Prognostic Role of Androgen Receptor Splice Variant 7 (AR-V7) in the Pathogenesis of Breast Cancer

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Abstract

Background:

The Androgen Receptor (AR) has emerged as an endocrine therapy target in Breast Cancer, exhibiting up to 80% expression in clinical cases. AR-V7, a constitutively activated splice variant of AR with a truncated ligand-binding domain (LBD), demonstrates ligand-independent transcriptional activity and resistance to nonsteroidal antiandrogens like Bicalutamide or Enzalutamide, targeting the LBD. In metastatic prostate cancer, elevated AR-V7 levels lead to therapeutic resistance and increased metastasis.

Methods:

In this study, we evaluated the expression of AR and AR-V7 in cell lines and a cohort of 89 patients undergoing surgical intervention for treatment-naïve breast cancer. Further clinicopathological correlations and survival analysis were performed to evaluate the relationship between the AR and AR-V7 expression and clinical outcomes.

Results:

AR-V7/AR-FL ratio was elevated in the TNBC cell line and downregulation of AR-FL upon AR antagonists' treatment led to a compensatory increase in AR-V7. Clinical samples showed significantly elevated expression of AR and AR-V7 in tumors compared to control cases. Further clinicopathological correlation revealed aggressive clinical traits, higher pathological grades, and poor survival with AR-V7 expression.

Conclusions:

Our study unravels AR-V7 as a marker for poor clinical outcomes, predicting breast cancer aggressiveness, and encourages consideration of AR-V7 as a probable target for therapeutic intervention.

INTRODUCTION

Breast Cancer (BrCa) is a leading cause of cancer-related mortality in women worldwide, with 2.3 million newly registered cases in 2022.[1] Despite an improved 5-year survival rates, mortality remains high, emphasizing the need for early diagnosis and prognostic markers. Hormones like estrogen and progesterone play a substantial role in breast cancer development, binding to Estrogen receptors (ER) and Progesterone receptors (PR), respectively.[2–5] The presence of theranostic biomarkers, ER, PR, and human epidermal growth factor receptor-2 (HER2) determines BrCa clinical management. Triple-negative breast Cancer (TNBC) is characterized by an aggressive phenotype, high metastasis, low overall survival

(OS), and limited treatment options.[6, 7] Systemic management of TNBC involves chemotherapy as a standard of care, which, however, frequently results into chemoresistance.[8] Further, the lack of ER, PR, and HER2 renders this subtype insensitive to endocrine and targeted therapy, necessitating the search for alternative therapeutic targets.

The Androgen Receptor (AR) is a 110 kDa steroid hormone receptor that mediates the action of androgens such as testosterone and dihydrotestosterone. It functions as a ligand-activated transcription factor, including an N-terminal domain (NTD), DNA-binding domain (DBD), hinge region, and carboxyl-terminal Ligand Binding Domain (LBD).[9] AR is expressed in 60% – 80% of breast carcinoma, making it a potential drug target.[10–12] In Prostate Cancer (PCa), AR promotes growth, development, and differentiation upon androgen activation. Androgen Deprivation Therapy (ADT) is the mainstay of treatment for metastatic PCa. Even though initially effective from ADT, tumors perpetually relapse and advance to the Castration-Resistant Prostate Cancer (CRPC) stage, a primary cause of PCa mortality.[13] Additionally, resistance against anti-AR therapies such as Enzalutamide and Abiraterone Acetate is encountered partly due to alterations and structural rearrangement in AR.[14, 15]

Alternative RNA splicing generates constitutively active AR variants (AR-Vs), with AR-V7 being extensively studied.[16–18] AR-V7 originates from splicing AR exons 1, 2, and 3 with cryptic exon 3b, lacking the C-terminal LBD but retaining the transcriptionally active NTD.[17, 19] The detailed transcript and protein structure of AR and its splice variants are represented in Fig. 1. This variant acquires ligand-independent transcriptional activity catalyzing the expression of androgen-responsive genes regardless of androgen levels. The LBD truncation further makes it resistant to drugs that directly target the LBD (Bicalutamide and Enzalutamide), competing with its natural ligands (testosterone and dihydrotestosterone) and inhibiting entry into the nucleus, thus effectively halting the transcriptional programming of AR.[20–22] In a study conducted by Antonarakis et al., AR-V7 expression in circulating tumor cells of CRPC patients has demonstrated a limited response to enzalutamide and abiraterone.[22] Hickey et al., in their work, An Australian study showed that AR-V7 is the predominantly expressed AR variant in clinical breast samples and plays a key role in response to ADT.[23] Another research involving a large cohort of BrCa patients reported that TNBC and metastatic/recurrent subtypes have more expression of AR-V7 associated with apocrine morphology.[24]

Now, the consensus in PCa is that elevated AR-V7 expression leads to resistance against the ADT, increased risk of relapse, shorter overall survival, and ultimately progression to CRPC.[16, 18, 25] With the prominent pleiotropic expression of AR in BrCa, the importance of AR and its splice variants AR-V7 as a prognostic and therapeutic biomarker in clinical breast cancer requires further exploration.

This study attempts to check the expression of AR and AR-V7 in breast cancer in Indian women and to correlate its expression with patient outcome, clinicopathological parameters, ER, PR, and HER2 status. Herein, we demonstrate the varied expression pattern of AR and AR-V7 in clinical breast cancer patients from India, and AR-V7 is clinicopathologically correlated with poor clinical outcomes in breast cancer

patients. We recommend the application of AR-V7 as a screening biomarker for the aggressive phenotype in breast cancer.

METHODS

Cell culture:

The human BrCa cell lines MDA-MB-231, MCF7, and MDA-MB-453 were procured from the American Type Culture Collection (ATCC), Manassas, VA, USA, and PCa cell line LNCaP was purchased from the National Centre for Cell Science, Pune, Maharashtra, India. MDA-MB-231 and MDA-MB-453 were cultured in Leibovitz's L-15 Medium, MCF7 in Minimum Essential Medium Eagle (MEM) along with 0.01 mg/ml human recombinant insulin, and LNCaP cells in RPMI-1640 media (Catalog: AL120A, HiMedia). The culture media was supplemented with Fetal Bovine Serum to a final concentration of 10% and 1% Antibiotic Antimycotic Solution (v/v). The culture media and associated components were purchased from HiMedia Laboratories, Mumbai, India. The cell lines were maintained in an incubator with normoxic conditions, as per ATCC recommendations. The representative images of breast cancer cells and their receptor status is attached in Figure S1.

AR antagonists’ treatment:

For treatment of Bicalutamide (14250, Cayman Chemical, MI, USA) and Enzalutamide (11596, Cayman), the three breast cancer cell lines were seeded into 90 mm Petri dishes. At ~60% confluence, the culture media was replenished with phenol red-free RPMI-1640 media (Catalog: AT120, HiMedia) supplemented with charcoal-stripped FBS (Catalog: RM10416, HiMedia). At ~75% confluence, cells were treated with Bicalutamide and Enzalutamide at 1 μM concentration. 24 hours post-stimulation, cells were harvested to extract RNA and protein for further experiments. The experiments were conducted independently in three biological replicates.

Clinical study design and assessments:

This was a cross-sectional study conducted in the Department of Biochemistry, All India Institute of Medical Sciences (AIIMS), New Delhi, in a closed collaboration with the departments of Surgical Disciplines, Surgical Oncology, and Pathology, AIIMS, New Delhi. The ethics committee approved this study from an ethical perspective for postgraduate research of AIIMS, New Delhi, vide Ref. No. IECPG-177/27.03.2019, RT-08/22.04.2019 dated April 29, 2019.

Enrollment of Patients and baseline data collection:

Participants were identified from a cohort of primary breast cancer patients undergoing surgical intervention for *de novo* breast carcinoma at the AIIMS, New Delhi, a tertiary healthcare center in Northern India. Informed consent from participants was obtained from the subjects prior to the recruitment into the study. All the participants were of Indian origin and 18+ years of age. We reviewed the demographic and clinical data (age, menopausal status, localization, BI-RADS score, tumor size (pathological), lymph node involvement, ER, PR, HER2 status, Ki67 scores, and histological grades from the hospital patient record for clinical correlation. Patients were followed up primarily through outpatient department services or with hospital records for survival assessment.

Processing of Patient's tissue samples:

The tumor core and adjacent benign tissue were collected in RNA later solutions (AM7021, Invitrogen) from the recruited subjects post-operatively. The Tumor specimens were processed for subsequent RNA and whole-cell lysate isolation. The matched tissues were formalin-fixed, paraffin-embedded (FFPE) for routine histopathological examination. These FFPE blocks were used for immunohistochemical evaluations of AR and AR-V7 after sectioning (5 µm) and adherence to poly-L-lysine-coated glass slides.

RNA extraction and cDNA synthesis:

RNA was isolated from cell lines and tissue specimens using ReliaPrep RNA Miniprep Systems (Z6011, Promega) according to the manufacturer's directions. The samples were treated with deoxyribonuclease to rule out any DNA contamination. Complementary DNA was reverse transcribed from total RNA (2 µg) using random hexamers following Verso cDNA Synthesis Kit (AB1453A, Thermo Scientific) protocol.

Reverse transcription-quantitative PCR analysis:

Real-time quantitative PCR was performed on AriaMx Real-time PCR system (G8830A, Agilent) using DyNAmo ColorFlash SYBR Green qPCR Kit (F416L, Thermo Scientific) according to the manufacturer's instructions. Primers were designed specifically for AR-targeting all transcript variants, AR-FL, and AR-V7 only. The primers used are listed in Table 4 in the supplementary information. PCR cycling conditions consisted of an initial denaturation step (7 minutes at 95°C) and 40 cycles of PCR (10 seconds at 95°C, 20 seconds at 60°C, and 20 seconds at 72°C). All qRT-PCR assays were conducted in two technical replicates and three biological replicates. Gene expression analysis was performed using Vandesompele *et al.*, which offers a normalization strategy that uses multiple reference genes and takes primer efficiency into account.[26]

Western blot analysis:

Cells and tissue specimens (followed by crushing and sonication) were lysed in RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, and 1% Triton X) supplemented with protease and phosphatase inhibitor cocktails (#786-108 and #786-782, G Biosciences). The concentration of extracted protein was determined by bicinchoninic acid assay. Protein extracts (40 µg) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrotransferred onto a Nitrocellulose membrane. Membranes were blocked, and antibodies were diluted in 5% skimmed milk (#GRM1254, HiMedia) followed by overnight incubation with primary antibodies at 4°C and subsequent secondary antibodies incubation for 2 hours at room temperature. The primary antibody used for AR and AR-V7 western blotting was anti-AR mAb (MA5-16412, Invitrogen 1:1000). The secondary antibody used was horseradish peroxidase-tagged anti-rabbit IgG. Immunodetection was performed as per the manufacturer's procedure using an enhanced chemiluminescence reagent (34580, ThermoFisher, Scientific). Blots were stripped off and reprobed with a rabbit polyclonal GAPDH antibody (ITT5052, G-Biosciences 1: 1500) as loading control and normalization. AR immunoblots were quantified using the ImageJ software package (NIH, USA) for densitometric analysis. The western blotting densitometry data was normalized to control tissues and reference protein (GAPDH).

Immunohistochemistry:

Immunohistochemical analysis was performed on tissue specimens against AR-FL and AR-V7 using anti-AR mAb (ab227678, Abcam 1:400) and anti-AR-V7 mAb (ab198394, Abcam 1:200). Tissue slices were deparaffinized using xylene. Antigen retrieval was attained by microwaving slides in citrate buffer (pH 6.0) / Tris-EDTA buffer (pH 9.0) for 30 minutes, followed by endogenous peroxidase blocking using 3% H_2O_2. Slides were incubated overnight at 4°C with respective antibodies. The staining was visualized using the 3, 3-diaminobenzidine, and hematoxylin as a counterstain. Pathologic evaluation and semi-quantitative scoring were performed by an expert breast cancer pathologist blinded to clinical data. Tumors observed with nuclear staining of 1% or more with intensities of 1+ to 3+ were deemed positive for AR or AR-V7, consistent with previous studies.[27,28]

Statistical analyses:

Statistical analyses were conducted using GraphPad Prism 9.5 and Stata for clinical statistics. The cell lines-based data were parametric and expressed as the mean values and SD derived from three biological replicates. The clinical data in this study were non-parametrically distributed and expressed in median and all individual values. The comparison among different groups was performed using the Mann-Whitney U-test. Any group of outliers were identified using Rout’s method (Q = 1%) and were removed from the study.

Kaplan-Meier analysis was employed to delineate the survival outcomes within the cohort. Survival data were censored up to May 07, 2024. The Hazard Ratio (logrank) was utilized to evaluate the magnitude of

influence of AR or AR-V7 expression on survival outcomes. Statistical significance was determined through the log-rank (Mantel-Cox) test. For clinicopathological correlation, univariate analysis was executed with Fisher's exact test (if n = ≤4) and Pearson's chi-square test (if n ≥5) tests. The p-value of ≤0.05 was considered statistically significant.

RESULTS

1. AR and AR-V7 are expressed in the breast cancer cell lines

The androgen receptor and its splice variant AR-V7 were assessed for their expression in the different cell lines via qRT-PCR and western blotting. It was observed as shown in Figure 2 that AR (all primary transcripts), AR-FL, and AR-V7 transcript were significantly enriched in the HER2 enriched cell line MDA-MB-453 cells followed by luminal type cell line MCF7 and TNBC cell line MDA-MB-231. Following the qRT-PCR pattern, western blotting also revealed a similar expression profile of AR-FL and AR-V7 in cells. Notably, MDA-MB-231 cells have negligible AR-FL expression. However, it was observed that these cells had a noticeable expression of AR-V7 even in the absence of AR-FL. We assessed the AR-V7/AR-FL ratio, which exhibited the highest value in the MDA-MB-231 (1.93), followed by MCF7 (1.05) and MDA-MB-453 (0.93) cell lines. The prostate carcinoma cell line LNCaP showing high expression of AR and AR-V7 was used as a positive control.[29]

2. AR antagonists increase the AR-V7 expression in cell lines

Several findings reported that suppression of AR-FL signaling using anti-AR drugs resulted in the increased expression of AR-Vs, including the AR-V7 in the prostate cancer cell lines.[19,30–32] However, to date, the effect of AR antagonists on AR-V7 expression levels in breast cancer has not been explored.

To evaluate the effectiveness of AR antagonists on AR spliced variants, the three cell lines were treated with clinically used AR antagonists, Bicalutamide (nonsteroidal antiandrogen) and Enzalutamide (second-generation, more potent AR inhibitor). The qRT-PCR data shows a significant decrease in the AR expression after antagonists' treatment in all the cell lines. In the investigation of AR-FL and AR-V7 expression by qRT-PCR, it was noted that, while antagonists' treatment downregulated AR-FL expression, there was a significant increase in the expression of AR-V7. The pattern of AR-V7 upregulation was consistent for all the cell lines irrespective of receptor status as shown in Figure 3. These results demonstrate the compensatory increase in the AR-V7 after treatment with Bicalutamide and Enzalutamide.

3. Baseline demographics of study subjects

From an 89-sample cohort, case distribution was symmetrical in the 41-50, 51-60, and 61-70 age groups, with a median age of 54 years (ranging from 21 to 77 years). Immunohistochemical analysis of ER, PR, and HER2 according to the ASCO-CAP guidelines [33,34] facilitated the categorization of cases into molecular subtypes. A significant majority was Luminal type (ER-positive, PR ±, and HER2-negative), comprising 56% of fifty samples. The HER-enriched category (ER-negative, PR-negative, and HER2-positive) accounted for 21% of twenty-one cases. Eighteen cases (20%) showed no expression of markers (ER-negative, PR-negative, and HER2-negative). No significant change was observed in the age distribution among the molecular subtype of the subjects. Based on this segregation, the cases were further stratified and analyzed for the expression of AR and AR-V7.

Baseline demography is presented in Table 1.

4. AR and AR-V7 are adequately expressed in clinical breast cancer

AR, AR-FL, and AR-V7 were determined by RNA and protein levels by qRT-PCR and western blotting in breast cancer samples. The qRT-PCR analysis revealed significant upregulation of AR, AR-FL, and AR-V7 in tumors compared to distant control tissue. TCGA breast cancer cohorts showed prominent expression of AR-FL while AR-V7 was less expressed (image attached in Figure S2). The assessment of proliferation status using the MKI67 gene displayed highly significant upregulation in tumors, indicating a robust Ki-67 index in the tumor core relative to normal tissues. Western blotting for AR-FL and AR-V7 demonstrated parallel trends, with a significantly higher cumulative expression in tumor tissues. These results are represented in Figure 4.

Immunohistochemistry represented in Figure 5 showed strong nuclear staining of AR in different subtypes of breast cancer. Most cases were positive for AR-FL (77.6%) and AR-V7 (68.4%) with varying intensities. AR-FL was expressed in 84% of luminal type and HER2 enriched cases and 46.2% of TNBC cases. AR-V7 expression was positive in 63.6% of luminal type, 73.7% of HER2 enriched, and 76.9% of TNBC cases. The higher expression of AR-V7 compared to AR in TNBC is represented in one case that shows positivity for AR-V7 irrespective of AR status. AR and AR-V7 expressions were relatively higher in the HR and HER2-positive breast cancers.

5. Correlation of AR-FL and AR-V7 expression with clinicopathological parameters

The association of AR with a particular tumor's biological characteristics and clinicopathological correlation was further investigated. AR was correlated with advanced age (≥50) ($p = 0.033$), strongly associated with ER-positivity ($p = 0.006$), PR-positivity ($p = 0.007$), and breast cancer subtypes, like luminal type (84.1%), HER2 enriched (84.2%) ($p = 0.023$). It was noted that AR-FL was least expressed in

the TNBC category, aligning with the previous reports.[35] The mean tumor diameter of AR-positive cases was 31.56 mm ± 13.5 mm (range 19-75 mm), while in AR-negative cases, it was 29.64 mm ± 13.08 mm (range 11-65 mm). We also observed that patients with AR-positivity had more than 2 lymph nodes infiltrated with tumors (93.7%), albeit non-significantly (p = 0.095). Table 2 summarizes the correlations between AR expression and clinicopathological factors.

Following this, the association of AR-V7 expression and clinicopathological correlation in BrCa patients was further investigated. As exhibited by AR, AR-V7 was also correlated with higher age (≥50) (p = 0.033). AR expression was strongly correlated with tumor size (≥T3) (p = 0.004). The mean tumor diameter of AR-V7-positive cases was 32.16 mm ± 14.90 mm (range 14-75 mm), while in AR-V7 negative cases, it was 28.57 mm ± 8.7 mm (range 11-45 mm). The mean diameter difference between AR-V7-positive and negative cases is 3.59 mm, exceeding that observed in the AR fraction, which is 1.91 mm. Patients with AR-V7-positivity had more lymph node infiltration (≥N2) (p = 0.014), and two or more lymph nodes were positive for tumor (0.028). AR-V7 was strongly associated with higher grades (3) (p = 0.033) and marginally significant for survival (0.0501). Table 3 shows the correlations between AR-V7 expression and clinicopathological factors.

6. Survival analysis according to AR and AR-V7 status

With a median follow-up period of 25 months, seventy-six patients were assessed for the survival parameters' evaluation. From the breast cancer patients analyzed in this study, fifty-six patients were alive (73.7%), Fourteen patients (18.4%) died during the study, while six patients (7.9%) had their cancer metastasized as of May 2024.

For overall survival, death due to any cause was considered an event, while distant metastasis and death were considered events for disease-free survival. Based on the expression of AR and AR-V7 in these cases, Kaplan-Meier analysis was performed to generate OS and DFS. The hazard ratio (HR) for OS and DFS between the AR-positive and negative groups was close to 1, suggesting AR has no impact on the risk of events. AR positivity correlated with poor OS and DFS, although no statistical significance was reached in either category. On the contrary, AR-V7 expression was associated with poor overall (logrank p = 0.0309, HR: 6.83) and disease-free (logrank p = 0.0211, HR: 4.725) survival. The HR for AR-V7-positive cases versus negative ones in OS and DFS suggests a pronounced risk of events associated with AR-V7 positivity. High AR-V7 correlated with poor survival in prostate cancer patients from TCGA data as shown in supplementary Figure S3. Kaplan-Meier survival plots of OS and DFS for both AR and AR-V7 are represented in Figure 6.

DISCUSSION

Our study aimed to evaluate the expression of AR-FL and AR-V7 in clinical breast cancer and to correlate their expression with clinicopathological features. A significant elevation of AR-FL and AR-V7 expression was noted in the HER2-enriched cell line MDA-MB-453, followed by MCF7 and MDA-MB-231. The

antibody used for AR western blotting targeted AR-NTD, and hence, AR-V7 was invariably detected in the immunoblots. In the TNBC cell line MDA-MB-231, AR-FL (110 kDa protein) was visible only after high chemiluminescence exposure, while AR-V7 was detectable under normal conditions. We observed a higher proportion of AR-V7/AR-FL in the TNBC cell line than luminal and HER2-enriched ones. The increase in the AR-V7 and AR-V7/AR-FL ratio has been documented for the onset of CRPC and is indicative of a more aggressive disease and shorter survival.[36, 37]

In BrCa cells treated with AR antagonists, a decrease in AR-FL expression and a significant increase in AR-V7 levels were observed. This compensatory increase has previously been reported in the prostate cancer cell lines [19, 36, 38] and is implied to be an adaptive shift towards AR-V7-mediated signaling. This phenomenon may also compromise the therapeutic efficacy of the antagonists under consideration (those who target the AR-LBD). Even though the application of anti-AR drugs in breast cancer is in limited clinical settings,[39] this compensatory increase in the AR-V7 must be considered. Through basic and clinical research, AR-V7 has closely been linked to drug resistance against Enzalutamide in CRPC.[40] Notably, AR-V7 not only forms heterodimers with AR-FL,[41] it can also act independently of AR-FL [42] suggesting its activity irrespective of AR ligands. Thus, recognizing the significance of AR-V7 in CRPC progression, research is underway to develop new drugs focussing on AR-V7,[43] including targeting AR-NTD.[44] EPI-7386, one such novel drug, is currently in clinical trials to evaluate its efficacy, both as a monotherapy and in combination with enzalutamide, for AR-V7-positive CRPC patients.[43]

In the clinical phase, 89 treatment-naïve patients were uniformly recruited, largely post-menopausal with higher BI-RADS scores. About three-quarters patients came from urban settings, consistent with Indian cancer registry data,[45] likely influenced by the concentration of cancer treatment facilities in urban regions.[46] Most cases were grade 2 and 3 ductal carcinomas, predominantly pathological T2 in size, with 60% showing positive lymph nodes for tumor infiltration. Through western blotting and qRT-PCR AR, AR-FL, and AR-V7 were found to be statistically significantly upregulated in tumors compared to the control cases. MKI67, as a proliferation marker, was used to assess the actively growing cancer cells and was consistently elevated in all the cases.

The clinicopathological correlation was conducted based on AR and AR-V7 expressions in clinical breast cancer cases. AR was found to be positive in 77% (59 out of 76) cases, aligning with previous studies reporting 60–80% AR positivity in breast cancer.[47] AR positivity correlated significantly with higher age groups (≥ 50) and ER, PR positivity consistent with findings by Anand et al., in the Indian breast cancer cohort.[48] Strong correlations of AR with ER-positive, luminal type, and HER2 enriched cases were observed, as previously reported in several other studies, including the TCGA database.[49–51] TNBC showed a relatively lower AR prevalence at 46%, indicating its preferential association with other hormone receptors.

Regarding AR-V7, it exhibited a significant association with higher age, similar to AR. AR-V7 correlated significantly with larger tumor sizes (T3 and above), increased lymph node involvement (≥ N2), and

higher histological grades of 3. These correlations suggest a potential link between AR-V7 and more aggressive phenotypes and advanced stages of breast cancer. These associations may lead to extensive tumor growth, more likelihood of metastasis, and a poorly differentiated and aggressive tumor. AR-V7 has been associated with resistance to anti-hormone therapy, unfavorable prognosis, and aggressive growth characteristics in prostate cancer.[52, 53] In contrast to AR, which was found to be positive in less than 50% of TNBC cases, AR-V7 was more prevalent, with a prevalence of 77% in TNBC. Additionally, AR-V7 was marginally significant for its association with survival. Taken together, AR-V7 can be linked to more aggressive clinical features in breast cancer cases.

Sample recruitment commenced between April 2019 and August 2022, with a hiatus in 2020 and 2021 due to the COVID-19 pandemic. Among the eighty-nine cases recruited, sixteen showed mortality, mainly from a higher age group and recruited in 2019 and early 2020, aligning with better 5-year survival in breast cancer patients.[54, 55] These parameters were correlated with AR and AR-V7 expression, and survival analysis revealed a trend in poor survival with AR positivity, though not significant. Survival for AR-V7-positive cases was notably worse than AR-positive ones, with higher hazard ratios for OS and DFS, indicating an increased risk of mortality and disease progression, respectively. Interestingly, the Kaplan – Meier curve for OS and DFS for AR positivity was insignificant but turned out significant for AR-V7, evidencing towards aggressive outcomes and poor survival associated with AR-V7.

In summary, a few noteworthy findings came from the current study. First, we showed the compensatory increase in the AR-V7 expression in breast cancer cell lines after treatment with Bicalutamide and Enzalutamide. Second, this is the first study establishing AR-V7 expression in breast cancer cases from India. Third, in line with the findings of prostate cancer, we established the association of AR-V7 expression with aggressive clinicopathological features and predictive biomarker of poor survival.

This study has certain limitations. We recruited eighty-nine *de novo* breast cancer patients diagnosed with primary breast cancer, which was insufficient for validation. Ki67 staining wasn't performed for all cases, preventing stratification into luminal A and B subtypes. Abundant adipose tissue in adjacent control tissue hindered matched control analysis for all the cases. Additional clinicopathological data assessment and a longer patient follow-up duration would strengthen the data and complete the survival analysis for OS and DFS. Considering over 20 AR isoforms reported,[56] evaluating them in clinical breast cancer could provide further insights. Multicentric collaboration across India could mitigate racial and ethnic biases associated with single-center collection from North India.

CONCLUSIONS

Despite recent therapeutic progress, breast cancer, particularly TNBC, remains a challenging cancer. The constitutively active AR isoform, AR-V7, does not require ligand binding and is resistant to standard anti-AR drugs. There is a pressing need to identify biomarkers associated with aggressive phenotypes and adverse clinical outcomes. Overall, the present study has attempted to check the expression and clinical correlation of AR and its constitutively expressed splice variant AR-V7 in clinical breast cancer. To the

best of our knowledge, this is the first of its type of study involving the AR-V7 in clinical breast cancer tissue from India. The findings suggest that AR-V7 may function as an independent biomarker for an aggressive phenotype, proposing its potential utility as a therapeutic target.

Abbreviations

Acronym	Expanded Form
AR	Androgen Receptor
AR-FL	Androgen Receptor Full-Length
AR-V7	Androgen Receptor Splice Variant 7
BCS	Breast-Conserving Surgery
BIRADS	Breast Imaging Reporting and Data System
CRPC	Castration-Resistant Prostate Cancer
DBD	DNA-Binding Domain
DFS	Disease-Free Survival
ER	Estrogen Receptor
FFPE	Formalin-Fixed Paraffin-Embedded
GEPIA	Gene Expression Profiling Interactive Analysis
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hormone Receptor/Hazard Ratio
LBD	Ligand-Binding Domain
MRM	Modified Radical Mastectomy
NTD	N-Terminal Domain
OS	Overall Survival
PR	Progesterone Receptor
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation Assay
SDS	Sodium Dodecyl Sulfate
TCGA	The Cancer Genome Atlas
TNBC	Triple-Negative Breast Cancer

Declarations

Ethics approval and consent to participate

This study was ethically approved from the Institute Ethics Committee for Postgraduate Research All India Institute of Medical Sciences, New Delhi, vide Ref. No. IECPG-177/27.03.2019, RT-08/22.04.2019 dated April 29, 2019. Informed written consent from participants was obtained from the subjects prior to the recruitment into the study. The c

Consent for publication

Not applicable

Availability of data and materials

The list of primers is attached in the supplementary file. qRT-PCR gene-expression data and other clinical details used in the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TPS collected the samples, performed the experiments, analyzed the data, and wrote the manuscript drafts. SA, IG, JT, and AR analyzed the data and reviewed the manuscript. AS, RP, and SD assisted in the clinical specimen and reviewed the manuscript. SRM helped in the execution of this study's histopathological examination. AG provided crucial clinical information. AS, RP, SD, SRM, and AG were major contributors in modifying the first draft of the manuscript. RD and SK conceptualized, monitored, funded, and oversaw the whole study. All authors read and approved the final manuscript.

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Tables

Table 1. Baseline demographics of study subjects

Sl. No.	Characteristics	Number of cases (percentage)
1	**Median age (range)**	54 years (21 to 77)
2	**Age groups**	
	≤30	2 (2.3%)
	31-40	11 (12.4%)
	41-50	23 (25.8%)
	51-60	2 (24.7%)
	61-70	23 (25.8%)
	≥71	8 (9.0%)
3	**Geographic setting**	
	Rural	14 (15.7%)
	Semi-urban	10 (11.2%)
	Urban	65 (73.1%)
4	**Laterality**	
	Left	46 (52.9%)
	Right	41 (47.1%)
5	**Menopause status**	
	Premenopausal	33 (37.1%)
	Postmenopausal	56 (62.9%)
6	**Localization**	
	Ductal	69 (89.6%)
	Lobular	4 (5.2%)
	Mixed	4 (5.2%)
7	**BIRADS**	
	≤3 group	4 (5.9%)
	4 group	24 (35.3%)
	5 group	29 (42.6%)
	≥6 group	10 (16.2%)
8	**Tumor size**	

Sl. No.	Characteristics	Number of cases (percentage)
	≤T1	5 (6.0%)
	T2	58 (69.9%)
	T3	13 (15.7%)
	≥T4	7 (8.4%)
9	**Lymph node**	
	N0	33 (39.8%)
	N1	36 (43.4%)
	N2	7 (8.4%)
	≥N3	7 (8.4%)
10	**ER status**	
	Positive	59 (66.3%)
	Negative	30 (33.7%)
11	**PR status**	
	Positive	52 (58.4%)
	Negative	37 (41.6%)
12	**HER2 status**	
	Positive	21 (23.6%)
	Negative	68 (76.4%)
13	**Ki-67 status (20%)**	
	Low	14 (48.3%)
	High	15 (51.7%)
14	**Histological Grade**	
	1	3 (3.4%)
	2	43 (48.3%)
	3	43 (48.3%)
15	**Surgical intervention**	
	MRM	68 (81.9%)
	BCS	15 (18.1%)

Table 2. Correlation between AR-FL expression and clinicopathological parameters of breast cancer patients

Sl. No.	Characteristics	Cases	AR-positive (n = 59)	AR negative (n = 17)	p-value
1	Age groups (years)				
	≤49	**28**	**18 (64.29%)**	**10 (35.71%)**	**0.033**
	≥50	**48**	**41 (85.82%)**	**7 (14.58%)**	
2	Menopause				
	Premenopause	27	18 (66.67%)	9 (33.33%)	0.089
	Postmenopause	49	41 (83.67%)	8 (16.33%)	
3	Localization				
	Ductal	65	48 (73.85%)	17 (26.15%)	0.187
	Lobular or mixed	7	7 (100.00%)	0 (0.00%)	
4	BI-RADS				
	≤4	26	19 (73.08%)	7 (26.92%)	0.764
	≥5	34	26 (76.47%)	8 (23.53%)	
5	Primary tumor (TNM)				
	≤T2	60	43 (71.67%)	17 (28.33%)	0.746
	≥T3	15	12 (80.00%)	3 (20.00%)	
6	Lymph node (TNM)				
	≤N1	59	43 (76.81%)	16 (23.19%)	0.560
	≥N2	13	12 (66.67%)	1 (33.33%)	
7	Number of lymph nodes				
	≤2	56	40 (71.43%)	16 (28.57%)	0.095
	≥3	16	15 (93.75%)	1 (6.25%)	
8	ER status				
	Positive	**52**	**45 (86.54%)**	**7 (13.46%)**	**0.006**
	Negative	**24**	**14 (58.33%)**	**10 (41.67%)**	
9	PR status				
	Positive	**48**	**42 (87.50%)**	**6 (12.50%)**	**0.007**
	Negative	**28**	**17 (60.71%)**	**11 (39.29%)**	

Sl. No.	Characteristics	Cases	AR-positive (n = 59)	AR negative (n = 17)	p-value
10	**HER2 status**				
	Positive	19	16 (84.21%)	3 (15.79%)	0.537
	Negative	57	43 (75.44%)	14 (24.56%)	
11	**Ki67 (20%)**				
	Low	13	10 (76.92%)	3 (23.08%)	1.000
	High	14	10 (71.43%)	4 (28.57%)	
12	**Molecular Subtypes**				
	Luminal type	**44**	**37 (84.09%)**	**7 (15.91%)**	**0.023**
	HER2 enriched	**12**	**16 (84.21%)**	**3 (15.79%)**	
	TNBC	**13**	**6 (46.15%)**	**7 (53.85%)**	
13	**Histological grade**				
	≤2	37	26 (70.27%)	11 (29.73%)	0.134
	≥3	39	33 (84.62%)	6 (15.38%)	
14	**Survival**				
	Alive	56	42 (75.00%)	14 (25.00%)	1.000
	Dead	14	11 (78.57%)	3 (21.43%)	
For n = <5, Fisher's exact test and n ≥5, Chi-square test; p ≤ 0.05 considered statistically significant.					

Table 3. Correlation between AR-V7 expression and clinicopathological parameters of breast cancer patients

Sl. No.	Characteristics	Cases	AR-V7 positive (n = 52)	AR-V7 negative (n = 24)	p value
1	Age groups (years)				
	≤49	**31**	**15 (53.57%)**	**13 (46.43%)**	**0.033**
	≥50	**45**	**37 (77.08%)**	**11 (22.92%)**	
2	Menopause				
	Premenopause	27	16 (59.26%)	11 (40.74%)	0.202
	Postmenopause	49	36 (73.47%)	13 (26.53%)	
3	Localization				
	Ductal	65	46 (70.77%)	19 (29.23%)	1.000
	Lobular or mixed	7	5 (71.43%)	2 (28.57%)	
4	BI-RADS				
	≤4	26	17 (65.38%)	9 (34.62%)	0.668
	≥5	34	24 (70.59%)	10 (29.41%)	
5	Primary tumor (TNM)				
	≤T2	**57**	**36 (63.16%)**	**21 (36.84%)**	**0.004**
	≥T3	**15**	**15 (100.00%)**	**0 (0.00%)**	
6	Lymph node (TNM)				
	≤N1	**59**	**38 (64.41%)**	**21 (35.59%)**	**0.008**
	≥N2	**13**	**13 (100.00%)**	**0 (0.00%)**	
7	Number of lymph nodes				
	≤2	**56**	**36 (64.29%)**	**20 (35.71%)**	**0.028**
	≥3	**16**	**15 (93.75%)**	**1 (6.25%)**	
8	ER status				
	Positive	52	34 (65.38%)	18 (34.62%)	0.402
	Negative	24	18 (75.00%)	6 (25.00%)	
9	PR status				
	Positive	48	30 (62.50%)	18 (37.50%)	0.146
	Negative	28	22 (78.57%)	6 (21.43%)	

Sl. No.	Characteristics	Cases	AR-V7 positive (n = 52)	AR-V7 negative (n = 24)	p value
10	**HER2 status**				
	Positive	19	14 (73.68%)	5 (26.32%)	0.569
	Negative	57	38 (66.67%)	19 (33.33%)	
11	**Ki67 (20%)**				
	Low	13	7 (53.85%)	6 (46.15%)	0.842
	High	14	7 (50.00%)	7 (50.00%)	
12	**Molecular Subtypes**				
	Luminal type	44	28 (63.64%)	16 (36.36%)	0.653
	HER2 enriched	12	14 (73.68%)	5 (26.32%)	
	TNBC	13	10 (76.92%)	3 (23.08%)	
13	**Histological grade**				
	≤2	**37**	**21 (56.76%)**	**16 (43.24%)**	**0.033**
	≥3	**39**	**31 (79.49%)**	**8 (20.51%)**	
14	**Survival**				
	Alive	56	36 (64.29%)	20 (35.71%)	**0.0501**
	Dead	14	13 (92.86%)	1 (7.14%)	
For n = <5, Fisher's exact test and n ≥5, Chi-square test; p ≤ 0.05 considered statistically significant.					

Table 4. List of primers used in this study

Transcript	Primer sequence (5' to 3')		Application	Annealing temperature (°C)	Product size (bp)
GAPDH	F:	ACGGATTTGGTCGTATTGGG	qRT-PCR	57.97	214
	R:	CGCTCCTGGAAGATGGTGAT		59.53	
HPRT1	F:	ACCAGTCAACAGGGGACATAA	qRT-PCR	58.66	190
	R:	CTTCGTGGGGTCCTTTTCACC		61.15	
ACTB	F:	CCTCGCCTTTGCCGATCC	qRT-PCR	60.9	70
	R:	CGCGGCGATATCATCATCC		58.71	
RNA18S	F:	GTAACCCGTTGAACCCCATT	qRT-PCR	58.09	151
	R:	CCATCCAATCGGTAGTAGCG		57.93	
AR_Pan	F:	CATGTACGCCCCACTTTTGG	qRT-PCR	59.47	114
	R:	TCTTCAGTGCTCTTGCCTGC		59.83	
AR-FL	F:	CGAGCTAGCCGCTCCAGT	qRT-PCR	61.52	176
	R:	TGCTTCCTCCGAGTCTTTAGC		59.8	
AR-V7	F:	AAGACCTGCCTGATCTGTGG	qRT-PCR	59.38	231
	R:	GCCAACCCGGAATTTTTCTCC		60.07	
MKI67	F:	GCCTGTACGGCTAAAACATGGAG	qRT-PCR	61.78	133
	R:	ACGTGCTGGCTCCTGTTCAC		63.00	

Figures

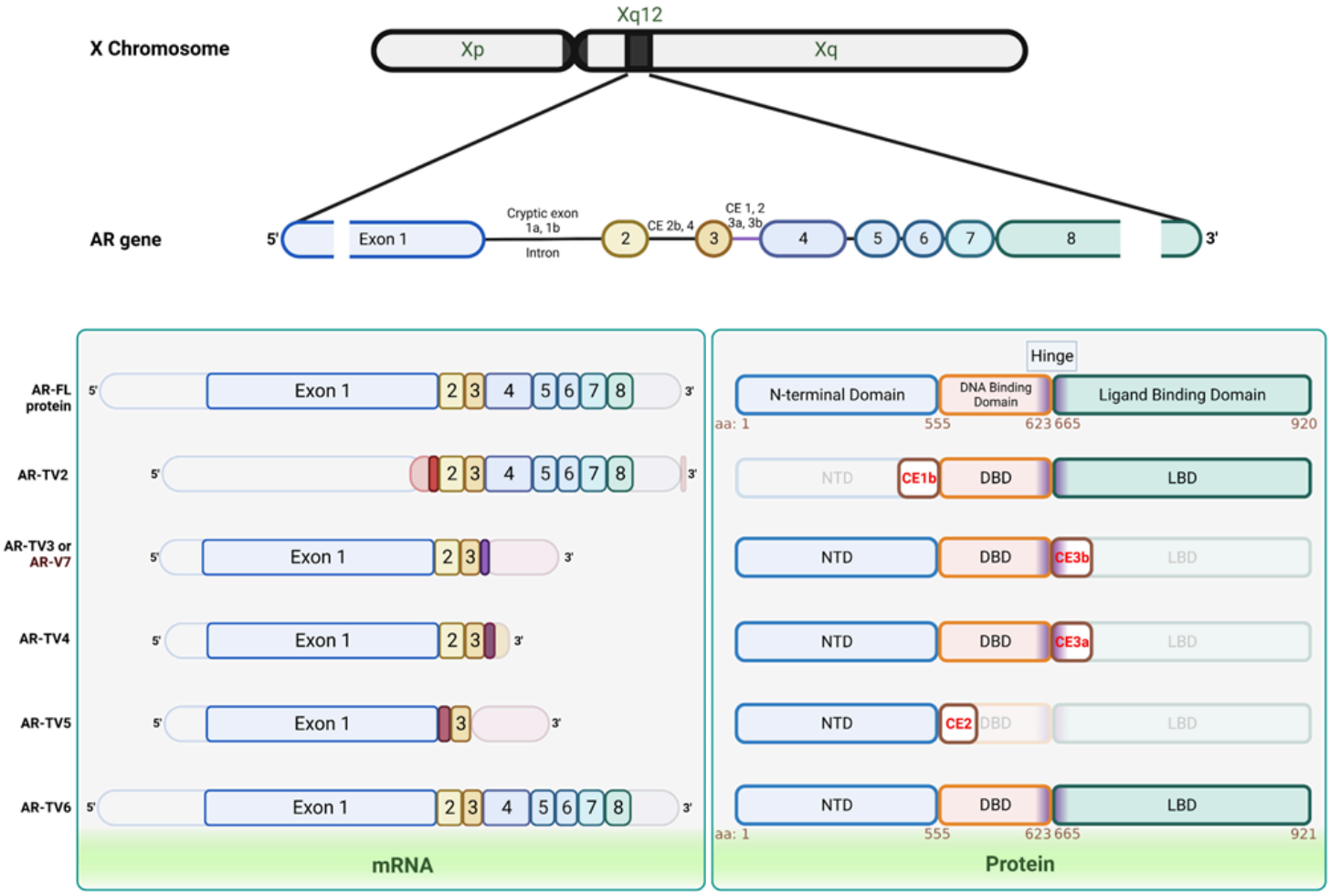

Figure 1

Transcript structures for AR full-length and the splice variants annotated in NCBI. The human AR gene consists of 8 canonical exons out of which exon 1 encodes for NTD (blue), exons 2 and 3 for DBD (orange), a hinge region (purple), and exons 4-8 for LBD (green). Notably, AR-V7 retains exons 1-3, followed by cryptic exon 3CEb (brown). Splicing pattern for AR-2, AR-TV4, AR-TV5, and AR-TV6 is also mentioned. Labeling of peptides is in accordance with the latest Human Genome Reference Consortium GRCh38.p14 (hg38). (Map not to scale). Created with BioRender.com.

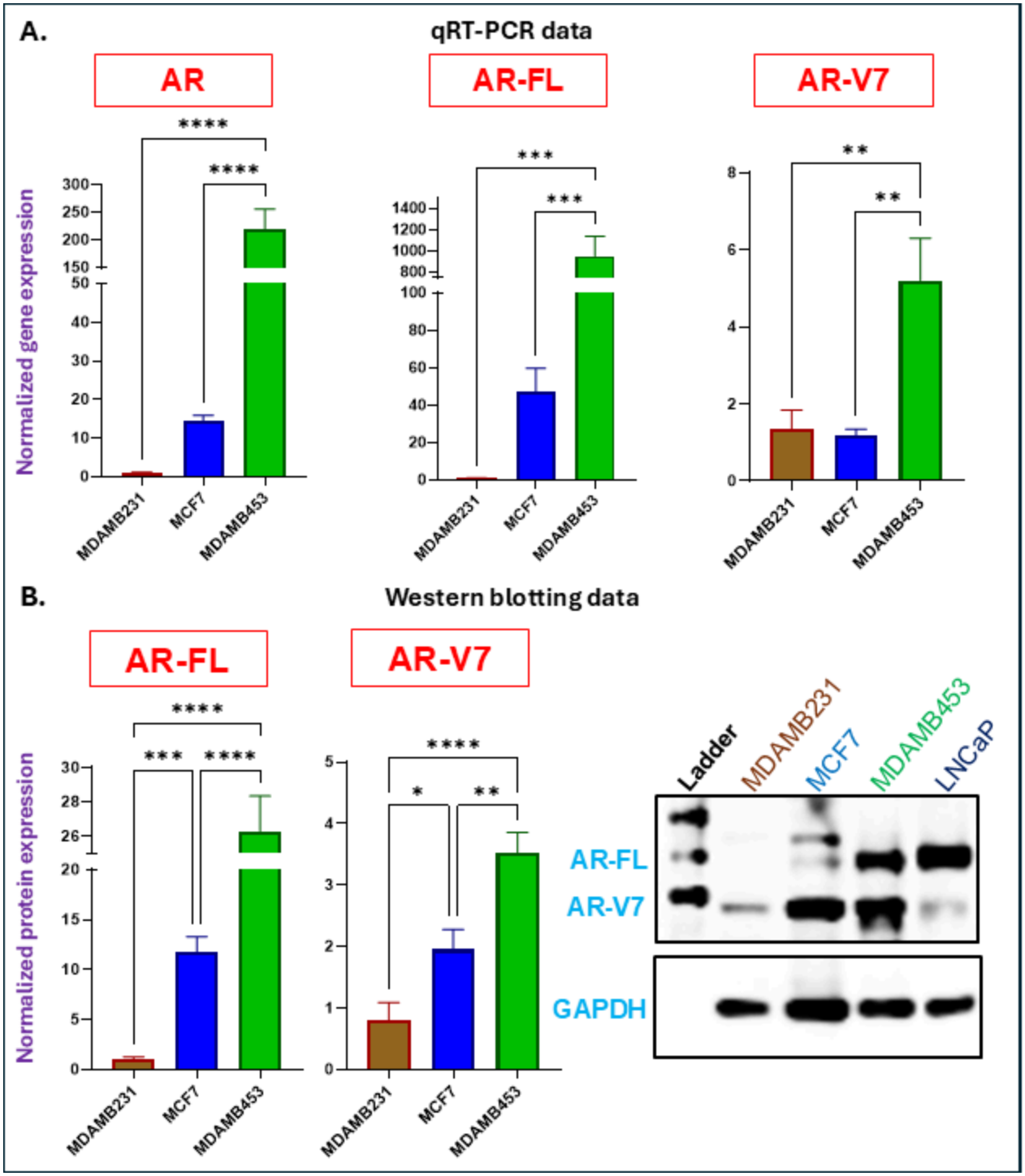

Figure 2

Relative mRNA and protein expression of AR, AR-FL, and AR-V7 its breast cancer cells. The upper panel (A) shows qRT-PCR of AR (targeting all primary transcripts), AR-FL, and AR-V7 in three cell lines. B. Western blotting of breast cancer cell lines along with prostate cancer cell line, LNCaP indicated expression of AR-FL in all but MDA-MB-231 cells. However, AR-V7 was detected in this cell line more prominently than AR-FL.

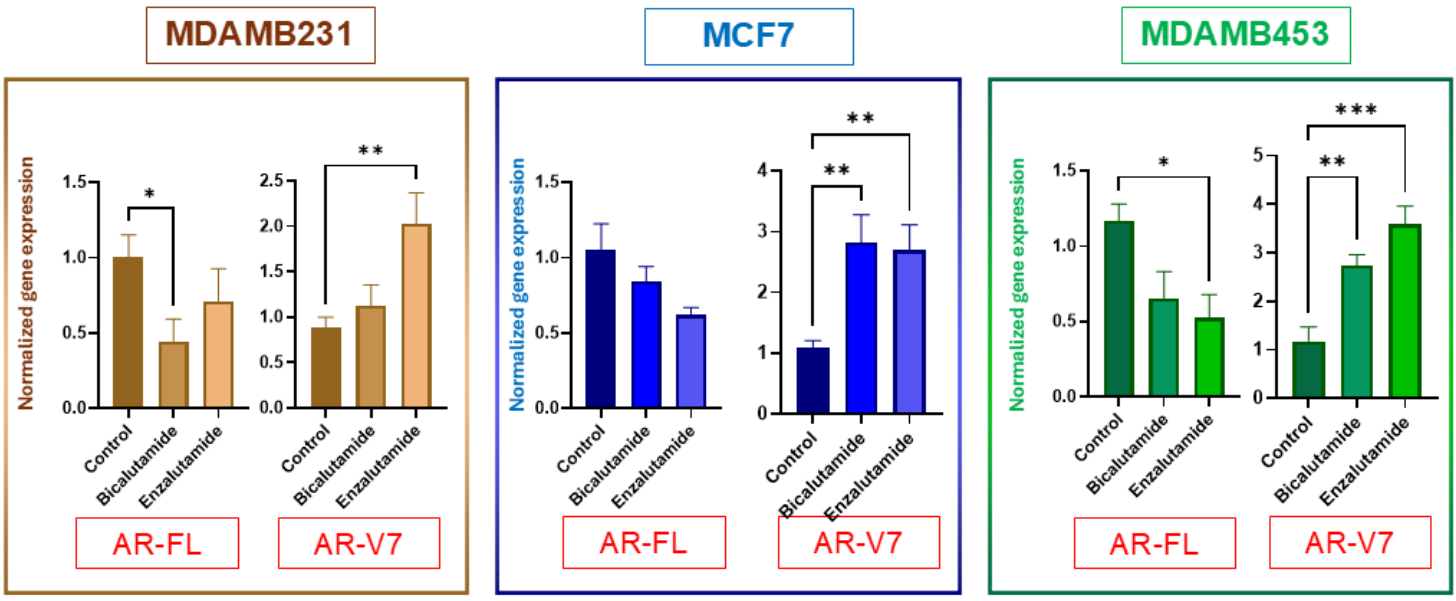

Figure 3

Elevation in the AR-V7 levels by treatment of breast cancer cell lines with AR antagonists. It can be observed that while AR-FL consistently goes down with the antagonists' treatment, there is an increase in the AR-V7 expression.

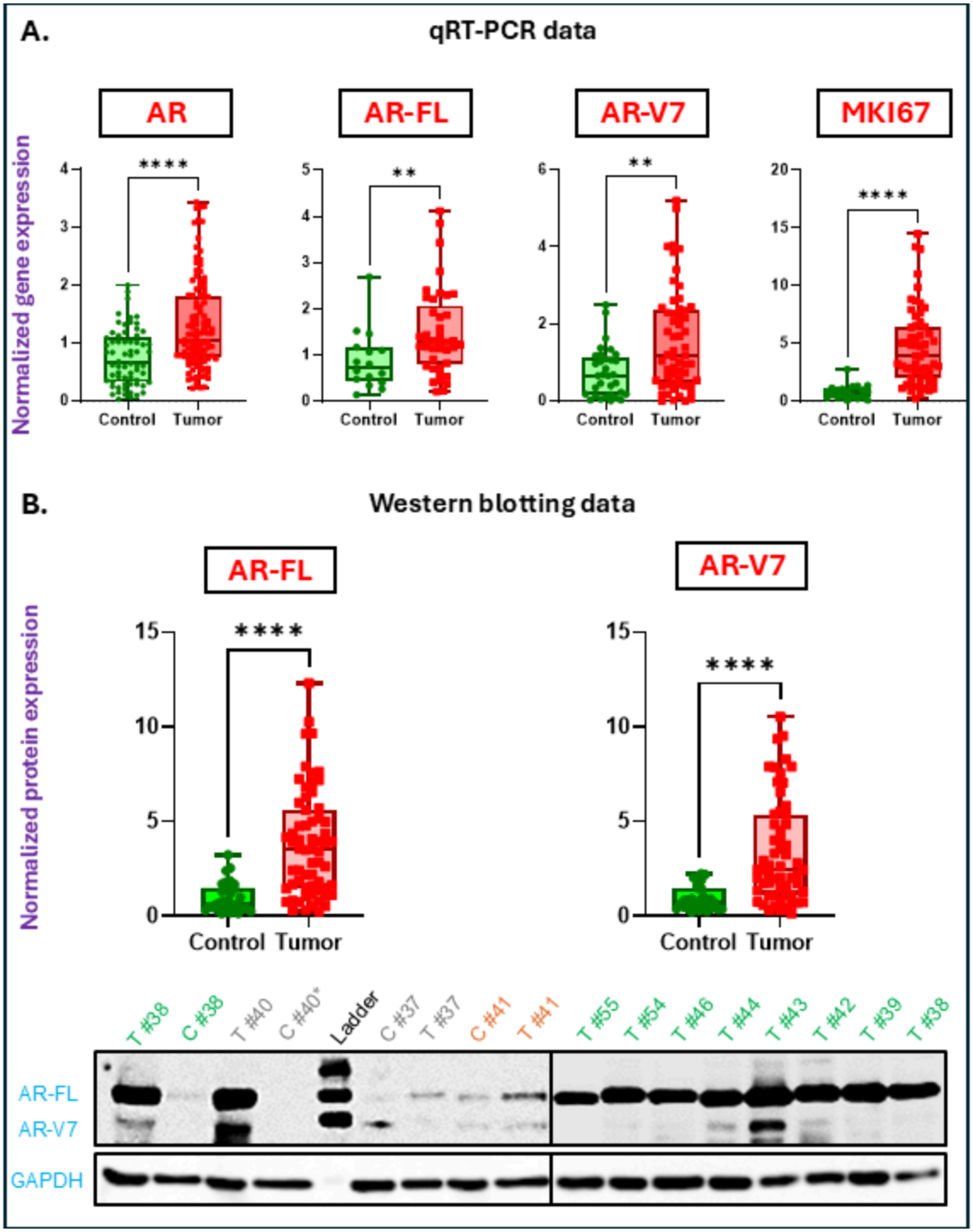

Figure 4

Normalized expression of AR-Vs in clinical samples. A. Upper panel shows qRT-PCR data of AR, AR-FL, and AR-V7 along with MKI67 genes. B. Lower panel denotes cumulative densitometry data of western blotting. Representative immunoblots are attached below. P value < 0.05 was considered statistically significant through the Mann–Whitney U test.

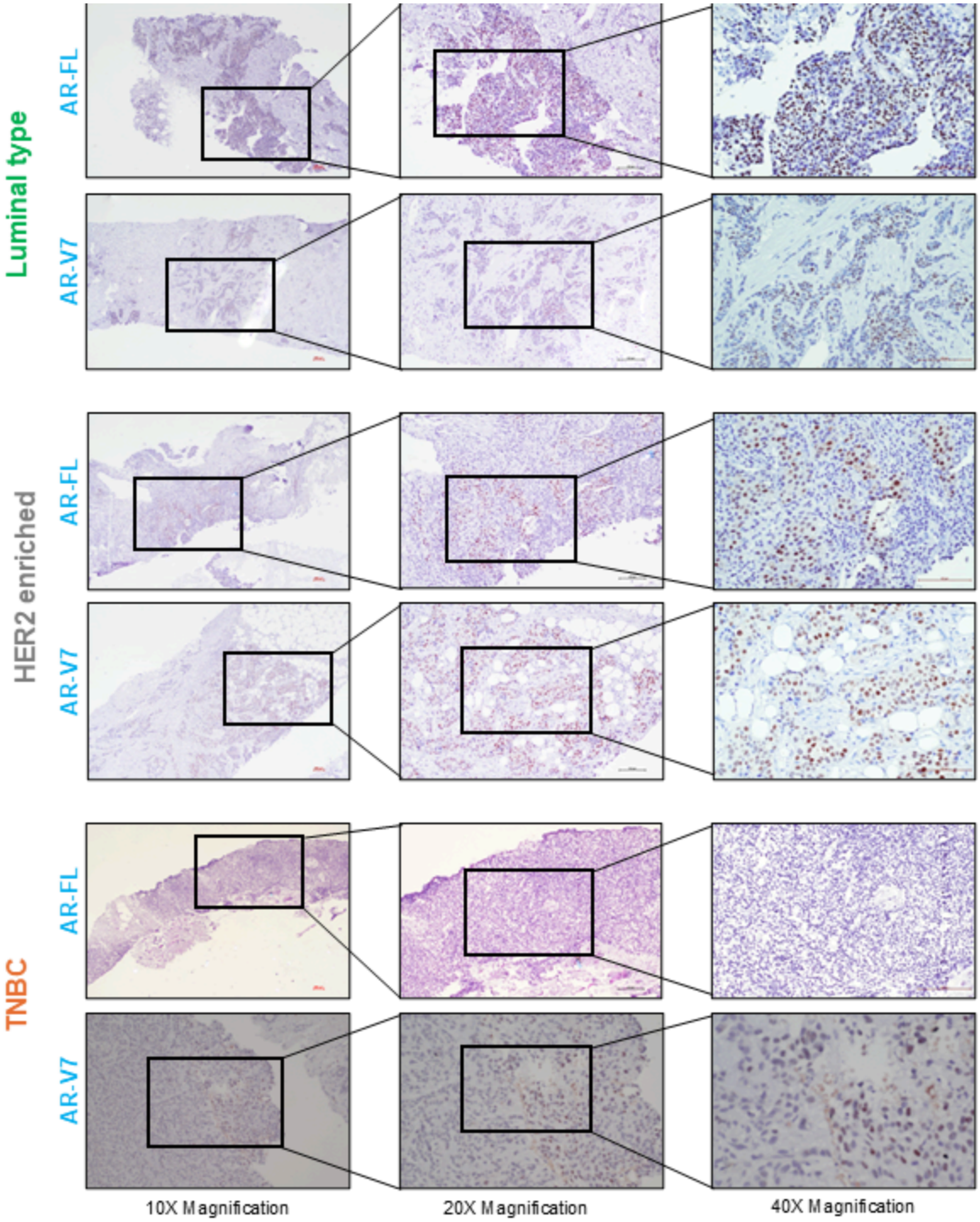

Figure 5

Immunohistochemical staining of AR-FL and AR-V7 in different subtypes of breast cancer tissues. IHC of AR exhibited strong nuclear staining while AR-V7 was weakly stained. It was interesting to note that in the attached TNBC, AR was negative while AR-V7 shows mild positivity for the same case. Images at the magnifications 10X, 20X and 40X, respectively.

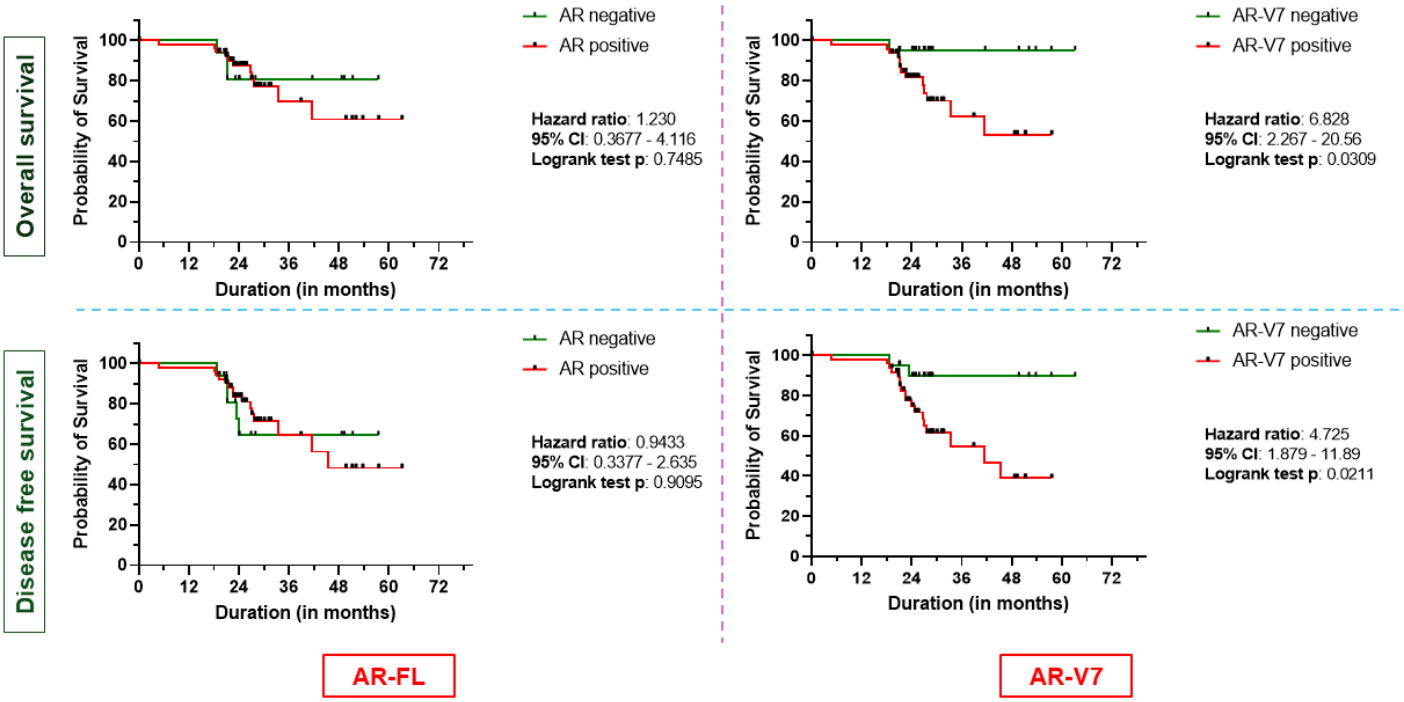

Figure 6

Kaplan–Meier curve depicting the relationship between AR and AR-V7 expression and probability of overall and disease-free survival. The KM curve shows pronounced association with the risk of events with AR-V7 positivity. Tick marks indicate censored data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryARV7paper.docx