CHAPTER-4

4. ASSOCIATION OF ANDROGEN RECEPTOR AND STEMNESS IN BPH AND PCA PATIENTS.

Androgen Receptor (AR) plays a pivotal role in the growth and development of benign and malignant tumours. Recent discoveries have also revealed the vital role of stem/progenitor cells in the development of BPH and PCa. However, the presence of AR in stem/progenitor cells and its molecular significance is still obscure. Hence, we aimed to correlate the expression of AR with stem/progenitor markers. Molecular assessment of several patient-derived benign tumours showed a significant positive correlation between AR with LGR4, β-CATENIN, OCT4 proteins, and Ki-67 cell proliferation marker. Moreover, the TCGA prostate adenocarcinoma dataset also showed a similar positive correlation with AR. Further, BPH patients with increased AR levels (AR^{hi}) have been found with increased stem/progenitor phenotype against BPH patients with lower AR levels (AR^{lo}). The patients in the AR^{hi} group showed early benign tumour onset (~62 years) with an increase in Ki67 expression as compared to patients in AR^{lo} (~67 years) group. Thus, the correlation of AR with both stem/progenitor and luminal marker confirms the significant association of AR with the luminal progenitor phenotype.

4.1 Introduction

The dependency of BPH on Androgens and AR has been discovered over a century ago by White JW.[1] Decreasing serum androgen levels in aging males is a well-documented fact, but patients with prostate pathology have increased androgen levels as compared to healthy individuals.[2, 3] With an increase in androgens, activation of AR increases causing enhanced cell proliferation of the gland. Thus, in the hyperplastic nodules of the prostate, the expression of nuclear AR was found to be relatively higher in proliferating epithelial cells than in stromal cells.[4] Moreover, the transition zone (TZ) of the gland expressed a greater number of the epithelial cell content than in the peripheral zone (PZ) during BPH condition.[5] In the 1080s, castration of the BPH patients showed decreased androgens and AR activation that remarkably reduced the volume of prostate.[6] Similar AR expression pattern was observed in the PCa condition, where the progression of the clinical-stage is associated with gain of epithelial AR and loss of Stromal AR .[7] Additionally, variety of growth factors like, IGFs, FGFs, KGFs, TGF-β, and EGF are involved in the progression of BPH and PCa by inducing rapid cell proliferation.[8] (*Figure 4.1*)

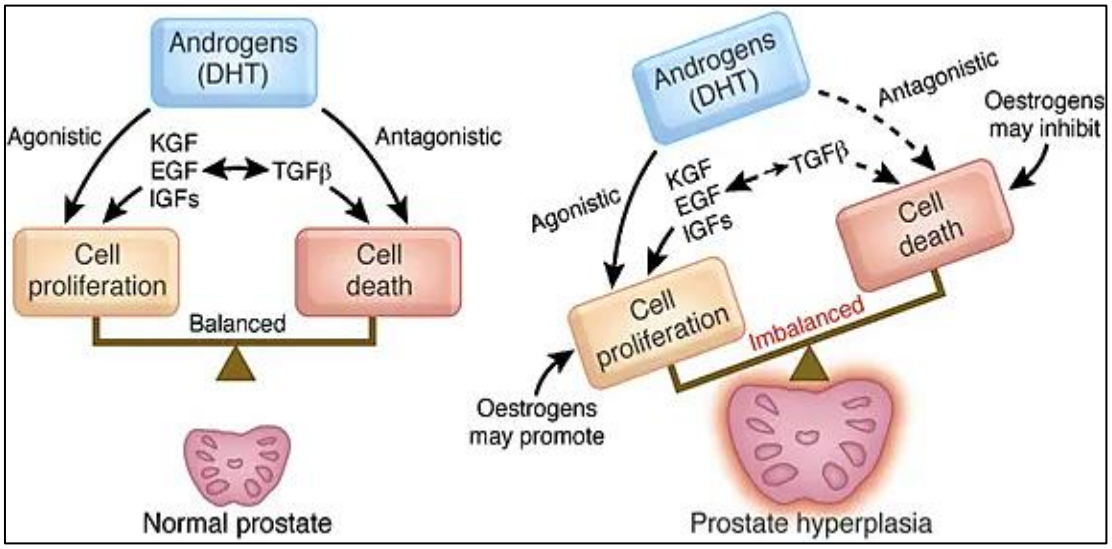


Figure 4. 1: Molecular imbalance through Androgens causes BPH condition in Prostate gland: Increasing availability of androgens and growth factors and decreasing cell death develops a molecular imbalance that progresses towards BPH condition. (Adapted[9])

Due to increased epithelial AR, regulation of AR targeted genes and proteins increases during prostatic diseases. AR is abundantly expressed in luminal cells of prostatic Epithelial compartments. Moreover, traits of AR like site-specific phosphorylations on N-Terminal Domain (NTD) (s81, s213) and expression of AR variants, especially ARV7 (also known as AR3), has been identified as key prostate disease drivers of prostate disease pathogenesis. Further, AR and its ARV7 variant plays a crucial role in BPH development and PCa survival with drug-resistant tumor relapse.[10-13] Interestingly, the conditional knockout of AR from BSCs in mice caused malignant luminal tumours, suggesting the key regulatory role of AR to maintain the homeostasis within BSCs.[14] A progressive increase of AR expression in BSCs leads to its differentiation into luminal epithelial cells.[15] Lately, it has been found that BSC signature in PCa cells, defines an aggressive PCa phenotype.[16]

AR is one of the most essential proteins of the developing prostate and is expressed at low levels in BSCs, moderately in Luminal Progenitors, fibromuscular stroma, and highest in functional luminal cells.[14, 17] Several stem/progenitor surface markers like CD133, CD44, CD49b, CD49f, PSCA have been detected in the epithelial cells during BPH condition.[18, 19 20, 21] The proliferation of specific secretory luminal, BSCs, and stem-progenitor cell regions were also detected in BPH patients.[22] Additionally, the expression of core pluripotency transcription factors NANOG, OCT4, SOX2, MYC, etc were detected during

BPH condition.[23, 24] Overexpression of these markers have been proved to develop PCa. The expression of AR in these cells have shown many contradictions due to the presence of diverse subpopulation in the stem/progenitor cells. For instance, some of the CD133+ BSCs in the prostate have no or low AR expression, but certain CD133+ luminal progenitors have high nuclear AR expression.[25, 26] Yet, the presence of low AR in stem cells was found to be vital for the homeostasis of the prostate gland.[14] Hence, the role of AR in stem/progenitor cells of BPH and PCa conditions entail further elucidations.

Leucine-rich repeat G-Protein Coupled receptor (LGR)-4 is one the cell surface receptor that is expressed in the prostate epithelial cells. LGR4 is significantly involved in prostate stem cell maintenance and differentiation.[27] Its presence in the LPs became evident by Karthaus *et al*.[28] LGR4 has been recognized as one of the key drivers of PCa progression and metastasis by increasing the CSC phenotype.[29] Similarly, ΔNP63α is another key regulator of stemness in the prostate BSCs.[30, 31] However, their presence in BPH condition is not widely explored. Additionally, their association with AR levels is also unclear in BPH condition.

Previously, we have observed a strong expression of AR and stem-associated markers in epithelial cells isolated from BPH patients.[19] Also, malignant transformation of these isolated cells exhibited increased CSC phenotype with elevation in pluripotent markers in the isolated epithelial cells. (Unpublished) We have also discovered the co-expression of LGR4 and P63 expression in the primary screening of these AR-positive epithelial cells, but the presence and role of AR are yet inexplicable in these cells. In this context, we aimed to investigate the correlation of AR with LGR4, P63 (ΔNP63α) along with stem/progenitor and luminal markers in the surgically excised BPH patient tissues (n=20). To develop the correlation, the protein expression profile of AR and stem-associated makers were assessed in BPH patients. We have further investigated the correlation between AR and stem-associated markers in PCa patients (n=492) in TCGA datasets.

4.2 Plan of Work

Surgically excised BPH prostate tissue was collected from Gotri Medical Hospital, Vadodara after due ethical clearance and kept at 4°C until processing. The tissue chips of each patient have distributed in suitable vials for the histology, IHC, protein, gene & miRNA expressions. A molecular profile of gene, miRNA, and protein expression was developed to study the molecular circuitry of AR. Thus, a correlation was developed among AR, AR-V7, and pARs213 with signalling pathway and stem/progenitor markers based on the protein expression profile of BPH patients. Further, the patients were divided into two groups based on the mean AR protein levels; patients with high AR (AR^{hi}) expression. (>mean AR protein expression) and patients with low AR (AR^{lo}) expression (<mean AR protein expression) and molecular analysis was performed between these two groups to understand the association between AR and stem/progenitor markers in AR increased and decreased BPH condition. Further, we also performed an extended bioinformatic analysis of the association between AR and stem/progenitor in TCGA prostate cancer datasets using cBioPortal tool. (*Figure 4.2*)

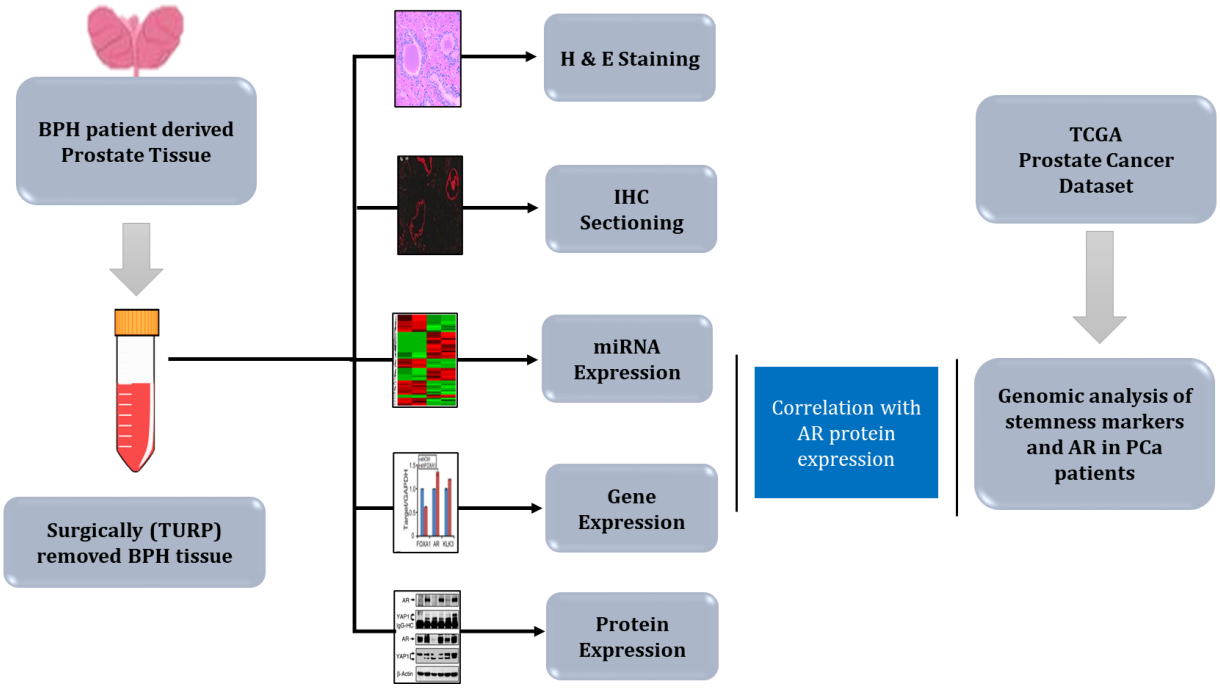


Figure 4. 2: Brief plan of work.

4.3 Results

4.3.1 BPH patients exhibit the expression of AR, LGR4, and P63 in epithelial cells.

Histological analysis of the BPH patient-derived tissue confirmed the BPH condition. The arrows marked in Figure 4.2A suggest the proliferated epithelial cells inside the prostatic acini suggesting the presence of BPH. Overall, the tissue morphology is intact, and visible invasive cells in the surrounding acini were absent, further confirming the BPH condition and absence of PCa condition. (*Figure 4.3A*) Immunohistochemistry of AR and Vimentin suggested the prominent expression of AR in epithelial cells of the tissue section, whereas Vimentin was prominent in the Stromal region of the BPH tissue. The expression profile of AR and P63 (4A4) showed strong cytoplasmic and nuclear localization of both the proteins in the epithelial compartment of the tissue. However, only few cells show co-expression of both these proteins. (marked with white arrow). Further, pARs213 and LGR4 proteins were not only expressed but also co-localized in the acinar epithelial cells of the BPH tissue. However, the expression of pARs213 did not appear as strong as total AR and LGR4 levels. (*Figure 4.3B*) Hence, results depict the presence of both AR and pARs213 in the BPH tissue along with P63 and LGR4 expression in the epithelial compartment.

4.3.2 Correlation of AR, AR-V7, and pARs213 with stem/progenitor markers.

To understand the correlation of AR attributes (full length-AR, AR-V7, and pARs213) with stem/progenitor cells in BPH condition, their protein expression profile was correlated with LGR4, ΔNP63, and OCT4 stem/progenitor markers in surgically excised BPH prostate tissue. (*Figure 4.4*) Densitometry of each protein suggested a positive correlation among AR, AR-V7, and pARs213 in BPH patients. As phosphorylation of AR at s213 residue is regulated by growth factors induced by AKT signaling, IGF1 and total AKT were found to be positively correlated with AR and AR-V7 levels, but no substantial correlation of pAKT was found with total AR and AR-V7 levels. Further, total AKT and pAKT levels were not correlated with pARs213 levels suggesting the involvement of other kinases that induce s213 phosphorylations.

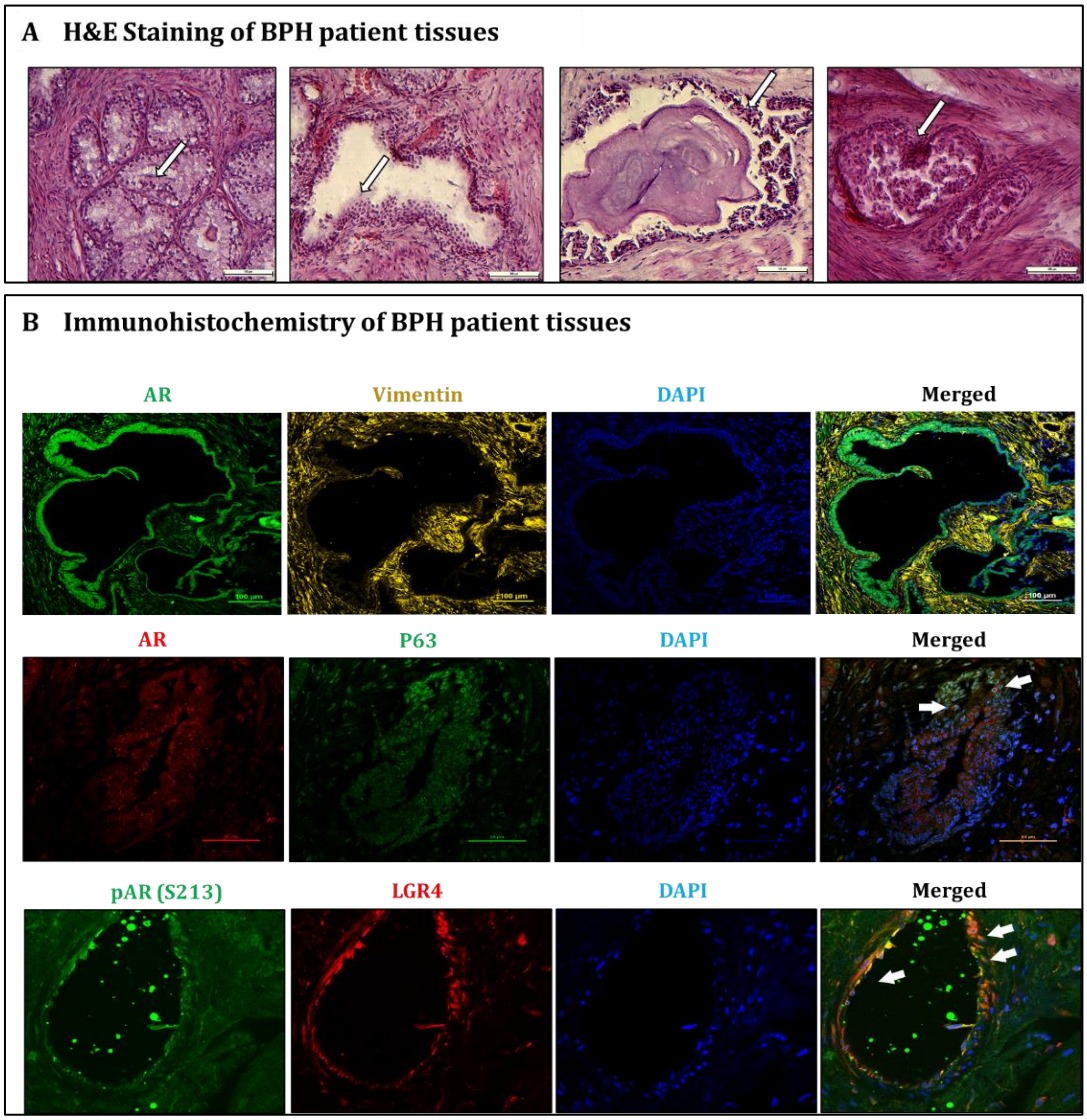


Figure 4. 3: Histology and immunohistochemistry of BPH patient tissues. (A) Histological section of surgically excised BPH prostate tissue; Scale 100µm, 20X. **(B)** Immunohistochemistry of AR (green) and Vimentin (Orange) Image:10X-100µm; AR (Red)-P63 (Green), AR(Red), and LGR4 (Green), Image:20X-50µm in BPH patient tissue.

The stem/progenitor associated proteins like LGR4 and OCT4 were positively correlated with AR, AR-V7, and pARs213 protein levels in BPH tissues. But, the downstream signal transducer of LGR4, β-CATENIN, was associated with the expression of AR and AR-V7, suggesting a plausible synergy of LGR4, β-CATENIN with AR and/or AR-V7 in the BPH tissue. Further, protein levels of ΔNP63α were not associated with the total AR, AR-V7, and pARs213 levels in BPH patients, suggesting no association of AR with BSCs in the BPH condition. The protein expression of cell proliferation marker, Ki-67 was positively correlated with AR, AR-V7, and pARs213 levels in the BPH patients. (Table 4.1) Cumulatively, all three traits of AR, AR (Full length), AR-V7, and pARs213 were found

to be correlated with LGR4 and OCT4 but not with ΔNP63α. Hence, results suggest that with increasing AR expression, the levels of ARV7 and androgen-independent activation of AR, pARs213, were also increased. The levels of stem/progenitor markers like LGR4/β-CATENIN and OCT4 were also associated with an elevation in AR. Moreover, they are associated with increased cell proliferation marker, Ki-67, suggesting higher proliferation with increasing AR expression in BPH patients.

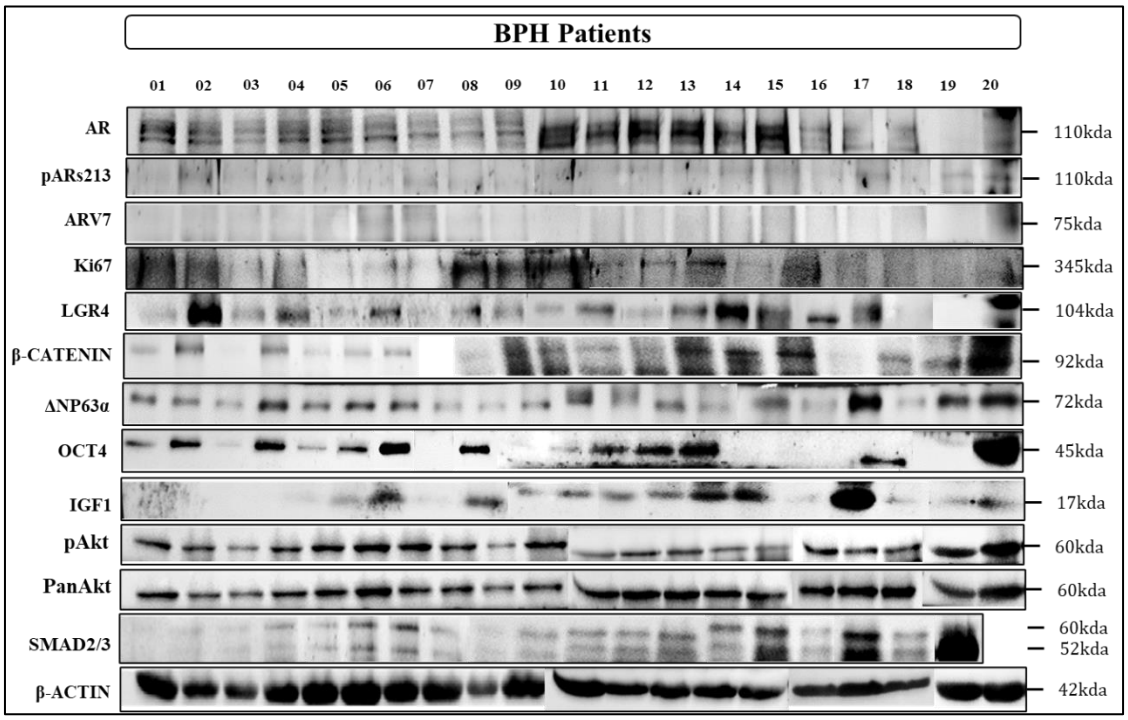


Figure 4. 4: Immunoblot image of protein expression profile in BPH patients. The protein expression of AR, pARs213, ARV7, Ki67, LGR4, β-CATENIN, ΔNP63α, OCT4, IGF1, pAKT, PanAKT, SMAD2/3 in BPH patients (N=20). *β-CATENIN and SMAD2/3 has N=19.

AR	**AR-V7**	**pAR (s213)**	**IGF1**	**Ki-67**	**pAKT**	**PanAKT**	**Smad2/3**	**LGR4**	**β-CATENIN**	**OCT4**	**ΔNP63α**
Pearson r	0.6661	0.6162	0.4806	0.5077	0.1176	0.3966	0.3666	0.4776	0.4673	0.4506	0.1595
95% confidence interval	0.3169 to 0.8563	0.2386 to 0.8319	0.04828 to 0.7613	0.06945 to 0.7817	-0.3428 to 0.5325	-0.05581 to 0.7139	-0.1385 to 0.7204	0.04441 to 0.7597	0.01650 to 0.7602	0.009934 to 0.7447	-0.3046 to 0.5624
P value (one-tailed)	0.0007	0.0019	0.016	0.0132	0.3107	0.0417	0.0739	0.0166	0.0218	0.0231	0.2509
P value summary	***	**	*	*	ns	*	ns	*	*	*	ns
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes	Yes	No	Yes	No	Yes	Yes	Yes	No
R squared	0.4437	0.3797	0.231	0.2578	0.01383	0.1573	0.1344	0.2281	0.2184	0.203	0.02545

ARV7	**AR**	**pAR (s213)**	**IGF1**	**Ki-67**	**pAKT**	**PanAKT**	**Smad2/3**	**LGR4**	**β-CATENIN**	**OCT4**	**ΔNP63α**
Pearson r	0.6661	0.5576	0.6178	0.5753	0.08608	0.411	0.605	0.6143	0.5441	0.6922	0.1732
95% confidence interval	0.3169 to 0.8563	0.1526 to 0.8022	0.2412 to 0.8327	0.1638 to 0.8163	-0.3707 to 0.5093	-0.03859 to 0.7223	0.1753 to 0.8411	0.2358 to 0.8310	0.1193 to 0.8005	0.3598 to 0.8687	-0.2917 to 0.5720
P value (one-tailed)	0.0007	0.0053	0.0018	0.005	0.3591	0.0359	0.005	0.002	0.008	0.0004	0.2326
P value summary	***	**	**	**	ns	*	**	**	**	***	ns
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No
R squared	0.4437	0.3109	0.3817	0.3309	0.00741	0.169	0.3661	0.3774	0.2961	0.4791	0.03001

pAR (S213)	**AR**	**AR-V7**	**IGF1**	**Ki-67**	**pAKT**	**PanAKT**	**Smad2/3**	**LGR4**	**β-CATENIN**	**OCT4**	**ΔNP63α**
Pearson r	0.6162	0.5576	0.3857	0.7037	-0.1023	0.11	0.1804	0.746	0.3786	0.4593	0.09048
95% confidence interval	0.2386 to 0.8319	0.1526 to 0.8022	-0.06864 to 0.7075	0.3667 to 0.8775	-0.5213 to 0.3565	-0.3496 to 0.5270	-0.3288 to 0.6084	0.4529 to 0.8935	-0.09147 to 0.7107	0.02089 to 0.7495	-0.3668 to 0.5126
P value (one-tailed)	0.0019	0.0053	0.0465	0.0004	0.3339	0.3222	0.2441	P<0.0001	0.055	0.0208	0.3522
P value summary	**	**	*	***	ns	ns	ns	***	ns	*	ns
Is the correlation significant? (alpha=0.05)	Yes	Yes	Yes	Yes	No	No	No	Yes	No	Yes	No
R squared	0.3797	0.3109	0.1488	0.4953	0.01047	0.0121	0.03256	0.5564	0.1433	0.2109	0.008188

Table 4. 1: Correlation analysis of protein expression profile in BPH patients. AR, AR-V7, and pAR(s213) correlation with the expression of other proteins in BPH patients.

4.3.3 BPH patients with higher AR expression exhibit increased AR-V7 and pAR213 levels.

To comprehend the association of AR with the molecular profile more efficiently, patients with higher levels of AR (AR^{hi}) (n=12) and lower levels of AR (AR^{lo}) (n=8) were evaluated. Though the levels of ARV7 and pARs213 were minimal, the AR^{hi} group of BPH patients depicted higher levels of ARV7 and pARs213 were significantly higher in the as compare to AR^{lo} patients. (*Figure 4.5*) The overexpression of ARV7 is associated with the neoplastic transformation of the prostate cells and PCa aggression. Moreover, increased pARs213 suggests the activation of AR via growth factors and cytokines that potentiate pARs213 of AR in an androgen-independent manner in AR^{hi} BPH patients. Thus, the molecular profile of AR expression suggests that higher AR levels support the expression of ARV7 and pARs213 in the BPH tissue cells to support the aggressive growth of the cells.

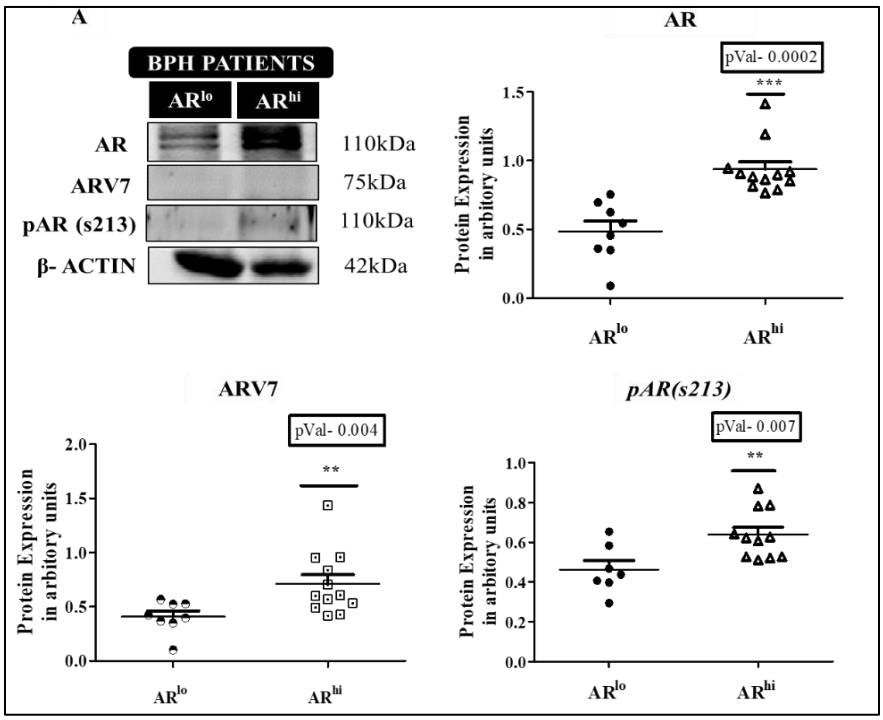


Figure 4. 5: Protein expression of AR, ARV7, and pARs213 in BPH patients. The image represents immunoblots and densitometric analysis of AR, ARV7, and pARs213 in graphs. N=20 (AR^{lo}=08; AR^{hi}=12) Mean±SEM; AR^{lo} v/s AR^{hi} patients; **p≤0.01, ***p≤0.001.

4.3.4 miRNA profile of AR^{hi} and AR^{lo} BPH patients

To regulate the gene expression in the target cells, AR regulates many of the microRNA (miR) expression via binding to their promoter directly. Hence to comprehend the regulatory role of AR over miRs targeting some of the stem-markers, we have identified few miR that regulates the expression of prostate stemness using TargetScan, Exicon, and miR-base genomic tools. (*Figure 4.6A*) Among the eight miRs screened in AR^{hi} (n=6) and AR^{lo} (n=6) groups of BPH patients, miR-34b-5p, miR-34c-5p, and miR-142-3p did not show expression in BPH patients. Expression of miR-35a-5p, Let-7d-5p, and Let-7f-5p targetting LGR4, MYC, and other stemness factors depicted an increasing trend in AR^{lo} patients but remained non-significant as compare to AR^{hi} patients. Nonetheless, SOX2 and LGR4 targetting miR-21-5p and miR-27a-3p were significantly downregulated in AR^{hi} against AR^{lo} BPH patient group. (*Figure 4.6B*) Interestingly, miR-21 exhibited a conspicuous difference in expression pattern in BPH and PCa conditions despite its direct regulation by AR and could serve as a molecular marker to differentiate between BPH and PCa. However, further molecular exploration of direct regulation of AR over miR-21 and miR-27a is required to corroborate this mechanism in BPH condition.

A Stemness markers targeting miRNA identification

Sr. No.	Target Genes	hsa-miR (Accession number)	Status in PCa	pCt Value	Reference
1	CD133	hsa-miR-142-3p (MIMAT0000434)	Validated	0.14 (8-mer)	Bissels U. et al., 2011; Waltering et al, The prostate, 2011
2	LGR4 MYC	hsa-mir-34b-5p (MIMAT0000685)	Predicted	pCt-NA (7-mer)	Waltering et al, The prostate, 2011
3	SOX2	hsa-mir-21-5p (MIMAT0000076)	Validated	0.25 (7-mer)	Waltering et al, The prostate, 2011
4	LGR4	hsa-miR-27a-3p (MIMAT0000084)	Predicted	0.37 (7-mer)	-
5	LGR4	hsa-let-7f-5p (MIMAT0000067)	Predicted	0.87 (7-mer)	-
6	MYC	hsa-let-7d-5p (MIMAT0000064)	Validated	0.98 (7-mer)	Kim et al, Genes and Developments, 2009
7	LGR4, MYC, KLF4, NANOG, OCT4	hsa-mir-34a-5p (MIMAT0000255)	Validated	0.85 (8-mer) 0.88 (8-mer) 0.34 (8-mer) NA NA	Q Hou, PlosOne, 2016; Frenzel, Genes and Cancer, 2010; Ng et al, cell death and diseases, 2014; Liu et al, Nature Madicine, 2011; Q et al, Cell Mol Biol Lett. 2014
8	LGR4	hsa-mir-34c-5p (MIMAT0000686)	Validated	0.85 (8-mer)	Zhang et al, Asian-Australas J Anim Sci. 2015
9	ΔNP63	hsa-mir-203 (MIMAT0031890)	Validated	pCt-NA (8-mer)	Lena et al, Cell Death & Differentiation, 2008

pCt: The probability of conserved targeting (Tool: TargetScan)

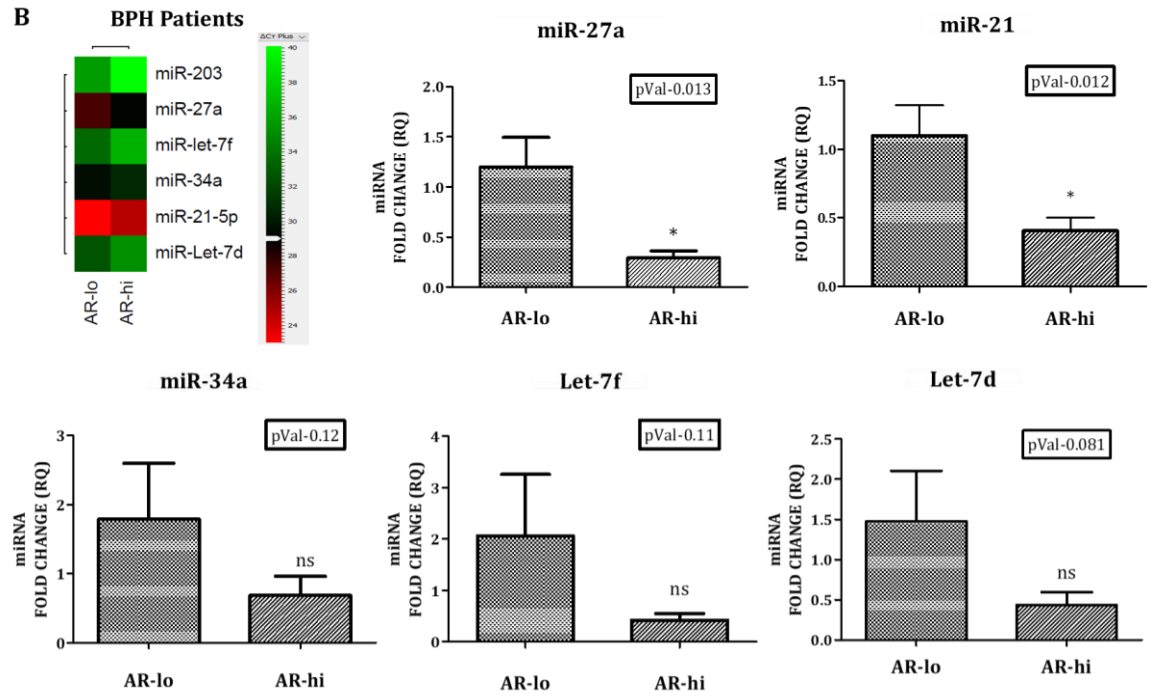


Figure 4. 6: miRNA expression profile in BPH patient tissues and BPH stem/progenitor cells. (A) Identification of miRNAs targeting stem/progenitor gene transcripts using TargetScan, mirBASE, and Exicon^{TM} tools, **(B)** miRNA expression of miR-27a, miR-21, miR-34a, Let7f, and Let-7d in BPH patient tissues; Graph represents AR^{lo} patients, Mean±SEM, N=6; AR^{hi} expressing patients N=6; *p<0.05.

4.3.5 BPH patients with higher AR have a distinct gene expression profile.

To understand the transcriptional profile of the AR^{hi} and AR^{lo} BPH patients, we have assessed two key AR targeted genes; *PSA* and *NKX3.1* which showed a significant decrease in *PSA* transcript levels and an increase in *NKX3.1* transcript level in AR^{hi} patients as compare to AR^{lo} patients. The assessment of stem cell gene expression showed that AR^{hi} patients had higher transcript levels of *β-CATENIN*, *LGR4*, and *cMYC*, whereas, decreased transcript levels of *SOX2* and ΔNP63α as compare to AR^{lo} patients. However, the expression of the *OCT4* gene remained non-significant between the two groups. (*Figure 4.7A*) The increased gene expression of LGR4 and β-CATENIN corresponded to the protein expression levels in AR^{hi} patients. Strikingly, OCT4 and ΔNP63α demonstrated contradicted gene expression with increased protein levels in AR^{hi} patients. (*Figure 4.7B*) The gene and protein expression pattern ascertain the influence of AR on stem/progenitor markers, LGR4, β-CATENIN, OCT4, and ΔNP63α, which correlated with the miR expression where LGR4 targeting miR-27a was decreased in AR^{hi} BPH patients and P63 targeting miR-203 did not express in the BPH patient tissues.

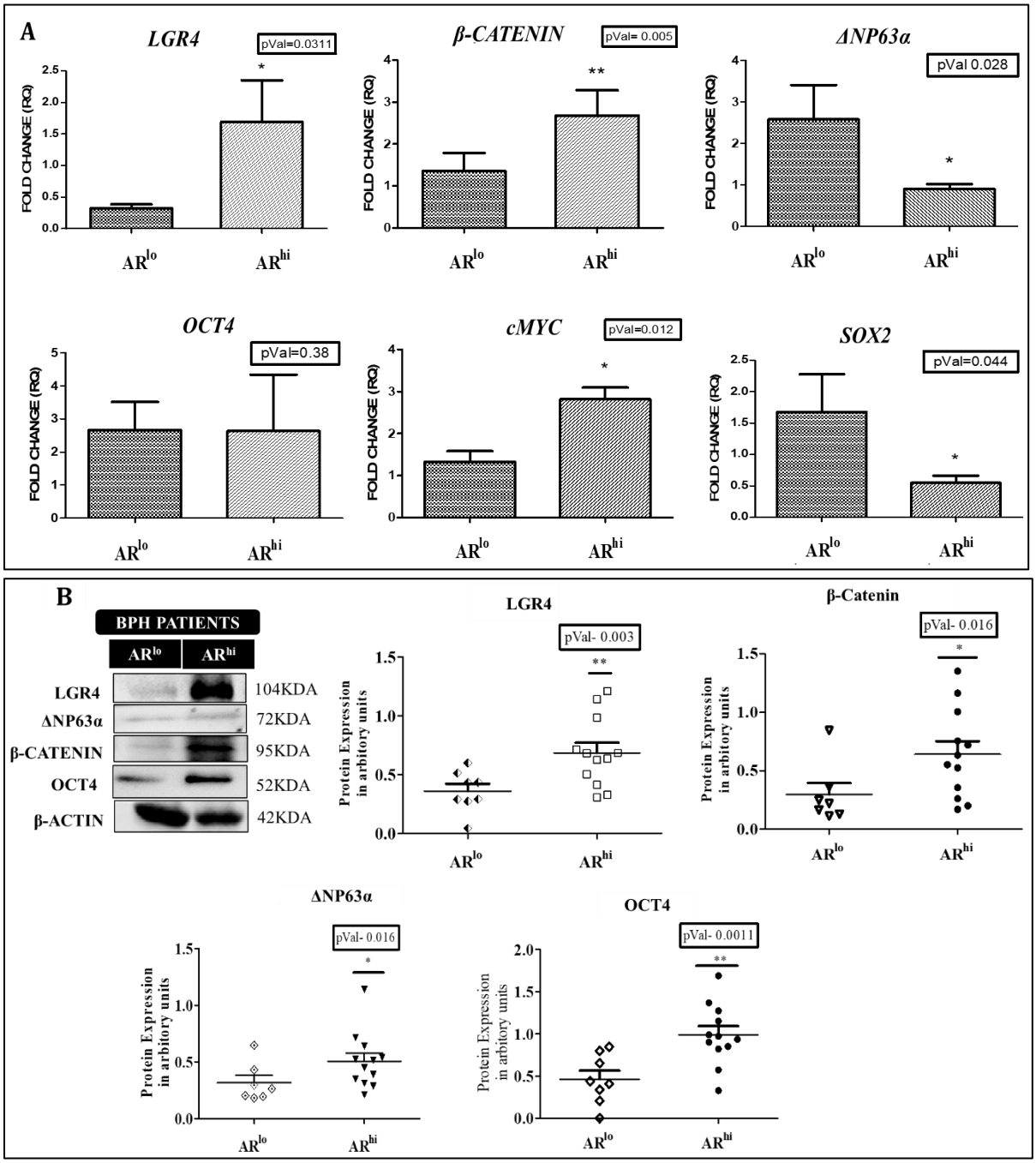
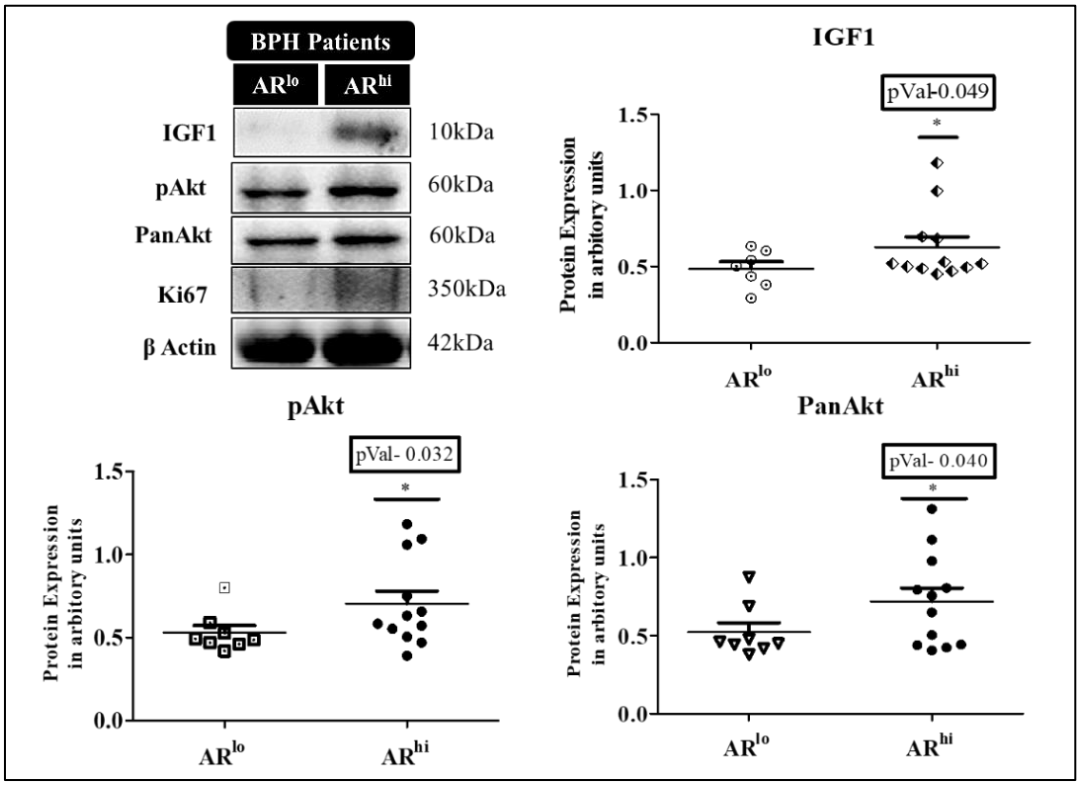


Figure 4. 7: Gene and protein expression profile of BPH patient tissues based on AR expression pattern. (A) Gene expressions of *LGR4, β-CATENIN, ΔNP63α, OCT4 (POU5F1), cMYC,* and *SOX2* in BPH patients with high and Low AR protein expression conditions; AR^{lo} patients N=6; AR^{hi} expressing patients N=9; **(B)** Protein expression of LGR4, β-CATENIN, ΔNP63α, and OCT4 in BPH patients and densitometric analysis represented in graphs; N=20 (AR^{lo}=08; AR^{hi}=12). Graph represents Mean±SEM; AR^{lo} v/s AR^{hi} patients; *p≤0.05, **p≤0.01, ***p≤0.001.

4.3.6 Patients with higher AR levels have increased cell proliferation and early BPH development

BPH condition is characterized by increased prostatic volume due to the proliferation of epithelial and fibroblast/myofibroblast cells. Further, due to the increased muscular volume of the prostate, the intraprostatic secretion of IGF1, a potent mitogenic growth factor, was found to be increased in the AR^{hi} group as compare to the AR^{lo} group. As IGF1 majorly acts via the AKT pathway, a significant increase in the levels of phosphorylated AKT and total AKT were observed in the AR^{hi} group of patients as compared to AR^{lo} groups. AKT signaling strongly imposes the activation of proliferation and survival of the target cells. Hence, to determine the proliferation state of AR^{lo} and AR^{hi} groups, protein expression of Ki67, which accelerates cell proliferation, showed elevated levels in AR^{hi} patients as compared to AR^{lo} patients suggesting more proliferative cells in AR^{hi} patient group. Further, it was also observed that AR^{hi} patients have significantly early BPH developments (~62 years) as against AR^{lo} patients (~67 years). (*Figure 4.8*) Thus, increased AR expression is associated with the early disease manifestation in elderly men.



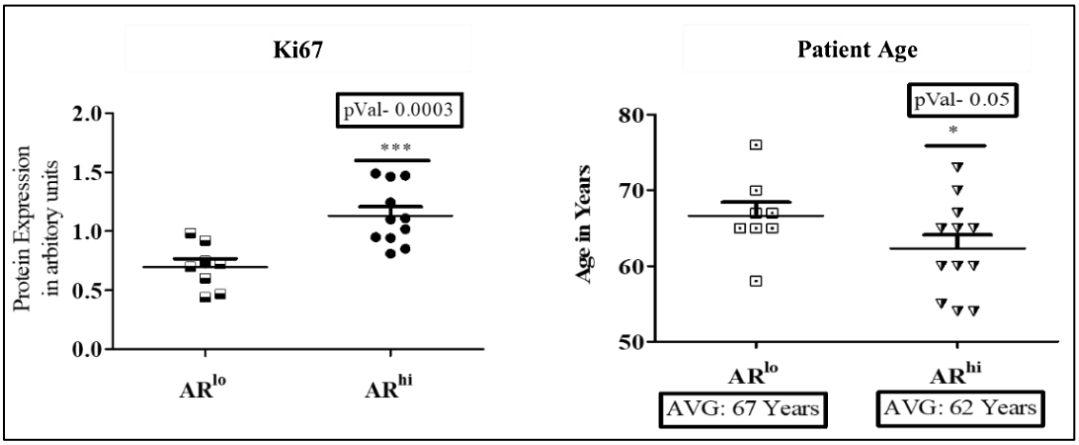


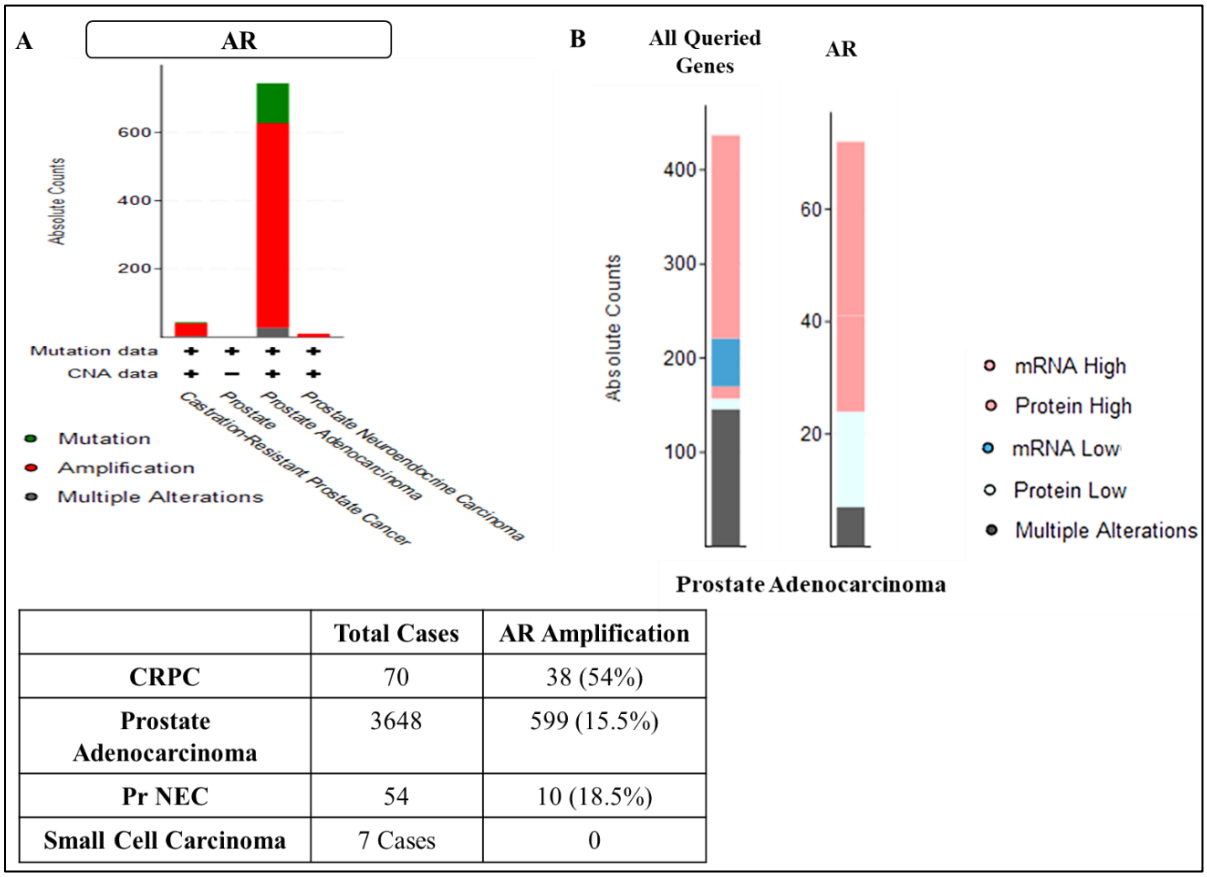
Figure 4. 8: Protein Expression profile of AR^{lo} and AR^{hi} patients. The image shows the protein expression of AKT, pAKT, IGF1, and Ki67 in AR^{lo} and AR^{hi} patients. The graph also shows the age of the BPH patient subjects in AR^{lo} and AR^{hi} patients. N=20 (AR^{lo}=08; AR^{hi}=12) Mean±SEM; *p<0.05, ***p≤0.001.

4.3.7 PCa patients exhibit co-expression of AR-LGR4-β-CATENIN

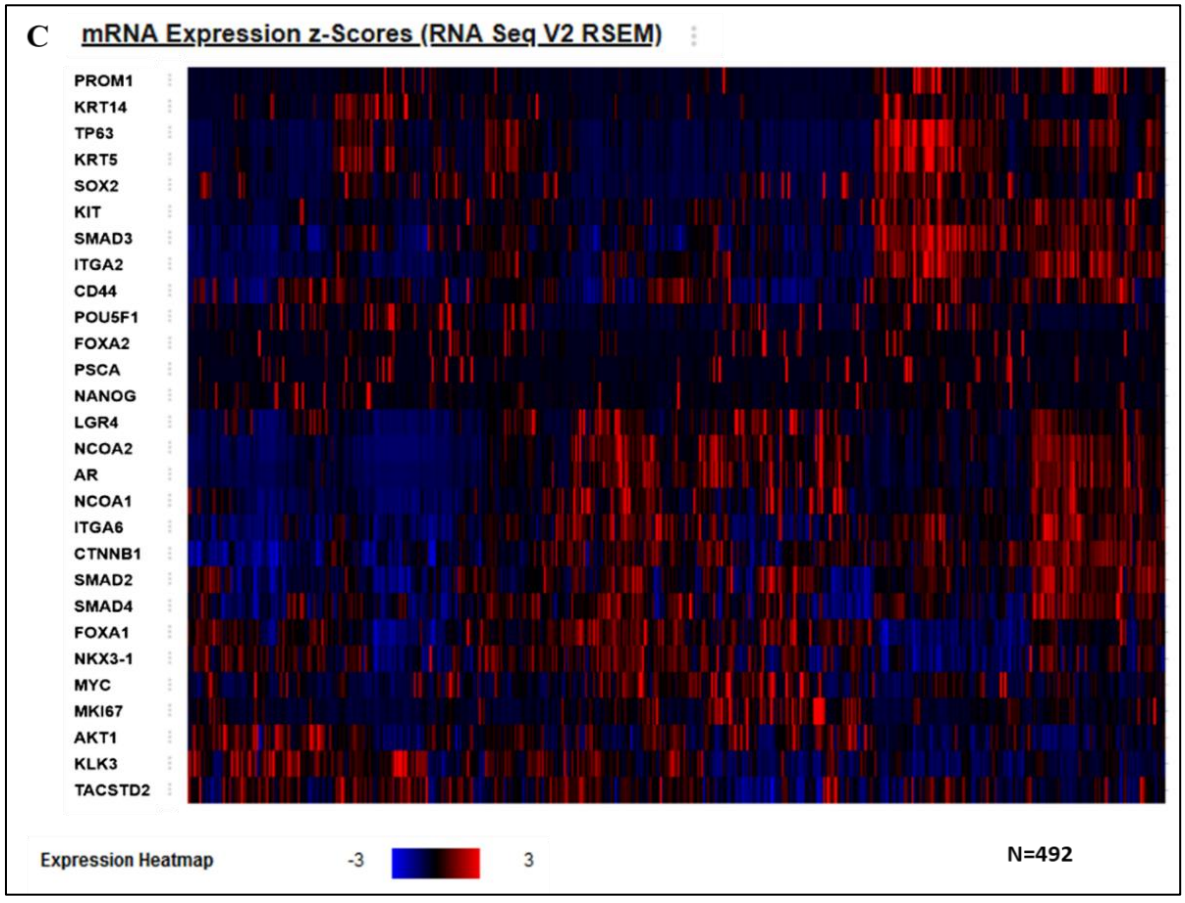
To understand the role of AR involved in different stages of PCa, AR expression pattern was explored in different PCa types using the TCGA data set. The alterations in the AR gene (Missense, Not mutated, Amplification, Gain, Diploid, Shallow deletion, deep deletion) was found to be around 18% in 720/4103 PCa patients. Approximately 600 patients were found to have amplified expression of AR in the Prostate Adenocarcinoma condition. (*Figure 4.9A*) To understand the role of AR in the regulation of stem/progenitor in PCa patients, a TCGA PCa dataset was further explored.[32] The study of about 28 "Queried genes" were comprised of *AR,* its co-activators (*NCOA1, NCOA2, FOXA1, FOXA2*) BSC Markers (*ITGA2 (CD49b), ITGA6 (CD49f), PROM1 (CD133), CD44, KIT (CD117), PSCA, CK5, CK14, LGR4, TP63, TACSTD2 (TROP2), POU5F1 (OCT4), SOX2, NANOG, MYC*), key regulatory signaling network genes (*AKT1, SMAD2/3/4, CTNNB1 (β-CATENIN)*), known AR targeted genes (*KLK3 (PSA), NKX3-1*) and cell proliferation marker *MKI67 (Ki-67)* in TCGA PRAD study. Analysis of the "Queried 28 genes" in TCGA PCa datasets suggested 93% alterations in 492 PCa patients, where AR was found to be altered in ~16% (~80) of the PCa patients. (*Figure 4.9B*) These genes were further assessed to understand the PCa expression pattern by generating an RNA-Seq heatmap. Analysis of gene expression datasets showed two distinct groups, one with very low or no *AR* mRNA and another with moderate to high *AR* mRNA expression. The PCa patients with low or no *AR* mRNA expression appeared to have higher transcript levels of stem/progenitor markers PROM1, TP63, KIT, CD44, CK5, CK14, ITGA2, and SOX2.

Whereas the PCa patients with higher AR transcripts showed increased mRNA levels of AR coactivators *NCOA1*, *NCOA2*, and *FOXA1* transcripts along with *LGR4* and *ITGA6 (CD49f)* stem cell markers. Importantly, elevated transcript levels of LGR4/Wnt pathway effector, CTNNB1 (*β-CATENIN)*, and TGF-β signal transducers *SMAD2*&*4* were also observed. However, there was a small group of the patient cluster within the high AR transcript levels which exhibited elevated transcript levels of TP63, PROM1, KIT, ITGA2, and CD44 suggesting the presence of luminal progenitor populations within the tumor. Strikingly, the elevation of the stem/progenitor phenotype in both groups had increased *SMAD3* mRNA levels in a distinct manner. (*Figure 4.9C*)

To investigate the coexpression of AR and stem/progenitor markers, AR protein levels were correlated with the RNA seq data of the 27 genes in PCa patients. The results demonstrated its weak but significant positive correlation with *LGR4*, *FOXA1*, *MYC*, *NKX3.1*, and *KLK3* and negative correlation with PROM1, CD44, KIT, TP63, KRT5, and SMAD3 transcripts in PCa patients. (*Figure 4.9D*) The stemness associated miRs explored in BPH patients, were also assessed in TCGA prostate adenocarcinoma subjects, retrieved from the S-MED Oncomir database.[33, 34] The results suggested significant downregulation of miR-27a, which supports the elevated translation of LGR4 transcripts in BPH and PCa conditions. However, strong and elevated expression of miR-21, miR-34a, Let-7d, and Let-7f suggests a decrease in the translation of stem/progenitor associated genes in the initial phase of the disease. Despite AR-mediated direct positive regulation of miR-21, its expression exhibited contrasting expression patterns, downregulated in AR^{hi} BPH subjects but highly elevated in TCGA adenocarcinoma subjects. (*Figure 4.9E*) We have also depicted the correlation between AR protein levels and protein expression of some of the markers available in the database. Intriguingly, AKT1 and β-CATENIN protein levels showed a substantial positive correlation, whereas KIT, MYC, and SMAD3 protein levels showed a negative correlation with AR protein levels in PCA patients. (*Figure 4.9F*) Thus, we confirm that the majority of AR rich PCa tumors have a negative or poor expression of basal markers. Further, the coexpression of AR and LGR4/β-CATENIN was observed in PCa patients similar to present data of BPH patients.

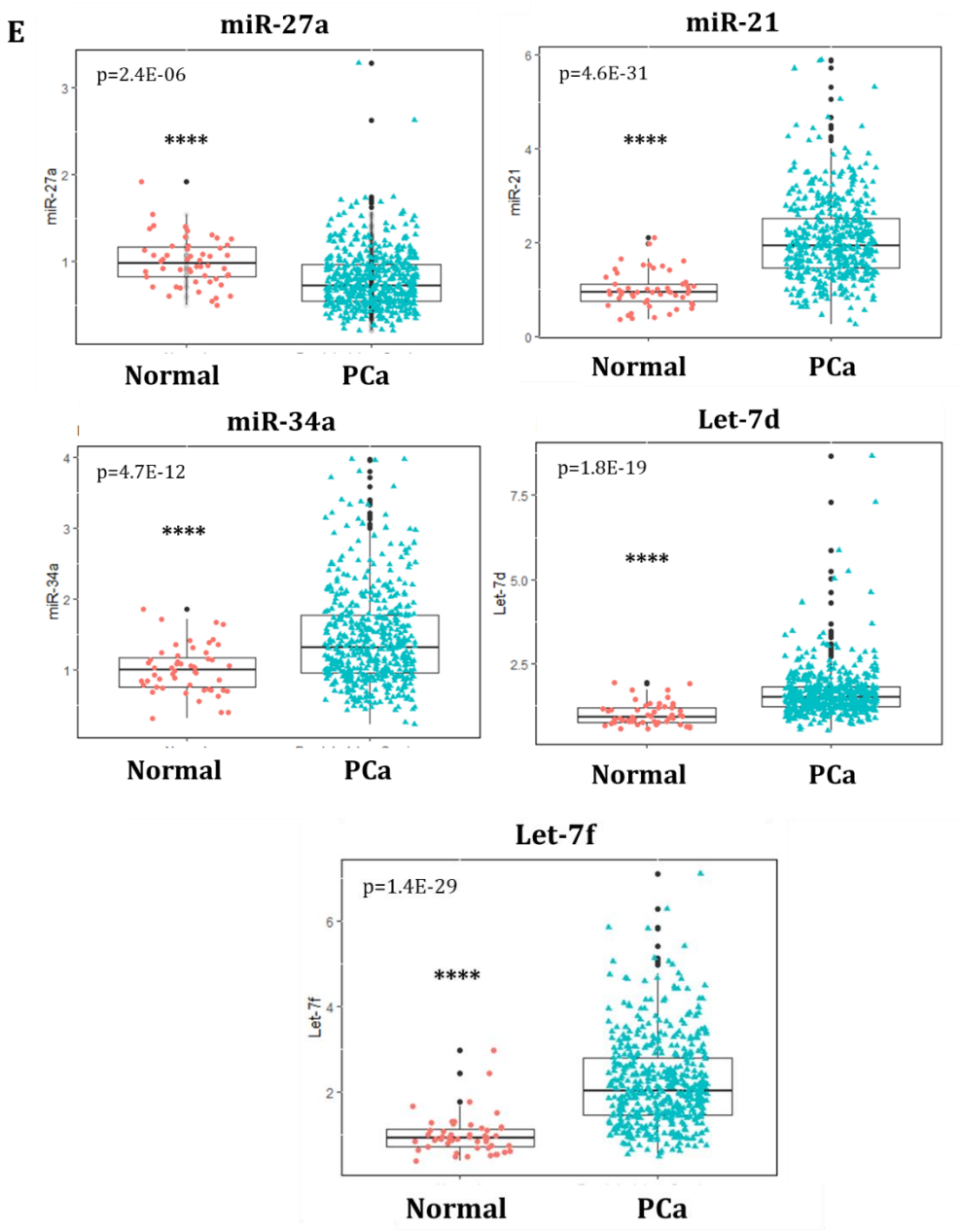


	Total Cases	AR Amplification
CRPC	70	38 (54%)
Prostate Adenocarcinoma	3648	599 (15.5%)
Pr NEC	54	10 (18.5%)
Small Cell Carcinoma	7 Cases	0

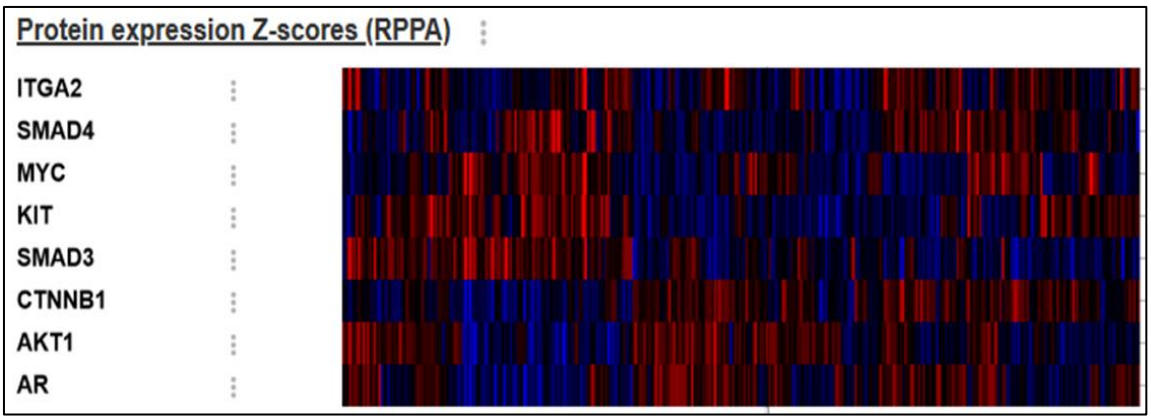


D. Correlation between AR protein levels vs mRNA levels in PCa patients

X (Protein)	Y (mRNA)	Pearson Correlation (X-Y)	P-Value	Correlation
AR	***PROM1***	**-0.18**	**5.479e-4**	**Neg**
	CD44	**-0.18**	**8.669e-4**	**Neg**
	LGR4	**0.14**	**7.809e-3**	**Pos**
	ITGA2	-0.05	0.310	na
	ITGA6	0.0	0.97	na
	KIT	**-0.17**	**1.442e-3**	**Neg**
	PSCA	-0.02	0.667	na
	KRT5	**-0.17**	**1.645e-3**	**Neg**
	KRT14	-0.10	0.0653	na
	TP63	**-0.16**	**2.124e-3**	**Neg**
	MYC	**0.14**	**6.767e-3**	**Pos**
	POU5F1	-0.01	0.864	na
	SOX2	-0.12	0.0310	na
	NANOG	-0.10	0.0645	na
	TACSTD2	-0.06	0.234	na
	CTNNB1	0.02	0.661	na
	FOXA1	**0.19**	**2.708e-4**	**Pos**
	FOXA2	-0.01	0.785	na
	NCOA1	0.02	0.677	na
	NCOA2	0.07	0.215	na
	SMAD2	0.0	0.93	na
	SMAD3	**-0.16**	**3.457e-3**	**Neg**
	SMAD4	0.13	0.0112	na
	AKT1	0.03	0.614	na
	NKX3.1	**0.19**	**2.597e-4**	**Pos**
	KLK3	**0.15**	**5.375e-3**	**Pos**
	MKI67	**0.15**	**6.437e-3**	**Pos**



F. Correlation between AR protein levels vs protein levels in PCa patients



X (Protein)	Y (Protein)	Pearson Correlation (X – Y)	P-Value	Correlation
AR	ITGA2	0.06	0.235	na
	KIT	**-0.34**	**1.05e-10**	**Neg**
	MYC	**-0.31**	**5.91e-9**	**Neg**
	CTNNB1	**0.44**	**4.97e-18**	**Pos**
	SMAD4	-0.07	0.209	na
	AKT1	**0.47**	**1.62e-20**	**Pos**
	SMAD3	**-0.28**	**1.04e-7**	**Neg**

Figure 4. 9: AR Expression analysis in PCa tissue samples (TCGA-Dataset) on the cBioPortal tool. (A) AR expression and mutation pattern in different types of PCa Patients (N=3779) **(B)** Alterations of "Queried gene Set" in TCGA Prostate Adenocarcinoma dataset (N=492) **(C)** Heatmap of mRNA expression of "Queried Gene set"; **(D)** Analysis of AR protein expression vs mRNA expression of queried genes (N=492); **(E)** Graphs represent the relative expression of miR-27a, miR-21, miR-34a, Let-7d, and Let-7f in normal (N=52) v/s prostate adenocarcinoma (N=499) subjects analyzed from TCGA dataset (S-MED); Mean±SD, ****p<1E-05, prostate adenocarcinoma v/s normal subjects; **(F)** Correlation analysis of AR protein expression v/s protein expression of queried genes in PCa patients (N=347). The correlation was performed using Pearson correlation; p<0.001 was considered significant. Pos: Positive Correlation, Neg: Negative Correlation, na: no association.

4.4 Discussion

In the present study, the immunohistology profile of BPH tissue suggested the prominent AR expression in the epithelial cells within the acini than in stromal cells, which is supported by an earlier report of Izumi *et al.*[35] Further, BPH patients showed trivial expression of ARV7 and pARs213, yet significantly associated with total AR levels in AR^{hi} BPH patients. Recently, increased AR-V7 mRNA expression has been reported in BPH patients as compared to normal tissue, which was markedly lower as compared to PCa patients.[10] The expression of AR-V7 is one of the highly expressed proteins in Castrate-resistant Prostate Cancer (CRPC) patients than in BPH and PCa patients.[36] Thus with disease progression, increasing AR-V7 levels occupies androgen-independent transcriptional regulation in the diseased cells. Further, phosphorylation of AR on S213 residue of NTD is regulated through androgen-independent activation of AR by kinases like AKT. The expression of AKT was also increased with AR expression in BPH patients and was strongly correlated with AR protein levels in PCa dataset. The S213

phosphorylation is known to get induced by the IGF1/AKT signaling pathway.[37] We have also demonstrated significantly increased levels of IGF1, pAKT, and total AKT in AR^{hi} patient groups, which supports the increased S213 phosphorylation of AR through IGF1/pAKT.[38]

LGR4 is one of the key proteins present in certain BSCs which is required for the developing prostate. Also, LGR4 regulates Wnt/β-CATENIN signaling to maintain stem/progenitor and its differentiation potential.[39] We have found a positive correlation of AR protein levels with LGR4/β-CATENIN and their gene and protein expression in AR^{hi} BPH patients, suggesting their significant co-expression and a simultaneous upsurge in the BPH tissue, which is also evident in the TCGA PCa dataset. Increased levels of β-CATENIN in BPH patient tissue has been reported previously by Bauman *et al.*[39] However, this is the first report depicting the upsurge of LGR4/β-CATENIN in BPH patients with AR^{hi} condition.

The stem/progenitor cells specific transcription factors, OCT4, ΔNP63, SOX2, NANOG, MYC also play a pivotal role in the development of BPH and PCa which are regulated by Wnt/β-CATENIN and AKT signaling pathways. [40, 41] The expression of OCT4 and ΔNP63 transcription factors were also determined in the developing prostate and adult prostatic BSCs.[42, 43] A patient study discovered that Benign Prostate tumors have higher expression of ΔNP63α as compare to primary and metastatic PCa.[44] Further, a study by Sotomayor *et al* demonstrated increased expression of rare OCT4A positive epithelial cells in both BPH and PCa.[45] Strikingly, we have observed positive association of OCT4 and ΔNP63α proteins with increased AR protein levels in BPH patient tissues. Additionally, increased expression of *OCT4A, SOX2,* and *MYC,* pluripotency markers were also detected during BPH condition.[46] SOX2 is highly expressed in PCa tumors and drives lineage plasticity and drug resistance in patients.[47-49] Immunohistological studies depicted the mild expression of SOX2 and NANOG during BPH.[24, 50] AR plays the suppressive role over *SOX2* gene expression,[51] which supports the negative association of *SOX2* transcripts with AR protein levels in both BPH patients and TCGA PCa dataset. Further, a gradual increase in the expression of cMyc oncogene was found from normal to benign to PCa depending upon increasing the Gleason score.[52] Moreover in the previous study, the gene expression profile of metastatic PCa patients exhibited a positive correlation between AR and cMyc transcript levels.[53] A Similar association between AR and cMyc was also seen in the BPH

and PCa patients included in the present study. However, it has been discovered that cMyc antagonizes the transcriptional activities of AR to diverge the cells from androgen dependence during aggressive CRPC.[54, 55] Further previous observation by Williamson *et al* supports the findings of the TCGA-PCa dataset suggesting a clear negative correlation of AR protein with the transcripts of BSC markers.[56]

The researchers have identified the numerous specific miRNAs targeting diverse cell activity to promote tumor growth in benign and malignant conditions. miR-21 and miR-27a were propagating a positive impact on tumor growth by inhibiting cell proliferation during PCa, and both serve as a diagnostic biomarker in PCa condition.[57-61] Overexpression of miR-21 directly mediates the repression of cellular apoptosis to promote invasive PCa growth, which also severed as a serum diagnostic biomarker for invasive PCa conditions.[61, 62] Repression of miR-21 also impairs the expression of CSC related SOX2, CD44, and EpCAM, leading to repressed prostasphere formation in PCa cells.[63] The expression of miR-21 is directly under the control of AR, which controls its overexpression in androgen-dependant PCa condition.[64, 65] Similar to miR-21, elevation in AR regulated onco-miR-27a levels inhibits cell cycle inhibitors and apoptotic factors in PCa cells, ultimately decreased disease aggressiveness.[66] Further, Zeng *et al* revealed that miR-27a expression declines the activity of OCT4/SOX2 axis in oral squamous carcinoma cells,[67] which supports the elevated OCT4 levels in BPH patients in the present study. Additionally, Lena *et al* previously found that mir-203 decreases the stemness via suppressing ΔNP63 expression in epithelial cells.[25] Here, we found a complete absence of mir-203 expression in BPH patient tissues and BPH stem/progenitor cells. As the present work focuses on BPH epithelial cells, the overexpression in miR-21 and miR-27a was not substantially evident as compared to PCa cells. Thus, BPH stem/progenitor cells and BPH patient tissues exhibited decreased levels of miR-21 and miR-27a expression with elevated AR levels despite the direct regulation of miR-21 by AR. Thus, we envisage higher transcript to protein expression rate of LGR4/β-CATENIN in the BPH stem/progenitor cells and AR^{hi} patient group due to lower levels of miR-27a. Though, the phenomenon has to be experimentally validated further.

The activity of AR requires co-activators like FoxA1, NCoA1 during early stages of PCa and FoxA2, NCoA2 during metastatic PCa.[68-71] The TCGA dataset in the present study showed a positive association between AR protein levels and FoxA1 transcripts,

suggesting their synergy in adenocarcinoma of the prostate. Activation of AR in the target prostate cell initiates the expression of luminal specific markers NKX3.1 and PSA in normal, BPH, and PCa conditions.[72, 73] BPH patient group with high AR levels were expressing elevated *NKX3.1* and declined *PSA* transcripts, as against PCa patients, where both *NKX3.1* and *PSA* are positively correlated with AR protein levels. It has been discovered that Nkx3.1 is also expressed in luminal progenitors of the prostate, but not PSA.[74] Hence, the difference in the association of AR with PSA and NKX3.1 in BPH and PCa patients suggest slightly different regulation via AR transcriptional activity.

Additionally, CD49b, CD49f, CD133, and CD44 are the most explored prostate BSC markers and several investigations have proven its significant involvement in the development of metastatic, CRPC carcinomas with their co-expression.[75-81] The TCGA-PCa dataset suggested upregulation of the transcript levels of CD49b (*ITGA2*), CD49f (*ITGA6*), CD133, and CD44 in few patients. An investigation by Kalantari *et al* suggested a remarkable increase in the expression of CD133 and CD44 in BPH, High grade prostatic intraepithelial neoplasia (HGPIN), and PCa patient tissues.[81] Further, previous findings in PCa patients and cell-lines suggested a negative correlation between AR and CD133 levels in BSCs.[56,75,81] The majority of the stem/progenitor markers like CD44/CD117(KIT) /CD49b/CD49f are the regulators of CSC phenotype and aggressiveness of the disease during PCa.[80, 82] Further, No correlation of AR with CD49f/CD117 stem/progenitor markers has been reported in BPH and PCa patients. Yet, *in-vitro* studies on PCa cell-lines reported their negative correlation in prostate BSCs except in few luminal progenitor subpopulations.[28, 83] These reports corroborate with the findings of the present TCGA-PCa study showing negative or no association of AR with CD49b, CD49f, CD133, and CD44 expression in patients.

Previously, it has been observed that Ki-67 levels increase with the aggressiveness of the disease from BPH to adenocarcinoma to metastatic PCa.[84] Moreover, Cindolo *et al* demonstrated the association between AR and Ki-67 with the Gleason score of PCa patients.[85] Further, Testosterone mediated AR activation in BPH-1 cells increased the expression of Ki-67 generating a proliferative outcome in the BPH cells.[86] Hence, These evidences support the fact that activation of AR supports the rapid growth of the cells in BPH and PCa patient study. This is the first study to highlight the positive association between AR and cell proliferation marker, Ki-67, and age of BPH occurrence in elderly

men. It is also clear that higher AR and Ki-67 levels suggest comparatively more proliferative tumor cells in BPH and PCa.

The study strongly reveals the explicit role of AR in BPH and PCa etiopathogenesis. We also report the strong association of AR levels with the growth and prevalence of BPH. This is the first report to depict the molecular association of NKX3.1, LGR4, β-CATENIN, OCT4, and ΔNP63α with AR protein levels in BPH patients. Despite AR-mediated direct regulation of miR-27a and miR-21, the expression pattern of miR-27a depict a similar profile, yet miR-21 expression differs among both the pathologies, suggesting disease-specific actions of AR in the prostate cells. We have also determined that the elevation of AR and LGR4/β-CATENIN were common in both BPH and PCa patients, which could be a linking factor between the diseases. However, the correlation was negative between AR protein levels and OCT4 and TP63 gene expression in the PCa patients that contradict the correlation in BPH patients exhibiting positive association. These evidences suggested a potential role of AR in increased luminal progenitor phenotype in the BPH tumors but not in PCa tumors, which highlights the AR dependant molecular divergence in BPH and PCa patients.

4.5 Summary

Overall, BPH patients with High AR phenotype have increased expression of LGR4, β-CATENIN, *cMYC*, and *NKX3.1* markers and decreased *PSA* gene expression, suggesting increased luminal progenitor phenotype in BPH patients. A similar correlation was also observed in the TCGA-PCa dataset, except *PSA* gene expression which is positively correlated with AR protein levels. Intriguingly, increased stem/progenitor associated protein levels of LGR4, ΔNP63α, and OCT4 in AR^{hi} BPH patients indicated a possible regulatory role of AR over these markers during BPH condition. One of the important signaling proteins of the stem cells, β-CATENIN was positively associated with AR protein levels in BPH patients and the PCa dataset. Also, BPH patients with elevated AR levels have increased Ki-67 cell proliferation marker expression, and thus have 5 years of early BPH development as compare to low AR expression group. Further, we have also demonstrated miR-27a and miR-21 could serve as a biomarker to determine the presence of BPH and PCa due to disease-specific AR expression patterns. (*Figure 4.10*)

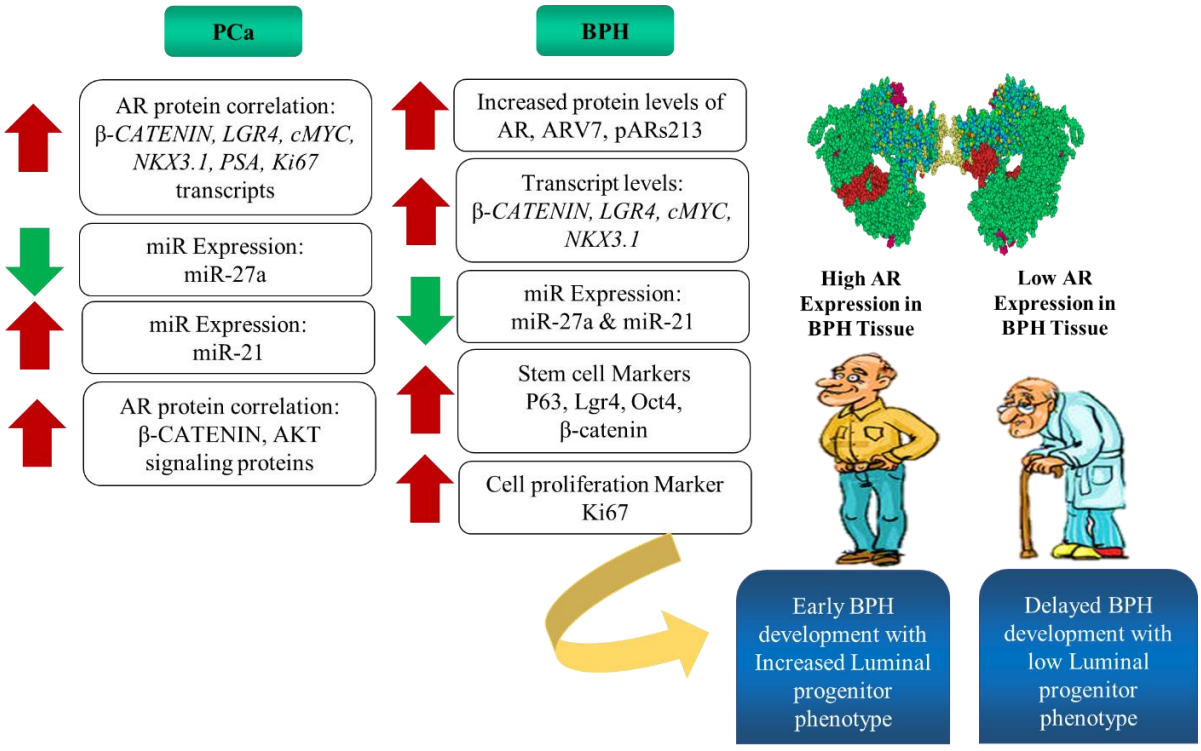


Figure 4. 10: Graphical summary. AR expression and its correlation in BPH and PCa Patient studies.

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