USA); the detailed methodology of which has been described in our previous publication [39], primary antibody for AR (Clone AR 441, DAKO, dilution at 1:75) was used with positive and negative controls run for each batch. Two pathologists (JSP and SP) scored the staining for AR protein independently and arrived at a final score. Nuclear staining in more $\geq$1% of tumor cells was considered as positive expression.

We also accessed tissue microarray sections of an independent cohort ($N = 107$) and examined the presence of AR and ZEB1 using the primary antibody for ZEB1 (Clone E2G6Y, Rabbit mAb, CST, Cat #70,512) at a dilution of 1:200. Any staining in $\geq$1% of tumor cells or tumor associated fibroblasts was taken as positivity for ZEB1. Stromal and tumor epithelial cells were differentiated in comparison to their corresponding haematoxylin & eosin stained sections.

Estimation of testosterone

The estimation of total testosterone in serum samples of the selected BC patients was done by a chemiluminescence based immunoassay method using the Abbott Architect ci8200 (Integrated) & i2000 (Immunoassay) instrument, detailed methodology of which has been described in our previous publication [39]. The serum samples were collected prior to surgery or after surgery from 154 BC patients.

Gene expression by quantitative real time PCR (qPCR)

Total RNA extraction was done using the Tri Reagent protocol according to manufacturer's instructions (Sigma Aldrich #T9424) from two 20 μm sections from the selected tumor blocks following the methods published previously(41). 500 ng of total RNA was reverse transcribed to cDNA using high-capacity cDNA conversion kit from Thermofisher scientific (Cat #4322171) as per manufacturer's instruction.

Primers were designed for the AR regulated genes (*CYP4Z1, TFAP2B, ABCC11, SOCS2, GADD45G, ZNF689, ID1, PIP, UGT2B11, KCNMA1,* SEC*14L2 and DOCK2*), proliferation related genes (*BIRC5, ANLN, CENPF* and *UBE2C*), EMT related genes (*SNAI2, TWIST1, ZEB1* and *ZEB2*) and genes coding for enzymes involved in androgen and estrogen synthesis (*SRD5A1* and *CYP19A1*) using primer 3 plus software and further validated on ensemble genome browser, NCBI blast and UCSC genome browser. The primers were synthesized by Juniper Life sciences, Bangalore, India. The details of the primer sequences are given in the supplementary table S4. The methods used for nucleic acid extraction, qPCR and selection of housekeeping genes (HKG) and the quality control criteria for inclusion of samples in the analysis has been described in detail in a previous publication [41]. Relative normalised expression was calculated for each gene as previously published [42].

To derive a proliferation score, a logistic regression model was constructed using proliferation related genes namely, *BIRC5, ANLN, CENPF, UBE2C* and *Ki67* protein as determinant. EMT score was derived as mean expression of chosen key EMT genes namely, *TWIST1, ZEB1, ZEB2* and *SNAI2*. These four EMT genes are transcription factors and considered to be the master regulators of EMT. They belong to the Snail, Zeb and Twist gene families and are involved in promoting EMT and cancer metastasis [43,44]. We also accessed the ER probability score for these tumors in the cohort derived according to methods described previously [40].

Public data sets accessed for analysis and validation

Gene expression data (cDNA microarray profiling, Illumina HT-12 v3 platform) from Molecular Taxonomy of BRCA International Consortium (METABRIC) project was retrieved from the cBioPortal [45](www.cbioportal.org/). The TCGA database (https://www.cancer.gov/tcga) was also accessed to validate our findings.

Calculation of EMT score in the external datasets

We derived an EMT score using a previously validated signature of 77 genes [46]. This is a pan-cancer EMT gene signature derived from 1934 tumors including breast, lung, colon, ovarian, and bladder cancers (total of 11 cancer types). Briefly, the EMT score is determined by subtracting the mean expression of epithelial markers from the mean expression of mesenchymal markers for each sample. A higher EMT score is assumed to be associated with more mesenchymal expression pattern.

Cell lines and culture

MDA-MB-453 cells were seeded in six-well dishes (0.3×10^6 cells per well) in RPMI 1640 and treated with 10 nM DHT alone and in combination with 10 μM bicalutamide (Bic) for 48 or 72 h. Appropriate solvent controls were included for all assays. RNA was extracted from the treated cells as described previously [47] and gene expression of the AR regulated and the EMT genes was assessed by qPCR.

Migration assay

MDA-MD-453 was treated with 10 nM DHT alone and in combination with 10 μM Bic for 72 h as described above. After 72 hours, the media was replaced with low serum media (0.2% Foetal Bovine Serum) and after 6 h, a wound was created by scratching the monolayer of cells using a pipette tip and images were captured at the initiation time (0 hour) and after 24 hours. The migratory ability was quantified and normalized by relative gap distance and compared between solvent control and treated cells.

Western blot

Protein was extracted from MDA-MD-453 treated with 10 nM DHT alone and in combination with 10 μM Bic for 48 or 72 h and expression was assayed as reported previously [47]. The details of the antibodies for E-cadherin, GCDFP-15 and β -actin with dilutions are as follows: anti-β Actin (Sigma; mouse monoclonal,1:1000), anti-E-cadherin (Abcam; Rabbit monoclonal, EP700Y, 1:500) and anti-GCDFP-15 (Abcam: Rabbit monoclonal, EP1583, 1:200). Densitometric analysis was performed using quantity one software (Bio-Rad).

Evaluation of cellular proliferative ability

1×10^4 cells were seeded in 96-well plates and treated with 10 nM DHT alone and in combination with 10 μM Bic as described above. Cell proliferation was assessed by MTT assay as described previously [48]. The assay was performed immediately after treatment (0 hour), 24, 48,

72 hours post-treatment. In an individual experiment, proliferation was studied in decuple, and the overall experiment repeated thrice.

Immunofluorescence

Cells were grown in 4-well slide chambers and subjected to DHT and bicalutamide treatment as described above. Immunofluorescence was performed as reported previously [48]. The cells were incubated in primary antibody anti-E-cadherin (Abcam; Rabbit monoclonal EP700Y, 1:500) and anti-Slug (CST; Rabbit monoclonal C19G7, 1:200) overnight at 4 °C and then labelled with the secondary antibody Alexa Fluor 488 Donkey Anti-Rabbit IgG (H + L) (Invitrogen). The slide was then mounted on gold antifade reagent with DAPI and examined under a fluorescent microscope (Olympus BX51).

Statistical methods

Descriptive analysis was done to evaluate the characteristics of the cohort between high and low AR score groups. Difference in the clinical variables between high and low AR groups was tested by independent Student's *t*-test or Mann-Whitney U test for continuous variables and chi-square test was done for categorical variables. Concordance between the AR regulated score and protein was estimated by receiver operating characteristic (ROC) curve analysis. Kaplan-Meier survival curves and log rank tests were used to compare the disease-free survival between the high and low AR score groups. Disease-free survival was calculated as the time from the date of first diagnosis to the time when a local or distant recurrence occurred. Patients with no event or had death due to non-breast cancer related causes were right censored. All tests were two-tailed and p-value <0.05 was considered statistically significant. All statistical analyses were done using statistical software XLSTAT version 2022.1.2 and R software version 3.6.3.

Results

Deriving the AR regulated gene score

We derived the AR regulated genes through a bioinformatic pipeline as described in the methods from publicly available microarray data sets obtained from ER+AR+ BC cell line (ZR-75-1) and ER-AR+ (MDA-MB-453) BC cell line, treated with DHT. Genes identified through the method were classified based on the context of ER expression (in presence of ER, independent of ER and absence of ER) as shown in Fig. 1. Pathway analysis showed that the commonly regulated genes among the two cell lines (ER+AR+ and ER-AR+) were involved in pathways related to AR and androgen signaling, while gene sets derived from only ER-AR+ cell line were involved in pathways unrelated to AR or androgen signaling. Therefore, AR regulated genes "in presence of ER" (35 genes) and AR regulated genes "independent of ER" (19 genes) were taken ahead for further analysis. A total of 54 genes were chosen and absolute fold change cut-off of 2 with a significant adjusted p-value was used to arrive at the final set of 12 genes. We further evaluated the transcript abundance of the chosen set of 12 genes (gene list in supplementary table S4) in the retrospective BC cohort described earlier.

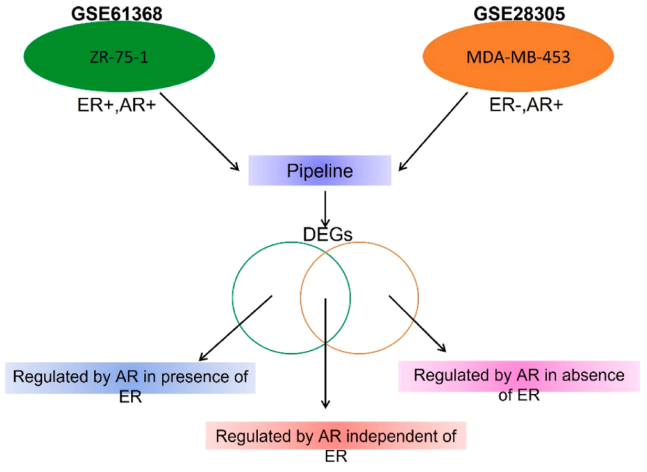

Fig. 1. Bioinformatic workflow to arrive at the three sets of AR regulated genes under different context of ER.

Six of the twelve genes selected (*CYP4Z1, TFAP2B, ABCC11, PIP, KCNMA1* and SEC*14L2*) had a higher fold change and a significant positive correlation with AR transcript levels ($p<0.05$) and all the six genes had a significant higher expression in the AR protein positive tumors ($p<0.05$), except *KCNMA1* ($p = 0.113$). The gene expression values of the six genes have been included in supplementary data S5. Interaction between AR and the twelve AR related genes was derived from the HumanBase [49] (https://hb.flatironinstitute.org), specifically in the mammary epithelium. Ten of the twelve chosen genes are primary interaction protein partners with AR, while only *PIP* and *CYP4Z1* are secondary protein interaction partners with one degree of separation (Supplementary figure 2). AR regulated gene score was calculated as average expression of these genes and this score had significant concordance with AR protein by ROC analysis (AUC-0.65, $p = 0.001$) in our BC cohort.

Prognostic value of AR regulated gene score

The AR regulated gene score ranged from 4.66 to 17.62 with a mean value of 10.51 and median of 10.6 in our BC cohort. The mean cut-off was taken to divide the tumors into AR high and AR low. 53% (133/249) tumors were classified as AR high by the gene score and were observed to be associated with significantly better clinical parameters such as higher age, smaller tumor size, lower grade, lower stage (all $p<0.05$), and a higher proportion of these were associated with post-menopausal status. These tumors also correlated with a lower proliferation score ($p = 0.016$) and higher ER probability score ($p = 0.007$) as shown in Table 1 and supplementary Fig. 3A & 3B

We then examined the prognostic relevance of AR protein in our

Table 1
Comparison of clinical variables between high and low AR groups in all tumors ($N = 249$).

Clinicopathological characteristics		AR high ($N = 133$) N (%)	AR Low ($N = 116$) N (%)	p-value
Age	Mean	58.14	54.16	**0.008**
	Median	59	53.5	
T-size	Mean	3.23	3.4	0.424
	Median	3	3	
	T1	44(35)	23(21)	**0.048**
	T2	70(55)	76(67)	
	T3	13(10)	14(12)	
Lymph Node	Positive	75(59)	73(65)	0.339
	Negative	53(41)	40(35)	
Stage	Low (I, II)	95(71)	65(56)	**0.011**
	High (III, IV)	38(29)	51(44)	
Grade	Low (I, II)	81(65)	53(47)	**0.005**
	High (III)	43(35)	59(53)	
Estrogen Receptor	Positive	102(77)	72(62)	**0.012**
	Negative	31(23)	44(38)	
Progesterone Receptor	Positive	93(70)	68(59)	0.063
	Negative	40(30)	48(41)	
HER2	Positive	32(24)	19(16)	0.166
	Negative	90(68)	81(70)	
	Equivocal	11(8)	16(14)	
Menopausal status	Pre	30(23)	39(34)	0.052
	Post	103(77)	77(66)	
ER probability score	Mean	0.664	0.524	**0.016**
Proliferation score	Mean	0.561	0.603	**0.007**

$p<0.05$ - Statistically significant (represented in bold).

cohort. AR protein status from IHC was available for 165/249 tumors. Of the 165 tumors, only 60 tumors were positive for AR protein (36%). As reported earlier [39], a disease-free survival analysis between AR positive and negative patients showed no significant difference in survival between the two groups in our cohort, indicating that evaluation of AR protein alone may not be of prognostic implication in these tumors. Next, we examined the survival difference between the AR groups (N = 225), 13 patients presented in stage IV and 6 patients lost to follow-up were excluded from original cohort of 244 patients included in the study. On Kaplan-Meier survival analysis, patients with high AR regulated gene score had significantly better survival when compared to the AR low tumors (mean survival time of 86.13 vs 72.69 months, log rank p = 0.032), clearly demonstrating that this score may be a better prognostic indicator than the AR protein (Fig. 2). A subtype specific DFS analysis in HR+HER2- and TNBC tumors however did not show difference in survival.

AR regulated TNBC have high expression of SRD5A1

We first examined the circulating levels of total testosterone between the AR high and low group of tumors and observed no difference between them (p = 0.839). Intracrine levels of the steroid hormones are known to influence the signaling through activation of steroid receptors [50]. Studies have also shown that intracrine androgen levels are higher within breast tissue than in circulation and is often not reflected by circulating androgens but rather by androgen metabolites and conjugates [51]. Therefore, we examined the expression levels of androgen synthesizing enzyme, *SRD5A1* and estrogen synthesizing enzyme *CYP19A1* within all tumors. *SRD5A1* catalyses the conversion of testosterone to DHT and *CYP19A1* catalyses the conversion of testosterone to estradiol.

We observed AR high group of tumors had a significant higher expression of *SRD5A1* within all tumors (p = 0.03, Fig. 3A). Further examination of the *SRD5A1* within subtypes showed no changes in HR+HER2- (p = 0.214) or HER2+ tumors and the difference was confined to TNBC tumors alone (p = 0.04, Fig. 3B). Expression level of *CYP19A1* however did not differ between the AR high and low tumors (p = 0.413), indicating estrogen metabolism was not altered in these tumors.

Tumors with high AR regulated gene score have high expression of EMT markers

AR is shown to induce EMT within prostate cancer while its association with EMT in BC is not well defined. We examined the distribution of EMT score (taken as the average expression of EMT related genes, details in methodology section), among the high and low AR driven tumors. It was observed that the tumors with a high AR regulated gene score were associated with a significantly higher EMT score than the low AR tumors (p = 0.017) as seen in Fig. 4A.

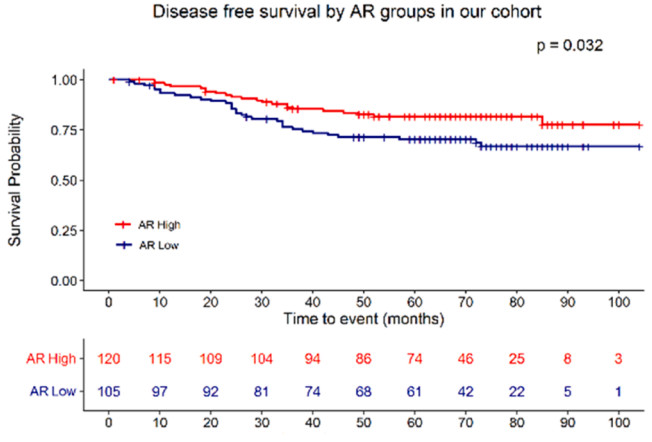

Fig. 2. Kaplan-Meier survival analysis showing the disease-free survival between AR high and AR low groups in all tumors in our cohort.

Next, to verify if the observed association of AR with EMT is confined to any subtype of BC, we divided the tumors into HR+HER2- (N = 132), HER2+ (N = 51) and TNBC (N = 66). Subset analysis within HR+HER2- and HER2+ tumors did not show any significant association between the two scores and no difference was observed in the EMT score between the AR high and low tumors (p = 0.190 and p = 0.455 respectively).

In the TNBC tumors (N = 66), 36% (24/66) had a high AR regulated score and these tumors were associated with favorable features such as higher age, smaller tumor size and significant lower proliferation score (p = 0.008) [52,53] and higher ER probability score (p<0.0001) (Table 2 & supplementary figure 4A & 4B), suggestive of luminal associated features. However, these tumors correlated with a significant higher EMT score (p = 0.024) compared to the AR low tumors (Fig. 4B), indicating that the association of AR with EMT was confined to TNBC tumors alone.

Crosstalk between the EMT regulating transcription factor ZEB1 and AR has been reported [54] previously. The positive association of EMT score in AR high tumors led us to examine the expression of ZEB1 in these tumors. A significant positive correlation was observed between *ZEB1* transcript and AR regulated gene score within all BCs (Pearson's r = 0.329, p<0.0010) including the TNBC subset (Pearson's r = 0.409, p = 0.001). Similarly, a significant positive correlation (p<0.05) was observed between AR regulated gene score and the transcript expression of other EMT markers in all tumors, except *SNAI2* (Pearson's r = 0.318, 0.286 for *ZEB1, TWIST1* respectively) and within TNBC tumors (Pearson's r = 0.370,0.287,0.374 for *ZEB1, TWIST1, SNAI2* respectively). We also examined the distribution of ZEB1 protein on the BC tumor cores in an independent cohort of tumors and found its expression predominantly in the stromal compartment of the tumor (Fig. 5). 52/99 (53%) of tumors in the tissue microarray (TMA) showed positivity for AR expression. Comparison of the expression pattern of ZEB1 between AR positive and negative tumors showed a higher expression of ZEB1 in AR positive tumors (p = 0.065). Only 2/19 (11%) of TNBC tumors were AR positive and no differences were observed in ZEB1 expression between the AR positive and negative TNBC tumors.

DHT treatment of MDA-MB-453 increases expression of EMT associated markers

We next examined the ability of DHT to induce EMT within the AR expressing BC cell line MDA-MB-453. In-vitro validation of the AR regulated score was performed by treatment of MDA-MB-453 with DHT and DHT+Bic as described in the methods. Activation of AR signaling and its subsequent repression using an antagonist (Bic) was validated by assessing the expression of AR downstream protein GCDFP-15. GCDFP-15 levels increased by 31.47% upon DHT treatment and (p = 0.022) was significantly repressed upon treatment with Bic (p = 0.028) as shown in Fig. 6A and 6B. Further, expression of the six AR regulated genes were analysed by qPCR and the mean of the gene expression value was calculated to derive the AR regulated gene score. A threefold increase in the AR regulated gene score was observed in the DHT treated cells (p = 0.11) and a slight decrease in expression was observed upon treatment with Bic (p = 0.44) (supplementary figure 5). However, when the six genes were examined individually, with DHT treatment, a significant increase in fold change (p<0.05) was observed in three genes namely, SEC*14L2, ABCC11* and *KCNMA1* and this expression was significantly repressed upon treatment with Bic (p<0.05, supplementary figure 6).

We observed morphological changes in MDA-MB-453 being more mesenchymal after treatment with DHT. Examination of the mRNA expression of EMT master regulator, *SLUG* showed an increase (fold change>3, p = 0.045) and this was significantly repressed (p = 0.003) with Bic treatment (Fig 6C). This was further confirmed by

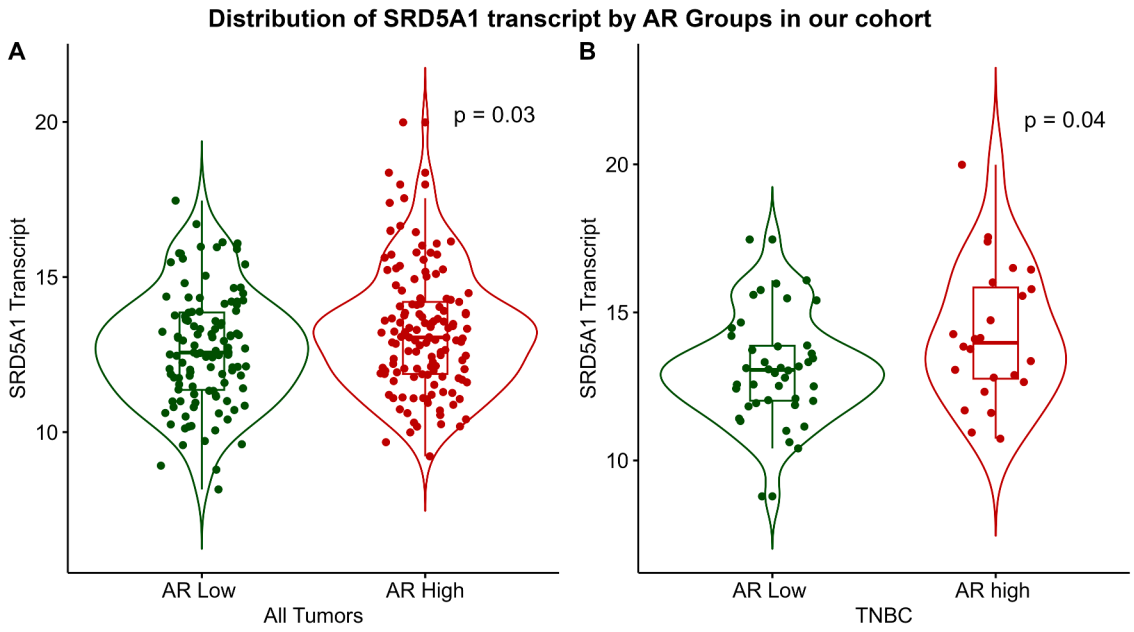

Fig. 3. *SRD5A1* levels in AR high and low groups in our cohort. (A) Distribution of *SRD5A1* transcript in all tumors (B) Distribution of *SRD5A1* transcript in TNBC tumors.

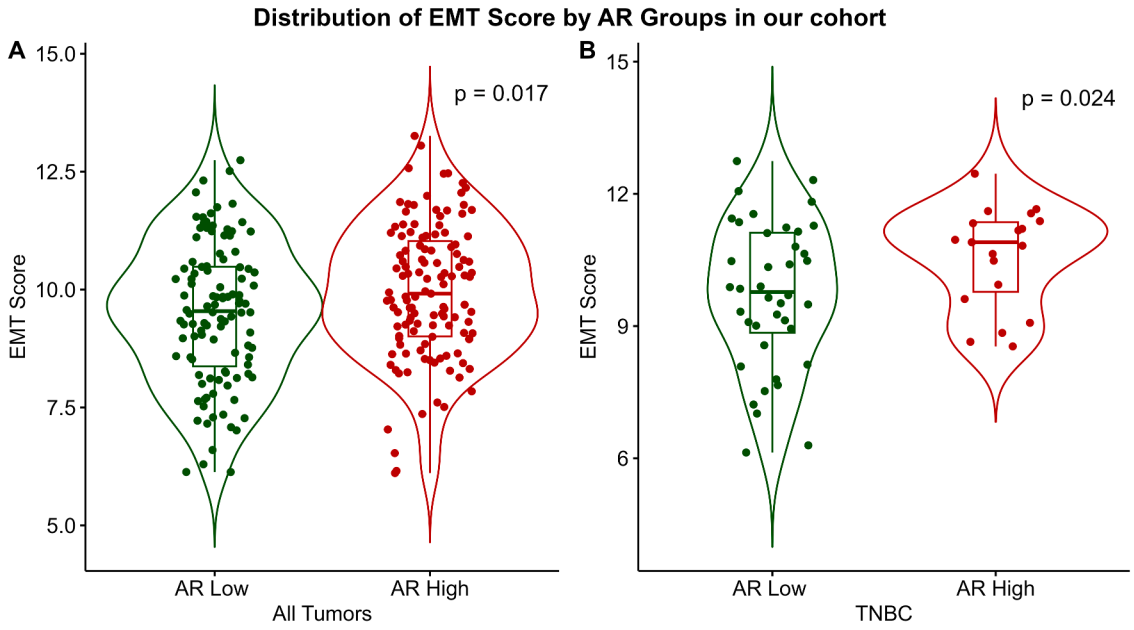

Fig. 4. EMT score in AR high and low groups in our cohort. (A) Distribution of EMT score in all tumors (B) Distribution of EMT score in TNBC tumors.

immunofluorescence where an increase in expression of SLUG protein in the DHT treated cells was observed by (Fig 6D). However, expression of the other EMT genes (*TWIST1, ZEB1 & ZEB2*) did not vary with the treatment conditions employed. Wound healing assay done to measure the migration ability of the cells showed a 22% increase in migration ($p = 0.027$) and Bic treatment reduced the migratory ability of the DHT treated cells ($p = 0.145$) as shown in Fig. 6E and 6F. A significant loss of E-cadherin protein (by 23.65%; $p = 0.024$) was also observed with DHT treatment and this was completely reversed upon when treated with Bic ($p = 0.047$) (Fig 6G & 6H). Findings were further confirmed by immunofluorescence (Fig 6I). We did not however observe any changes in the proliferative potential of the cells treated with DHT with or without Bic.

Validation in external cohorts

To validate our findings, we accessed the METABRIC cohort with a total of 1904 tumors. Of these, 1369 tumors were HR+HER2-, 299 were TNBC and 236 were HER2+. As observed in our cohort, the six AR regulated genes were significantly and positively correlated with AR transcript in the METABRIC cohort, and the AR regulated gene score was calculated as described above. This score ranged from 5.37 to 10.92 and mean cut-off at 7.77 was used to divide the tumors into AR high and AR low. 998/1904 (52%) were AR high and as observed in our cohort, these tumors had favourable clinicopathological features like low grade, low stage, smaller T-size and were mostly lymph node negative and post-menopausal ($p<0.05$) (supplementary table S6). The ER score and proliferation score was calculated by taking the average of the epithelial

Table 2
Comparison of clinical variables between high and low AR groups in TNBC tumors ($N = 66$).

Clinicopathological characteristics		AR high ($N = 24$) N (%)	AR Low ($N = 42$) N (%)	p-value
Age	Mean	55.33	50.97	0.143
	Median	55	52	
T-size	Mean	3.45	3.6	0.685
	Median	2.9	3.5	
	1	6(27)	6(15)	0.438
	2	14(64)	29(71)	
	3	2(9)	6(14)	
Lymph Node	Positive	12(52)	20(49)	0.794
	Negative	11(48)	21(51)	
Stage	Low (I, II)	15(63)	29(69)	0.587
	High (III, IV)	9(37)	13(31)	
Grade	Low (I, II)	12(55)	18(45)	0.472
	High (III)	10(45)	22(55)	
Menopausal status	Pre	7(29)	15(36)	0.585
	Post	17(71)	27(64)	
ER probability score	Mean	0.304	0.075	**<0.0001**
Proliferation score	Mean	0.596	0.687	**0.008**

$p<0.05$ - Statistically significant (represented in bold).

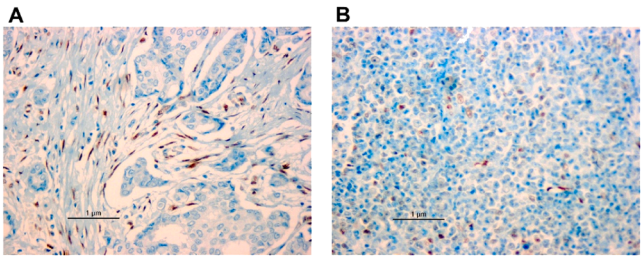

Fig. 5. Representative IHC image of ZEB1 protein expression at 20X (A) In stroma and (B) In tumor. Brown color indicates the positively stained tumor/stromal cells in the blue background stain of hematoxylin.

makers and proliferation related markers respectively. As seen in our cohort, the AR high tumors had a significantly higher ER score ($p<0.001$) and a lower proliferation score ($p<0.001$) across all tumors. Disease-free survival analysis showed that the tumors with high AR regulated gene score had significantly better DFS (mean survival time of 232.13 vs 211.46 months, log rank $p = 0.003$) when compared to the AR low tumors in the METABRIC cohort. On further analysis of the survival within each subtype of BC, a significant association of AR high tumors with good prognosis was observed only within the HR+HER2- (mean survival time of 247.43 vs 206.2 months, log rank $p<0.0001$), and no significant difference in survival between the AR groups was observed within the HER2+ and TNBC subtype.

Further, a subset analysis within the TNBC subtype showed that 51/299 (17%) of the tumors were AR high and were of a lower grade, mostly post-menopausal ($p<0.05$), were associated with a significantly high ER score and a low proliferation score ($p<0.0001$). No other clinicopathological features were significantly different between AR high and AR low tumors.

Next, EMT score (described in methods) was calculated based on a pan-cancer EMT gene signature and this score was compared between the AR high and low tumors. It was observed that the tumors with high AR gene score had a significantly higher EMT score (Fig. 7A and 7B). This correlation was seen in all tumors ($p<2.2e^{-16}$) as well as in the TNBC subtype ($p = 2.6e^{-06}$).

These results further confirmed the findings from our cohort that the TNBC tumors driven by AR may be associated with the traits related to EMT phenotype. In addition, levels of enzyme 5α-Reductase (*SRD5A1*) were also significantly higher in the AR high tumors ($p = 0.001$), an indication that these tumors may be driven by AR signaling. Similar trends were also observed in TCGA cohort (details in the supplementary data S7).

Discussion

The role of AR in breast cancer is very complex, context dependent based on ER status and studies have shown its dual behavior as a promoter of tumor growth in TNBC and inhibitor of tumor progression in HR+HER2- tumors [7,55,56]. The prognostic significance of AR and the perplexity regarding the protein expression in TNBC has been reported in a recent multi-institutional study [30]. Results suggest that AR alone is not a reliable marker as AR expression is population specific and the role of AR as a prognostic indicator is highly variable across different cohorts of patient samples, suggesting the need to derive alternate strategies for better identification of BC tumors driven by AR activity that could respond to anti-AR therapies. Since expression of AR is observed in less than half of BC as evident from data that has emerged from various Indian cohorts [57,58], we attempted to derive an AR regulated gene score using expression profiles of AR regulated genes as an indication of AR downstream activity. A systematic method to create a bioinformatic pipeline was used to arrive at the AR regulated genes using publicly available data from AR+ cell lines representing ER positive (ZR-75–1) and ER negative groups (MDA-MB-453), treated with the non-aromatizable androgen, DHT. This approach is unique as it takes into consideration the presence of ER which is known to highly influence the functional consequence of AR mediated signaling due to their crosstalk [59]. Use of gene expression profiles driven by AR have been largely confined to identification of luminal androgen receptor (LAR) subtype of TNBC and molecular apocrine tumors [60–64] within BC.

In line with previous studies, our method derived extensive gene sets, but this was narrowed down to a smaller set of markers to achieve the advantage of easier application in clinical settings. In our cohort, the AR regulated gene score identified 36% of the TNBC tumors as having a high AR score, whereas in METABRIC and TCGA, only 17% of the TNBC tumors had high AR score indicating population based differences in the molecular composition of TNBC based on ethnicity.

EMT is an evolutionarily conserved and complex process that plays a central role in tumor progression, aggression, invasion, metastasis, and resistance to therapy. Role of AR and androgens in inducing EMT has been well established in experimental systems of prostate cancer [15,65]. Though the regulatory role of AR in EMT was initially disputed, more recent studies have alluded involvement of transcription factors such as Slug [66], ELF5A2 [67,68] and splice variants of AR in inducing EMT and stemness in prostate cancer [69]. Evidence for the role of AR in EMT in BC was initially observed by Liu et al. [70] with downregulation of E-cadherin by AR activation in both metastatic (MDA-MB-435) and nonmetastatic (T47D) BC cells [70].Though induction of EMT by steroid nuclear receptors in BC was referred to by *in silico* approaches [64], more definitive mechanism was shown in experimental model system involving MDA-MB 453, a cell line representing the LAR subtype of TNBC. These cells acquired mesenchymal features when treated with AR agonist [16], mediated through β catenin and Wnt signaling and more recent work by the same group show regulation of EMT by RGS2, an AR mediated protein [18]. We observed similar findings of enhanced epithelial to mesenchymal traits in MDA-MB-453 cell line upon treatment with DHT.

Giovannelli et al., showed the overexpression of AR in MDA-MB-231 cells, could induce invasiveness through AR/SRC/PI3K complex [71], while Graham et al., showed that ZEB1 and AR regulate each other to promote cell migration or EMT in TNBC cell lines (MDA-MB-231 and MDA-MB-453), and have further demonstrated a suppressive effect of anti-AR drug Bic on ZEB1 expression [54]. Our results from a cohort of primary BC mirror these observations previously reported as we show that AR driven tumors have a higher expression of EMT markers within the TNBC subtype. Validation of these findings in larger public datasets,

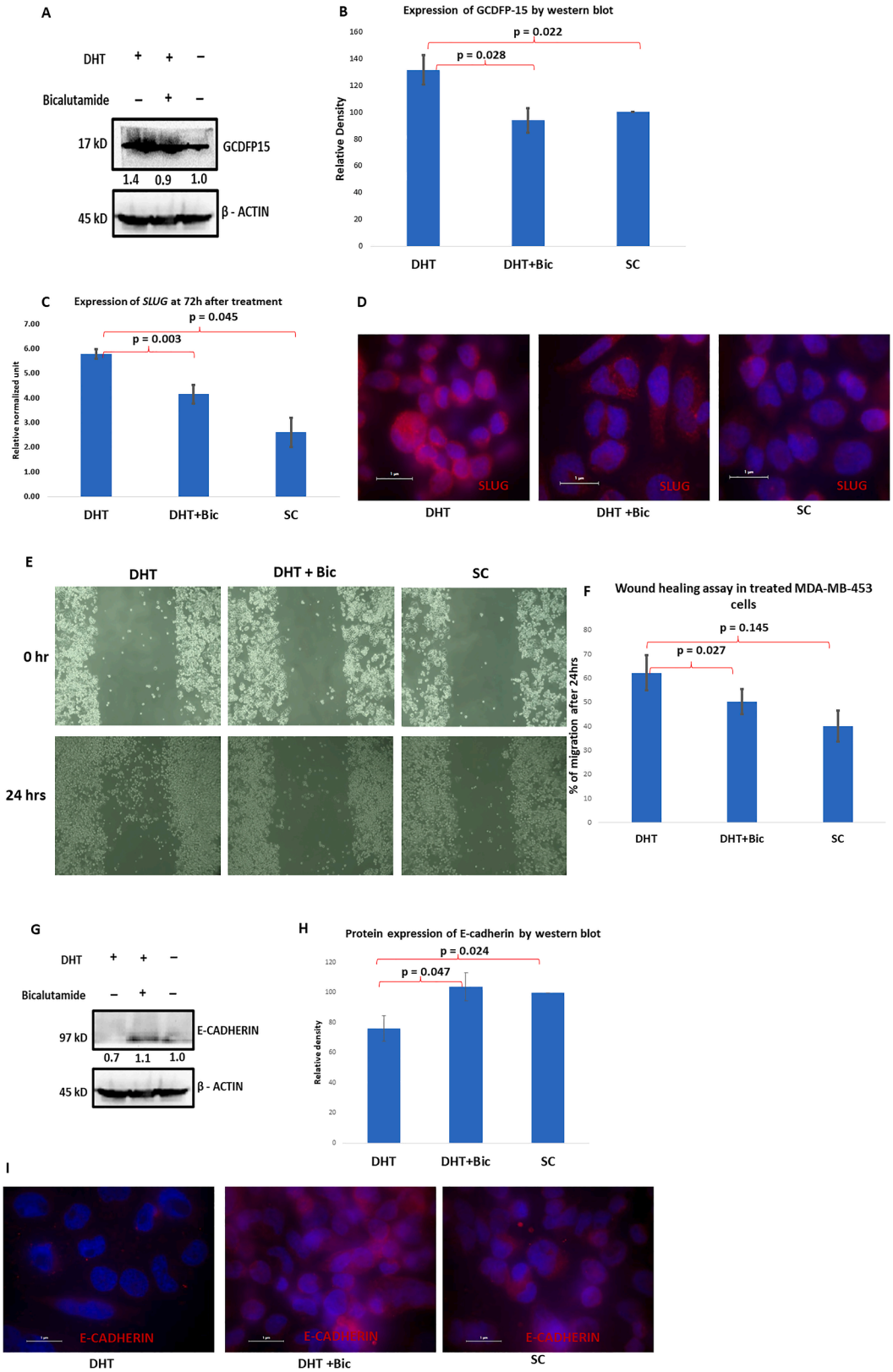

Fig. 6. A & B: Expression levels of GCDFP-15 and β-Actin by western blot in the treated MDA-MB-453 cells. C: Expression of *SLUG* transcript by qPCR at different treatment conditions, 72 h after treatment. D: Levels of SLUG protein in the treated MDA-MB-453 cells as reported by immunofluorescence. E: Wound healing assay showing MDA-MB-453 cells migrating under different treatment conditions calculated at 24 h after scratch, magnification at 6.7X. F: Graph depicting the percentage of migratory cells in different treatment conditions. G & H: Expression levels of E-cadherin and β-Actin by western blot in the treated MDA-MB-453 cells. I: Levels of E-cadherin protein in the treated MDA-MB-453 cells as reported by immunofluorescence. Values are mean ± SEM (n = 3). Statistical analysis was performed by the student's *t*-test compared with the solvent control.

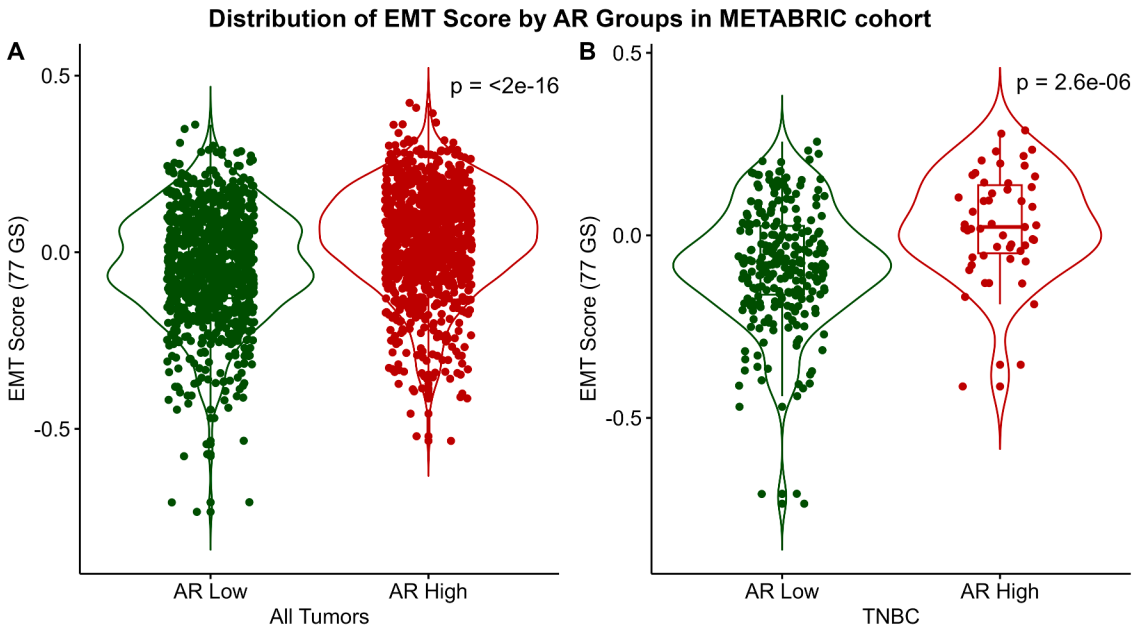

Fig. 7. EMT score in AR high and low groups in METABRIC cohort. (A) Distribution of EMT score in all tumors (B) Distribution of EMT score in TNBC tumors.

further confirm the association of AR with this process. Though the positive association between AR and EMT was observed across all tumors, subset analysis showed that the phenomenon was confined to TNBC tumors alone. The number of AR driven tumors was higher in HR+HER- and HER2+ tumors, but this trend was not observed in these subtypes suggesting AR is an insufficient driver in the presence of ER and HER2 and also, the pathways driven by AR may differ in the absence of these receptors.

EMT is a phenomenon that contributes to tumor progression. However, research in recent years suggests that EMT programs can be heterogenous and may have many different phenotypic manifestations. A recent publication by the "EMT International association" [72] shows the complexity of the process and states that the tissues under pathological conditions express both epithelial and mesenchymal markers suggesting "partial EMT" often exists in tumors as the norm rather than exception. Aggressive behavior of these hybrid phenotypes expressing both epithelial and mesenchymal features were observed in recent preclinical and clinical reports, including breast cancer [73,74]. The correlation of EMT with poor prognosis has also not been consistently observed in BC. Tan et al. observed that their generic EMT score derived using transcriptomic data [19] was not associated with poor DFS in BC, even though it identified poor survival in ovarian and colorectal cancer. Similar results were observed in a more recent study [75] that developed an algorithm to quantify the extent of EMT using gene expression analysis and found BC with lower EMT scores had poor prognosis. The association of many EMT-inhibiting factors such as GRHL2 with poorer survival [76] also emphasizes a context-specific role of EMT in mediating survival, rather than a universal association as previously thought, especially when contemplating of EMT as a linear and binary process. Association of the AR high regulated tumors with a better survival despite being associated with higher EMT features in our cohort are consistent with the published reports above.

Moreover, our results showed that the expression of *SRD5A1* is significantly higher in the AR driven tumors with high EMT score, which may be an indirect implication that active metabolites of androgens may be playing a role in inducing EMT in these tumors. Studies in other cancers have shown the involvement of SRD5A1 in cell migration [77], further supporting our finding. Consistent with the findings that a higher EMT score was observed only in AR driven tumors of the TNBC subtype, high *SRD5A1* levels was also observed only in TNBC and not in the other subtypes.

Deriving the AR regulated genes using cell lines probably is construed as a weakness of our method. Cell lines (MDA-MB-453 and ZR-75–1) have an intrinsic high expression of AR and treatment with DHT is more akin to mimicking the physiological condition of higher circulating levels of testosterone and we employed this method due to the absence of gene expression data from clinical samples with known AR status determined by IHC. We have derived the association of AR with EMT features by correlative analysis from cohort of BC patients and lack of mechanistic explanation by which AR induces EMT within TNBC may be a limitation of the study. However, the exact mechanism by which AR induces EMT is still not discernible despite being attempted in various experimental models. Though we derived our observation from a small number of TNBC tumors within our cohort, the results were validated in larger external datasets to confirm the validity of the AR gene score derived in our study.

Conclusion

The prognostic and predictive role of AR in BC is elusive due to the differential signaling of AR in ER positive when compared to TNBC tumors and due to the absence of well standardized methodologies for detection of AR. Approaches involving multiple downstream markers are better and are likely to identify tumors truly driven by AR than AR protein alone. Our results confirm the dual role of AR in different subtypes of BC and warrant further in-depth assessment of the functional relevance of AR in relation to cellular phenotypic processes such as EMT, to advance a more informed targeted approach for successful use of AR targeted treatments, specifically in TNBC.

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Ethics approval

All procedures performed in this study involving human participants were in accordance with the ethical standards of the Institutional Ethical Committee, St John's Medical College and Hospital, Bangalore and Sri Shankara Cancer Hospital and Research centre, Bangalore and approved by the same.

Consent to participate

Informed consent was obtained from all the patients to use their tissue and blood sample for research.

Consent for publication

None of the participants have any objection for publication.

Author contributions

SR: performance of all experiments, analysis of data and drafting the manuscript. SVP: performed bioinformatic analysis for the study. ACE: performance of IHC and sample collection. MGN, ADM & CMN: involved in cell culture experiments. SP: involved in histological analysis. VPN: performance of experiments. AA: patient consent and follow-up. MP, MKJ & RS: involved in gene score analysis. RSR: surgical oncologist. SBS: surgical oncologist. JSP: Analysis of data, conception and design of the study, performance of histological examination, and drafting the manuscript. All authors contributed to the article and approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2023.101761.

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