

ANALYSIS OF LONG NON-CODING RNAS IN ANDROGEN RECEPTOR SIGNALING OF PROSTATE CANCER

Dissertation

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1. List of abbreviations

ADT	Androgen deprivation therapy
AR	Androgen receptor
AREs	Androgen response elements
BAT	Bipolar androgen therapy
ceRNA	Competing endogenous RNA
ChIP-seq	Chromatin Immunoprecipitation sequencing
CRPC	Castration-resistant prostate cancer
DElncRNAs	Differentially expressed LncRNAs
DEmiRNAs	Differentially expressed miRNAs
DEmRNAs	Differentially expressed mRNAs
DE RNAs	Differentially expressed RNAs
ENZ	Enzalutamide
LncRNAs	Long non-coding RNAs
miRNAs	microRNAs
NTAT	Non tumoral adjacent tissues
ODM	Darolutamide
PART1	Prostate androgen-regulated transcript 1
PCa	Prostate cancer
PSA	Prostate-specific antigen
RIP	RNA immunoprecipitation
RNA-seq	RNA sequencing
SAL	Supraphysiological androgen levels
SASP	Senescence-associated secretory phenotype
TCGA	The Cancer Genome Atlas
TT	Tumor tissue

2. German Summary (Zusammenfassung)

Prostatakrebs (PCa) ist eine der häufigsten Krebserkrankungen bei Männern und steht in enger Verbindung mit der Androgenrezeptor (AR)-Signalübertragung. Jüngste Fortschritte in der Systembiologie haben zur Identifizierung wichtiger Gene und Signalwege geführt, die an der Prostatakarzinogenese beteiligt sind. In dieser Studie nutzten wir diese Methoden, um potenzielle konkurrierende endogene RNA (ceRNA)-Achsen aufzudecken, wobei ein besonderer Schwerpunkt auf der Bedeutung von *ADAMTS9-AS2/miR-150/PRKCA*, *ADAMTS9-AS2/miR-150/MMP14*, *MEG3/miR-150/PRKCA* und *MEG3/miR-150/MMP14* lag, die *miR-150* als zentrale Komponente hervorgehoben wurde. Überlebensanalysen zeigten, dass *miR-150* eine starke statistische Signifikanz aufweist, und weitere Untersuchungen der Expressionsniveaus mithilfe von TCGA-Datensätzen bestätigten, dass die identifizierten Gene im Vergleich zu normalem Gewebe signifikant mit PCa assoziiert sind, was ihr Potenzial als prognostische Marker und therapeutische Ziele etabliert.

Darüber hinaus identifizierten wir zwei gegensätzlich androgenregulierte lange nicht-kodierende RNAs (lncRNAs), *PART1* und *ADAMTS9-AS2*, die die AR-Signalübertragung vermitteln und mit Prostatakrebsproben sowie der Reaktion auf AR-Antagonisten oder supraphysiologische Androgenspiegel (SAL), die in der bipolaren Androgentherapie (BAT) verwendet werden, korrelieren. Unsere Analyse von Tumorgewebe (TT) und benachbartem normalem Gewebe (NTAT) von 50 Patienten zeigte eine antithetische Regulation von *ADAMTS9-AS2* und *PART1*. RNA-Sequenzierungen in Kombination mit ex vivo-Behandlungen von Prostatektomieproben der Patienten bestätigten die SAL-induzierte Regulation dieser lncRNAs. Knockdown-Experimente zeigten weiterhin, dass *ADAMTS9-AS2* und *PART1* die SAL-induzierte zelluläre Seneszenz vermitteln. RNA-Immunpräzipitation (RIP)-Assays detektierten AR-lncRNA-Interaktionen, und bioinformatische Analysen identifizierten ligandspezifische lncRNA- und miRNA-Interaktionen mit *PART1* und *ADAMTS9-AS2*, die durch ex vivo-Behandlungen weiter bestätigt wurden. Insgesamt deuten unsere Ergebnisse darauf hin, dass die SAL-regulierten lncRNAs *ADAMTS9-AS2* und *PART1* eine entscheidende Rolle bei der Kontrolle der AR-Signalübertragung spielen und als wertvolle therapeutische Ziele im Management von Prostatakrebs dienen könnten.

3. Summary

Prostate cancer (PCa) is one of the most prevalent cancers in males and is strongly associated with androgen receptor (AR) signaling. Recent advancements in systems biology have led to the identification of key genes and pathways involved in prostate carcinogenesis. In this study, we utilized these methods to uncover potential competing endogenous RNA (ceRNA) axes, with a particular focus on the importance of *ADAMTS9-AS2/miR-150/PRKCA*, *ADAMTS9-AS2/miR-150/MMP14*, *MEG3/miR-150/PRKCA*, and *MEG3/miR-150/MMP14*, highlighting *miR-150* as a central component. Survival analyses indicated that *miR-150* has strong statistical significance, and further examination of expression levels using TCGA datasets confirmed that the identified genes are significantly associated with PCa compared to normal tissue, establishing their potential as prognostic markers and therapeutic targets.

Moreover, we identified two oppositely androgen-regulated long non-coding RNAs (lncRNAs), *PART1* and *ADAMTS9-AS2*, which mediate AR signaling and correlate with prostate cancer specimens and responses to AR antagonists or supraphysiological androgen levels (SAL) used in bipolar androgen therapy (BAT). Our analysis of tumor tissue (TT) and adjacent normal tissue (NTAT) from 50 patients revealed antithetic regulation of *ADAMTS9-AS2* and *PART1*. RNA sequencing, coupled with ex vivo treatment of patient prostatectomy samples, confirmed SAL-induced regulation of these lncRNAs. Knockdown experiments further demonstrated that *ADAMTS9-AS2* and *PART1* mediate SAL-induced cellular senescence. RNA immunoprecipitation (RIP) assays detected AR-lncRNA interactions, and bioinformatic analyses identified ligand-specific lncRNA and miRNA interactions with *PART1* and *ADAMTS9-AS2*, further validated through ex vivo treatments. Collectively, our findings suggest that the SAL-regulated lncRNAs *ADAMTS9-AS2* and *PART1* play critical roles in controlling AR signaling and could serve as valuable therapeutic targets in prostate cancer management.

1. Introduction

1.1 Global Prevalence of Prostate Cancer

Prostate cancer (PCa) stands as one of the most prevalent malignancies affecting men worldwide (Bray et al. 2024). According to recent global cancer statistics, it ranks as the second most common cancer among males, contributing significantly to cancer-related morbidity and mortality (Siegel et al. 2024). With an increasing incidence observed in both developed and developing nations, PCa represents a critical public health challenge. Advances in diagnostic techniques and screening programs have led to early detection in many cases, yet disparities in healthcare access and treatment outcomes persist (Rawla 2019). This underscores the necessity for ongoing research, awareness, and improved healthcare strategies to combat the rising burden of PCa globally (Culp et al. 2020).

1.1.2 Significance of PCa Research

Given its high incidence and substantial impact on men's health, understanding the molecular mechanisms underlying PCa is crucial (Litwin und Tan 2017). Developing effective therapeutic strategies necessitates a deep insight into the biological processes driving its progression. Research in this field is pivotal not only for uncovering the genetic and environmental factors contributing to the disease but also for identifying biomarkers for early detection and prognosis (Sweeney et al. 2015). Furthermore, advancements in this area have the potential to lead to personalized treatment approaches, optimizing outcomes and minimizing side effects for patients.

1.1.3 Current Challenges in PCa Therapy

Despite significant advancements in treatment, managing PCa remains fraught with challenges, particularly in advanced stages where resistance to conventional therapies is common (Attard et al. 2016). Hormone therapies, chemotherapy, and radiation, while effective initially, often face diminishing returns as the cancer evolves, leading to treatment-resistant forms of the disease (Petrylak et al. 2004). The heterogeneity of PCa further complicates treatment, as it can manifest with varying degrees of aggression and responsiveness to therapy. Additionally, side effects from existing treatments can significantly impact patients' quality of life, necessitating a delicate balance

between efficacy and tolerability. These challenges underscore the urgent need for novel therapeutic approaches and a deeper understanding of the disease's underlying mechanisms.

1.2 The Role of Androgen Receptor (AR) Signaling in PCa

1.2.1 Mechanisms of AR Signaling in PCa Development

AR signaling plays a pivotal role in the development and progression of PCa. The AR, a type of nuclear hormone receptor, is activated by binding androgens, such as testosterone and dihydrotestosterone (Watson et al. 2015). Once activated, the AR translocates to the cell nucleus, where it binds to specific DNA sequences called androgen response elements (AREs). This binding regulates the transcription of genes involved in various cellular processes, including cell proliferation, differentiation, and apoptosis (Heemers und Tindall 2007).

In the context of PCa, AR signaling is crucial for tumor growth and survival. AR-driven transcriptional programs promote the proliferation of PCa cells while inhibiting apoptosis, thereby contributing to the progression of the disease. Moreover, alterations in AR signaling, such as AR gene amplification, mutations, and splice variant formation, can lead to continuous AR activation, even in the absence of androgens. This persistent activation is a key factor in the development of castration-resistant prostate cancer (CRPC), a stage of the disease that is particularly challenging to treat (Antonarakis et al. 2014).

Therapeutic interventions targeting AR signaling, such as androgen deprivation therapy (ADT) and AR antagonists, have been developed to inhibit this pathway and control tumor growth. However, resistance to these therapies often develops, necessitating ongoing research into the molecular mechanisms of AR signaling and the development of novel therapeutic strategies (Morgan et al. 2024).

1.2.2 Impact of AR Signaling on Cellular Processes

AR signaling significantly influences various cellular processes, including proliferation, differentiation, and cellular senescence, which are crucial for both normal prostate function and PCa progression. The AR, upon activation by androgens, binds to androgen response elements

(AREs) in the DNA, modulating the transcription of genes involved in these processes (Heemers und Tindall 2007).

Proliferation: AR signaling promotes the proliferation of prostate cells by upregulating genes involved in cell cycle progression, such as cyclins and cyclin-dependent kinases. This proliferative effect is essential for normal prostate development and maintenance. However, in the context of PCa, aberrant AR activation leads to uncontrolled cell division and tumor growth (Feldman und Feldman 2001).

Differentiation: AR signaling also plays a critical role in the differentiation of prostate epithelial cells. Proper differentiation is necessary for maintaining the specialized functions of the prostate gland. Disruptions in AR signaling can result in the loss of differentiation, contributing to the development of poorly differentiated and more aggressive cancer phenotypes (Balk und Knudsen 2008).

Cellular Senescence: Cellular senescence is a state of permanent cell cycle arrest that acts as a barrier to cancer progression. AR signaling can influence senescence pathways, and aberrations in this signaling can help cancer cells evade senescence, thereby promoting continued growth and survival (Hessenkemper et al. 2014, Kallenbach et al. 2022, Roediger et al. 2014).

In PCa, alterations in AR signaling pathways, such as mutations, amplifications, and the emergence of constitutively active AR splice variants, are frequently observed. These aberrations are often associated with more aggressive disease and poor clinical outcomes. For instance, the presence of AR splice variants like AR-V7, which remain active without androgens, is linked to resistance to conventional therapies and worse prognosis (Isaacs und Isaacs 2004).

Understanding the impact of AR signaling on these cellular processes is critical for developing targeted therapies aimed at disrupting these pathways. Therapeutic strategies that can effectively modulate AR signaling hold promise for improving outcomes in patients with advanced PCa.

1.2.3 Therapeutic Targeting of AR Signaling

Targeting AR signaling has become a cornerstone in PCa therapy. The development of AR antagonists and other targeted therapies aims to disrupt AR-mediated pathways and improve patient outcomes.

1.3 Emergence of Second-Generation AR Antagonists

1.3.1 Mechanism of Action of Second-Generation AR Antagonists

Second-generation AR antagonists, such as darolutamide and enzalutamide, represent a significant advancement in the treatment of PCa. These agents are designed to inhibit AR-mediated transactivation more effectively than their predecessors, thereby interfering with AR signaling and its downstream effects on PCa cells (Scher und Sawyers 2005).

Darolutamide and enzalutamide bind to the AR with high affinity, preventing androgens like testosterone and dihydrotestosterone from activating the receptor. By blocking androgen binding, these antagonists inhibit the AR from translocating to the nucleus, where it would normally bind to AREs on the DNA to regulate gene transcription. This inhibition disrupts the expression of AR target genes that are critical for cell proliferation, survival, and tumor growth (Fizazi et al. 2014).

Furthermore, second-generation AR antagonists are designed to overcome the limitations of first-generation therapies. For instance, enzalutamide has a higher binding affinity for the AR and can inhibit AR activity even in the presence of increased androgen levels. It also interferes with AR's ability to interact with co-activators and suppresses the transcriptional activity of constitutively active AR splice variants, such as AR-V7, which are often implicated in the development of CRPC (Armstrong et al. 2019).

Darolutamide, another potent AR antagonist, offers additional advantages by having a distinct chemical structure that minimizes penetration of the blood-brain barrier, reducing central nervous system side effects commonly associated with other AR antagonists. This makes darolutamide a particularly valuable option for patients who are sensitive to the neurological side effects of other treatments (Fizazi et al. 2014).

By effectively inhibiting AR signaling, second-generation AR antagonists like darolutamide and enzalutamide play a crucial role in controlling PCa progression, particularly in patients with advanced or castration-resistant disease. Their ability to target key mechanisms of AR action underscores their importance in the ongoing effort to improve therapeutic outcomes for PCa patients (Kokal et al. 2020).

1.3.2 Effects of Second-Generation AR Antagonists on Cellular Senescence

Recent studies indicate that second-generation AR antagonists, such as darolutamide and enzalutamide, can induce cellular senescence in PCa models. Cellular senescence is a state of stable cell cycle arrest in which cells cease to proliferate but remain metabolically active. This process serves as a crucial tumor-suppressive mechanism, preventing the proliferation of damaged or abnormal cells (Chen et al. 2006, Kokal et al. 2020).

The induction of senescence by second-generation AR antagonists represents a promising therapeutic effect beyond their primary role of inhibiting AR signaling. By promoting senescence, these agents can halt the growth of cancer cells and limit tumor progression. This is particularly significant in the context of CRPC, where traditional therapies often fail to maintain long-term efficacy (Sharifi 2013, Kallenbach et al. 2022).

The mechanisms through which second-generation AR antagonists induce cellular senescence involve several pathways. One key mechanism is the downregulation of AR target genes that promote cell cycle progression, such as those encoding cyclins and cyclin-dependent kinases. Inhibition of these genes leads to cell cycle arrest, a hallmark of senescence (Ghafouri-Fard et al. 2022). Additionally, AR antagonists can modulate the expression of senescence-associated secretory phenotype (SASP) factors, which reinforce the senescence state and influence the tumor microenvironment (Cao et al. 2014).

Understanding the senescence-inducing effects of second-generation AR antagonists is crucial for optimizing therapeutic strategies. For instance, combining these AR antagonists with other agents that target senescent cells or modulate the SASP could enhance therapeutic efficacy and prevent tumor relapse (Pungsrinont et al. 2020). Furthermore, biomarkers of senescence could be

developed to identify patients who are most likely to benefit from these therapies (Coppé et al. 2008).

By elucidating the role of cellular senescence in the action of second-generation AR antagonists, researchers can better harness this effect to improve treatment outcomes for PCa patients. Continued investigation into the molecular underpinnings of this process will pave the way for innovative therapeutic approaches that exploit the full potential of these advanced AR-targeting agents.

1.4 Introduction to SAL in PCa Therapy

Supraphysiological Androgen Levels (SAL) refer to androgen concentrations that exceed the normal physiological range. The concept of using SAL in PCa therapy is rooted in the observation that high-dose androgens can have paradoxical anti-tumor effects in certain contexts (Schweizer et al. 2016, Roediger et al. 2014).

1.4.1 Mechanism of Action of SAL

The use of SAL aims to exploit the differential responses of PCa cells to extreme androgen levels. At supraphysiological concentrations, androgens can induce cellular stress and apoptosis in PCa cells, particularly those adapted to low androgen environments typical of ADT (Denmeade und Isaacs 2010, Teply et al. 2018). The mechanisms include:

AR Overactivation: Excessive activation of the AR can lead to detrimental cellular outcomes, including DNA damage and apoptosis (Shiota et al. 2011).

Cell Cycle Arrest: High androgen levels can disrupt cell cycle regulation, leading to growth arrest and senescence (Yu et al. 2009).

Metabolic Stress: Elevated androgens can alter cellular metabolism, leading to energy depletion and cell death (Massie et al. 2011).

1.4.2 Potential Benefits of SAL in PCa Therapy

Overcoming Resistance: SAL can potentially overcome resistance mechanisms that develop during prolonged ADT, re-sensitizing tumors to subsequent treatments.

Cytotoxic Effects: By inducing stress and apoptosis in cancer cells, SAL can directly reduce tumor burden.

Combination Strategies: SAL can be combined with other therapies, such as AR antagonists or chemotherapeutics, to enhance overall treatment efficacy.

1.4.3 Clinical Applications and Research on SAL in PCa

1.4.3.1 Bipolar Androgen Therapy (BAT)

BAT is a therapeutic approach that alternates between SAL and androgen deprivation. This cyclical treatment aims to maximize the anti-tumor effects of androgen fluctuations. Clinical trials have shown that BAT can stabilize disease and reduce prostate-specific antigen (PSA) levels in some patients with CRPC. For example, the clinical trial identified by NCT02090114 is evaluating BAT's efficacy in asymptomatic men with CRPC and has demonstrated that BAT can stabilize disease and reduce PSA levels in some patients (Denmeade et al. 2023). High-dose androgen monotherapy is being explored as a treatment option for certain PCa patients. Initial studies suggest that this approach may be effective in specific patient populations, particularly those with tumors still responsive to androgen signaling (Teply et al. 2018).

1.4.4 Challenges and Considerations in Using SAL

The use of supraphysiological androgen levels carries risks, including cardiovascular events and potential stimulation of androgen-sensitive tissues. Identifying patients who are likely to benefit from SAL is crucial. Biomarkers and genetic profiling may help tailor this therapy to individuals most likely to respond. The durability of responses to SAL and the potential for long-term control of PCa require further investigation (Schweizer et al. 2016).

By advancing our understanding of SAL and its application in PCa therapy, researchers aim to develop more effective treatment strategies that improve patient outcomes and quality of life.

1.5. Long non-coding RNAs (lncRNAs)

1.5.1 Overview of lncRNAs and Their Functions

lncRNAs are a class of RNA molecules longer than 200 nucleotides that do not encode proteins. Despite lacking coding potential, lncRNAs play crucial roles in regulating gene expression at various levels, including chromatin modification, transcription, and post-transcriptional processing. They are involved in diverse biological processes such as cell differentiation, proliferation, and apoptosis (Ghafouri-Fard et al. 2020). LncRNAs can act as molecular scaffolds, decoys, guides, or enhancers, influencing cellular function and contributing to disease pathogenesis, including cancer. In cancer, lncRNAs can function as oncogenes or tumor suppressors, and their dysregulation is often associated with tumor initiation, progression, metastasis, and resistance to therapy (Ghafouri-Fard et al. 2019). LncRNAs can modulate cancer cell behavior by interacting with chromatin, transcription factors, or other RNA molecules, thereby affecting the expression of cancer-related genes. Their aberrant expression profiles in tumors make lncRNAs potential biomarkers for cancer diagnosis, prognosis, and therapeutic targets (Ghafouri-Fard et al. 2021).

1.5.2 LncRNAs in PCa

PCa is one of the most common malignancies affecting men globally. Emerging evidence highlights the significant role of lncRNAs in the development and progression of PCa. LncRNAs in PCa can be broadly categorized into those that are upregulated and those that are downregulated, each contributing to the disease through distinct mechanisms (Taheri et al. 2023a).

1.5.2.1 Upregulated lncRNAs in PCa

Upregulated lncRNAs in PCa often act as oncogenes, promoting tumor growth, invasion, and metastasis (Figure 1). They can enhance AR signaling, a critical pathway in PCa progression, by stabilizing AR mRNA, modulating AR target gene expression, or interacting with AR co-regulators.

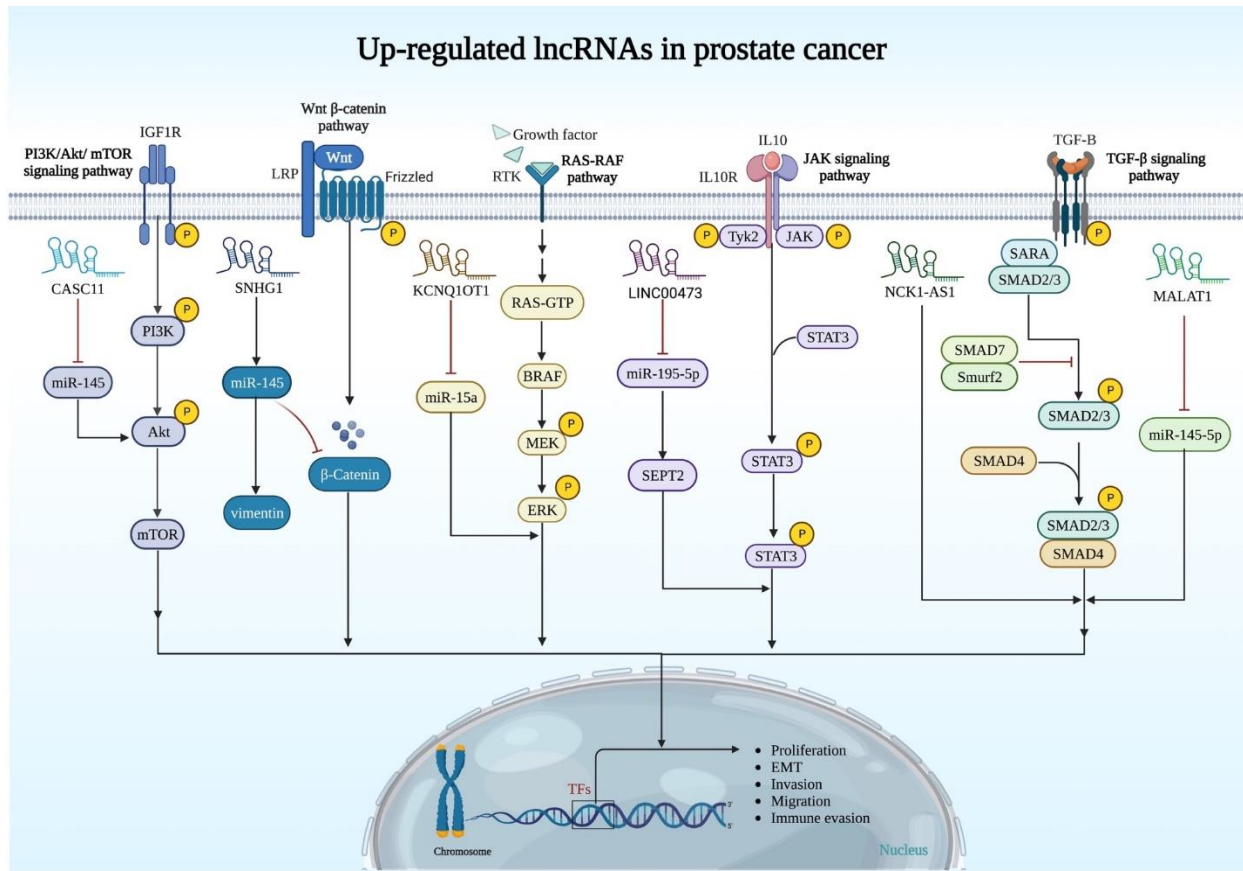


Figure 1. *Oncogenic lncRNAs upregulated in PCa*, specifically influencing the PI3K/AKT/mTOR, Wnt/β-catenin, RAS/RAF, JAK, and TGF-β pathways. (Reprinted from (Taheri et al. 2023a); an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>))

1.5.2.2 Downregulated lncRNAs in PCa

Conversely, downregulated lncRNAs in PCa often function as tumor suppressors. Their reduced expression can lead to the loss of regulatory control over cell growth and differentiation, contributing to tumorigenesis. These lncRNAs may normally inhibit oncogenic pathways or support the integrity of genomic expression networks (Figure 2).

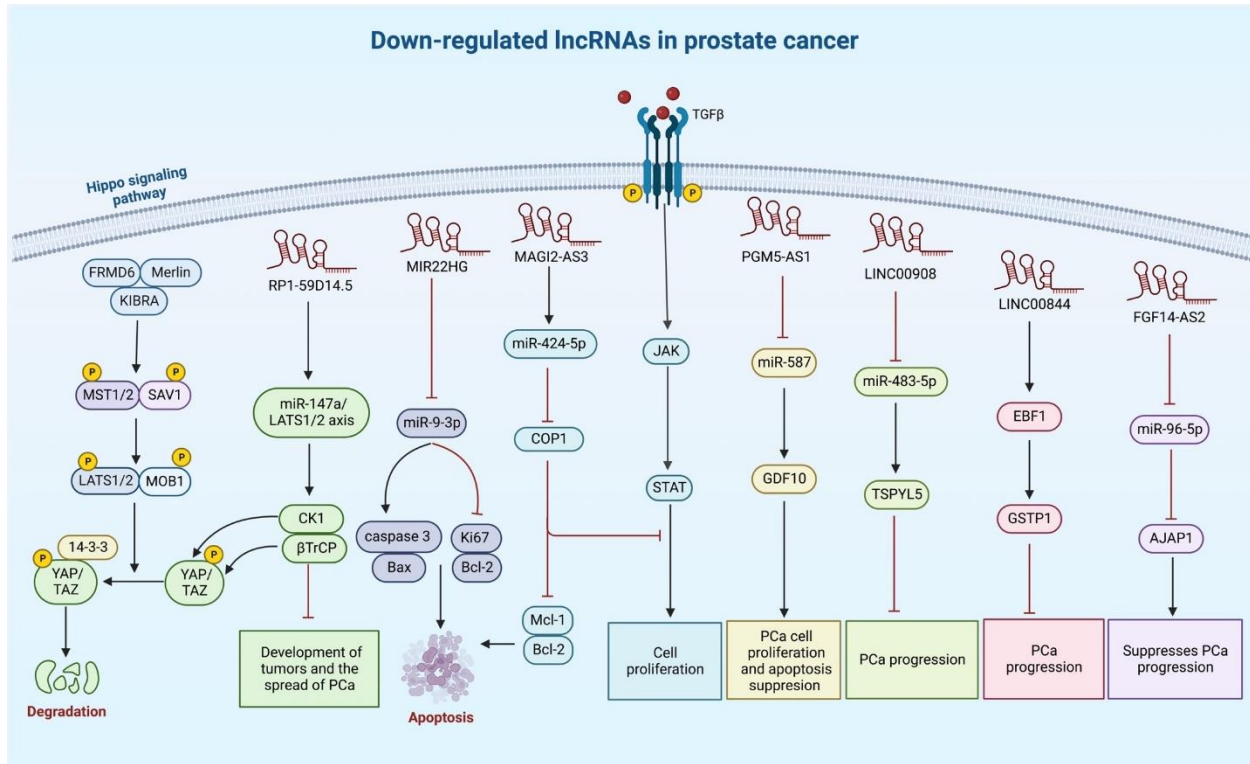


Figure 2. *An overview of the functions of lncRNA tumor suppressors in PCa.* Various lncRNAs can inhibit cell proliferation and invasiveness in PCa cells, primarily by acting as sponges for oncogenic miRNAs. (Reprinted from (Taheri et al. 2023a); an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>))

1.5.2.3 Specific lncRNAs in PCa

ADAMTS9 antisense RNA 2 (*ADAMTS9-AS2*) is a lncRNA that has been implicated in various cancers, including PCa. It is generally considered a tumor suppressor lncRNA. In PCa *ADAMTS9-AS2* is thought to inhibit cancer cell proliferation and metastasis by regulating the expression of its sense gene, *ADAMTS9*, which encodes a protease involved in the degradation of extracellular matrix components (Figure 3) (Ghafouri-Fard et al. 2023a).

The downregulation of *ADAMTS9-AS2* is associated with poor prognosis and advanced disease stages in PCa patients. Its restoration could represent a therapeutic strategy to inhibit tumor growth and spread.

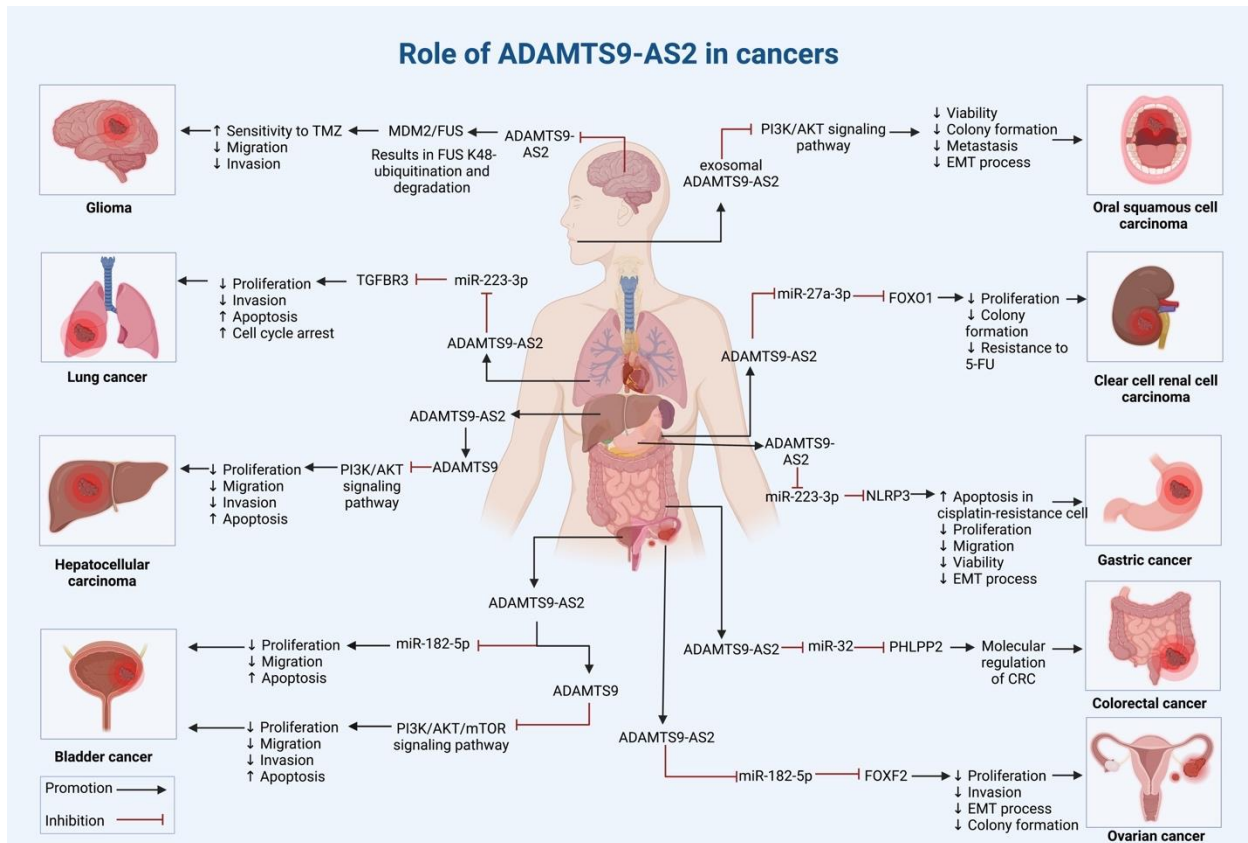


Figure 3. *Signaling pathways associated with ADAMTS9-AS2 in various cancers.* The long non-coding RNA *ADAMTS9-AS2* functions as a tumor suppressor by sequestering multiple oncogenic microRNAs. Additionally, *ADAMTS9-AS2* directly interferes with oncogenic signaling pathways, thereby reducing cancer cell proliferation, migration, and invasion, while promoting apoptosis. (Reprinted from (Ghafouri-Fard et al. 2023c); an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>)).

Prostate androgen-regulated transcript 1 (*PART1*) is another lncRNA that plays a role in PCa. *PART1* is involved in modulating AR signaling. It can enhance AR transcriptional activity, thereby promoting the expression of AR target genes that drive PCa cell proliferation and survival. *PART1* is often upregulated in PCa and is associated with tumor aggressiveness. It could serve as a potential biomarker for diagnosis and prognosis, as well as a target for therapeutic intervention to disrupt AR-driven oncogenic processes (Figure 4) (Ghafouri-Fard et al. 2023c).

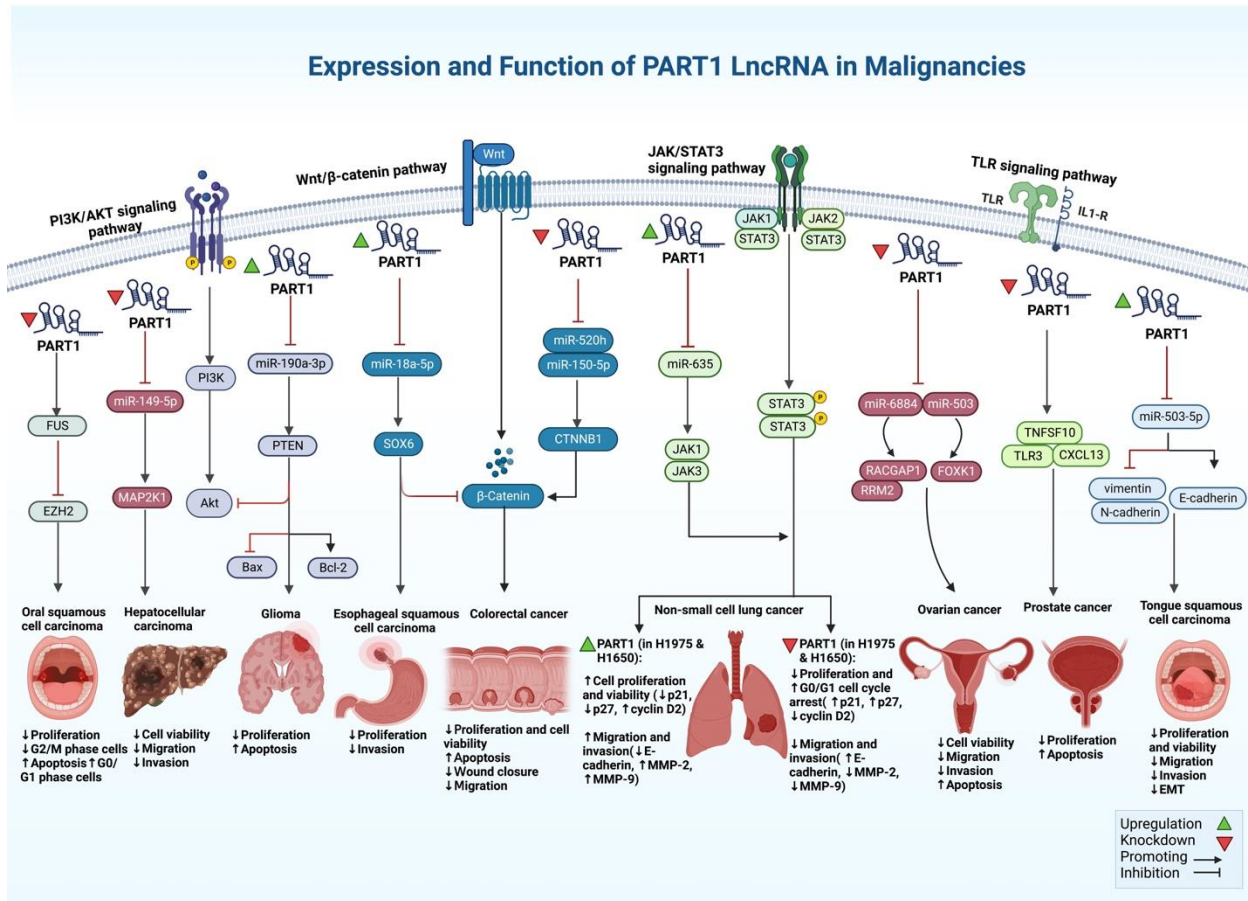


Figure 4. The illustration depicts the various signaling pathways of the PART1 lncRNA, highlighting its expression levels and functional roles across different cancer types. (Reprinted from (Ghafouri-Fard et al. 2023c); an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>).

The hypothesis is that PCa shows differential expression of these lncRNAs and AR targeted therapies of PCa may influence lncRNAs expression and their activities. Here, we used patient expression data, *ex vivo* treatment of PCa tissue samples, ChIP-seq and functional assays to identify novel androgen-regulated signaling in PCa including SAL controlled cellular senescence.

1.6 Unraveling the ceRNA Network: Mechanisms and Implications in Androgen Receptor Signaling of Prostate Cancer

The competing endogenous RNA (ceRNA) network represents a pivotal mechanism of gene regulation, where various RNA molecules, including long non-coding RNAs (lncRNAs),

microRNAs (miRNAs), and messenger RNAs (mRNAs), interact to control gene expression (Salmena et al. 2011). In the context of prostate cancer, ceRNA networks play a critical role in modulating AR signaling, a key pathway driving cancer progression. Through their ability to act as molecular sponges, lncRNAs can sequester miRNAs, thereby preventing them from inhibiting target mRNAs that are crucial for AR signaling (Prensner et al. 2013). This intricate balance within the ceRNA network not only influences the expression of AR target genes but also impacts tumor growth, therapy resistance, and disease outcomes. Understanding the mechanisms and implications of ceRNA networks in AR signaling provides valuable insights into the molecular underpinnings of prostate cancer and opens up new avenues for therapeutic intervention (Huarte 2015).

2. Aim of the study

The aim of this study is to investigate the impact of the AR ligand SAL on cellular senescence in PCa cells, encompassing both androgen-sensitive and CRPC variants. The research focuses on understanding how SAL induces cellular senescence and the precise pathways involved, given its established use in Bipolar Androgen Therapy (BAT) for metastatic PCa.

Another key objective is to examine the role of lncRNAs in mediating the effects of SAL on gene expression. RNA sequencing (RNA-seq) data reveals significant changes in the expression of specific lncRNAs, notably the up-regulation of *ADAMTS9-AS2* and the down-regulation of *PART1* in response to SAL treatment (Taheri et al. 2024, Taheri et al. 2022). These lncRNAs are linked to AR signaling and cellular senescence processes. The study aims to uncover the regulatory mechanisms of these lncRNAs, particularly how *ADAMTS9-AS2* may function as a direct AR target and the implications of *PART1*'s dual functionality as both an oncogene and a tumor suppressor in various contexts.

Additionally, the study will explore the interactions between lncRNAs and microRNAs (miRNAs), focusing on *miR-98-5p* and *miR-7-5p*, which are up-regulated in PCa and have anti-apoptotic properties. These miRNAs may interact with *ADAMTS9-AS2*, forming a regulatory network that influences AR signaling and PCa progression. By delving into these complex interactions, the study aims to provide new insights into the molecular mechanisms underlying BAT and AR pathways, potentially identifying novel therapeutic targets and strategies for PCa treatment. Understanding these intricate regulatory mechanisms may pave the way for improved therapeutic interventions and a better grasp of PCa biology.

3. Overview of manuscripts

LncRNA/miRNA/mRNA Network Introduces Novel Biomarkers in Prostate Cancer

Taheri M, Safarzadeh A, Hussen BM, Ghafouri-Fard S, Baniahmad A. *Cells*. 2022 Nov 25;11(23):3776.

The study presented in this manuscript focuses on the construction and analysis of a competing endogenous RNA (ceRNA) network to understand the role of differentially expressed genes in prostate cancer. Utilizing a suite of bioinformatics tools, we aimed to elucidate the significance of these molecular modules in predicting patient survival outcomes.

We commenced our research by analyzing microarray data from prostate cancer and normal samples using the Limma package. This analysis allowed us to identify differentially expressed RNAs (DE RNAs), which were categorized into mRNAs, long non-coding RNAs (lncRNAs), and microRNAs (miRNAs). Our findings revealed a total of 684 differentially expressed mRNAs (DEmRNAs), including 437 downregulated and 241 upregulated mRNAs. Notable downregulated DEmRNAs included *TGM4* and *SCGB1A1*, while upregulated examples comprised *TDRD1* and *CRISP3*. Additionally, we identified 6 differentially expressed lncRNAs (DElncRNAs) with 1 downregulated (*H19*) and 5 upregulated (such as *PCA3*, *PART1* and *PCGEM1*), along with 59 differentially expressed miRNAs (DEmiRNAs), which included 30 downregulated (such as *hsa-miR-1274a* and *hsa-miR-1274b*) and 29 upregulated (such as *hsa-miR-1268* and *hsa-miR-1207-5p*).

The construction of the ceRNA network incorporated 5 miRNAs, 5 lncRNAs, and 17 mRNAs. Through this network, we identified key hub genes, including *hsa-miR-17*, *hsa-miR-93*, *hsa-miR-150*, *hsa-miR-25*, *PART1*, *hsa-miR-125b*, *PCA3*, *H19*, *RND3*, and *ITGB8*. These hub genes demonstrated significant potential in diagnostic applications, as confirmed by the high diagnostic value revealed through ROC analysis of their expression levels.

In summary, our research introduces several novel diagnostic biomarkers for prostate cancer. The identification and validation of these biomarkers within the ceRNA network contribute valuable insights into the molecular mechanisms underlying prostate cancer and provide a foundation for improved diagnostic and therapeutic strategies.

An in-silico Approach for Recognition of Long non-coding RNA-Associated Competing Endogenous RNA Axes in Prostate Cancer

Taheri M, Safarzadeh A, Ghafouri-Fard S, Baniahmad A. **Urology Journal**. 2024 Feb 28;21(01):57-73.

Prostate cancer is among the leading causes of cancer-related deaths worldwide, necessitating the development of novel therapeutic strategies. To address this need, we developed an in-silico method to identify competing endogenous RNA (ceRNA) networks that could reveal new molecular targets for treatment. By conducting comprehensive microarray data analyses on prostate tumor and normal specimens, we identified a total of 1,312 differentially expressed mRNAs (DEmRNAs). Among these, 778 DEmRNAs were down-regulated, including genes such as *CXCL13* and *BMP5*, while 584 DEmRNAs were up-regulated, including *OR51E2* and *LUZP2*. In addition, we detected 39 differentially expressed long non-coding RNAs (DElncRNAs), with 10 being down-regulated (e.g., *UBXN10-AS1*, *ADAMTS9-AS2* and *FENDRR*) and 29 being up-regulated (e.g., *PCA3* and *LINC00992*). We also identified 10 differentially expressed microRNAs (DEmiRNAs), comprising 2 down-regulated (e.g., *MIR675* and *MIR1908*) and 8 up-regulated (e.g., *MIR6773* and *MIR4683*).

Using these differentially expressed RNAs, we constructed a ceRNA network to map the interactions between mRNAs, lncRNAs, and miRNAs in prostate cancer. This network serves as a crucial framework for understanding how these RNAs regulate each other and influence cancer progression. By integrating these transcripts into a cohesive network, we were able to evaluate the related signaling pathways and assess the significance of these RNAs in predicting patient survival outcomes. Our analysis highlighted the potential roles of specific RNAs in modulating critical cancer-related processes, thereby underscoring their importance as biomarkers and therapeutic targets.

Overall, this study provides valuable insights into the molecular mechanisms underlying PCa and introduces novel candidates for developing targeted treatment strategies. The ceRNA network we constructed offers a comprehensive view of the complex regulatory interactions in PCa, paving the way for future research and clinical applications. By identifying key differentially expressed

RNAs and their roles in cancer biology, our findings contribute to the advancement of precision medicine approaches, aiming to improve patient prognosis and treatment efficacy in prostate cancer.

Interaction between non-coding RNAs and Androgen Receptor with an Especial Focus on Prostate Cancer

Taheri M, Khoshbakht T, Jamali E, Kallenbach J, Ghafouri-Fard S, Baniahmad A. **Cells**. 2021 Nov 16;10(11):3198.

The manuscript explores the androgen receptor (AR), a crucial member of the nuclear receptor superfamily, focusing on its structure, functions, and interactions. AR comprises three main domains: the N-terminal domain with strong transactivation functions, the DNA-binding domain, and the C-terminal domain, which interacts with androgens and antiandrogens used in prostate cancer treatment. The receptor's functions extend beyond DNA binding, involving interaction with other transcription factors and initiating non-genomic activities through pathways such as ERK, AKT, and MAPK.

The review highlights the significant role of non-coding RNAs, including microRNAs, long non-coding RNAs, and circular RNAs, in interacting with AR. These interactions are critical in the pathogenesis of various human cancers, especially prostate cancer. The manuscript compiles and analyzes current data on how non-coding RNAs influence AR expression and signaling and vice versa. Understanding these complex interactions has potential implications for developing new treatment strategies and enhancing the efficacy of existing therapies.

Unveiling four axes *ADAMTS9-AS2|MEG3/hsa-miR-150|PRKCA|MMP14* within prostate cancer through establishment of the ceRNA network

Taheri M, Safarzadeh A, Baniahmad A. Manuscript submitted for publication in **International Journal Of Surgery**.

In this study, we utilized systems biology approaches to identify potential competing endogenous RNA (ceRNA) networks in prostate cancer.

Our analysis highlighted the significance of the following ceRNA axes: *ADAMTS9-AS2/miR-150/PRKCA*, *ADAMTS9-AS2/miR-150/MMP14*, *MEG3/miR-150/PRKCA*, and *MEG3/miR-150/MMP14*. Notably, *miR-150* emerged as a central component within these networks and demonstrated strong statistical significance in survival analyses. Additionally, expression levels from The Cancer Genome Atlas (TCGA) datasets showed that the identified genes are significantly associated with prostate cancer compared to normal tissue, supporting our bioinformatic findings.

Consequently, these genes can be considered as prognostic markers for prostate cancer, and the identified pathways present promising targets for therapeutic interventions.

The lncRNAs PART1 and ADAMTS9-AS2 act in an antithetic manner on AR signaling and induction of cellular senescence in prostate cancer cells

Taheri M, Schindler A, Baniahmad A. Manuscript submitted for publication in **International Journal Of Biological Macromolecules**.

PCa is fundamentally linked to AR signaling, and therapeutic strategies targeting this pathway include AR antagonists and BAT, which involves SAL. This manuscript investigates the role of two long non-coding RNAs (lncRNAs), *PART1* and *ADAMTS9-AS2*, which are regulated in opposing manners by androgens and are associated with PCa progression and response to AR-targeted therapies.

The study involved a comparative analysis of tumor tissue (TT) samples and adjacent normal tissue (NTAT) from 50 patients, revealing antithetic regulation of *ADAMTS9-AS2* and *PART1* expression. RNA sequencing and ex vivo treatment of patient prostatectomy samples confirmed that SAL regulates these lncRNAs. Functional assays demonstrated that knockdown of *PART1* and *ADAMTS9-AS2* mediates SAL-induced cellular senescence. Furthermore, RNA immunoprecipitation (RIP) experiments detected interactions between AR and these lncRNAs.

Bioinformatics analyses identified specific interactions between ligands, lncRNAs, and microRNAs (miRNAs) with *PART1* and *ADAMTS9-AS2*, which were further validated through ex vivo treatments. The findings suggest that *ADAMTS9-AS2* and *PART1*, which are oppositely regulated by SAL, play crucial roles in modulating AR signaling in the context of BAT.

3.1 Manuscript I



Article

LncRNA/miRNA/mRNA Network Introduces Novel Biomarkers in Prostate Cancer

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Abstract: The construction of a competing endogenous RNA (ceRNA) network is an important step in the identification of the role of differentially expressed genes in cancers. In the current research, we used a number of bioinformatics tools to construct the ceRNA network in prostate cancer and identify the importance of these modules in predicting the survival of patients with this type of cancer. An assessment of microarray data of prostate cancer and normal samples using the Limma package led to the identification of differentially expressed (DE) RNAs that we stratified into mRNA, lncRNA, and miRNAs, resulting in 684 DE mRNAs, including 437 downregulated DE mRNAs (such as TGM4 and SCGB1A1) and 241 upregulated DE mRNAs (such as TDRD1 and CRISP3); 6 DE lncRNAs, including 1 downregulated DE lncRNA (H19) and 5 upregulated DE lncRNAs (such as PCA3 and PCGEM1); and 59 DE miRNAs, including 30 downregulated DE miRNAs (such as hsa-miR-1274a and hsa-miR-1274b) and 29 upregulated DE miRNAs (such as hsa-miR-1268 and hsa-miR-1207-5p). The ceRNA network contained a total of 5 miRNAs, 5 lncRNAs, and 17 mRNAs. We identified hsa-miR-17, hsa-miR-93, hsa-miR-150, hsa-miR-25, PART1, hsa-miR-125b, PCA3, H19, RND3, and ITGB8 as the 10 hub genes in the ceRNA network. According to the ROC analysis, the expression levels of 19 hub genes showed a high diagnostic value. Taken together, we introduce a number of novel promising diagnostic biomarkers for prostate cancer.

Keywords: prostate cancer; ceRNA; lncRNA; miRNA; regulatory network

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1. Introduction

Prostate cancer is the most frequent malignancy in males worldwide, accounting for 27% of all cancer diagnoses [1]. During the period from 2014 to 2018, the incidence of prostate cancer has stayed stable, yet the annual incidence for advanced prostate cancer has increased by 4–6% since 2011 [1]. This means that the percentage of prostate cancer cases being diagnosed at advanced stages has increased by approximately two times over the past decade. Consequently, it is necessary to find appropriate markers for an early diagnosis of this common cancer. The prostate-specific antigen (PSA), as the mostly used marker, has some limitations, including the poor interchangeability of PSA results which are acquired from diverse tests. The current gaps should be filled by arranging commutable reference materials for calibration immunoassay tests, identifying analytical features

that can clarify the different performance of assays, and giving more focus on laboratory tests when clinical practice guidelines are organized [2].

It is also important to mention that high-grade prostate cancer is likely on a genetic basis, whereas low-grade disease is more likely to be associated with environmental factors. In fact, the vivid alteration in stage from the time when PSA was introduced as a screening test has been accompanied by a more modest shift in Gleason grade, suggesting that grade may be established in the early phases of tumor pathogenesis [3]. Meanwhile, metastatic prostate cancers exhibit histological and immunophenotypical heterogeneity, necessitating the conduction of individualized therapeutic regimens [4]. For reasons of cost-effectiveness, this panel should be applied to individuals at risk of high-grade prostate cancer, likely based on PSA levels. According to current guideline recommendations, more recently, a PSA-based algorithm for predicting high-grade tumors has been developed [5].

The recent decade has witnessed a revolution in the application of high throughput data analysis tools, leading to the construction of several interaction networks between different types of biomolecules including RNA, DNA, and protein in different disease contexts, particularly cancer. This type of study has led to the identification of promising biomarkers for the early detection of cancer. In the field of prostate cancer, several efforts have been made. For instance, the re-analysis of high throughput expression data has led to the identification of several differentially expressed genes (DEGs) between cancerous and normal tissues. Further functional enrichment analyses have shown the relationship between these genes and clinical outcomes of patients [6]. Similar approaches have identified differentially expressed circular RNAs in this type of cancer and the main signaling pathways being controlled by these transcripts [7].

The competing endogenous RNA (ceRNA) network being constructed between long non-coding RNAs (lncRNAs), microRNAs (miRNAs), and mRNAs has been shown to contribute to the pathobiology of different cancers and regulate fundamental processes, both within cancer cells and inside the tumor microenvironment [8,9]. Identification of this type of interaction between different RNA molecules can introduce novel biomarkers for cancer diagnosis. In the current research, we used a number of bioinformatics tools to construct the ceRNA network in prostate cancer and identify the importance of these modules in the prediction of survival of patients with this type of cancer.

2. Methods

2.1. Microarray Data Collection

The Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) (accessed on 12 September 2022) was used to obtain the human expression data for GSE88808 (Illumina HumanHT-12 WG-DASL V4.0 expression beadchip (gene summary, GenomeStudio report), Duarte, CA, USA), GSE200879 (HTA-2_0 Affymetrix Human Transcriptome Array 2.0 (Genosplise), Créteil, France), and GSE60117 (Agilent-021827 Human miRNA Microarray (V3), Palo Alto, CA, USA). There were 98, 137, and 77 samples in each of these datasets, respectively. For further analysis, we chose 113 prostate cancers and 9 normal tissues from GSE200879, 49 prostate tumors and 49 adjacent normal samples from GSE88808, and 56 prostate tumors and 21 normal tissues from GSE60117 (Table 1). Moreover, we selected prostate tumor samples with Gleason scores of 7 or above. The expression data included the expression signatures of lncRNAs, miRNAs, and mRNAs.

Table 1. Information of datasets.

Datasets.	Platform	Use	Patient	Control	Tissue
GSE88808	GPL22571	DEmRNA-DElncRNA	49	49	Prostate
GSE200879	GPL32170	DEmRNA-DElncRNA	128	9	Prostate
GSE60117	GPL13264	DEmiRNA	56	21	Prostate

2.2. Microarray Data Processing, Integrative Meta-Analysis and Assessment of Data Quality

Normalization is a crucial stage in the integration of heterogeneous data since the described datasets contain various and trendy platforms. The statistical programming language R was used for the processing and integration of microarray data. Data were initially normalized independently for pre-processing using the preprocessCore package's normalizeQuantiles function (<https://bioconductor.org/packages/release/bioc/html/preprocessCore.html>) (accessed on 14 September 2022). Next, data from both platforms were combined using the R software. The ComBat function from the R Package Surrogate Variable Analysis (SVA) was utilized to exclude batch effects (non-biological differences) [10]. The batch-effect removal was assessed using PCA and a boxplot. A unit expression matrix was the outcome of the meta-analysis (the combination of three datasets of this study).

2.3. Analysis of Differentially Expressed lncRNAs, miRNAs and mRNAs

We used the Limma package in R language [11] to identify differentially expressed mRNAs (DEmRNAs), lncRNAs (DElncRNAs), and miRNAs (DEmiRNAs) between prostate and normal samples. GSE88808 and GSE200879 were used to obtain DEmRNAs and DElncRNAs. GSE60117 was utilized to acquire DEmiRNAs. The cut-off criteria for evaluating DEGs were $|\log_2$ fold Change (FC)| > 0.5 and false discovery rate (FDR; adjusted p value) < 0.05. Following that, we used the HUGO gene nomenclature committee to identify DElncRNAs.

2.4. Two-Way Clustering of DEGs

We determined the gene expression levels of significant DEGs. We used this data in the pheatmap package in R language [12] to complete the two-way clustering based on the Euclidean distance.

2.5. TCGA Data Collection and Processing

We included a total of 500 PRAD samples and 52 control samples for further analysis. We used the TCGAbiolinks package to download the transcriptome profiling data (TCGA-PRAD), and limma and edgeR packages to analyze the data. As a result, DEGs were evaluated with the cut-off criteria of the false discovery rate (FDR; adjusted p value) < 0.05 and $|\log_2$ fold Change (FC)| > 0.5. Finally, we identified the genes that were present in both the TCGA and GEO datasets.

2.6. Gene Ontology (GO) Enrichment Analysis

We used the clusterProfiler R package [13] to perform gene ontology (GO) enrichment analysis to investigate the functions of the remarkably upregulated and downregulated DEGs that we discovered. The functional category criteria were established at an adjusted p -value of 0.05 or below.

2.7. Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

To determine the potential roles of DEGs that took part in the pathways based on the KEGG database, KEGG pathway analyses of these genes were conducted [14].

2.8. PPI Network Construction and Hub Genes Identification

The PPI network for DEGs was built using the STRING database [15]. The highest level of confidence (confidence score > 0.9) and text mining, experiments, and database sources were used to establish the interactions parameter. The interactions between the proteins were examined using the Cytoscape software v3.9 [16]. Finally, the Cytohubba plugin [17] of Cytoscape was used to calculate the degree of connectivity of nodes to find the top 20 DEGs as hub genes.

Figure 1. The workflow of the study.

3. Results

3.1. Microarray Data Processing, Integrative Meta-analysis, and Assessment of Data Quality

The boxplot of unprocessed raw data and normalized data after batch effect removal is shown in Figure 2. These boxplots demonstrate the accuracy of the normalization and quality of the expression data. A total of 220 samples are displayed in the PCA plot (Figure 3) on the 2D plane covered by their first two main components (PC1 and PC2). This figure shows the integration of two samples and the elimination of the batch effect.

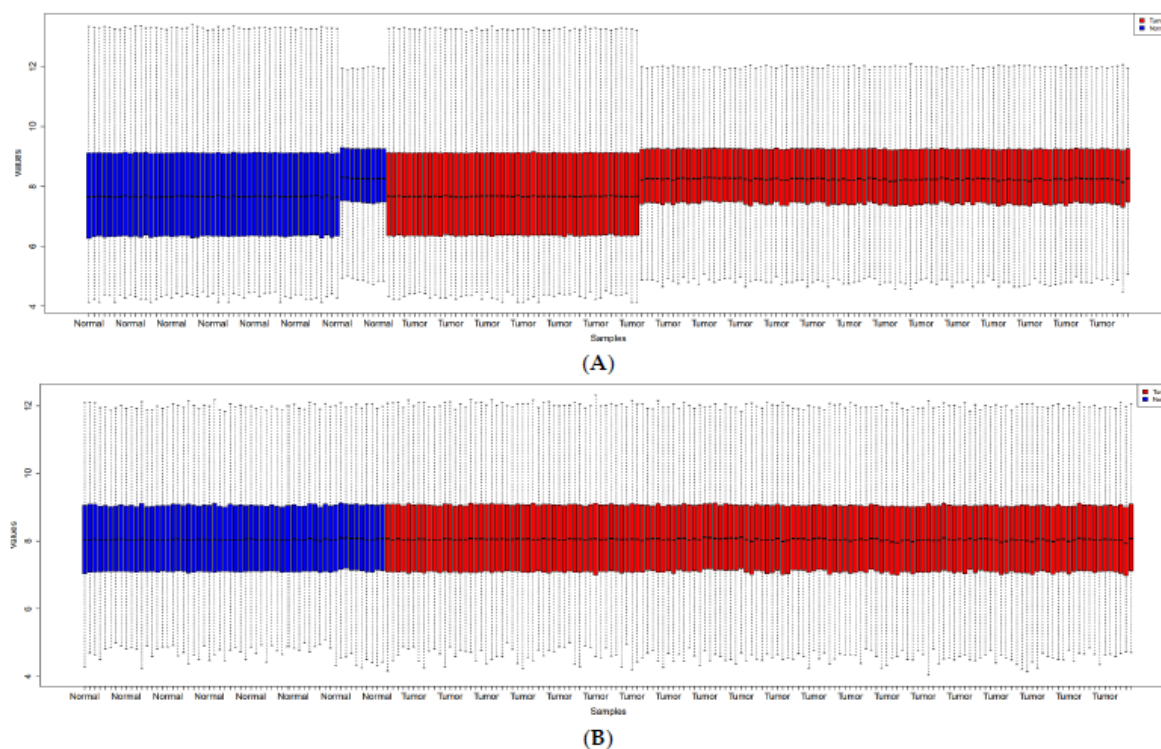


Figure 2. Boxplots show the raw data (A) and the normalized data following the removal of the batch effect (B). Prostate cancer samples are indicated by red boxes, whereas normal samples are shown by blue boxes.

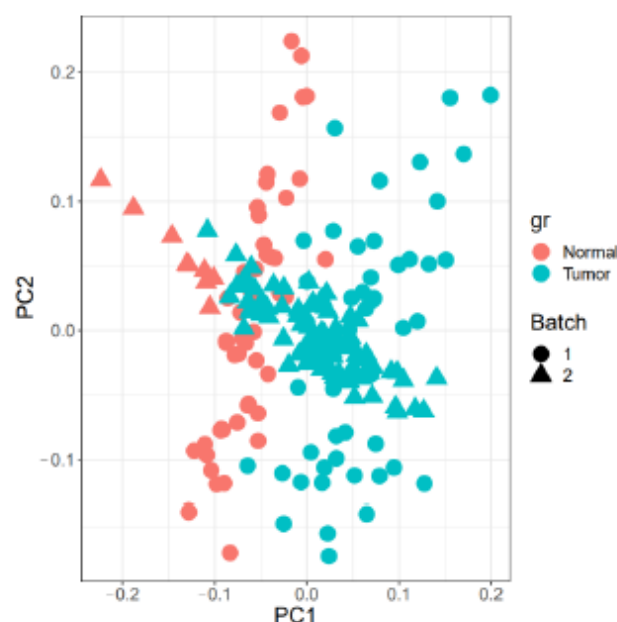


Figure 3. PCA plot. The batch implies that there are two platforms in the data. In addition, two groups of normal and tumor samples were created.

3.2. DEGs Analysis

The Limma package (version 3.52.3) was used to analyze microarray data from prostate cancer and normal samples. This analysis resulted in the identification of 684 DEmRNAs, including 437 downregulated DEmRNAs (such as TGM4 and SCGB1A1) and 241 upregulated DEmRNAs (such as TDRD1 and CRISP3); 6 DElncRNAs, including 1 downregulated DElncRNA (H19) and 5 upregulated DElncRNAs (such as PCA3 and PCGEM1); and 59 DEMiRNAs, including 30 downregulated DEMiRNAs (such as hsa-miR-1274a and hsa-miR-1274b) and 29 upregulated DEMiRNAs (such as hsa-miR-1268 and hsa-miR-1207-5p). Tables 2–4 present the most substantially up- and down-regulated DEmRNAs, DElncRNAs, and DEMiRNAs, respectively.

Table 2. The top 10 up- and down-regulated DEmRNAs between prostate and normal samples.

Down-Regulated			Up-Regulated		
DEmRNA	Log FC	Adjusted <i>p</i> Value	DEmRNA	Log FC	Adjusted <i>p</i> Value
TGM4	−1.81445890301	$1.88134991221065 \times 10^{-6}$	TDRD1	1.96438775509	$2.94051508709133 \times 10^{-13}$
SCGB1A1	−1.79767675328	$1.1360308699886 \times 10^{-12}$	CRISP3	1.87368847071	$3.24726996164822 \times 10^{-7}$
CYP3A5	−1.65579619243	$3.31725286897387 \times 10^{-17}$	SIM2	1.74655085898	$4.647251888494 \times 10^{-19}$
SLC14A1	−1.65191691277	$4.64209378540325 \times 10^{-18}$	OR51E2	1.70867540991	$6.13073882233394 \times 10^{-15}$
UPK1A	−1.63034519258	$7.18623407849526 \times 10^{-17}$	ERG	1.61588303371	$7.65718475641531 \times 10^{-9}$
DAPL1	−1.53220612346	$1.9314597808472 \times 10^{-11}$	LUZP2	1.56541422855	$2.35873230610011 \times 10^{-16}$
DUOXA1	−1.51899377232	$2.05445836986054 \times 10^{-26}$	OR51F2	1.42414934014	$7.53873927742971 \times 10^{-12}$
VSNL1	−1.47785936294	$1.15093949751989 \times 10^{-20}$	SERPINA3	1.36101547484	$5.22437085901616 \times 10^{-10}$
CD177	−1.45668628491	$6.69536496429305 \times 10^{-11}$	SLC45A2	1.32890185136	$6.82938830563377 \times 10^{-13}$
KRT15	−1.45323249293	$8.37913282536119 \times 10^{-13}$	DLX1	1.28252404614	$3.49922286189955 \times 10^{-14}$

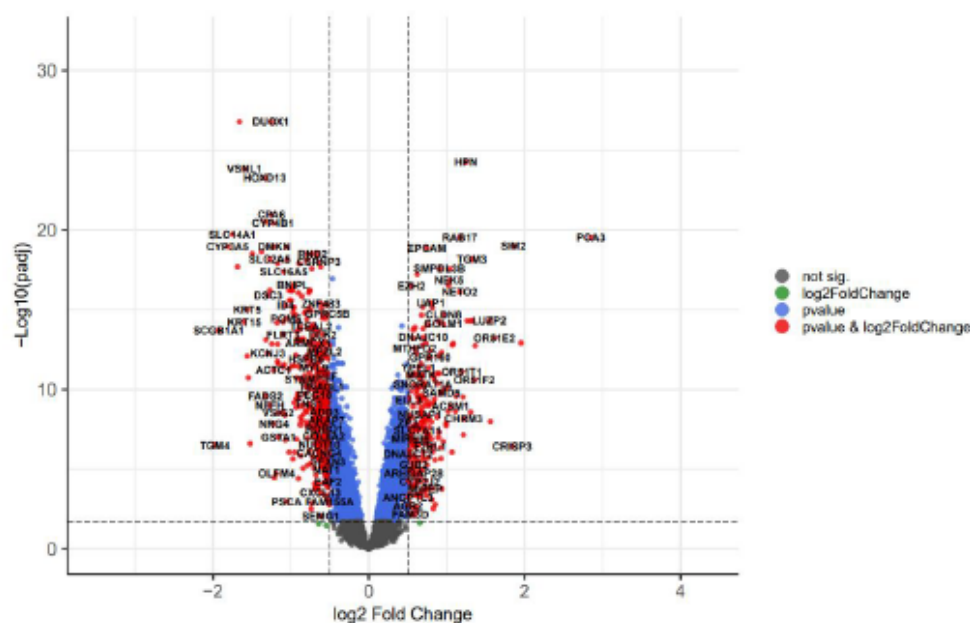
Table 3. The up- and down-regulated DElncRNAs between prostate and normal samples.

Down-Regulated			Up-Regulated		
DElncRNA	Log FC	Adjusted <i>p</i> Value	DElncRNA	Log FC	Adjusted <i>p</i> Value
H19	-1.0381434825	$4.13318956179463 \times 10^{-8}$	PCA3	3.0652524078	$2.06486869720981 \times 10^{-28}$
			PCGEM1	0.6952150040	0.0165229458698012
			KIAA0087	0.6870034897	$8.50318057764249 \times 10^{-6}$
			TERC	0.6575339188	$6.93708899641187 \times 10^{-7}$
			PART1	0.5929794137	$1.36877756290811 \times 10^{-5}$

Table 4. The top 10 up- and downregulated DE miRNAs between prostate and normal samples.

Down-Regulated			Up-Regulated		
DEmiRNA	Log FC	Adjusted <i>p</i> Value	DEmiRNA	Log FC	Adjusted <i>p</i> Value
hsa-miR-1274a	-1.6191209	1.72×10^{-10}	hsa-miR-1268	1.7455355	1.87×10^{-9}
hsa-miR-1274b	-1.5536019	1.38×10^{-8}	hsa-miR-1207-5p	1.3106131	1.96×10^{-6}
hsa-miR-1260	-1.3607157	1.32×10^{-11}	hsa-miR-205	1.0448365	0.0245
hsa-miR-21	-1.2456683	3.34×10^{-20}	hsa-miR-338-3p	1.0091872	2.56×10^{-7}
hsa-miR-1308	-1.2394549	8.32×10^{-8}	hsa-miR-638	1.0089855	1.33×10^{-5}
hsa-miR-142-3p	-1.1264728	3.62×10^{-7}	hsa-miR-134	0.917789	6.14×10^{-6}
hsa-miR-720	-1.0837599	8.61×10^{-7}	hsa-miR-320d	0.905801	4.53×10^{-15}
hsa-miR-146b-5p	-1.0641932	3.47×10^{-7}	hsa-miR-197	0.8431693	4.21×10^{-18}
hsa-miR-30b	-1.0514609	1.03×10^{-14}	hsa-miR-149	0.8239717	1.47×10^{-6}
hsa-miR-150	-0.8801092	1.64×10^{-5}	hsa-miR-214	0.7540948	4.05×10^{-7}

In order to compare the variation in miRNA, lncRNA, and mRNA expressions between prostate cancer and normal samples, we utilized the volcano plot using the EnhancedVolcano package [24] in R (Figure 4). In addition, two heatmaps demonstrated that 20 clearly distinct DEmRNA expression patterns between prostate and normal samples were identifiable (Figure 5A). The expression of DElncRNAs is also shown in a heatmap (Figure 5B).



total = 10941 variables

(A)

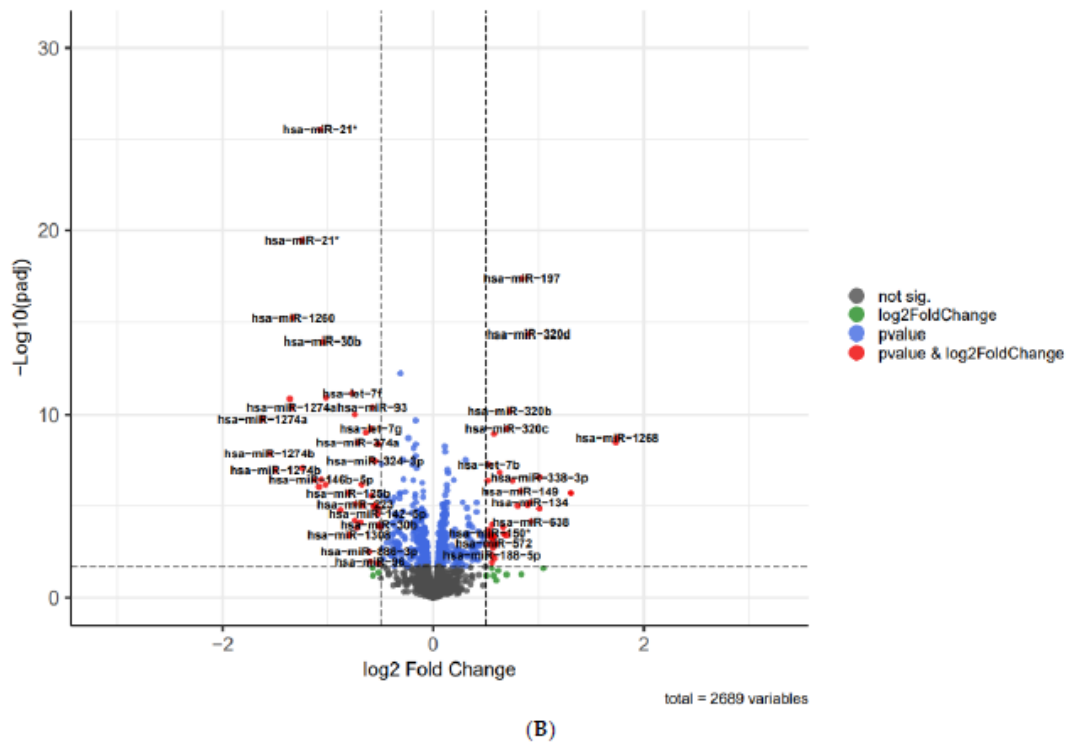
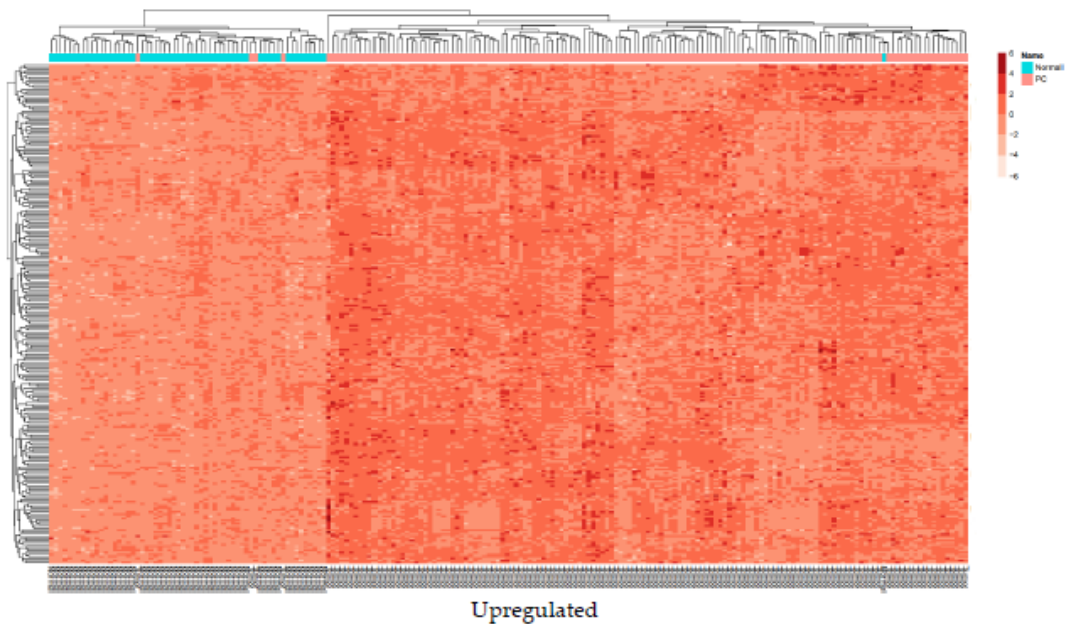


Figure 4. The volcano plot of DElncRNAs, mRNAs DEmRNAs (A) and DEmiRNAs (B); horizontal axis, $\log_2(FC)$; vertical axis, $-\log_{10}(\text{adjusted } p \text{ value})$.



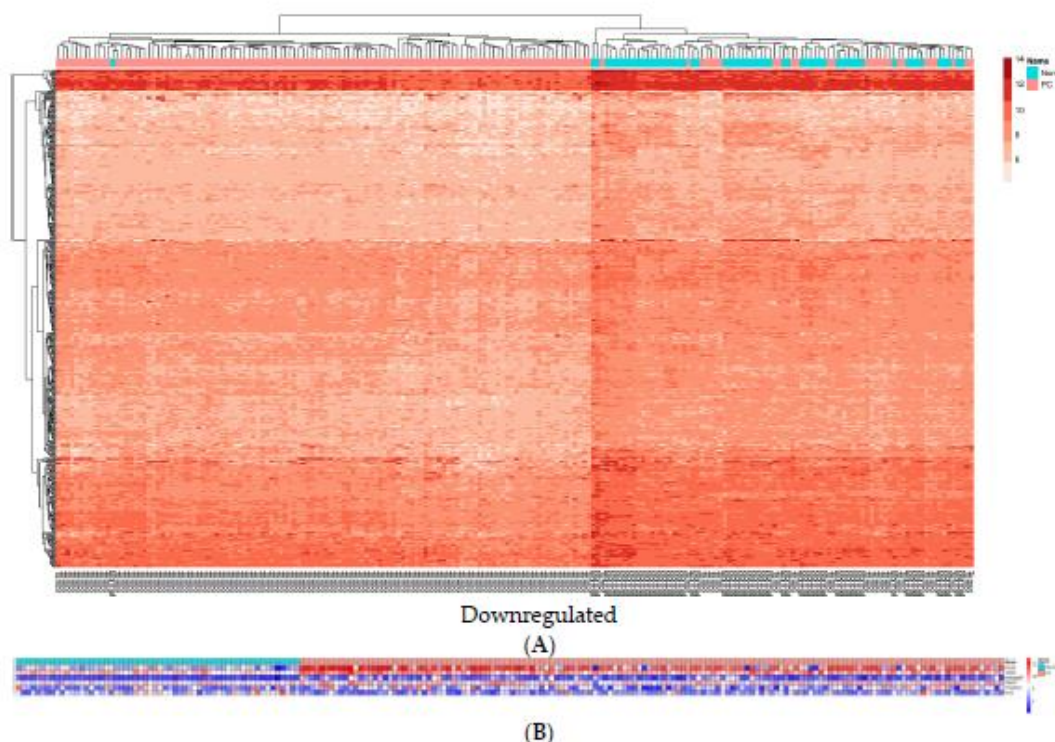


Figure 5. (A) Two heatmaps of DEmRNAs between prostate samples and normal samples; horizontal axis, the samples; vertical axis, DEmRNAs. (B) The heatmap depicts the expression of DEmcRNAs.

3.3. TCGA Data Analysis

All available TCGA data on PRAD were obtained from the TCGA data portal using TCGAbiolinks package in R programming language. In September 2022, there were RNAseq data on 552 PRAD samples, including 500 primary solid tumor and 52 solid tissue normal samples. We analyzed this data using limma and edgeR packages to retrieve DEGs. DEGs were evaluated with the cut-off criteria of false discovery rate (FDR; adjusted p value) < 0.05 and $|\log_2$ fold Change (FC)| > 0.5 . Finally, we identified the genes that exist in both GEO datasets and the TCGA dataset (Figure 6). As a result, we found out that there were 193 upregulated and 416 downregulated DEGs in both GEO datasets and the TCGA dataset. We continued the analysis with these genes.

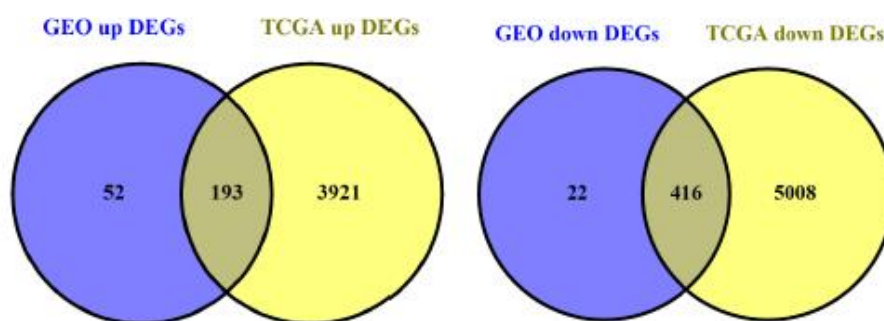


Figure 6. Venn diagram. The number of hub genes in PPI and ceRNA networks and TCGA-PRAD DEGs.

3.4. GO Enrichment Analysis of DEGs

For the analysis, the clusterProfiler package (version 4.4.4) was employed. In GO functional enrichment analysis, 604 GO entries reached an adjusted p value of less than 0.05, most of which were biological processes (BP), followed by cellular component (CC) and molecular function (MF). The first 30 entries are collagen-containing extracellular matrix (CC), sarcolemma (CC), cell-substrate junction (CC), focal adhesion (CC), basement membrane (CC), extracellular matrix binding (MF), muscle system process (BP), muscle contraction (BP), muscle tissue development (BP), urogenital system development (BP), ear development (BP), membrane raft (CC), membrane microdomain (CC), gland development (BP), muscle cell differentiation (BP), contractile fiber (CC), regulation of epithelial cell proliferation (BP), cell-substrate adhesion (BP), epithelial cell proliferation (CC), response to xenobiotic stimulus (BP), mesenchyme development (BP), regulation of smooth muscle cell proliferation (BP), smooth muscle cell proliferation (BP), laminin binding (MF), cell junction assembly (BP), extracellular matrix structural constituent (MF), collagen binding (MF), inner ear development (BP), mesenchymal cell differentiation (BP), and muscle organ development (BP). Figure 7 shows the bar plots of the top 10 enriched functions.

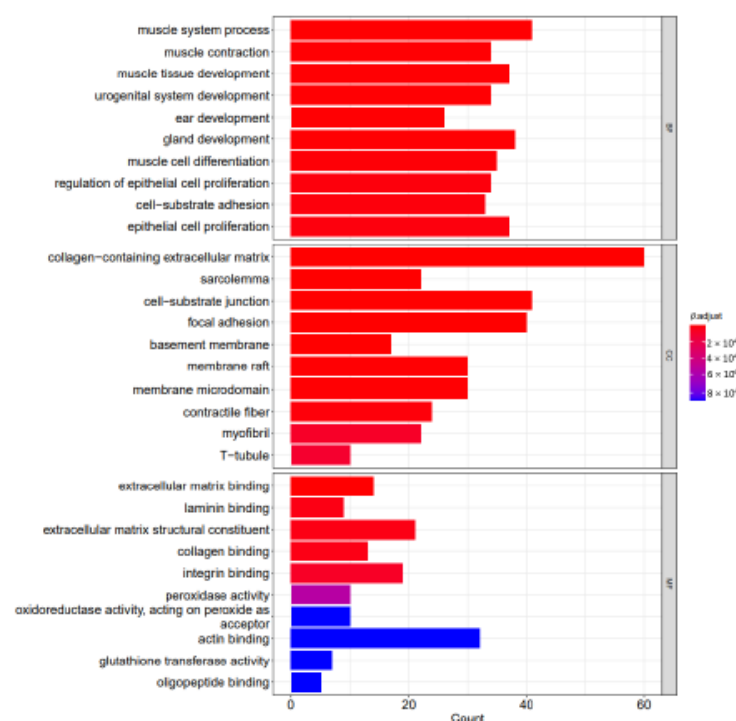


Figure 7. The bar plots of the top 10 enriched functions include BP (biological process), CC (cellular component), and MF (molecular function). The gene-set count is shown on the X axis, while the gene-set function is shown on the Y axis; the bar color, which ranges from red (most significant) to blue (least significant), indicates the adjusted p value.

The dot plot of the top 10 enriched functions and the enriched GO-induced graph are, respectively, visualized in Figures 8 and 9.

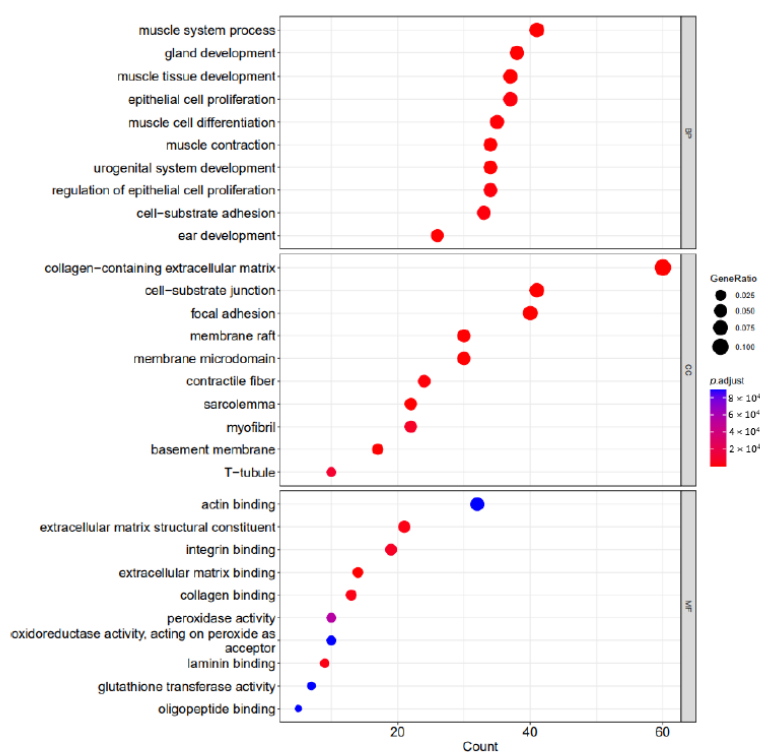
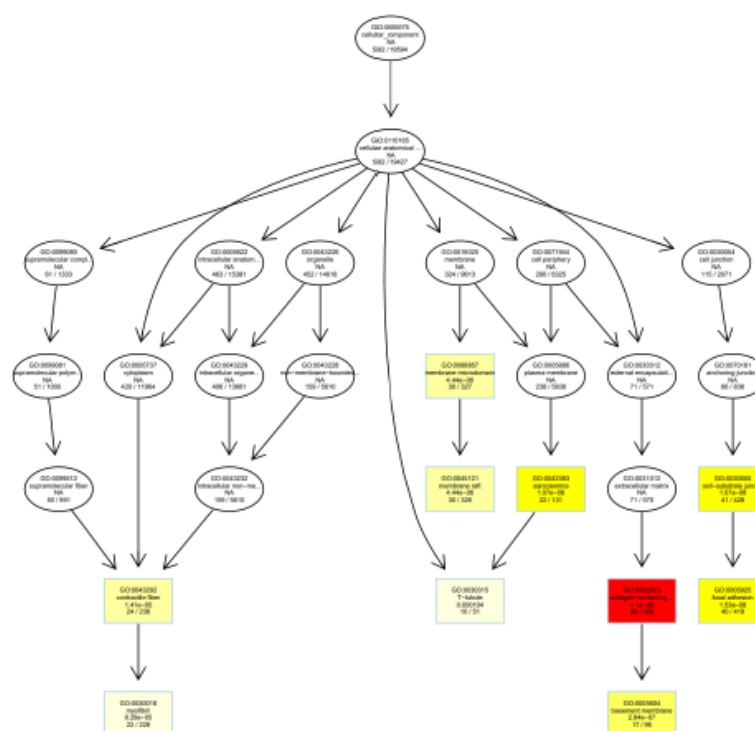
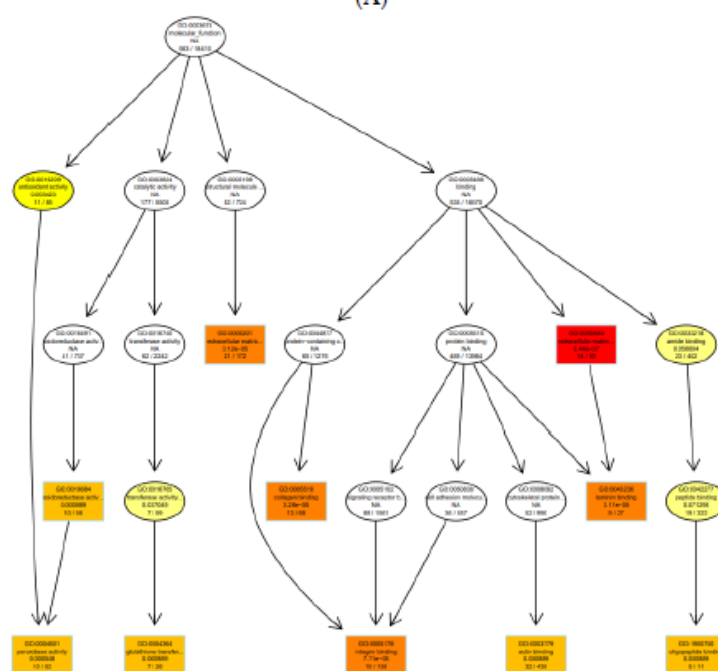


Figure 8. The dot plots of top 10 enriched functions. The gene-set count is shown on the X axis, while the gene-set function is shown on the Y axis. The color of the dot reflects the adjusted p value, and ranges from dark blue (most significant) to red (least significant). GeneRatio is represented by the dot size.



(A)



(B)

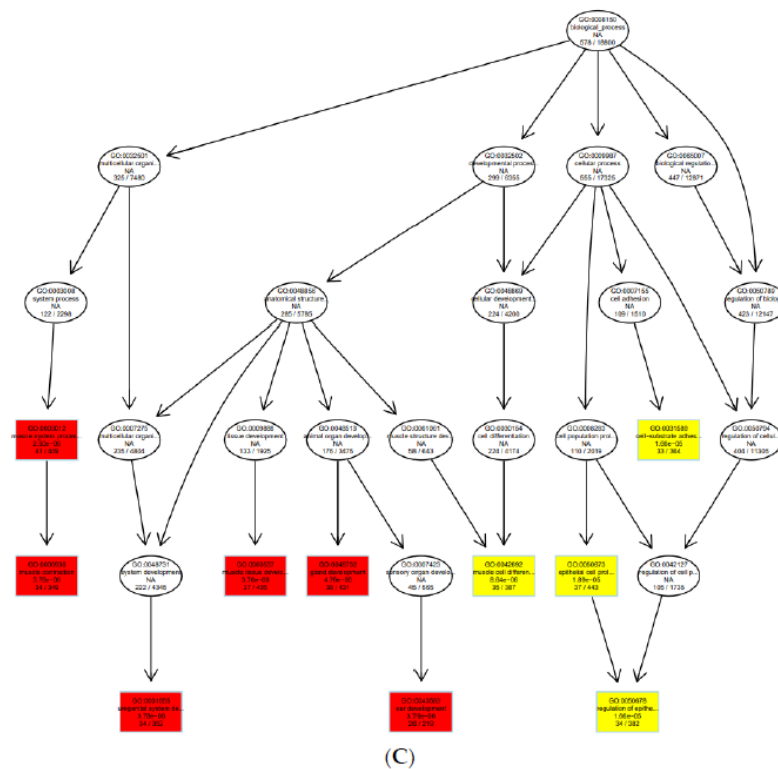


Figure 9. GO graph visualization of the top GO terms enriched. (A) A GO sub-graph has been created using the top 30 GO keywords in the category “Cellular Component”. (B) A GO sub-graph has been created using the top 30 GO keywords in the category “Molecular Function”. (C) A GO sub-graph has been created using the top 30 GO keywords in the category “Biological Process”. Boxes indicate the most significant terms. From dark red (most significant) to light yellow (least significant), the color of the box indicates the relative significance.

Figure 10 indicates a network of GO terms and Figure 11 shows the gene-concept network of the top five GO terms.

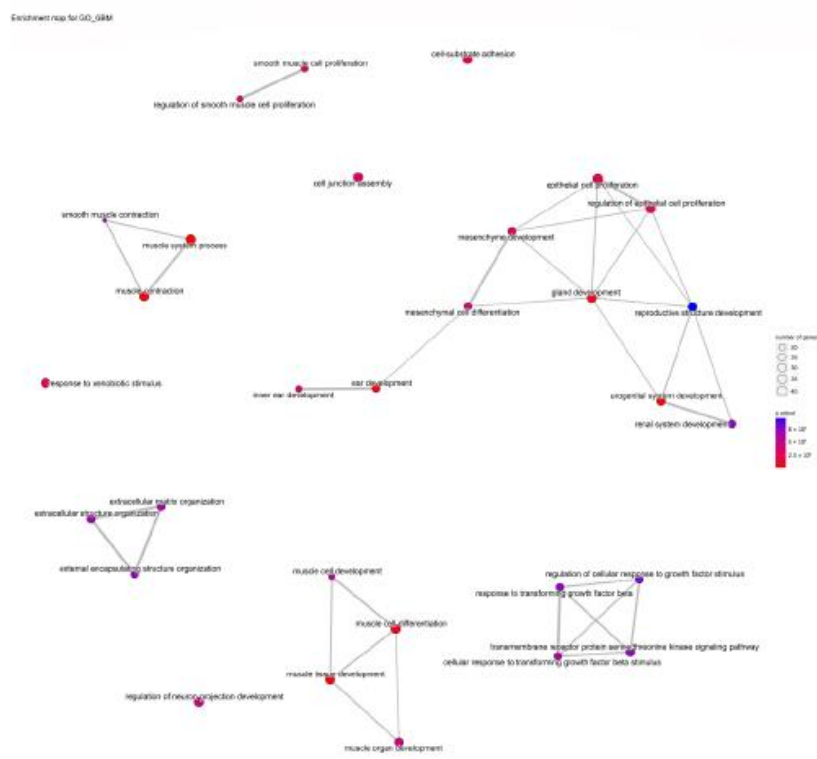


Figure 10. A network of GO terms.

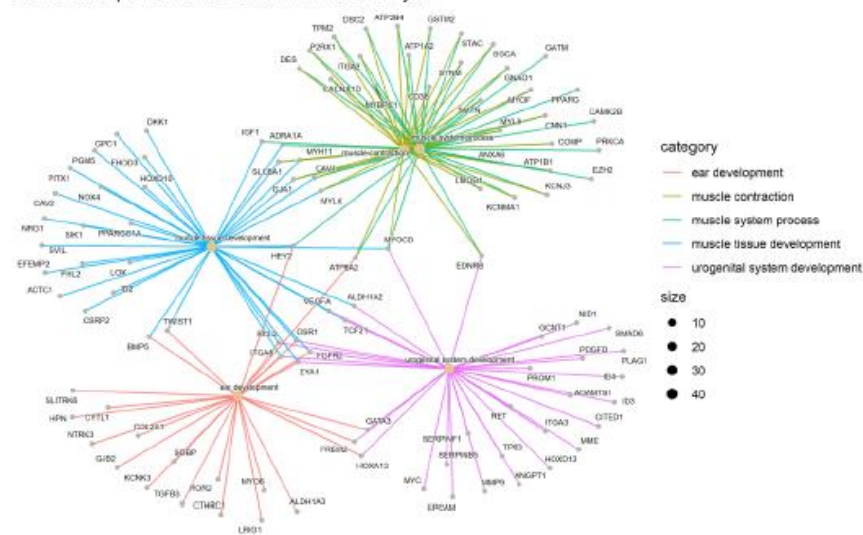
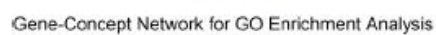


Figure 11. Top 5 GO terms as a network plot. These GO terms are connected to genes in this graph. The connection of genes to the corresponding GO is marked with a special color; there are more genes for a specific GO term if the dot relating to it is bigger.

In Figure 12, the intersection of the top 10 GO phrases was represented by the UpSet plot. It highlights the gene overlap between several gene sets.

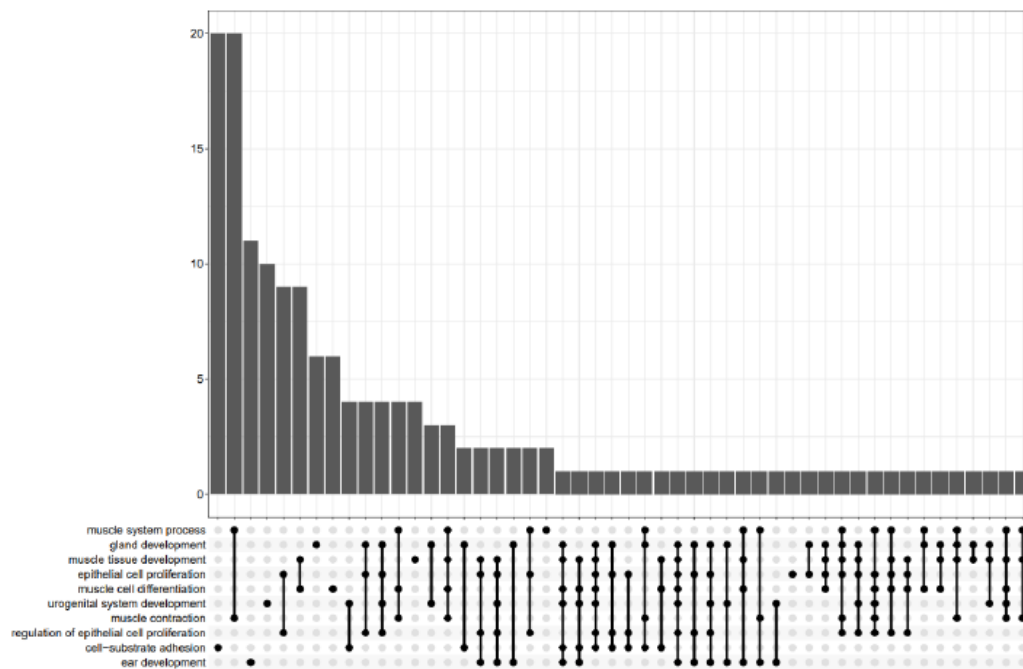


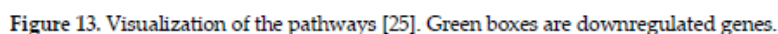
Figure 12. Upset plot of 10 GO terms.

3.5. Pathway Analysis

Using Pathview [25] and gage [26] packages in R, to find the probable functional genes, the KEGG pathway analysis of 241 upregulated and 437 downregulated DEGs was carried out (Table 5 and Figure 13).

Table 5. Down-regulated and up-regulated pathways.

Down-Regulated		Up-Regulated	
Pathway	p Value	Pathway	p Value
Glutathione metabolism	0.009743969		
Regulation of actin cytoskeleton	0.041300676		



3.6. Identification of Genes Related to Cell Senescence

We used the CellAge database (<https://genomics.senescence.info/cells/>) (accessed on 24 September 2022) to find the genes associated with cell senescence. A total of 279 genes associated with cell senescence were found in this database. As a result, 21 DEGs, including TP63, ID4, ITGB4, CAV1, ID1, TLR3, TGFB1I1, MYLK, NTN4, ETS2, LGALS3, GNG11, IGFBP3, SIK1, VEGFA, EZH2, MATK, HJURP, NOX4, MYC, and MMP9 were discovered to be involved in cell senescence (Figure 14).

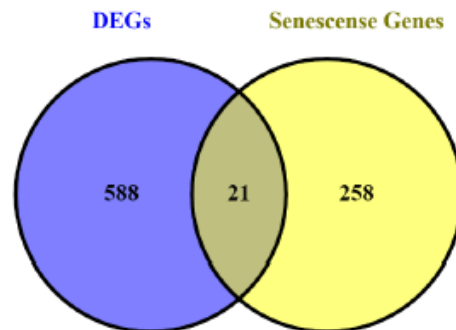
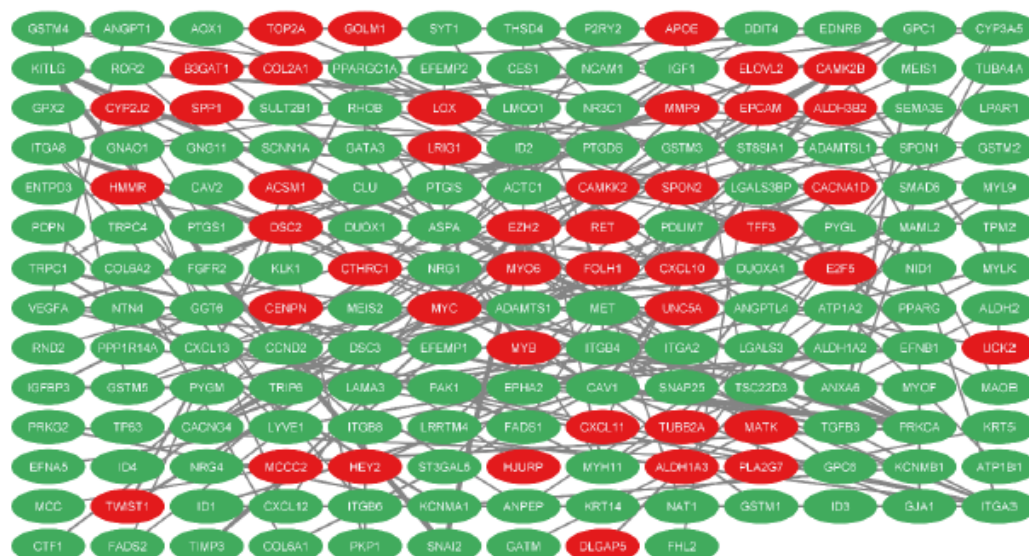


Figure 14. A total of 21 DEGs are related to cell senescence.

3.7. PPI Network Construction and Selection of Hub Genes

To find the hub genes, a PPI network of DEGs (Figure 15) with 178 nodes and 185 edges created from STRING was imported into the Cytohubba plugin of Cytoscape 3.9. ITGA2, ITGA3, CAV1, PRKCA, ITGB4, ITGA8, MET, VEGFA, GPC1, MMP9, CAMK2B, LAMA3, PAK1, MYH11, and ITGB6 were the 15 hub genes with the highest degree of connectivity. Table 6 shows information about these hub genes. The greatest degree to the lowest degree is used to order these hubs.



(A)

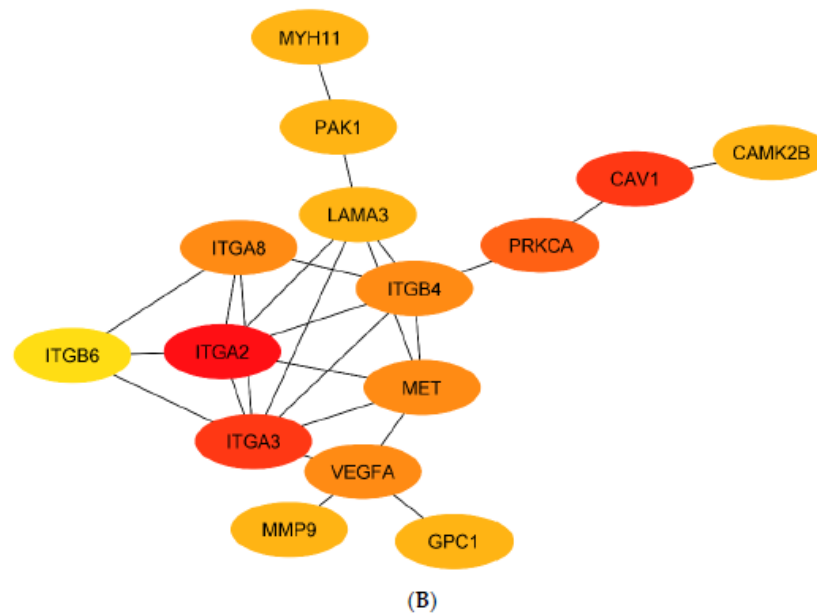


Figure 15. PPI network of DE mRNAs. (A) Total PPI network, (B) subnetwork of hub genes.

Table 6. The information of hub genes in PPI network.

Hub Gene	Adjusted <i>p</i> Value	Log2FC	Degree	Closeness Centrality	Betweenness Centrality
ITGA2	$6.0533584885592 \times 10^{-8}$	-0.5208186242	10	0.23939393939393938	0.09017016738535721
CAV1	$8.91282635281604 \times 10^{-13}$	-0.8409077603	9	0.29924242424242425	0.4624758504505339
ITGA3	$7.60154283187703 \times 10^{-10}$	-0.5037277367	9	0.24687499999999998	0.06612958068654272
PRKCA	$1.16610481754651 \times 10^{-8}$	-0.5112308295	7	0.3038461538461538	0.407488292298419
ITGB4	$7.82620045760532 \times 10^{-13}$	-0.7272225278	6	0.27430555555555556	0.16944869476515048
VEGFA	$1.39662632691052 \times 10^{-5}$	-0.6752232048	6	0.2532051282051282	0.1266603298248868
ITGA8	$8.22863554284887 \times 10^{-6}$	-0.5563216624	6	0.23099415204678364	0.057575617069287974
MET	$2.90476617068553 \times 10^{-11}$	-0.6406154443	6	0.2438271604938272	0.03557286595261279
MMP9	0.00114089353601013	0.5468156204	5	0.26158940397350994	0.16307398649170796
MYH11	$9.37807316500015 \times 10^{-5}$	-0.5708966261	5	0.20954907161803715	0.037650113599480686
GPC1	$8.55395903416247 \times 10^{-9}$	-0.5393742260	5	0.2438271604938272	0.11728875906091096
CAMK2B	$1.18776167986803 \times 10^{-7}$	0.6565135653	5	0.2507936507936508	0.2030725954776588
LAMA3	$6.50066323396685 \times 10^{-5}$	-0.5027987850	5	0.24687499999999998	0.05840017928625522
PAK1	$4.26292437544967 \times 10^{-8}$	-0.5319966868	5	0.24085365853658536	0.09575277043631478
ITGB6	$5.08198308078253 \times 10^{-13}$	-1.0073168769	4	0.20256410256410257	0.0

3.8. Inspection of the Regulatory Network of miRNA-Hub Genes

The miRNAs that target hub genes were collected from the NetworkAnalyst web database (Figure 16). Both Hsa-miR-26b-5p and Hsa-miR-1-3p were considered important miRNAs because they interacted with hub genes at the highest level (degree 5) possible.

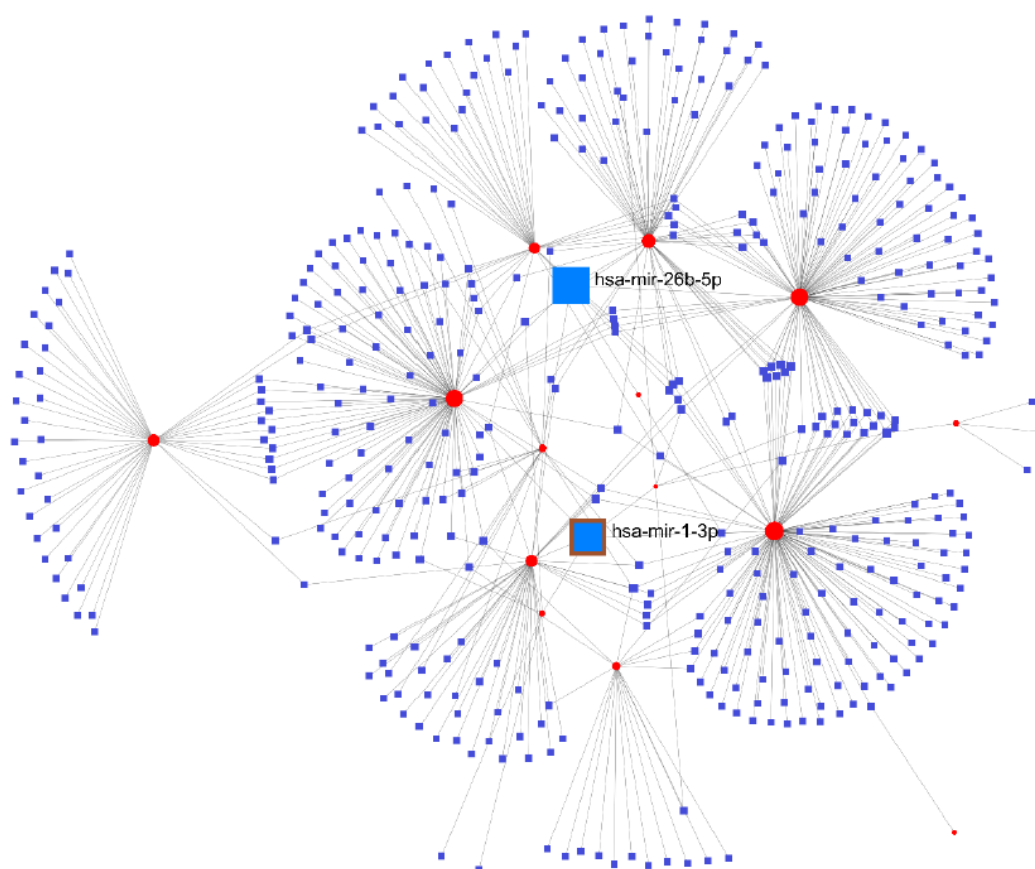


Figure 16. The network of miRNA-hub genes. Circles represent the hub gene, while the squares represent miRNAs targeting the hub genes.

3.9. Examination of the Regulatory Network of TF-Hub Genes

By using the NetworkAnalyst database, we were able to acquire TFs that target hub genes (Figure 17). The TF-hub gene network revealed that SUZ12 regulates 12 hub genes and may play a significant role in the development of prostate cancer.

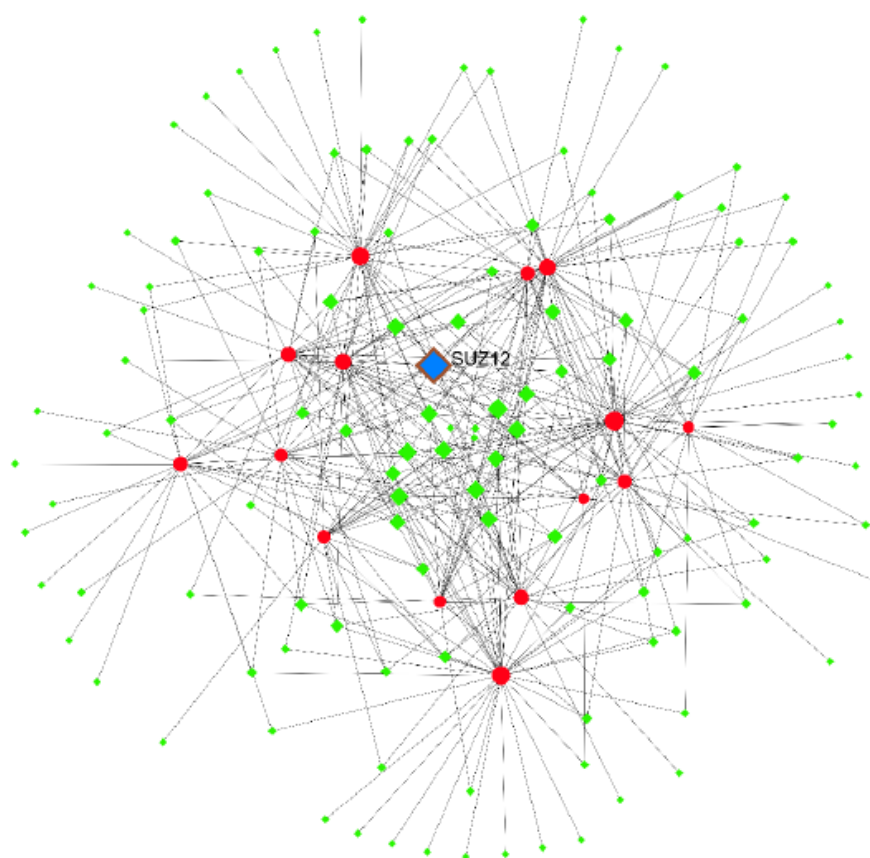


Figure 17. The network of TF-hub genes. Circles show the hub gene, while the diamonds represent TFs targeting the hub genes.

3.10. ceRNA Network Construction in Prostate Cancer

The interaction between lncRNAs and miRNAs was evaluated using miRcode. This step showed that 5 of the 6 lncRNAs may target 20 of the 59 PC-specific DE miRNAs (Table 7). Then, to determine which mRNAs were targeted by these 20 miRNAs, we utilized miRWalk in combination with the miRTarBase, TargetScan, and miRDB filters. According to the research, 5 of the 684 mRNAs may be targeted by 5 miRNAs (Table 8). If miRNA-targeted mRNAs were not found in DE mRNAs, they were eliminated. The information from Tables 7 and 8 was utilized to construct the lncRNA–miRNA–mRNA ceRNA network in Cytoscape 3.9. The ceRNA network contained a total of 5 miRNAs, 5 lncRNAs, and 17 mRNAs (Figure 18). We displayed this ceRNA network using a Sankey diagram generated by the ggalluvial R package (Version: 0.12.3) [27] in order to better understand the impact of lncRNAs on mRNAs in prostate, of which is mediated by their interaction with miRNAs (Figure 19). Finally, we determined the nodes' degrees using the cytohubba app, and we displayed the top 10 nodes in the network with the highest degree centrality (Figure 20). As 10 hub genes in the ceRNA network, we identified hsa-miR-17, hsa-miR-93, hsa-miR-150, hsa-miR-25, PART1, hsa-miR-125b, PCA3, H19, RND3, and ITGB8.

Table 7. The MiRcode database showed interactions between 5 DElncRNAs and 5 DEMiRNAs.

lncRNA	miRNA
PCA3, PART1, KIAA0087, PCGEM1	hsa-miR-150
PCA3	hsa-miR-425
PCA3, KIAA0087, PART1	hsa-miR-199a-5p
PCA3, H19	hsa-miR-17
PCA3, PART1	hsa-miR-143
PART1	hsa-miR-25
PART1	hsa-miR-125b
H19, PCA3	hsa-miR-93
PCA3, KIAA0087	hsa-miR-96
PCA3, KIAA0087, PART1	hsa-miR-214
H19, PCA3	hsa-miR-22
KIAA0087, PART1	hsa-miR-30b
KIAA0087, PCGEM1	hsa-miR-375
KIAA0087, H19	hsa-miR-338-3p
KIAA0087, PART1	hsa-miR-10a
KIAA0087, PART1	hsa-miR-133b
PART1	hsa-miR-25
PART1	hsa-miR-142-3p
PART1	hsa-miR-30c
PART1, PCGEM1	hsa-miR-205

Table 8. miRWalk (miRTarBase, TargetScan and miRDB filters) database showed interactions between 5 DEMiRNAs and 17 DEMRNAs.

miRNA	mRNA
hsa-miR-150	PRKCA, MYB
hsa-miR-17	ITGB8, E2F5, ELAVL2, PLAG1, RND3, NETO2
hsa-miR-25	DSC2, ROBO1, GALNT7, EZH2
hsa-miR-125b	BCL2
hsa-miR-93	ITGB8, PRRG4, RND3, FRMD6, SIK1, NETO2, SMAD6

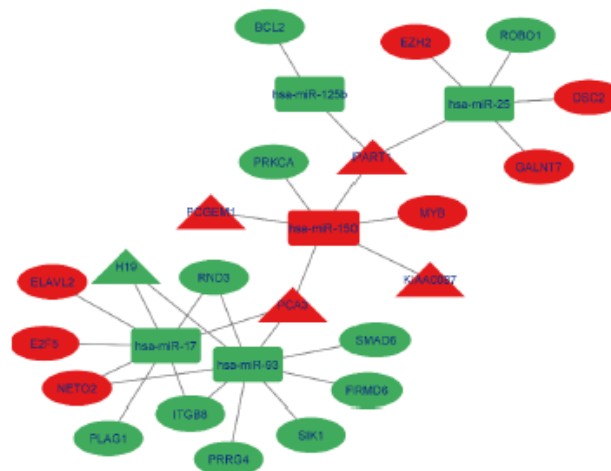


Figure 18. Red nodes signify a strong expression level, while green nodes signify a low level of expression. Ellipses signify protein-coding genes; rectangles signify miRNAs; triangles represent lncRNAs; gray edges specify lncRNA-miRNA-mRNA interactions.

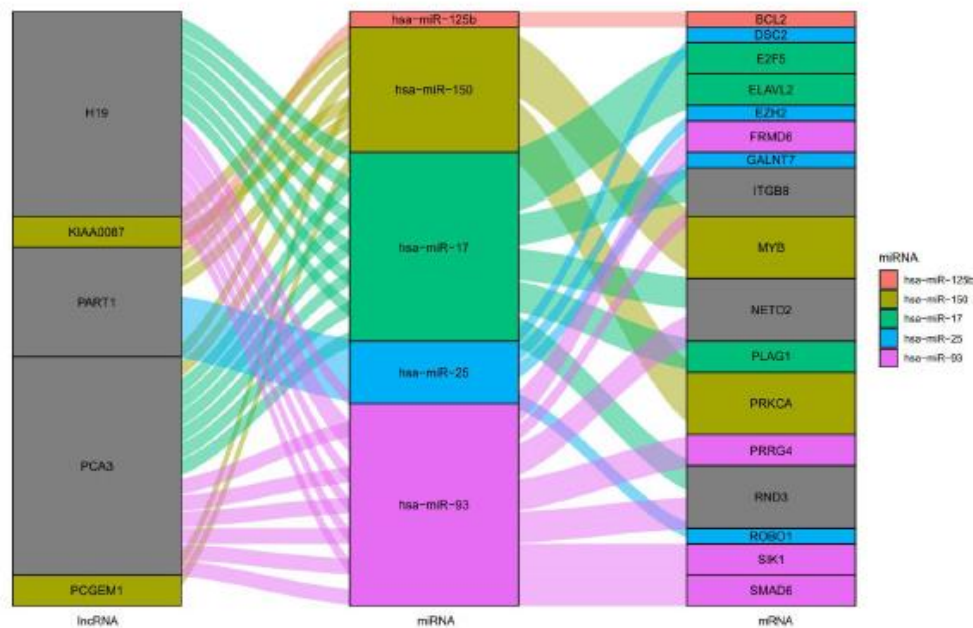


Figure 19. The ceRNA network in prostate cancer is shown by a Sankey diagram. Each rectangle represents a gene, and depending on the size of the rectangle, the degree of relationship between each gene is shown.

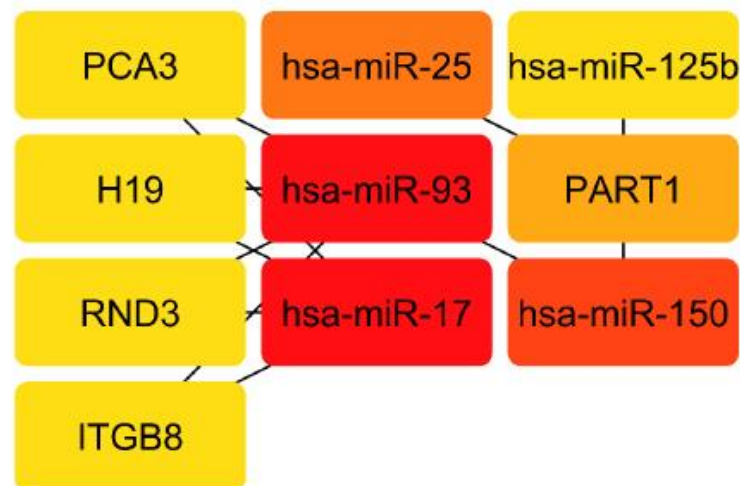
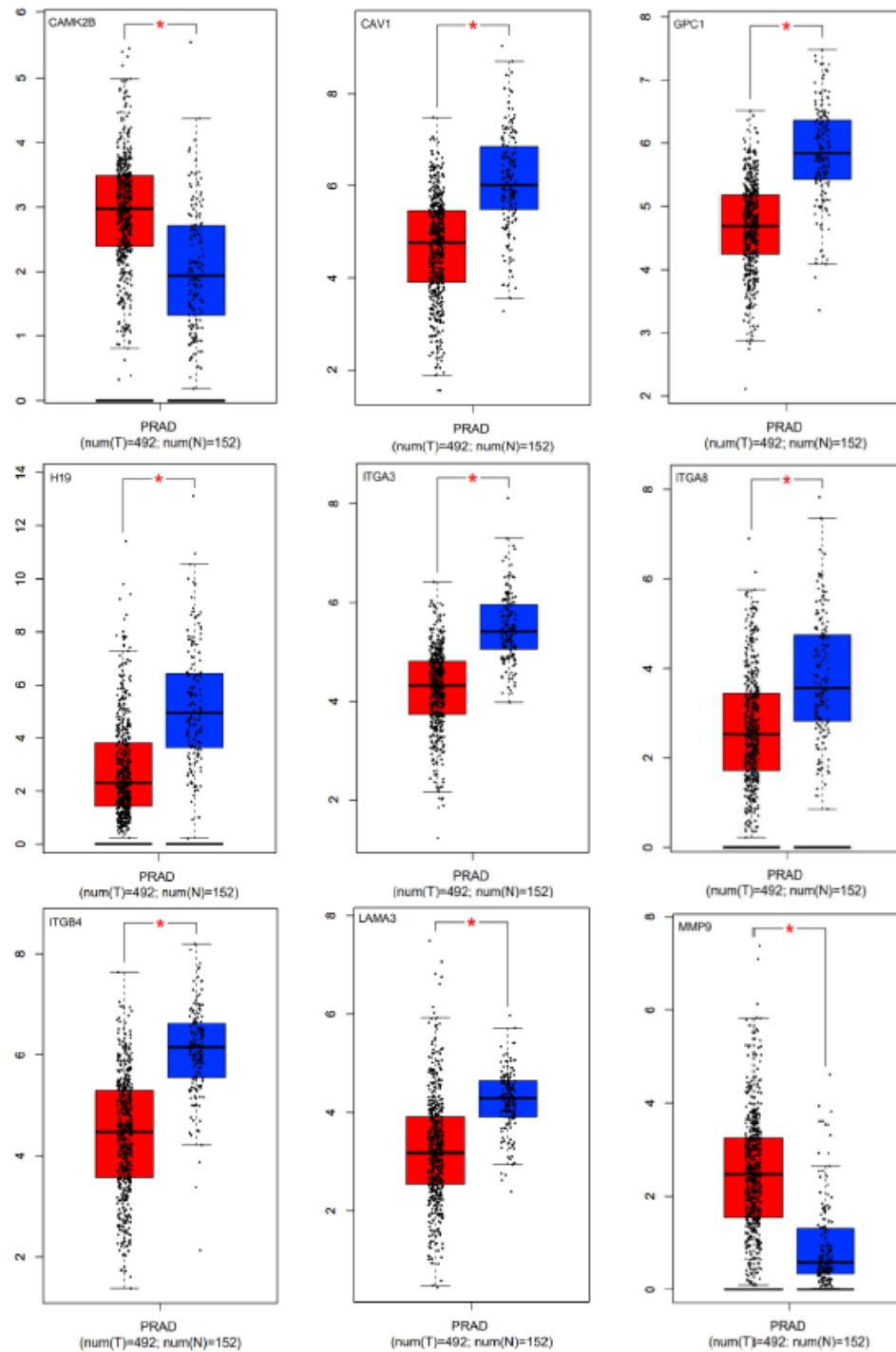


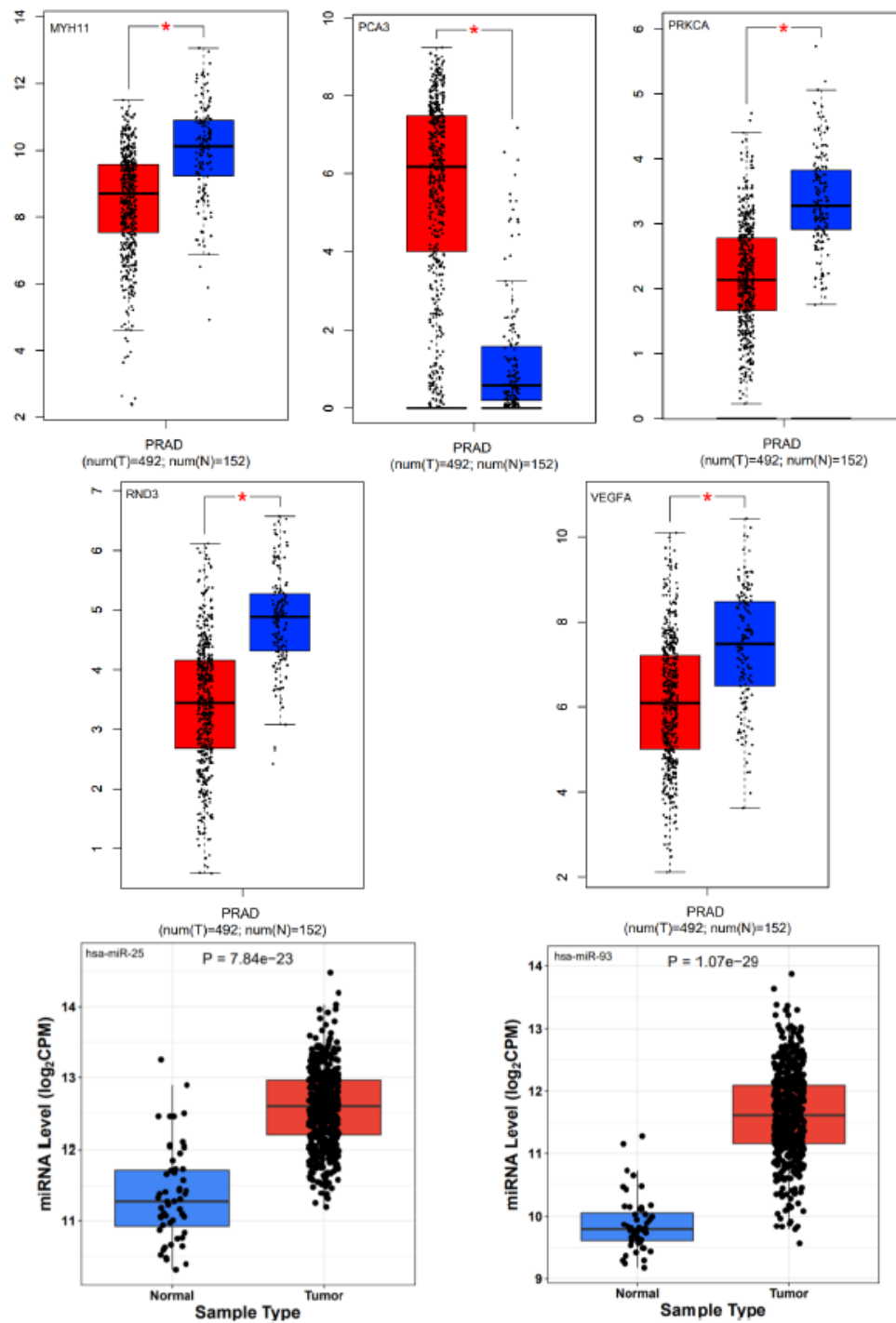
Figure 20. Top 10 genes with highest closeness centrality in ceRNA network.

3.11. Validation of Hub Genes via Expression Value

The expression value of hub genes was assessed using the Gepia (<http://gepia.cancer-pku.cn/>). Additionally, we used CancerMIRNome (<http://bioinfo.jialab-ucr.org/CancerMIRNome/>) to evaluate the hub miRNA gene expression value. As a result, CAMK2B, CAV1, GPC1, H19, ITGA3, ITGA8, ITGB4, LAMA3, MMP9, MYH11, PCA3, PRKCA,

RND3, VEGFA, hsa-miR-25, hsa-miR-93, hsa-miR-125b, and hsa-miR-17 indicated good statistical significance (Figure 21).





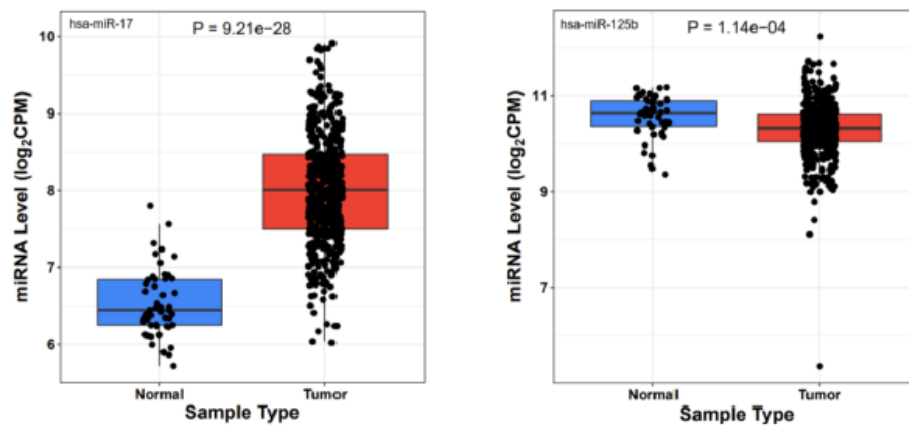
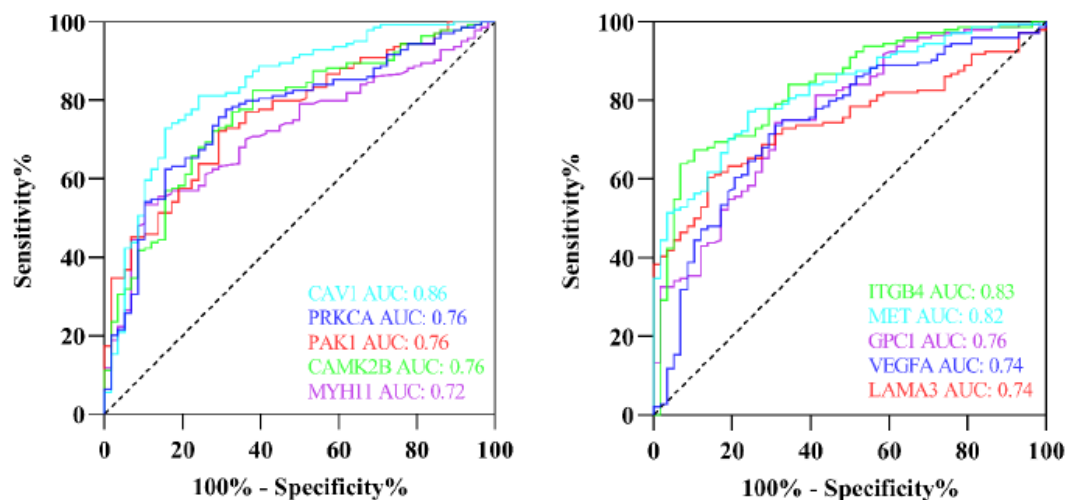


Figure 21. Box plots of the gene expression of hub genes in prostate and normal samples based on TCGA. Red and blue boxes show the gene expression of hub genes in prostate and normal samples, respectively. The red star in some boxplots means that the expression difference between the samples is significant.

3.12. Validation of Hub Genes via ROC Curve

We used graphpad prism 9.0 and CancerMIRNome to construct ROC curves. The ROC curve was used to evaluate how accurately the hub genes predicted outcomes. AUC was used to compare the diagnostic values of these hub genes. ROC curves and AUC values of the dataset are shown in Figure 22. The computed AUC values in this study, which were based on the findings, varied from 0.7 to 1—this is considered to have high discriminative power. The expression levels of 22 hub genes had a high diagnostic value, according to the ROC analysis.



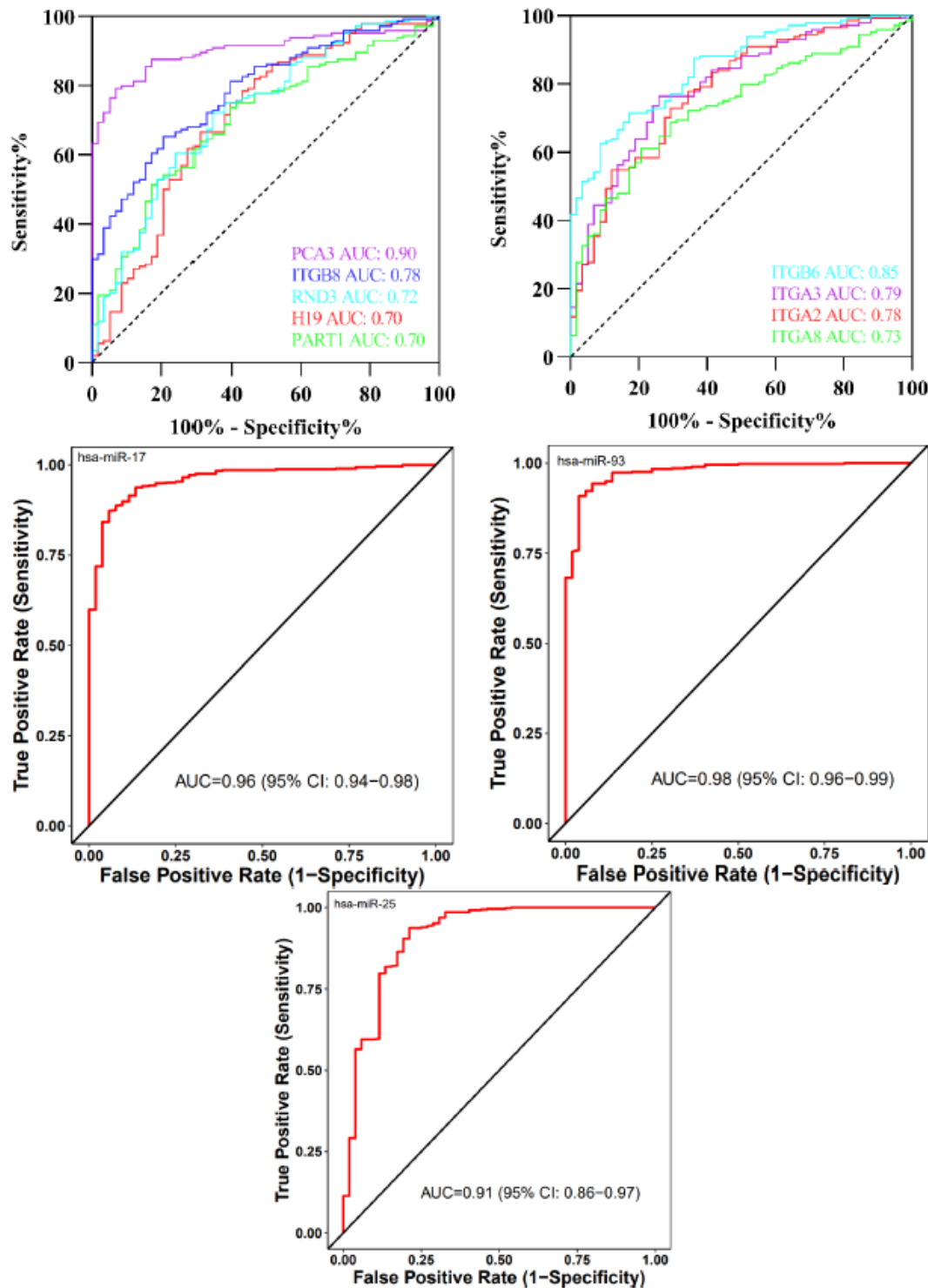


Figure 22. Appraisal of the hub genes. ROC curves for 22 hub genes. The calculated AUC area under the curve for dataset ranged from 0.7 to 1.

4. Discussion

In order to find novel biomarkers for prostate cancer, we used a bioinformatics approach and constructed the ceRNA network in this context. We also valued the importance of the identified hub genes in the pathogenesis of prostate cancer and found their association with signaling pathways.

In the first step, we identified several DEGs. *TGM4* and *SCGB1A1* have been among down-regulated mRNAs in this type of cancer. *TGM4* gene codes for transglutaminase 4, a protein with a restricted pattern of expression toward prostate. The encoded protein has been shown to regulate the interactions between prostate cancer cells and vascular endothelial cells through bypassing the ROCK pathway [28]. Moreover, *TDRD1* and *CRISP3* have been among upregulated mRNAs. *TDRD1* is responsible for coding a protein containing a tumor domain that suppresses transposable elements during spermatogenesis. The encoded protein has been shown to be expressed in most human prostate tumors, in spite of its absence in normal prostate tissues [29]. *CRISP3* is found in low quantities in seminal plasma. The over-expression of *CRISP3* in addition to the down-regulation of *PTEN* illustrates a subgroup of prostate cancer patients with a high probability of biochemical recurrence [30]. We also reported the down-regulation of *H19* and up-regulation of a number of lncRNAs, such as *PCA3* and *PCGEM1*. Notably, *PCA3* is probably the most important lncRNA biomarker for prostate cancer [31].

Key factors known to be involved in prostate cancer tumorigenesis were identified with this approach. However, there are limitations. Importantly, the bioinformatic prediction is strongly dependent on the patient-derived data sets and number of data sets. We have used here a large number of data sets and from different original sources to minimize off-target findings. Still, it may be possible that using other patient-derived data sets will provide results that do not show a complete overlap of the ceRNA network.

KEGG pathway analysis has revealed glutathione metabolism and the regulation of actin cytoskeleton as the mostly down-regulated pathways. DE miRNAs have also been reported to participate in a number of critical signaling pathways that affect prostate carcinogenesis. The ceRNA network contained a total of 5 miRNAs, 5 lncRNAs, and 17 mRNAs. We identified *hsa-miR-17*, *hsa-miR-93*, *hsa-miR-150*, *hsa-miR-25*, *PART1*, *hsa-miR-125b*, *PCA3*, *H19*, *RND3*, and *ITGB8* as the 10 hub genes in the ceRNA network. According to the ROC analysis, the expression levels of 19 hub genes showed a high diagnostic value. Therefore, the constructed ceRNA network has been shown to affect important cellular pathways in prostate carcinogenesis and influence the prognosis of patients with this type of cancer. Taken together, we introduce a number of novel, promising diagnostic biomarkers for prostate cancer. This ceRNA-based panel can be applied as a second-level test to patients with certain PSA levels. This level should be identified in future studies.

Author Contributions: M.T. and S.G.-F. wrote the manuscript and revised it. A.B. supervised and designed the study. A.S., B.M.H. and M.T. collected the data and performed the bioinformatic analysis. All authors read and approved the submitted version. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

PC	Prostate Cancer
lncRNA	Long non-coding RNA
miRNA	MicroRNA
mRNA	Messenger RNA
ceRNA	Competitive Endogenous RNA
DEG	Differentially Expressed Genes
GEO	Gene Expression Omnibus
SVA	Surrogate Variable Analysis
PCA	Principal Component Analysis
Limma	Linear Models for Microarray Data
FDR	False Discovery Rate
Log2FC	Log2 Fold Change
HGNC	HUGO gene nomenclature
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
PPI	Protein-protein Interaction
TF	Transcription Factor
TCGA	The Cancer Genome Atlas
PRAD	Prostate Adenocarcinoma
BP	Biological Process
MF	Molecular Function
CC	Cellular Component
ROC	Receiver Operating Characteristic
AUC	Area Under the ROC Curve

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3.2 Manuscript II

UNCLASSIFIED

An in-silico Approach for Recognition of Long non-coding RNA-Associated Competing Endogenous RNA Axes in Prostate Cancer

Mohammad Taheri¹, Arash Safarzadeh², Soudeh Ghafouri-Fard^{2*}, Aria Baniahmad^{1**}

Purpose: Prostate cancer is among the most central sources of cancer-related mortalities. In order to find novel candidates for therapeutic strategies in this kind of cancer, we developed an in-silico method for identification of competing endogenous RNA network.

Methods: According to the microarray data analyses between prostate tumor and normal specimens, we attained 1312 differentially expressed (DE)mRNAs, including 778 down-regulated DE mRNAs (such as CXCL13 and BMP5) and 584 up-regulated DE mRNAs (such as OR51E2 and LUZP2), 39 DE lncRNAs, including 10 down-regulated DE lncRNAs (such as UBXN10-AS1 and FENDRR) and 29 up-regulated DE lncRNAs (such as PCA3 and LINC00992) and 10 DE miRNAs, including 2 down-regulated DE miRNAs (such as MIR675 and MIR1908) and 8 up-regulated DE miRNAs (such as MIR6773 and MIR4683).

Results: We constructed the ceRNA network between these transcripts. We also evaluated the related signaling pathways and the significance of these RNAs in prediction of survival of patients with prostate cancer.

Conclusion: This study provides novel candidates for construction of specific treatment routes for prostate cancer.

Keywords: prostate cancer; ceRNA; lncRNA; miRNA

INTRODUCTION

Long non-coding RNAs (lncRNAs) are a group of transcripts with sizes more than 200 nt. They have diverse regulatory roles in expression of genes. This kind of epigenetic regulators influence epigenetic marks mainly in the nucleus, thus affecting gene transcription⁽¹⁾. In addition, they can serve as molecular sponges for microRNAs (miRNAs)⁽²⁾, thus regulating the expression of miRNA targets. This mode of action leads to establishment of lncRNA/miRNA/mRNA axes that contribute in the several physiological processes. Dysregulation of lncRNAs can lead to several disorders through induction of imbalances in these molecular axes. LncRNAs that act as molecular sponges are called competitive endogenous RNAs (ceRNAs). Evaluation of the activity of ceRNA networks has practical significance, particularly in unraveling the mechanism of carcinogenesis. Prostate cancer as one of the most important sources of cancer-related mortalities⁽³⁾, is one of the hot topics in the field of cancer-related ceRNA networks. For instance, Li et al. have constructed a prostate cancer-specific ceRNA network by incorporating lncRNA/miRNA/mRNA interactions based on experimental and in silico methods. Their method has led to identification of 42 significant prostate cancer-survival-associated triplets which make a condensed sub-network consisting of only 25 nodes. The latter finding shows the involvement of some nodes in many triplets.

MIR22HG/hsa-mir-21/TGFBR2 and MIR22HG/hsa-mir-21/BCL2 triplets have been recognized as two significantly survival-associated triplets with the greatest average degree in the detected subnetwork⁽⁴⁾. Similarly, Guo et al. have constructed a prostate cancer-specific core ceRNA network with the capability to be applied as diagnostic and prognostic marker in this type of cancer⁽⁵⁾.

The current study aimed at identification of the ceRNA network in prostate cancer using an in-silico approach. The related signaling pathways and the significance of these RNAs in prediction of survival of prostate cancer patients have also been evaluated.

MATERIALS AND METHODS

Microarray Data Assessment

The human expression profile of GSE69223, GSE46602, and GSE55945, all with [HG-U133 Plus 2] Affymetrix Human Genome U133 Plus 2.0 Array, which contained 30, 50, and 21 specimens, respectively, were obtained using the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>). We chose 15 prostate tumor and 15 normal tissue specimens from GSE69223, 36 prostate tumor samples from GSE46602 and 13 prostate tumor samples from GSE55945 for additional analyses. This data contained lncRNAs, miRNAs and mRNAs expression profile.

Data processing, meta-analysis and evaluation of data

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Table 1. The top 10 up- and downregulated DEMRNAs between prostate tumor and normal samples.

Down-regulated			Up-regulated		
DEmRNA	Log FC	Adjusted P value	DEmRNA	Log FC	Adjusted P value
CXCL13	-2.914284	0.0001	OR51E2	2.410149	0.002
BMP5	-2.549856	0.0002	LUZP2	2.205251	0.0005
WIF1	-2.453527	0.0001	HOXC6	2.178773	0.0001
NELL2	-2.383551	0.00003	HPN	2.004699	0.000000002
SLC14A1	-2.214827	0.00004	C15orf48	1.984213	0.01
DAPL1	-2.038041	0.002	TRPM4	1.971099	0.00000005
KRT23	-2.011459	0.00004	B3GAT1	1.834909	0.0002
LGR6	-1.843800	0.00001	PRCAT47	1.807160	0.01
CFD	-1.792887	0.00002	THBS4	1.804980	0.0001
PTGS1	-1.750086	0.00001	DLX1	1.804387	0.0001

quality

The statistical programming language R was used to analyse and combine all of the microarray data. Data from Affymetrix and Agilent was initially normalized individually for pre-processing using the preprocessCore package's normalizeQuantiles function (version 1.58.0). (<https://bioconductor.org/packages/release/bioc/html/preprocessCore.html>). With the purpose of exclusion of batch effects (non-biological differences), we used the ComBat function from the R Package Surrogate Variable Analysis (SVA) (version 3.44.0)⁽⁶⁾. Batch effect removal was then evaluated. We showed the result of the meta-analysis in a unit expression matrix.

Assessment of differentially expressed transcripts
We used the Limma package (version 3.52.3)⁽⁷⁾ in R language to find differentially expressed mRNAs (DEmRNAs), lncRNAs (DElncRNAs) and miRNAs (DEmiRNAs) between prostate tumor and normal specimens. DElncRNAs, DElncRNAs and DEmiRNAs were appraised with the cut-off criteria of false discovery rate (FDR; adjusted *p* value) < 0.05 and |log2 fold Change (FC)| > 0.5. Subsequently, we identified DElncRNAs and DEmiRNAs using HUGO gene nomenclature.

Two-Way Clustering of DEGs

Expression levels of significant DElncRNAs, DElncRNAs, and DEmiRNAs were obtained and used in the pheatmap package (version 1.0.12)⁽⁸⁾ in R language to conduct the two-way clustering based on the Euclidean distance.

Gene Ontology (GO) Enrichment

ClusterProfiler R package (version 4.4.4)⁽⁹⁾ was applied to conduct gene ontology (GO) enrichment and investigation of the functions of the significantly up-regulated and down-regulated DEGs. The functional category criteria were established at an adjusted *p*-value < 0.05.

KEGG Pathway Analysis

KEGG pathway analysis of considerable DEGs was performed using the KEGG database⁽¹⁰⁾.

PPI Network Construction

PPI network for DEGs was identified using the STRING database⁽¹¹⁾. Highest level of confidence was utilized to create the interactions parameter (confidence score > 0.9). Protein interactions were visualized using the Cytoscape software v3.9⁽¹²⁾. The top 20 DEGs related to hub genes were lastly detected using the Cytoscape

Table 2. The up- and downregulated DElncRNAs between prostate tumor and normal samples.

Down-regulated			Up-regulated		
DElncRNA	Log FC	Adjusted P value	DElncRNA	Log FC	Adjusted P value
UBXN10-AS1	-1.085041069	0.0002	PCA3	2.194085974	0.001
FENDRR	-1.001246896	0.0002	LINC00992	2.131068688	0.00008
MAGI2-AS3	-0.951283196	0.00003	CIQTNF3-AMACR	2.129359564	0.0001
MAGI2-IT1	-0.877464251	0.0002	PCAT18	1.444563038	0.03
BOLA3-AS1	-0.818242331	0.00004	ERVH48-1	1.384998066	0.02
ADAMTS9-AS2	-0.79674974	0.00003	DRAIC	1.351053821	0.006
HCG11	-0.765278673	0.0001	FOXP4-AS1	1.343377023	0.0009
TBX5-AS1	-0.74698408	0.00001	LINC00842	1.213140476	0.004
RBMS3-AS3	-0.714422829	0.0001	LINC00920	1.030649901	0.04
MEG3	-0.707122105	0.0002	DANCR	1.026630806	0.0005
			PRRT3-AS1	0.916404009	0.03
			SNHG19	0.900564908	0.0001
			PCAT7	0.827071849	0.02
			C5orf34-AS1	0.824415749	0.01
			CRNDE	0.733537617	0.01
			SNHG9	0.711240397	0.04
			LINC01351	0.708756468	0.03
			ENO1-AS1	0.689974397	0.01
			ZNF793-AS1	0.68123815	0.009
			MCF2L-AS1	0.645850215	0.007
			PRKAG2-AS1	0.640111087	0.02
			PCAT6	0.622871409	0.02
			POU6F2-AS2	0.618162182	0.03
			LINC00862	0.596493068	0.02
			RPARP-AS1	0.595574259	0.004
			LEF1-AS1	0.592353189	0.02
			LINC00665	0.538956003	0.002
			LINC00973	0.520799038	0.02
			LINC01128	0.507755709	0.005

Table 3. The significantly up- and downregulated DEmiRNAs between prostate tumor and normal samples.

Down-regulated DEmiRNA	Log FC	Adjusted P value	Up-regulated DEmiRNA	Log FC	Adjusted P value
MIR675	-1.461212788	0.003	MIR6773	1.110887917	0.0000363
MIR1908	-0.809060479	0.005	MIR4683	0.903634366	0.0003
			MIR7110	0.875949754	0.001
			MIR3658	0.746514424	0.0001
			MIR3185	0.670389146	0.01
			MIR6824	0.617549387	0.02
			MIR4647	0.599044810	0.004
			MIR4784	0.549181183	0.01

plugin⁽¹³⁾ of Cytoscape using the betweenness method.

ceRNA Network and Hub Genes

A ceRNA network was constructed through these steps: 1) Searching the miR2Disease database (<http://watson.compbio.iupui.edu:8080/miR2Disease/index.jsp>)⁽¹⁴⁾ utilizing the term "prostate cancer" for the prostate cancer (PC)-related miRNAs. 2) measuring the interactions between lncRNAs and miRNAs based on the PC-related miRNAs using miRcode (<http://www.mircode.org/>); 2) Application of miRDB (<http://www.mirdb.org/>)⁽¹⁵⁾, miRTarBase (<https://mirtarbase.cuhk.edu.cn/>)⁽¹⁶⁾, TargetScan (<http://www.targetscan.org/>)⁽¹⁷⁾ and miRWalk (<http://129.206.7.150/>)⁽¹⁸⁾ for predicting miRNAs-targeted mRNAs; 3) Discovery of the intersections of the DElncRNAs and DEMiRNAs, and formation of lncRNA/mRNA/miRNA ceRNA network using Cytoscape v3.9 and 4) predicting hub genes using cytohubba plugin based on degree approach.

Confirmation of hub genes via expression values

Expression value of hub genes was evaluated using the ualcan database⁽¹⁹⁾.

Survival analysis

Survival package (version 3.5.0) (<https://CRAN.R-project.org/package=survival>) in R was utilized to find survival curves. The clinical data for patients with prostate cancer was obtained from TCGA (PRAD-TCGA). Univariate survival analysis was performed using Kaplan-Meier curves. Statistics were considered significant for P -value < 0.05 . The start time to the end time in this analysis is from 0 to 5000 days.

RESULTS

Microarray Data Processing

Figure 1 depicts the boxplots of raw data, normalized data after batch effect removal and quantile normalization. These plots show the reliability of the quality of the expression data. Moreover, the boxplot of the pre-processed data had good normalization. Figure 2 shows the Euclidean distances between the samples after batch effect removal. In the PCA plot (Figure 3), 101 specimens are shown in the 2D plane traversed by their first two principal components (PC1 and PC2) According to

this plot, the samples had a good dispersion following the removal of batch effect.

DEGs Analysis

Based on the microarray data analysis between prostate tumor and normal samples by Limma, we analyzed differentially expressed mRNA, lncRNA and miRNAs and obtained 1312 DEMiRNAs, including 778 down-regulated DEMiRNAs (such as CXCL13 and BMP5) and 584 upregulated DEMiRNAs (such as OR51E2 and LUZP2), 39 DElncRNAs, including 10 downregulated DElncRNAs (such as UBXXN10-AS1 and FENDDR) and 29 upregulated DElncRNAs (such as PCA3 and LINC00992) and 10 DEMiRNAs, including 2 down-regulated DEMiRNAs (such as MIR675 and MIR1908) and 8 upregulated DEMiRNAs (such as MIR6773 and MIR4683). The most significantly upregulated and downregulated DEMiRNAs, DElncRNAs, and DEMiRNAs are shown in Tables 1-3, respectively.

Volcano plot was depicted with the EnhancedVolcano package (version 1.14.0)⁽²⁰⁾ in R to compare the variation in miRNA, lncRNA, and mRNA expression between prostate tumor and normal samples (Figure 4). Moreover, the two-way clustering showed 20 clearly distinct DEMiRNA expression patterns between prostate tumor and normal samples (Figure 5a). The expression of DElncRNAs and DEMiRNAs is demonstrated in two heatmaps (Figure 5b).

GO Enrichment Analysis of DEGs

The substantially DEGs were enriched in 497 GO terms. ClusterProfiler package was used for analysis. For performing this analysis, the all genes listed in the database in clusterProfiler package have been used as background. In GO functional enrichment analysis, 497 GO entries fulfil Adjusted P value < 0.05 , the majority of which are biological processes, followed by cellular component and molecular function. The first 30 entries are collagen-containing extracellular matrix (CC), extracellular matrix structural constituent (MF), extracellular matrix organization (BP), extracellular structure organization (BP), cell-cell junction (CC), basement membrane (CC), cell junction assembly (BP), cell-substrate adhesion (BP), collagen trimer (CC), sarcolemma (CC), muscle contraction (BP), muscle system process

Table 4. Down-regulated and Up-regulated Pathways

Down-regulated Pathway	P value	Up-regulated Pathway	P value
Focal adhesion	0.007574712	Purine metabolism	0.04602828
Protein digestion and absorption	0.014154194		
Vascular smooth muscle contraction	0.023808509		
ECM-receptor interaction	0.031880880		
Complement and coagulation cascades	0.049110016		

Table 5. The MiRcode database demonstrated interactions between 13 DElncRNAs and 13 DEMiRNAs.

lncRNA	miRNA
PCA3, ERVH48-1, ADAMTS9-AS2, RBMS3-AS3, MEG3	miR-96
PCA3, ERVH48-1, ADAMTS9-AS2, BOLA3-AS1, RBMS3-AS3, MEG3	miR-182
PCA3, CRNDE, ADAMTS9-AS2, MEG3	miR-221
PCA3, CRNDE, ADAMTS9-AS2, MEG3	miR-222
MCF2L-AS1, CRNDE, ADAMTS9-AS2, HCG11, MEG3	miR-205
ENO1-AS1, CRNDE, ERVH48-1, MAGI2-AS3, ADAMTS9-AS2, MEG3	miR-145
CRNDE, SNHG9, MAGI2-AS3, ADAMTS9-AS2, HCG11, MEG3	miR-31
CRNDE, ERVH48-1, MAGI2-AS3, ADAMTS9-AS2, HCG11, MEG3	miR-181b
CRNDE, ADAMTS9-AS2	miR-183
ERVH48-1, SNHG9, ADAMTS9-AS2, BOLA3-AS1, MEG3	miR-184
POU6F2-AS2, ADAMTS9-AS2,	miR-375
POU6F2-AS2, MEG3	miR-125b-5p
MAGI2-AS3, MEG3	miR-16

Table 6. miRWalk, miRDB and TargetScan databases revealed interactions between 10 DEMiRNAs and 24 DEMRNAs.

miRNA	mRNA
miR-96	TP53INP1, NIPA1
miR-182	FOXF2
miR-221	TRPS1, KIT
miR-222	STOX2, TRPS1
miR-205	LRRK2
miR-145	ADD3, TGF2, MYO6
miR-31	SPRED1
miR-181b	KLHL15, PLPP3, PLAG1
miR-16	RAB9B, GALNT7, PSAT1, TGFBR3, PDLIM5, SLC9A6
miR-125b-5p	MFSO9, STOX2, HK2

(BP), urogenital system development (BP), morphogenesis of a branching structure (BP), I band (CC), endoplasmic reticulum lumen (CC), extracellular matrix structural constituent conferring tensile strength (MF), contractile fiber (CC), regulation of cell-substrate adhesion (BP), Z disc (CC), mesenchyme development (BP), mesenchymal cell differentiation (BP), respiratory tube development (BP), myofibril (CC), membrane raft (CC), membrane microdomain (CC), gland morphogenesis (BP), renal system development (BP), glycosaminoglycan binding (MF) and sarcomere (CC). Figure 6 shows the barplots of top 10 enriched functions.

Pathway Analysis

Using Pathview (version 1.36.1)⁽²¹⁾ and gage (version 2.46.1)⁽²²⁾ packages in R, KEGG pathway analyses of 177 down-regulated and 177 up-regulated DEGs were performed to identify the potential functional genes (Table 4 and Figure 11). Figure 10 shows the schematic visualization of 3 pathways (1 up-regulated and 2 down-regulated pathways).

PPI network construction and selection of hub genes
In order to find the hub genes, a PPI network of DEGs (supplementary file) with 411 nodes and 555 edges that was acquired from STRING was loaded into the Cytoscape plugin of Cytoscape 3.9. The 20 hub genes with the highest betweenness of connectivity were EGF,

PRKCA, FLNA, CAV1, RGS9, RGS2, CD3EAP, RRM2, ITGA1, PPP1R12B, SDC2, MLC1, PRKG1, BIRC5, P4HB, FGFR2, POLR2H, VCL, PIK3R1 and RGS17.

LncRNAs can act as an endogenous "sponge" to regulate the expression of mRNA by adsorbing miRNA, according to the ceRNA theory⁽²³⁾. The lncRNA-miRNA-mRNA ceRNA network was built using upregulated or down-regulated miRNAs, as well as lncRNAs or mRNAs⁽²⁴⁾. DElncRNAs and DEMiRNAs networks did not interact in our research. We utilized the miR2Disease database as a result. In miR2Disease, we chose miRNAs that changed in prostate tumor tissues compared to adjacent normal tissue samples, both up- and down-regulating. We discovered 14 PC-related miRNAs using the miR2 Disease database. The relationship between lncRNAs and miRNAs was then evaluated using miRcode. This step showed that 13 of 14 PC-specific miRNAs may target to the 13 of 39 DElncRNAs (Table 5). Then we used miRDB, miRTarBase, TargetScan and miRWalk to predict targeted mRNAs by these 13 miRNAs to discover the relationship between miRNAs and mRNAs. We found that 10 miRNAs might target 24 out of the 1312 mRNAs (Table 6). If miRNA-targeted mRNAs were not found in DEMRNAs, they were eliminated. Using this information (Table 5 and 6), we used Cytoscape 3.9 to construct the lncRNA-miRNA-mRNA ceRNA network. Once the targeted DEMRNAs and DElncRNAs' expression patterns were reversed, DElncRNAs, targeted DEMRNAs, and the interacting miRNAs were all eliminated from the ceRNA network. A total of 8 lncRNAs, 4 mRNAs, and 3 miRNAs were included in the ceRNA network (Figure 13). At last, we computed nodes degrees and displayed the top 7 nodes with the highest degree in the network using cytohubba app (Figure 14). We found ENO1-AS1, hsa-miR-182, hsa-miR-125b-5p, hsa-miR-145, MEG3, FOXF2 and MYO6 as 7 hub genes in ceRNA network.

Confirmation of hub genes via expression value

The expression value of hub genes was evaluated using the ualcan database. As a result, all hub genes in PPI network and MIR182 in ceRNA network indicated good statistical significance (Figure 16 and Table 7).

Survival analysis

For survival analysis, we downloaded and analyzed transcriptome profiling of prostate cancer samples (TCGA-PRAD) using TCGAbiolinks (version 2.24.3)⁽²⁵⁾ and edgeR (version 3.38.4)⁽²⁶⁾ packages. Survival was analyzed based on Kaplan-Meier curves using surviv-

Table 7. Statistical significance of hub genes based on sample types in prostate cancer.

Hub genes	Statistical significance of expression value
POLR2H	<1E-12
CAV1	2.23E-08
ITGA1	4.22E-05
PIK3R1	3.79E-10
RGS9	5.79E-05
SDC2	1.25E-04
P4HB	<1E-12
PPP1R12B	1.13E-06
RRM2	2.32E-07
PRKCA	2.07E-06
VCL	7.55E-07
BIRC5	7.23E-11
CD3EAP	4.68E-08
PRKG1	4.22E-06
FLNA	3.27E-07
MLC1	1.03E-04
RGS2	3.40E-04
EGF	4.02E-13
RGS17	9.88E-05
FGFR2	<1E-12
MIR182	<1E-12

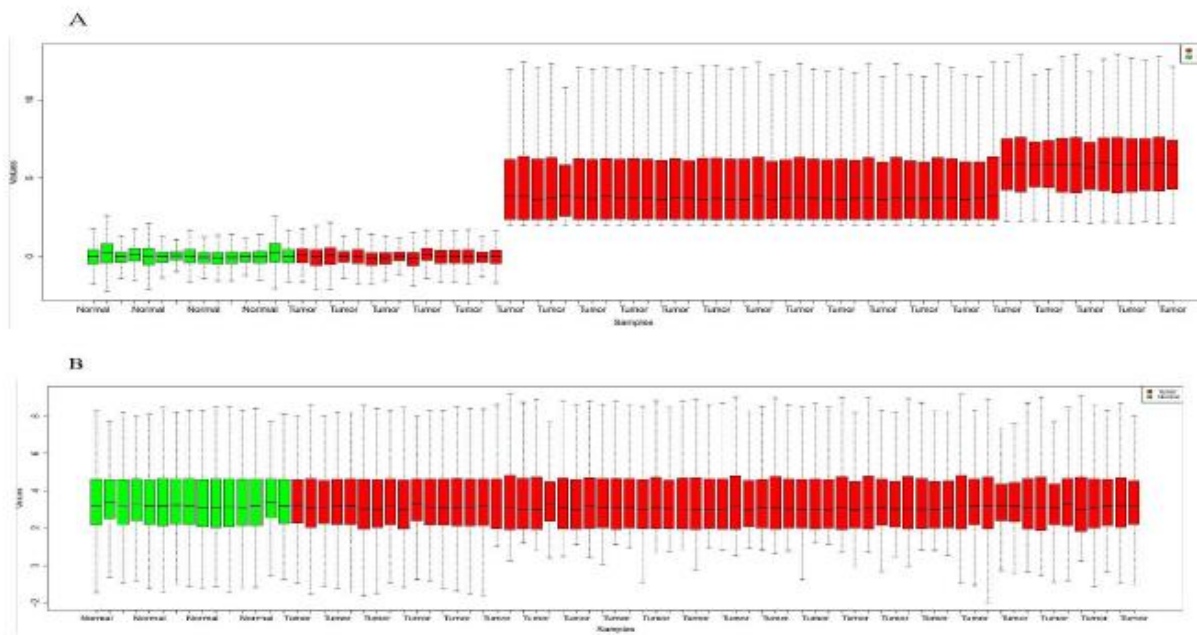


Figure 1. Boxplots after combining datasets. **A)** First box plot shows the combination of datasets **B)** The second boxplot shows the merged datasets after removing the batch effect removal.

al package in R. We performed survival analysis based on hub genes in PPI and ceRNA networks. The difference was statistically significant with log-rank $P < 0.05$. RRM2 and MYO6 showed a strong correlation with a reduced overall survival time in individuals with pros-

tate cancer (Figure 17).

DISCUSSION

ceRNA networks have been found to participate in the pathoetiology of several cancers, including prostate cancer. Unraveling the interactions between constitu-

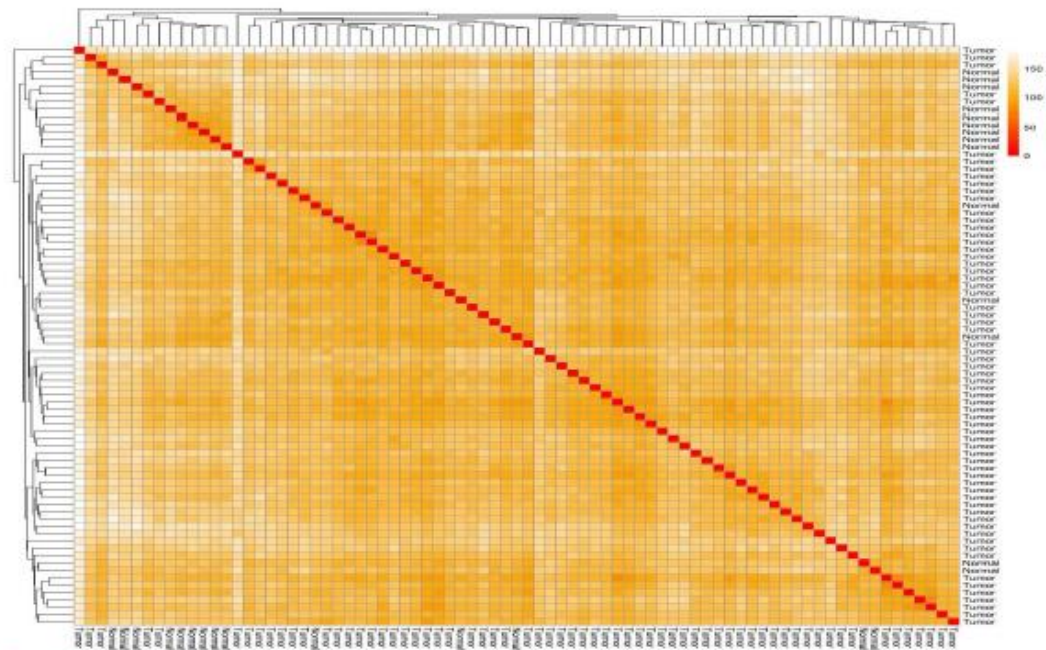
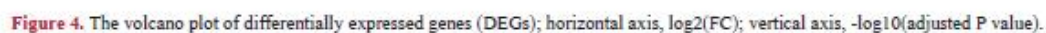
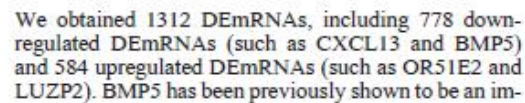


Figure 2. The Euclidean distances between samples. Based on the Euclidean distance, hierarchical clustering between the samples has been established; Legend shows the distance value between samples.



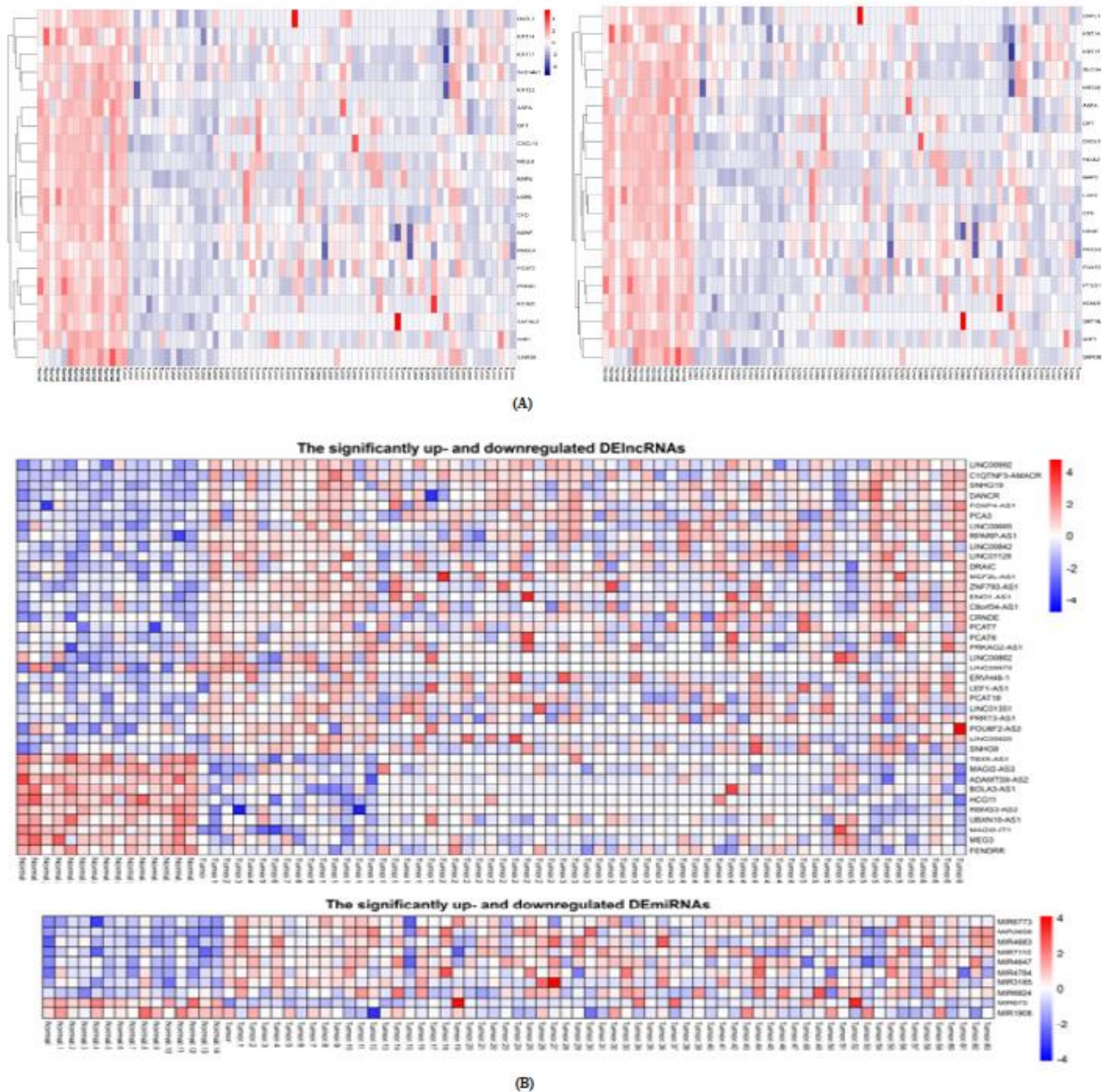


Figure 5. A. The two-way clustering of DEMiRNAs between prostate tumor samples and normal samples; horizontal axis, the samples; vertical axis, DEMiRNAs. B. Two heatmaps depicting expression of DElncRNAs and DEMiRNAs.

portant regulator of basal prostate stem cell homeostasis being involved in the initiation of prostate cancer⁽²⁷⁾. In addition, CXCL13 is an androgen-responsive gene participating in the androgen-regulated migration and invasion of prostate cancer cells⁽²⁸⁾. On the other hand, OR51E2 has been shown to inhibit proliferation and induce prostate cancer cell death⁽²⁹⁾. LUZP2 has been previously reported to be over-expressed in hormone-naïve

prostate cancer but its expression has been decreased in the course of evolution of hormone-naïve prostate cancer to castration-resistant ones⁽³⁰⁾. Moreover, we found 39 DElncRNAs, including 10 down-regulated DElncRNAs (such as UBXN10-AS1 and FENDRR) and 29 up-regulated DElncRNAs (such as PCA3 and LINC00992) and 10 DEMiRNAs, including 2 down-regulated DEMiRNAs (such as MIR675

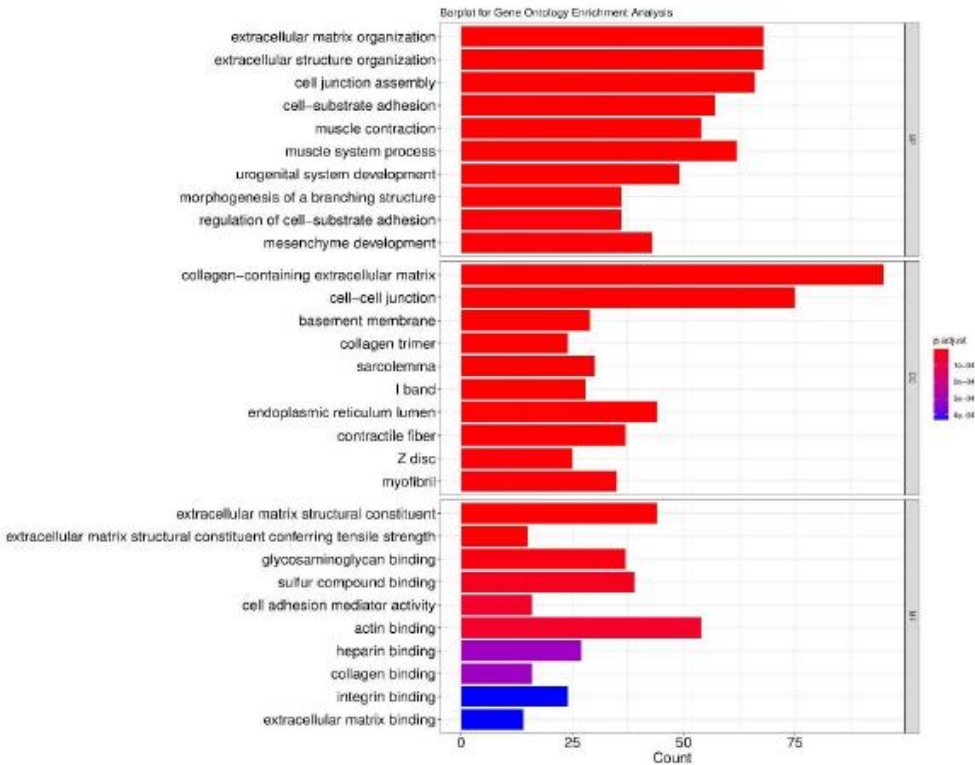


Figure 6. The barplots of top 10 enriched functions. BP (biological process), CC (cellular component) and MF (molecular function). X axis displays the count of geneset; Y axis displays the geneset function; Bar color signifies the adjusted P-value, ranging from red (most significant) to blue (least significant).

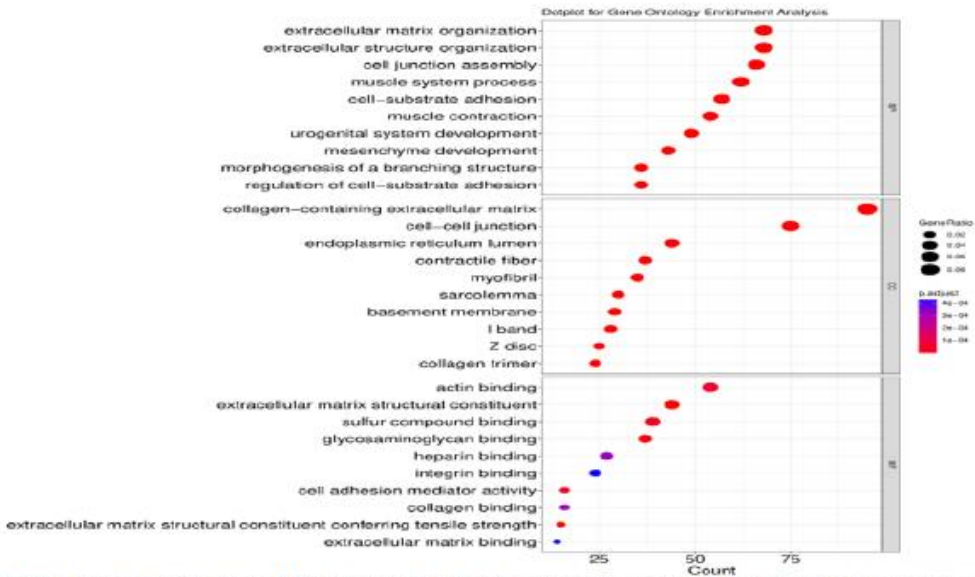


Figure 7. The dotplots of top 10 enriched functions. X axis displays the count of geneset; Y axis displays the geneset function; Dot color represents the adjusted P-value, ranging from dark blue (most significant) to red (least significant). Dot size represents the GeneRatio and The larger the size of the dot, the higher the value of the gene ratio.

Gene-Concept Network for GO Enrichment Analysis

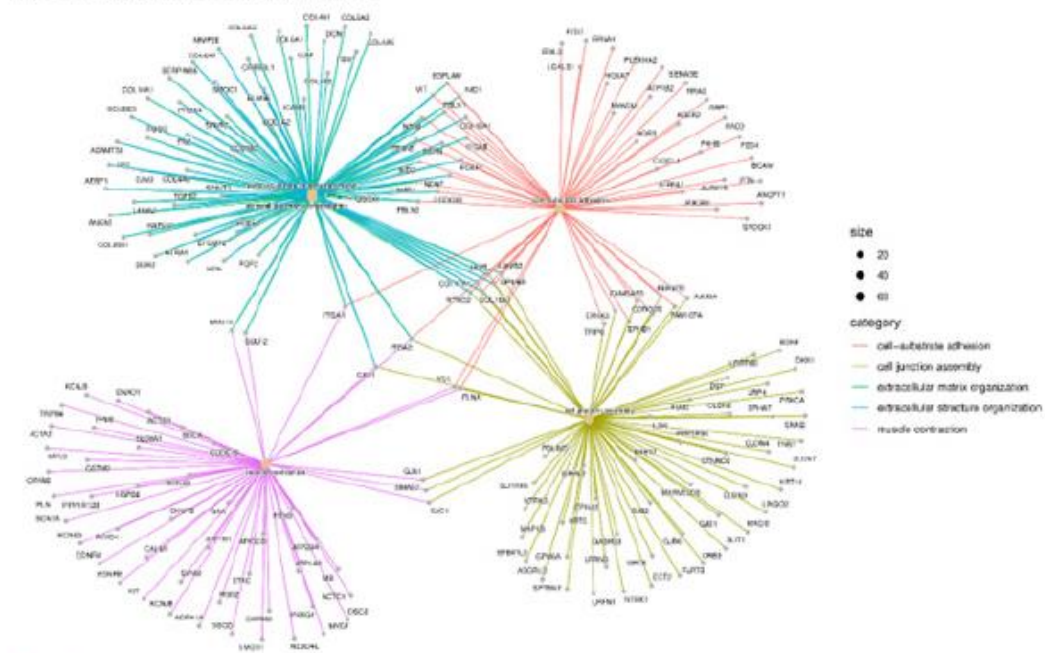


Figure 9. Network plot of top 5 GO terms. GO terms are linked with genes. There are more genes for a specific GO term if the dot relating to it is bigger.

In figure 10, the UpSet plot visualized the intersection between top 10 GO terms. It highlights the gene overlap between several gene sets.

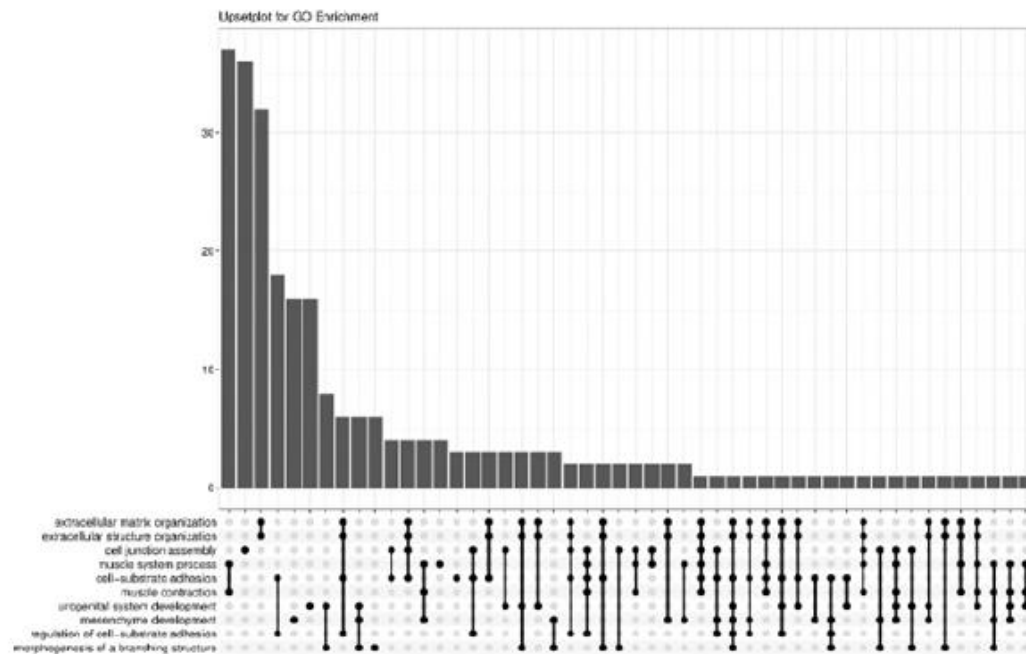
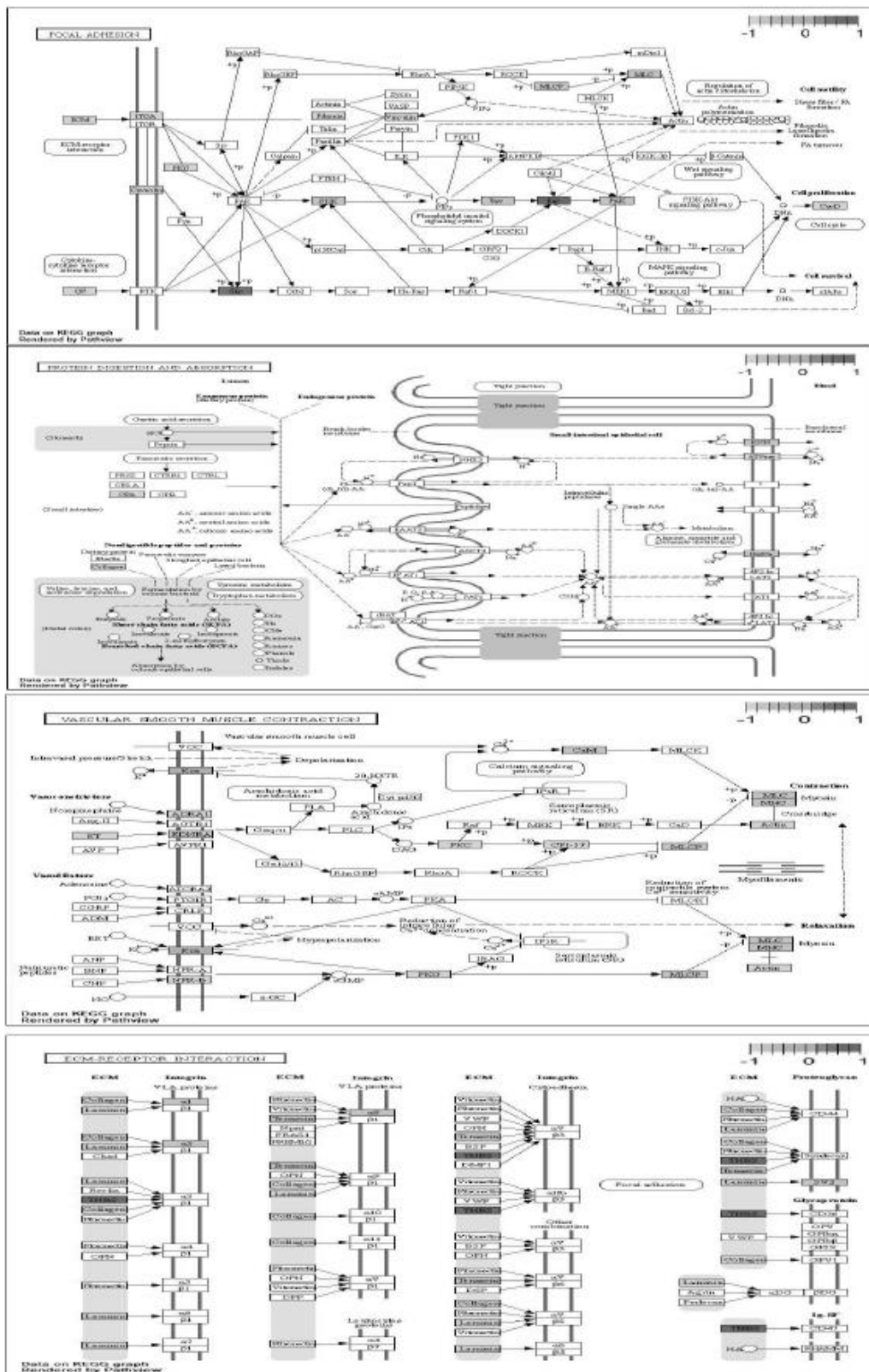


Figure 10. UpSetPlot of 10 GO terms.

Recognizing Lnc-RNA in PCa-Taheri et al.



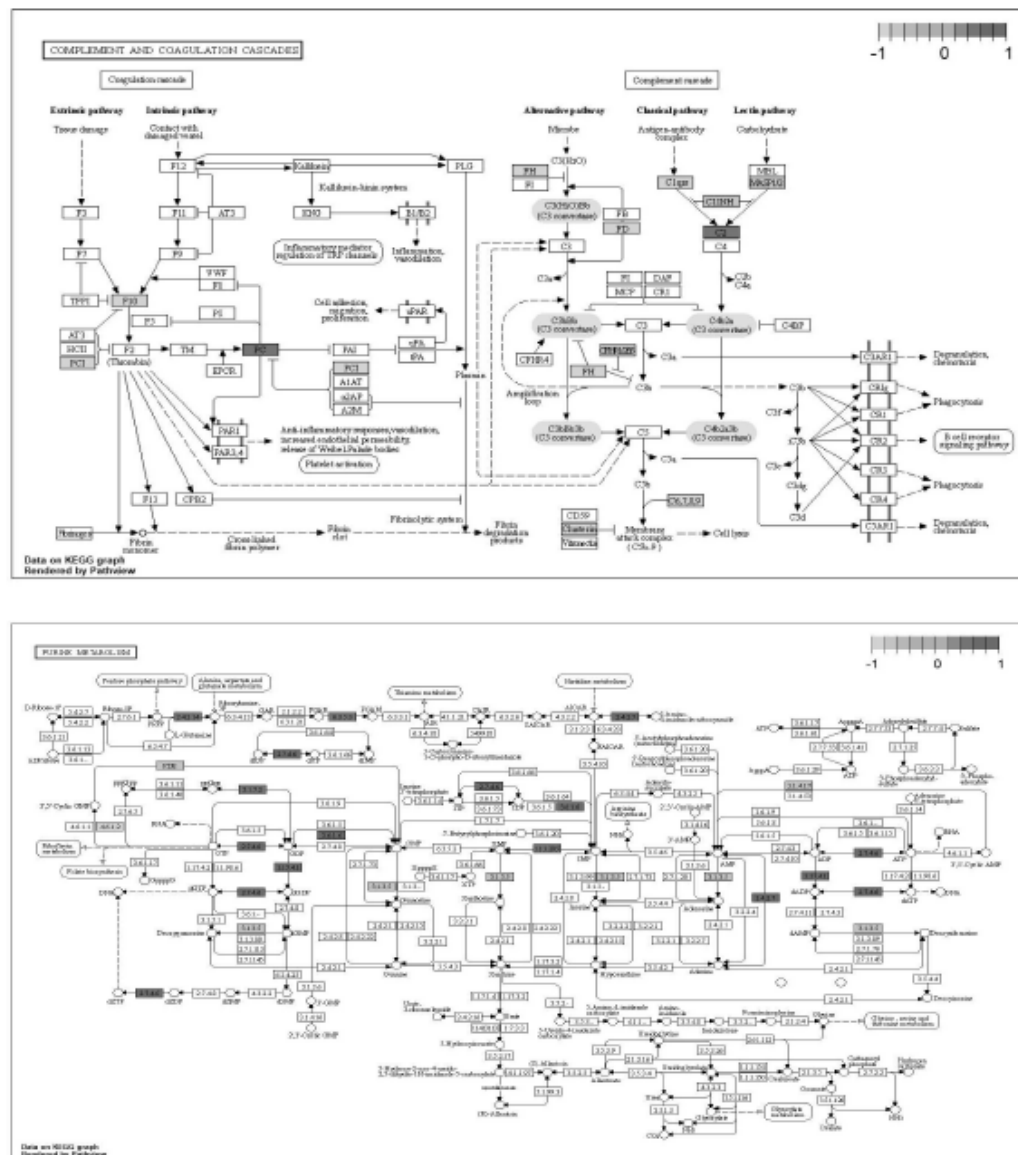


Figure 11. Visualization of pathways. Green boxes are downregulated genes and red boxes are upregulated genes.

and MIR1908) and 8 up-regulated DEMiRNAs (such as MIR6773 and MIR4683). PCA3 has been shown to regulate important pathways and targets and contribute in the development of prostate cancer⁽³¹⁾. We constructed the ceRNA network between these transcripts. A total of 13 lncRNAs, 24 mRNAs, and 13 miRNAs were included in the ceRNA network. Dysregulated pathways included focal adhesion, protein digestion and absorption, vascular smooth muscle contraction, ECM-receptor interaction, complement and coagulation cascades and purine metabolism. Dysregulation of focal adhesion is an important step in tumorigenesis leading to metastasis. In line with this, the smooth muscle contraction with myosin is known to regulate the

redistribution of actin-controlled factors during cell migration. Also, extracellular matrix (ECM) and ECM-receptor plays an important role in the cell-cell and cell-matrix. Changes and plasticity of ECM is suggested to control progression and invasion potential of prostate cancer cells (Luthold et al., 2022). Moreover, oncogenic activity of the complement cascade has been suggested to play a role in facilitating cancer cell proliferation and dysregulation of mitogenic pathways (Rutkowski et al., 2010). The biosynthesis of purines, as a basic component of nucleic acids, is linked to prostate cancer progression by providing the increased need accompanied with increased growth rate and proliferation of cancer cells (Khalafalla et al., 2022, BBA review). The purine

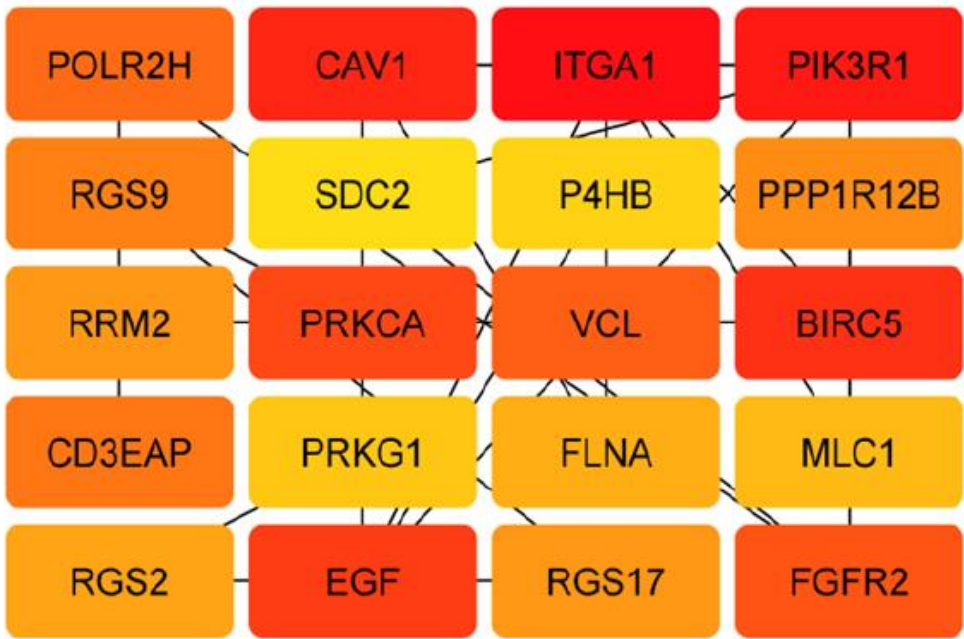


Figure 12. 20 hub genes with the highest betweenness of connectivity.

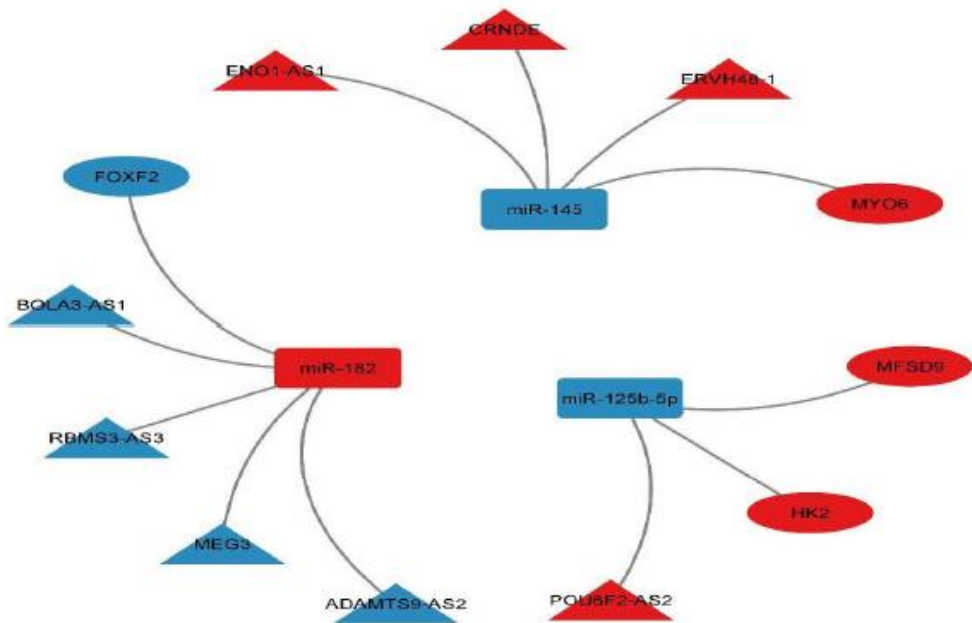


Figure 13. CeRNA network in prostate cancer. Red nodes mean a strong expression level, while blue nodes signify a low level of expression. Ellipses show protein-coding genes; rectangles show miRNAs; Triangles show lncRNAs; gray edges designate lncRNA-miRNA-mRNA interaction.

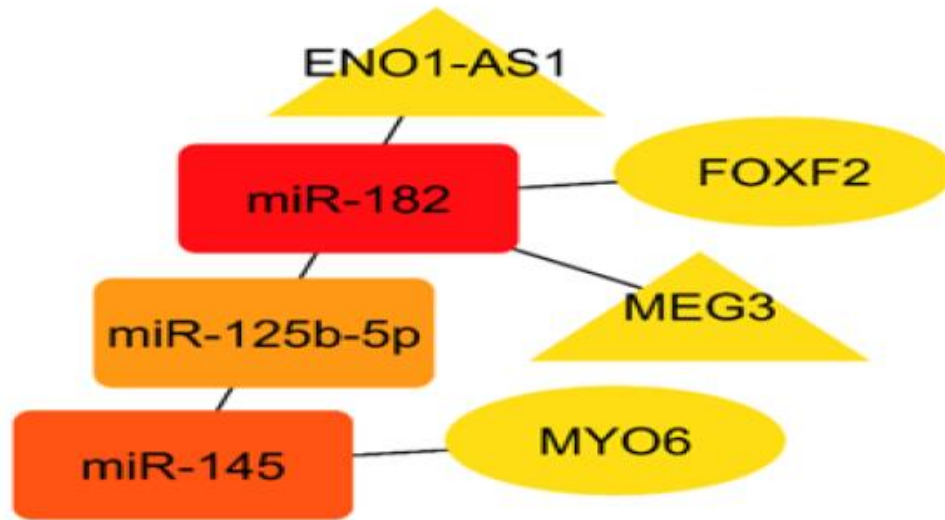


Figure 14. Top 7 genes with highest degree in ceRNA network.

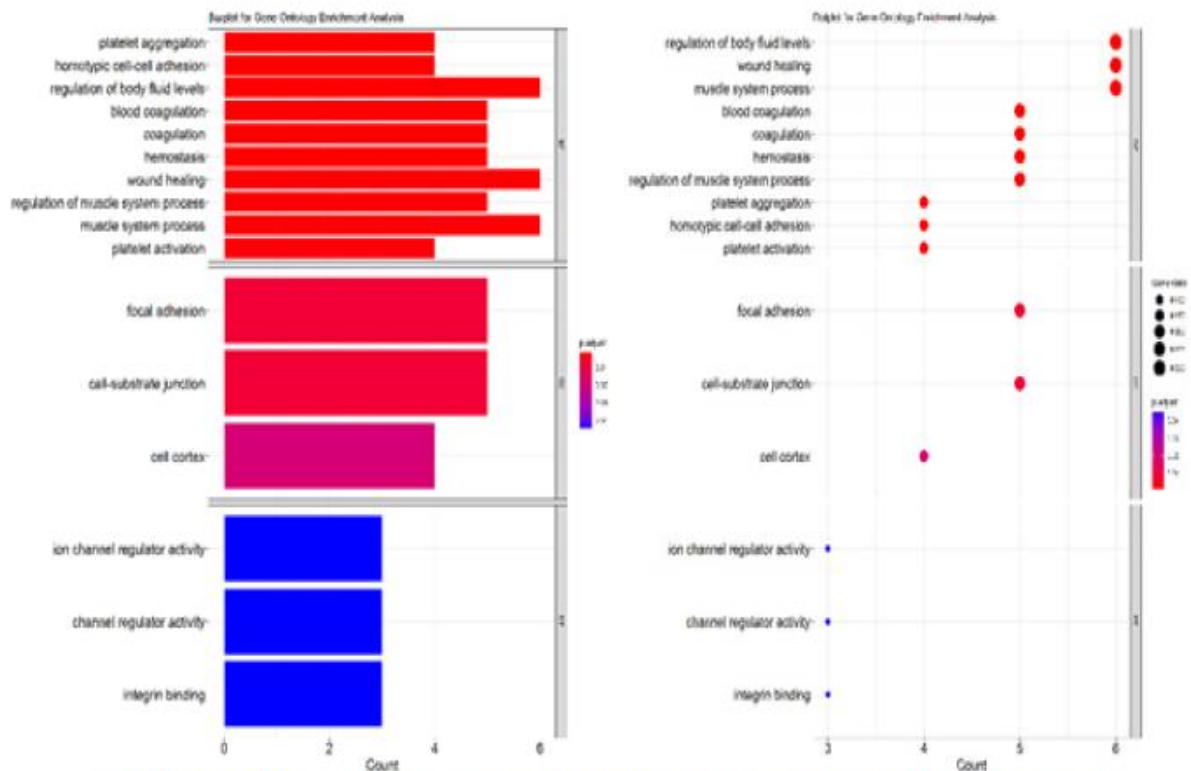


Figure 15. Gene ontology enrichment analysis of the target genes in the networks. Barplot and dotplot indicate top functional terms related to hub genes.

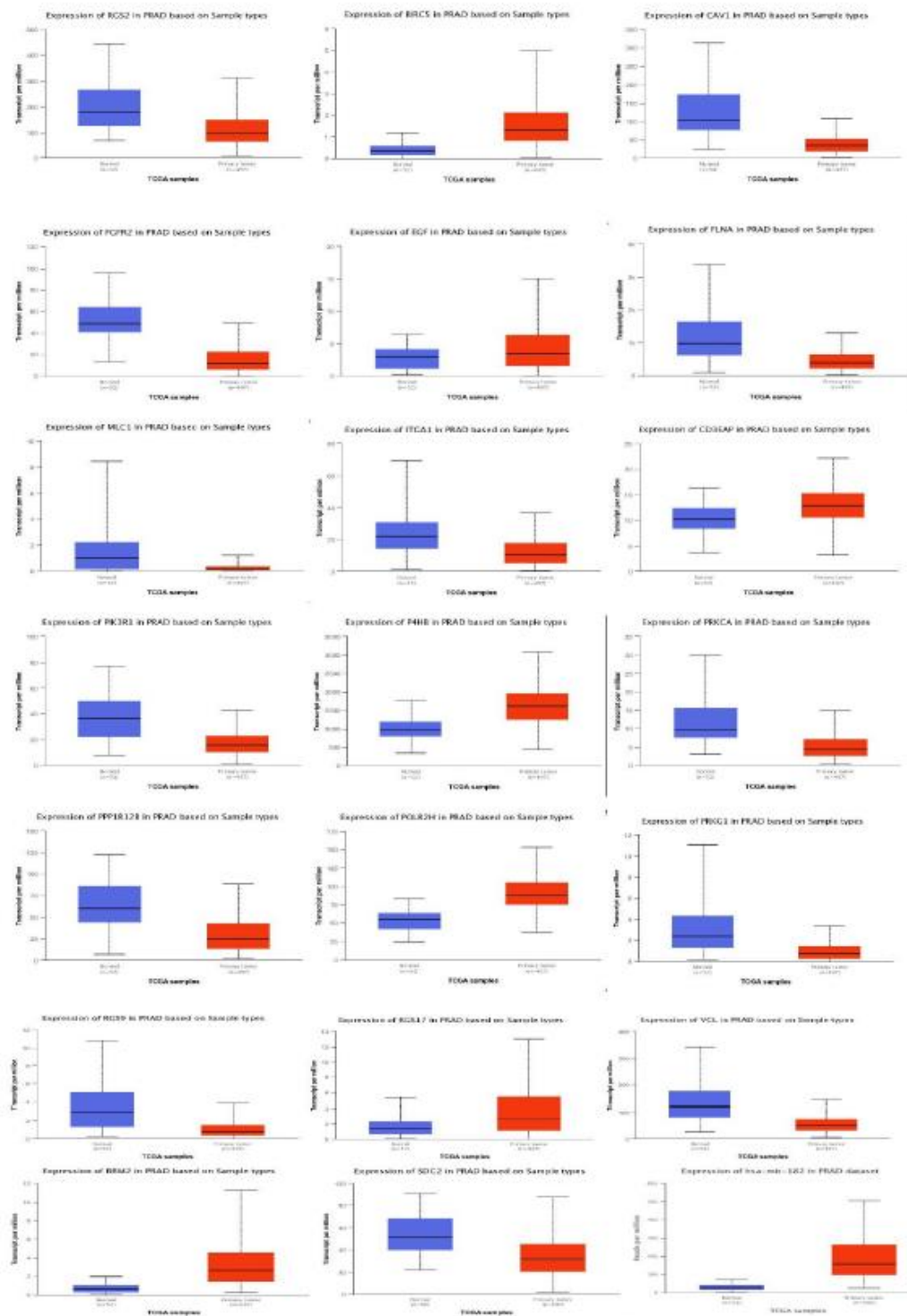


Figure 16. Box plots of gene expression of hub genes in prostate tumor and healthy samples based on TCGA. Red and green boxes show gene expression of hub genes in prostate tumor and healthy samples, respectively.

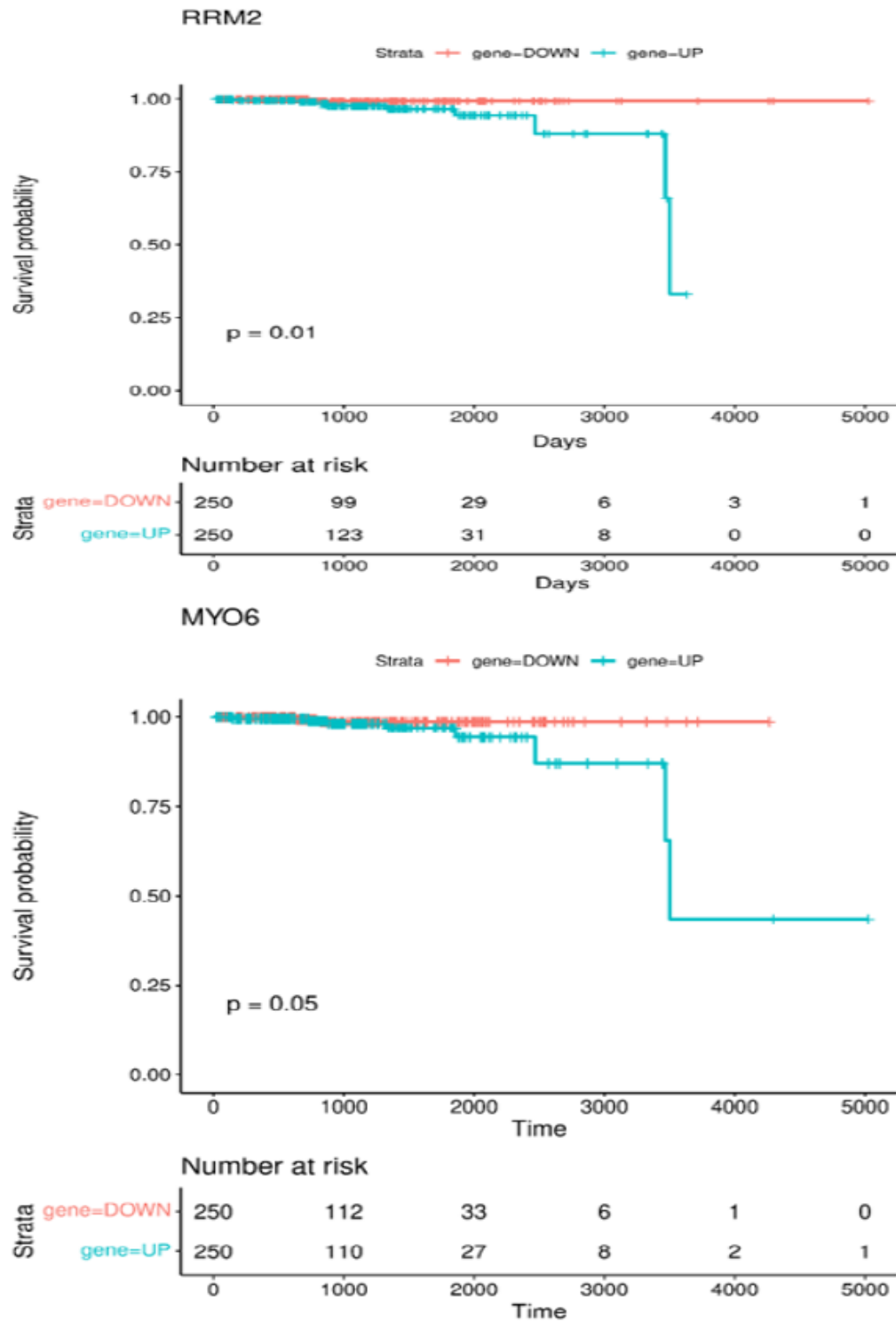


Figure 17. RRM2 and MYO6 Kaplan-Meier survival curves is related to patients with prostate cancer's overall survival.

metabolism by purinosome is a multi-enzyme complex located around mitochondria and microtubules. Purinosome has been emphasized for its therapeutic potential in cancers (Yin et al., 2018; *Frontiers Immunology*). These analyses point towards a novel identified ceRNA network of metastatic potential in prostate cancer. Thus, several important cancer-related pathways linked to each other are modulated by the identified ceRNA networks in the current study. We also evaluated the significance of these RNAs in the determination of survival of patients with prostate cancer. Among the dysregulated genes, RRM2 showed a strong correlation with a reduced overall survival time in individuals with prostate cancer. RRM2 codes one of two subunits of ribonucleotide reductase. This enzyme facilitates conversion of ribonucleotides to deoxyribonucleotides. Expression of the encoded protein by this gene is controlled during the progression of cell-cycle. This protein is up-regulated in several cancers and is involved in the gemcitabine metabolism. Thus, RRM2 has been suggested as a marker for chemotherapy response and prognosis. Its up-regulation can facilitate DNA damage repair and affect activity of signaling cascades⁽³²⁾. Future studies are needed to find the underlying mechanism of participation of this gene in the course of prostate cancer. This study provides novel candidates for design of specific treatment modalities for prostate cancer and broadens the current insight about the role of non-coding RNAs in the pathogenesis of prostate cancer.

AVAILABILITY OF DATA AND MATERIALS

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

COMPETING INTEREST

The authors declare they have no conflict of interest

APPENDIX

<https://journals.sbm.u.ac.ir/uroj/index.php/uj/libraryFiles/downloadPublic/59>

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3.3 Manuscript III



Review

Interaction between Non-Coding RNAs and Androgen Receptor with an Especial Focus on Prostate Cancer

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Abstract: The androgen receptor (AR) is a member of the nuclear receptor superfamily and has three functional domains, namely the N-terminal, DNA binding, and C-terminal domain. The N-terminal domain harbors potent transactivation functions, whereas the C-terminal domain binds to androgens and antiandrogens used to treat prostate cancer. AR has genomic activity being DNA binding-dependent or through interaction with other DNA-bound transcription factors, as well as a number of non-genomic, non-canonical functions, such as the activation of the ERK, AKT, and MAPK pathways. A bulk of evidence indicates that non-coding RNAs have functional interactions with AR. This type of interaction is implicated in the pathogenesis of human malignancies, particularly prostate cancer. In the current review, we summarize the available data on the role of microRNAs, long non-coding RNAs, and circular RNAs on the expression of AR and modulation of AR signaling, as well as the effects of AR on their expression. Recognition of the complicated interaction between non-coding RNAs and AR has practical importance in the design of novel treatment options, as well as modulation of response to conventional therapeutics.

Keywords: androgen receptor; miRNA; lncRNA; circular RNAs; prostate cancer

1. Introduction

The androgen receptor (AR), alternatively named as NR3C4 (nuclear receptor subfamily 3, group C, member 4), is a nuclear receptor [1] that is activated by a number of androgens, such as testosterone and its more active form, dihydrotestosterone [2]. AR has three protein domains, namely the N-terminal transcriptional regulation, DNA binding, and C-terminal ligand binding domain [3]. After being activated by its ligands in the cytoplasm, AR is transferred into the nucleus, where it exerts its main DNA-binding-dependent functions [4]. In fact, AR is a cytoplasmic protein in the absence of its ligands associated with chaperone proteins, such as heat shock proteins and co-chaperones. Androgen binding to the AR results in conformational changes, dissociating it from chaperone proteins [4]. After translocation to the nucleus, the androgen/AR complex dimerizes and binds to androgen response elements (AREs), which are present in the AR target genes. This process is involved in the regulation of the expression of target genes [5]. In addition to this canonical route of action, the androgen/AR complex has other functions that are mediated through non-DNA-binding-dependent routes [6]. Modulation of activity of ERK, AKT, and MAPK pathways are examples of this kind of function [6–8].

Notably, specific coregulators have been found to modulate the transcriptional activity of androgen/AR complex. The binding of coregulators with androgen/AR complex can either enhance (via coactivators) or suppress (via corepressors) its transactivation capability. This process is accomplished via the epigenetic changing of chromatin configuration and histone modifications [5].

A bulk of evidence indicates that non-coding RNAs (ncRNAs) have functional interactions with AR. This type of interaction is implicated in the pathogenesis of human malignancies, particularly prostate cancer. In the current review, we summarize the available data on the role of microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) on expression of AR, as well as the effects of AR on their expression.

2. Effects of miRNAs on AR

2.1. Acting on AR mRNA to Directly Negatively Regulate AR Expression

Several miRNAs have been found to suppress the expression of AR. In fact, most of these miRNAs have been shown to bind with 3'UTR of AR transcript, thus inducing its degradation or translation suppression. The interactions between miRNAs and AR mRNA, mainly through binding with its 3'UTR, have been mostly assessed in the context of prostate cancer. MiR-299-3p is one of the AR-interacting miRNAs. Expression of miR-299-3p has been reported to be decreased in prostate cancer samples, compared to noncancerous prostate samples. The restoration of miR-299-3p in prostate cancer cells has led to the induction of cell cycle arrest, reduction of cell proliferation and migration, and enhancement of levels of apoptotic markers. Moreover, the up-regulation of miR-299-3p leads to reduced expressions of AR, PSA, and VEGFA, suppressed epithelial mesenchymal transition (EMT), reduced levels of Slug, TGF- β 3, p-AKT, and p-PRAS40, and enhanced E-cadherin levels. Taken together, miR-299-3p exerts anti-tumor effects via affecting activity of AR and VEGFA pathways [9].

Another study in prostate cancer cells has identified miR-185-binding sites in the 3'UTR of the AR transcript. Notably, the suppression of AR expression by miR-185 has compromised the interaction between AR and ARE and decreased the levels of the AR target gene PSA [10]. Moreover, miR-185-mediated inhibition of AR has suppressed the proliferation of prostate cancer cells and enhanced their apoptosis. Thus, the miR-185 has been suggested as a negative regulator of AR signaling and tumor suppressor miRNA in LNCaP cells [10].

MiR-381 is another down-regulated miRNA in prostate cancer. Forced over-expression of miR-381 in LNCaP cells inhibited their proliferation, migratory aptitude, and invasion. Mechanistically, miR-381 suppresses AR mRNA expression by binding to its 3'UTR [11].

MiR-let-7c is another miRNA that decreases expression and activity of AR in prostate cancer cells. It modulates AR transcription through c-Myc. MiR-let-7c-mediated inhibition of AR can reduce the proliferation of prostate cancer cells [12].

Figure 1 depicts a number of tumor suppressor miRNAs that regulate expression of AR in prostate cancer cells.

2.2. Indirectly Regulate AR Expression or AR Signal

On the other hand, a handful of miRNAs have been found to exert oncogenic roles in prostate cancer, through the regulation of transcription of AR expression or signaling. Following androgen deprivation therapy, hormone-sensitive prostate cancer can evolve to castration-resistant prostate cancer (CRPC). MiRNAs can contribute to this process. For instance, miR-221/-222 has been shown to be up-regulated in bone metastatic CRPC samples. In vitro studies have demonstrated that stable overexpression of miR-221 induces the androgen-independent growth of prostate cancer cells, by releasing these cells from androgen deprivation-related G1 arrest. The up-regulation of this miRNA in LNCaP has led to the reduction of expression of a subclass of androgen-responsive genes, without influencing the expression of AR or integrity of AR-androgen. MiR-221 has been found to regulate the expressions of HECTD2 and RAB1A, two genes being capable of the induction

of CRPC phenotype in various prostate cancer cells. Further, the up-regulation of miR-221 has led to alterations in the expression levels of several cell cycle-related genes and the activation of EMT-related pathways. Taken together, it has been hypothesized that miR-221 has a major role in AR signaling reprogramming and the subsequent evolution of the CRPC phenotype [13].

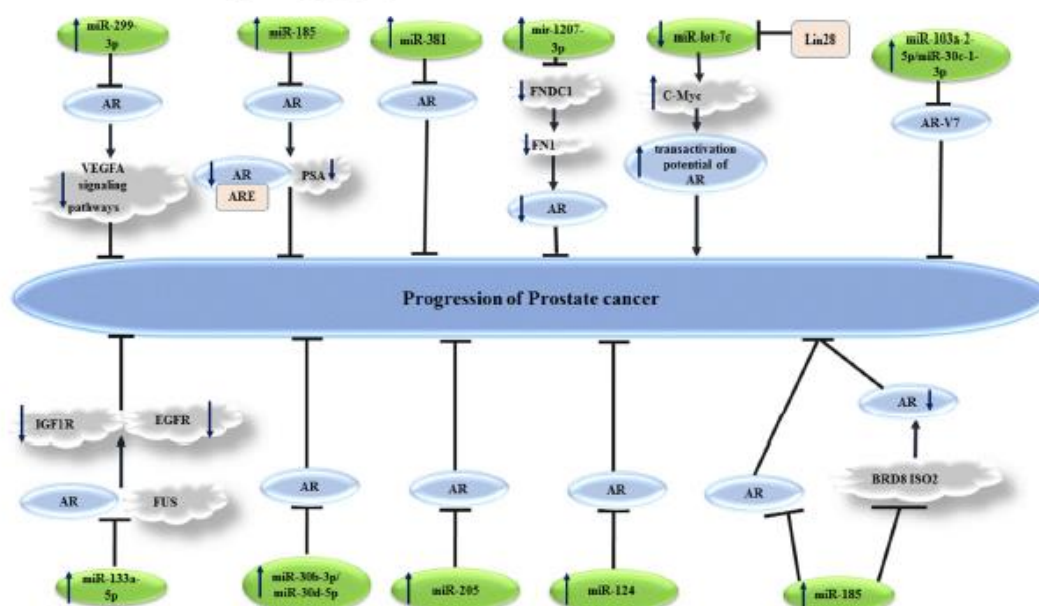


Figure 1. Several miRNAs have been shown to affect levels of androgen receptor (AR), thus influencing the progression of prostate cancer. Detailed information about these miRNAs is presented in Table 1. (⊥ reduction or inhibition of, ↑ increased levels of, ↓ decreased levels of).

Experiments in mice models have indicated the effect of surgical castration in the induction of an early upsurge in the serum levels of miR-125b. Moreover, bicalutamide-mediated AR blocking has resulted in the prompt release of this miRNA into the media of cultured prostate cancer cells. NCOR2, as a corepressor of AR, has been shown to be targeted by miR-125b. Thus, miR-125b has been suggested as a key regulator of AR, which alters the efficacy of anti-androgen therapies [14].

Mir-96 is another oncogenic miRNA that can target a RAR γ network to control AR signaling. Down-regulation of RAR γ , a member of the nuclear receptor superfamily, has been shown to significantly affect the viability of prostate cancer cells and gene signature of these cells. A gene network, comprising of numerous RAR γ target genes, such as SOX15, has been found to be correlated with poor disease-free survival of prostate cancer patients [15].

Mir-541 is another oncogenic miRNA that can affect prostate cancer course through modulation of AR signaling. In fact, infiltrating CD4(+) T cells, which are associated with poor clinical outcomes in this type of cancer, can increase FGF11 levels. Up-regulation of this growth factor leads to increase levels of miR-541. The subsequent down-regulation of AR signaling regulates MMP9 levels, in favor of tumor metastasis [16]. Figure 2 shows the effects of oncogenic miRNAs in the progression of prostate cancer, through the modulation of AR signaling.

In addition to above-mentioned miRNAs, several miRNAs can directly or indirectly affect AR signaling by binding with 3'UTR, the coding region of AR, or influencing the levels of AR co-activators/co-repressors (Table 1).

Table 1. The effects of different miRNAs on AR in prostate cancer (PCa), FUS: fused in sarcoma, AR-V7: androgen receptor variant 7, BCa: breast cancer, BPH: benign prostatic hyperplasia, ANCTs: adjacent non-cancerous tissues, PEITC: phenethyl isothiocyanate, Enz: enzalutamide, CIN: cervical intraepithelial neoplasia, AI: androgen-independent, 5-hmC: 5-Hydroxymethylated cytosine, ↓: decrease in, ↑: increase in).

miRNAs	Expression of miRNAs in PCa	Target Region of AR mRNA/Effect of miRNAs on AR	Targeted Pathway	Cell Line/Samples/Animal Models	Function of miRNAs in Cancer Cells	References
miR-299-3p	↓	↓	VEGFA signaling	LNCaP-104S, MDA-PCa-2b, 22Rv-1, C4-2B, PC-3, WPE-1/TCGA PRAD publication: 330 matching tumor and 51 normal samples	↓ proliferation, EMT process and migration, growth, ↑ cell cycle arrest, apoptosis, and drug sensitivity	[9]
miR-185	↓	↓ 3'UTR	ARE, PSA	LNCaP	↓ proliferation, ↑ apoptosis	[10]
miR-381	↓	↓ 3'UTR	—	LNCaP	↓ proliferation, migration, and invasion	[11]
miR-1207-3p	↓	↓	FNDC1, FN1, AR	WPE1-NA22, MDA-PCa-2b, PC-3, E006AA, E006AA-hT, LNCaP, C4-2B, RWPE-1	↓ proliferation, migration, ↑ apoptosis	[17]
miR-21	↑	↑	TGFBR2, Smad2/3	RWPE-1, MDA-PCa-2b, 22Rv1, PC-3, and LNCaP/male athymic nude mice	↓ tumor-suppressive activity of TGFβ pathway	[18]
miR-let-7c	↓	↓ suppression of AR at the level of transcription	Lin28, c-Myc	LNCaP, C4-2B/22 PCa samples/nude mice	↓ proliferation, ↓ transactivation, potential of AR	[12]
miR-133a-5p	↓	↓ 3'UTR	FUS, PSA, IGF1R, and EGFR	RWPE-1, VCaP, and LNCaP/TCGA database: 497 tumor tissue samples and 52 non-cancerous tissue samples	↓ proliferation and viability	[19]
miR-103a-2-5p/miR-30c-1-3p	↓	↓ 3'UTR AR-V7	—	VCaP	↓ cell growth and proliferation	[20]
miR-30b-3p and miR-30d-5p	↓	↓ 3'UTR	—	LNCaP, PC3, LAPC4/15 primary PCa samples, 15 adjacent normal prostate samples, and 15 metastatic CRPC samples	↓ cell growth	[21]
miR-31	↓	↓ coding region	—	RWPE-1, VCaP, LNCaP, 22Rv1, PC3, DU145, and HEK293	↓ proliferation, cell growth and colony formation, ↑ cell cycle arrest	[22]
miR-205	↓	↓ 3'UTR	—	DU145, PC3, 22Rv1, LNCaP/49 PCa, and 25 samples without PCa	↓ proliferation, colony formation and metastases, ↑ cell adhesion, overall survival	[23]
miR-124	↓	↓ 3'UTR	—	LNCaP, 22Rv1, DU145, PC-3, C4-2/male BALB/C nude mice	↓ proliferation, migration, and cell growth	[24]
miR-145	↓	↓	Ago2, PSA, TMPRSS2, KLK2	PC3, DU145, LNCaP, 22Rv1, VCaP, PNT2/49 PCa, and 25 samples without PCa	↓ proliferation, ↑ G1 arrest	[25]
miR-8080	—	↓ AR-V7 3'-UTR	IGF-1R and NIKK3.1	22Rv1 and VCaP/male TRAP rats and male nude mice	Luteolin treatment: ↑ miR-8080: ↓ proliferation, growth and oxidative stress, ↑ apoptosis, and Enz effects under castration conditions	[26]
miR-124	↓	↓ 3'UTR	p53	RWPE-1, pRNS-1-1, LNCaP, C4-2B, cds2, 22Rv1, and LAPC-4/8 matched pairs of CaP and BPH tissues/male athymic nu/nu mice	↓ cell growth, ↑ apoptosis	[27]
miR-124	↓	↓ 3'UTRs of AR-V4, -V7	EZH2 and Src	LNCaP, C4-2B, 22Rv1, and VCaP/male athymic nude mice	↓ proliferation and cell growth, ↑ apoptosis, sensitivity to Enz	[28]
miR-125b	↑	↑ indirectly by decreasing the co-repressor of AR	NCOR2	HEK293 and LNCaP/male nude mice	↑ cell growth and survival, ↓ apoptosis	[14]
miR-473p	↑	—	MEKK1	LNCaP/38 pairs of tumor tissues and ANCTs	↑ cell survival, ↓ docetaxel-induced apoptosis in AR+ prostate cancer cells	[29]

Table 1. Cont.

miRNAs	Expression of miRNAs in PCa	Target Region of AR mRNA/Effect of miRNAs on AR	Targeted Pathway	Cell Line/Samples/Animal Models	Function of miRNAs in Cancer Cells	References
miR-185	↓	↓ directly by binding 3'UTRs, ↓ indirectly by suppressing co-activator of AR	BRD8 ISO2	LNCAp, PC-3/10 pairs of tumor tissues and ANCTs	↓ proliferation and invasion	[30]
miR-449	–	↓ AR-v7	EZH2	CWR22Rv1 and VCaP/male nude mice	↓ cells growth and invasion, Enz resistance	[31]
miR-34b	↓	↓ 3'UTR	ETV1	MDA-PCa-2b, DU-145/143 PCa samples (from 3 different groups), and GEO analysis: GSE21032	↓ proliferation, ↑ apoptosis	[32]
miR-320a	↓	↓ 3'UTR	–	22Rv1, VCaP, and LNCAp/10 PCa samples/SD rats	OBP-801 treatment ↑ miR-320a: ↓ proliferation and cell growth	[33]
miR-17	↓	↓ indirectly by suppressing co-activator of AR	PCAF	RWPE1, LNCAp, PC-3, DU145, C4-2B, and ALVA31	PEITC treatment ↑ miR-17: ↓ cell growth	[34]
miR-141	↑	↑ AR-regulated transcriptional activity	Shp	RWPE-1, LNCAp, DU145, and C4-2B	PEITC treatment ↓ miR-141 and AR signaling activation	[35]
miR-449a	–	↓ 3'UTR	PSA	C4-2 and LNCAp	capsaicin treatment ↑ miR-449a: ↓ proliferation, ↑ G0/G1 cell cycle arrest	[36]
miR-331-3p	↓	↓ indirectly by regulating ERBB-2	ERBB-2, PI3K/AKT signaling pathway, PSA	LNCAp, 22Rv1, DU145/tumor tissues, and ANCTs	↓ indirectly AR pathway target genes via cross-talk between ERBB-2 and AR signaling pathways	[37]
miR-371	↓	↓ 3'UTR	KLK3	LNCAp and PC3/83 PCa samples and 6 BPH as controls/male nude mice	↓ proliferation and tumor growth	[38]
miR-1207-3p	↓	↓ indirectly by regulating FNDC1	FNDC1, FN1	RWPE-1, CM, WPE1-NA22, RWPE-1, MDA PCa 2b, PC-3, E006AA, E006AA-hT, LNCAp, C4-2b	↓ proliferation, migration, ↑ apoptosis	[39]
miR-301a	↑	↓ 3'UTR	TGF-β1/Smad/MMP9 signals	CWR22Rv1, 3T3-L1/21 pairs of tumor tissues, and ANCTs/male nude mice	Recruitment of pre-adipocytes: ↑ miR-301a: ↑ invasion and metastasis	[40]
miR137	↓	↓ indirectly by regulating AR cofactor complexes	NCoA2, KDM1A, KDM2A, KDM4A, KDM5B, KDM7A and MED1	PREC, LNCAp, LNCAp/C4-2, and PC-3/TCGA database	miR137: suppressor of androgen signaling by modulating expression of transcriptional coregulators	[41]
miR-361-3p	↓	↓ 3'UTR of ARv7	–	CW22Rv1, C4-2, and LNCAp/TCGA analysis/male nude mice	↑ Enz sensitivity	[42]
miR-2909	↑	↑	TGFBR2, TGFβ signaling, PSA	PC3 and LNCAp	↑ cell growth	[43]
miR-200a	↓	↓ AR-V7 indirectly by regulating BRD4	BRD4	LNCAp and C4-2B/10 ADPC tissue and 10 CRPC tissue samples	↓ proliferation, ↑ apoptosis	[44]
miR-135b	–	↓	MUC1-C	LNCAp	↑ invasion and EMT process	[45]
miR-17-5p	↓	↓ indirectly by regulating co-activator of AR	PCAF, PSA	RWPE1, LNCAp, C4-2B, PC3, and PrEC	↓ cell growth	[46]
miR-3162-5p	↑ in PCa tissues with higher Gleason grade	↓ 3'UTR	KLK3, PSA	LNCAp, PC3	↓ proliferation, migration, and colony formation	[47]

Table 1. Cont.

miRNAs	Expression of miRNAs in PCa	Target Region of AR mRNA/Effect of miRNAs on AR	Targeted Pathway	Cell Line/Samples/Animal Models	Function of miRNAs in Cancer Cells	References
miR-644a	↓	↓ 3'UTR (directly) and indirectly by regulating co-activators of AR	SRC-1, SRC-2, SRC-3, CCND1, CBP, and AR24	LNCAp, LAPC4, and 22Rv1/male athymic nude male mice	↓ invasion, EMT process, metastasis and Warburg effect, ↑ apoptosis	[48]
miR-221	↑	↓ indirectly by regulating co-activators of AR	HECTD2 and RAB1A	LNCAp and LNCAp-Abl, LAPC-4, PC-3, Du145, and 22Rv1	↑ AI cell growth, emt process, and metastasis	[13]
miR-29b	↑	↑ indirectly by regulating co-activators of AR	TET2, FOXA1, mTOR	LNCAp, BtR, VCaP, and 293T/male BALB/C nude mice	↑ 5-hmC-mediated tumour progression	[49]
miR-141-3p	–	↓ 3'UTR	–	LNCAp	↓ both mRNA and protein expression levels of AR	[50]
miR-96	↑	↓ indirectly by regulating co-activator of AR	RAR γ , TACC1	RWPE-1, RWPE-2, PNT2, HP1-AR, LNCAp, LAPC4, EAA006, MDAPCa2b, LNCAp-C42, 22Rv1, PC3 and DU145/36 PCa samples, and MSKCC dataset	↑ proliferation and viability	[15]
miR-185	↓	↓ indirectly by regulating co-activator of AR	SREBP signaling	LNCAp, C4-2B, RWPE-1/male athymic nude mice	↓ proliferation, clonogenicity, tumorigenicity, cell growth, migration and invasion, ↑ apoptosis	[51]
miR-342	↓	↓ indirectly by regulating co-activator of AR	SREBP signaling	LNCAp, C4-2B, RWPE-1/male athymic nude mice	↓ proliferation, clonogenicity, tumorigenicity, cell growth, migration and invasion, ↑ apoptosis	
miR-204	↓	↓ indirectly by regulating XRN1	XRN1, PSA, miR-34a	LNCAp, 22Rv1 and PC-3 and CL1/171 BPH, plus PCa samples/nude mice and rats	↓ growth and colony formation of LNCAp and 22Rv1 cells but ↑ growth and colony formation of CL1 and PC-3 cells	[52]
miR-541	↑	↓	FGF11, MMP9	LNCAp, CWR22Rv1 and C4-2/20 PCa samples/male nude mice	↑ invasion and metastasis (while infiltrated T cells co-cultured with PCa cells)	[16]
miR-205	↓	↓ indirectly by regulating SQLE	SQLE	LNCAp, C4-2, PC-3, DU145, RWPE-1, HEK293T, VCaP, and LNCAp Abl	↓ cell growth and de novo cholesterol biosynthesis	[53]
miR-130a	↓	↓ indirectly by regulating coregulators of AR	CDK1, PSAP, PSMC3IP, GTF2H1	LNCAp, PC-3, Du-145 and RWPE-1/5 low Gleason grade PCa samples, 6 high Gleason grade PCa samples, 3 recurrent PCa samples, and 6 nonmalignant samples	↑ apoptosis	[54]
miR-203	↓	↓ indirectly by regulating coregulators of AR	PARK7, MNAT1, TFIIF, NCOA4, CDK1	LNCAp, PC-3, Du-145 and RWPE-1/5 low Gleason grade PCa samples, 6 high Gleason grade PCa samples, 3 recurrent PCa samples, and 6 nonmalignant samples	↑ apoptosis and cell cycle arrest	
miR-205	↓	↓ indirectly by regulating coregulators of AR	PARK7, RAN, KHDRBS1	LNCAp, PC-3, Du-145 and RWPE-1/5 low Gleason grade PCa samples, 6 high Gleason grade PCa samples, 3 recurrent PCa samples, and 6 nonmalignant samples	↑ cell cycle arrest	
miR-212	↓	↓ (AR and AR-V7) indirectly by regulating hnRNPH1	hnRNPH1, SRC-3	LNCAp, MDA-PCa-2b and C4-2B/13 African American samples, and 17 Caucasian American samples/SCID mice	↓ cell growth and ↑ sensitivity to bicalutamide	[55]
miR-34a	↓	↓ 3'UTR	Notch-1	C4-2B, CWR22rv1, LNCAp, and VCaP	↓ proliferation and self-renewal capacity	[56]
miR-190a	↓	↓ indirectly by regulating the activator of AR	YB-1	LNCAp, C4-2, PC-3, DU-145, 22Rv1/mal nude mice	↓ proliferation and cell growth	[57]

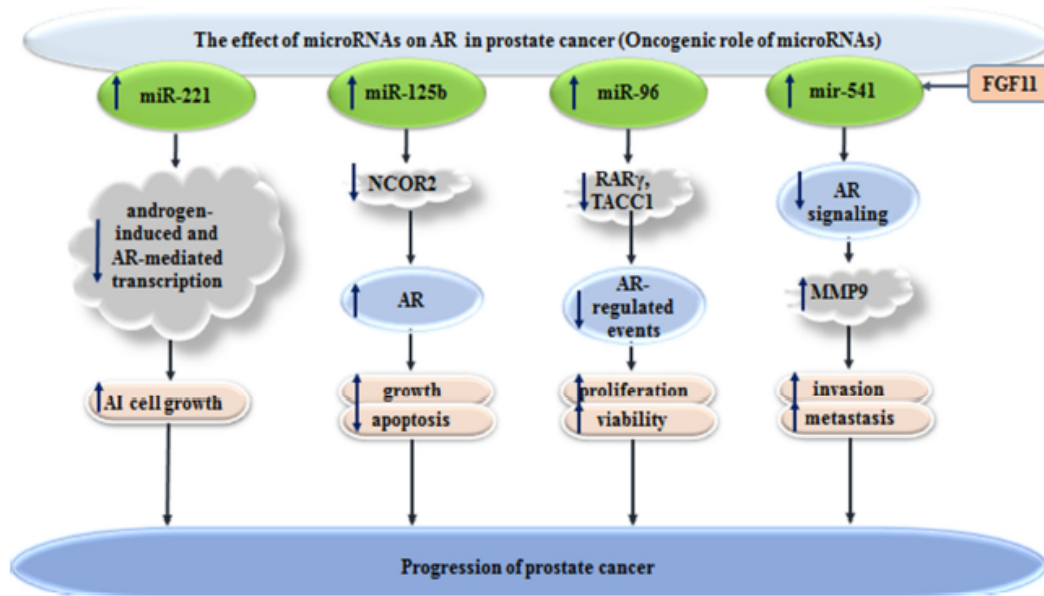


Figure 2. Effects of oncogenic miRNAs in progression of prostate cancer, through the modulation of AR signaling (↑ increased levels of, ↓ decreased levels of).

2.3. Effects of miRNAs on AR in Other Different Cancer Types

In addition to prostate cancer, the effects of miRNAs on AR have been investigated in other cancer types (Table 2). For instance, experiments in two AR-positive bladder cancer cell lines have shown that phenyl glucosamine can inactivate and degrade AR through the restoration of miR-449a expression. Lentivirus-mediated up-regulation of miR-449a has been shown to further suppress the proliferation of these cells via the induction of cell cycle arrest [58].

Table 2. Effects of miRNAs on AR in different cancer types (↓: decrease in, ↑: increase in).

Cancer Types	MiRNAs	Expression of miRNAs in Different Cancer Types	Target Region of AR mRNA/How microRNAs Affect AR	Molecular Mechanisms	Cell Line/Samples/Animal Models	Function of miRNAs in Cancer Cells	References
Bladder cancer	miR-449a	–	↓	–	UMUC3 and TCCSUP	ABDH1A treatment ↑ miR-449a ↓ proliferation, viability, ↑ cell cycle arrest	[58]
Breast cancer	miR-9-5p	↓	↓ 3'-UTR	–	MDA-MB-453, MCF-7, T-47D/11 pairs of tumor tissues and ANCIs	↓ proliferation and cell growth	[59]
Cervical cancer	miR-130a-3p	↑	↓ 3'UTR	–	20 CIN I, 20 CIN II, 30 CIN III tissue and 20 healthy tissue samples	↑ proliferation and invasion	[60]
Hepatocellular carcinoma	miR-135b-5p	↓	↓ 3'-UTR	HIF-2α, c-Myc, p27	SK-hep1, HepG2, SNK, Huh7 and HA22T	↓ proliferation, colony formation	[61]
	miR-92a-2-5p	↑	↓ 3'UTR	PHLPP/p-AKT/β-catenin signaling	SK-Hep1, Hep G2, HEK 293 T, THP-1, Hepa 1-6, HA22T/male nude mice	↑ invasion	[62]
	miR-367-3p	↓	↑ indirectly by regulating MDM2	MDM2/FKBP5/PHLPP/(pAKT and pERK) signals	SKhep1 and HA22T/126 HCC samples	↑ Sorafenib chemotherapy efficacy, ↓ invasion and metastasis	[63]
Glioma	miR-999	↓	↓ 3'UTR	circ-ASPH1, SOCS2-AS1	U251, U87MG, LN229	↓ proliferation, migration and invasion	[64]

In addition, miR-9-5p has been identified as a suppressor of AR expression in breast cancer. A feedback loop has been recognized between these genes in breast cancer cells, in which androgen agonists of AR can increase expression of miR-9-5p. In fact, miR-9-5p can inhibit the proliferation of breast cancer cells in a manner independent from the estrogen receptor (ER) status of these cells. Moreover, miR-9-5p can decrease the activity of AR-downstream signals, even in the conditions that breast cancer cells are induced by AR-agonists [59].

In cervical cancer, the oncogenic miR-130a-3p has been found to target both ER α and AR. MiR-130a-3p silencing, ER α up-regulation, and AR up-regulation have suppressed proliferation and invasion of cervical cancer cells. Besides, antagomiR-130a could decrease tumor bulk in animal models. Taken together, miR-130a-3p has a possible role in the progression of cervical cancer, through the suppression of ER α and AR [60].

In hepatocellular carcinoma, miR-135b-5p has been shown to suppress AR-mediated cell proliferation, through the regulation of HIF-2 α /c-Myc/P27 axis [61]. Moreover, macrophage-derived miR-92a-2-5p-containing exosomes could increase the invasiveness of liver cancer cells, through the modulation of AR/PHLPP/p-AKT/ β -catenin axis [62]. Finally, miR-367-3p could increase the effectiveness of sorafenib in the suppression of liver cancer metastasis via the modulation of AR signals [63].

In glioma, the circ-ASPH/miR-599/AR/SOCS2-AS1 axis has been identified as a molecular mechanism for cancer progression. In fact, circ-ASPH could mediate this function by sponging miR-599 [64].

3. Regulatory Impact of lncRNAs on AR

3.1. Regulation of AR Expression

PCGEM1 is a lncRNA with important roles in splicing events. This lncRNA has been found to interact with the splicing factors heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and U2AF65. Experiments have shown correlation between PCGEM1 and AR3, a principal and clinically important alternatively spliced form of AR in prostate cancer. Besides, androgen deprivation leads to enhanced expression of PCGEM1 and its accretion in nuclear speckles. Androgen deprivation-induced PCGEM1 has a role in regulation of the competition between two splicing factors for AR pre-mRNA [65]. Another study has identified HORAS5 as a CRPC-promoting lncRNA through assessment of patient-derived xenografts, clinical information with subsequent *in vitro* and *in vivo* confirmation studies. This lncRNA is a cytoplasmic lncRNA, which increases proliferation and viability of prostate cancer cells via sustaining AR activity even in androgen-depleted settings. Notably, HORAS5 silencing has reduced AR expression, as well as expression of oncogenic targets of AR, including KIAA0101. In clinical samples, up-regulation of HORAS5 has been associated with poor survival. Taken together, HORAS5 has been identified as targetable contributor in the induction of CRPC phenotype through maintaining oncogenic activity of AR [66]. Recent investigations identified a novel lncRNASAT1 as AR-interacting partner. The expression of this lncRNA is down-regulated in PCa tumor tissue compared to non-tumor tissue indicating a tumor suppressive function. lncRNASAT1 is up-regulated by the treatment with supraphysiological androgen level (SAL) in PCa cells and human PCa tissue *ex vivo* and mediates the SAL-induced cellular senescence [67]. Further, it has been shown that lncRNASAT1 interacts with AR on chromatin level regulating AR transactivation and AR target gene expression [68]. Another study has identified a feed-forward regulatory circuit between AR and PlncRNA-1, which enhances progression of prostate cancer [69]. In addition, PCAL7 lncRNA is another lncRNA that enhances progression of this type of cancer through promoting AR signaling [70]. Figure 3 depicts the role of a number of lncRNAs in progression of prostate cancer through modulation of AR signaling.

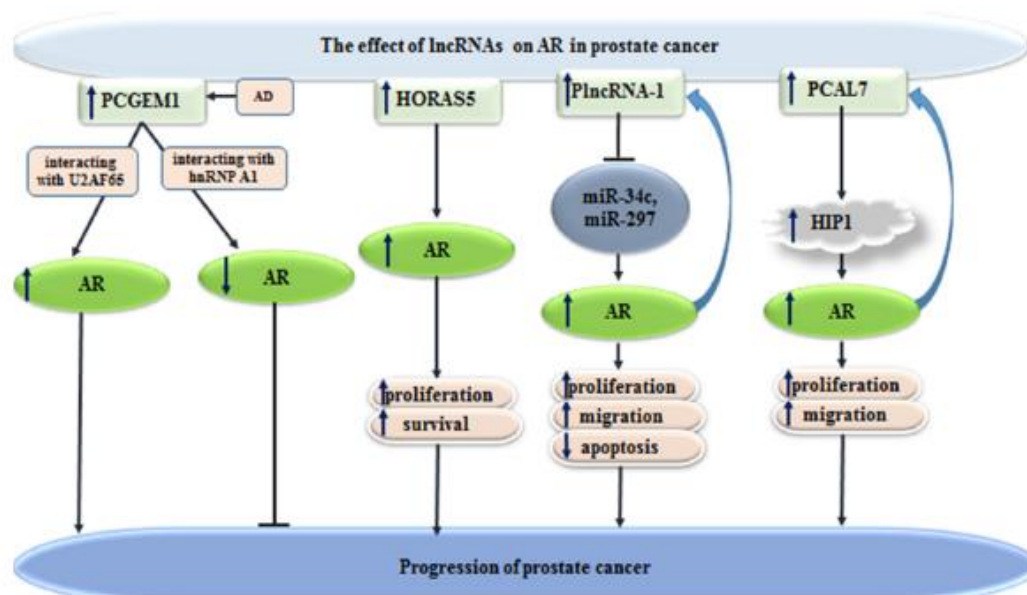


Figure 3. Effects of lncRNAs on AR in prostate cancer (↓ reduction or inhibition of, ↑ increased levels of, ↓ decreased levels of).

3.2. Regulation of AR Activity as AR-Interacting Partner

Several other oncogenic lncRNAs have been found to regulate AR signaling. For instance, HOTAIR increases AR-mediated transcriptional program and induces CRPC phenotypes [71]. Moreover, MALAT1 has been shown to suppress cell cycle progression in this type of cancer through regulation of AR signaling [72]. LINC00844 is another lncRNA that affects migration and invasion of prostate cancer cells through modulation of this route [73].

In brief, lncRNAs can affect AR levels through interacting with AR on chromatin level, regulation of AR transactivation and modulation of AR target gene expression. Some lncRNAs can also regulate stability of AR transcripts and preventing its ubiquitination. Table 3 shows the effects of different lncRNAs on AR in prostate cancer.

Table 3. Effects of different lncRNAs on AR in prostate cancer (ANCT: PCa: prostate cancer, DHT: dihydrotestosterone, CRPC: castration-resistant prostate cancer, ADPC: androgen-dependent prostate cancer, AD: androgen deprivation, DIM: 3,3'-Diindolylmethane, SAM: Synergistic activation mediator, LBD: ligand-binding domain, ↓: decrease in, ↑: increase in).

lncRNAs	Expression of lncRNAs in PCa	Target Region of AR mRNA/How lncRNAs Affect AR	Molecular Mechanisms	Cell Lines/Samples/Animal Models	Function of lncRNAs in Cancer Cells	References
ARLNC1	↑	stabilizing AR transcript	–	VCaP and LNCaP/11 benign prostate samples, 85 localized prostate cancer samples, and 37 from metastatic PCa samples/athymic nude mice	↑ Proliferation and cell growth, ↓ apoptosis	[74]
PRNCR1 and PCGEM1	↑	interact with, and increase its ligand-independent activation	DOT1L	LNCaP, RWPE, WPE, CWR22Rv1/BPH and PCa tissues male athymic Nu/Nu mice	↑ Proliferation and cell growth	[75]
HOTAIR	↑	↑ By preventing AR ubiquitination and blocking its interaction with MDM2	–	LNCaP; C4-2B/GEO analysis: GSE39988 and GSE21034	↑ cell growth and invasion	[71]
MALAT1	↑	↑ indirectly by inhibiting miR-320	miR-320	DU145, 22Rv1, PC3, LNCaP/BALB/cA-nu mice	DHT treatment ↑ proliferation and cell cycle progression	[72]
LINC00844	↓	modulated AR binding to chromatin	NDRG1	LNCaP, VCaP, and 22Rv1/GEO database: GSE109336	↓ migration and invasion	[73]
LINC00675	↑	directly modulate AR interaction with MDM2, inhibited AR's ubiquitination, ↑ indirectly by regulating the co-activator of AR	MDM2, GATA2	LNCaP-SE, LNCaP-JP, LNCaP, LNCaP-C4-2b, 293T/male BALB/c nude mice	↑ tumor formation, tumor growth and drug resistance	[76]

Table 3. Cont.

LncRNAs	Expression of LncRNAs in PCa	Target Region of AR mRNA/How LncRNAs Affect AR	Molecular Mechanisms	Cell Lines/Samples/Animal Models	Function of LncRNAs in Cancer Cells	References
CCAT1	↑	↑ by binding to P68	DDX5 (P68), miR-28-5P	PC3, DU145, and LNCaP/8 ADPC tissues and 4 CRPC samples/BALB/C nude mice	↑ proliferation, colony formation, and cell cycle progression, ↓ apoptosis	[77]
PCGEM1	↑ in AD	↑ AR3 by interacting with U2AF65, ↓ AR3 by interacting with hnRNP A1	U2AF65, hnRNP A1	LNCaP, CWR22Rv1, LNCaP95, HECK293T/male SCID mice	↑ castration resistance	[65]
SOCS2-AS1	↑ Castration-resistant Prostate Cancer Cells	↑ by regulating cofactor recruitment for epigenetic controls	TNFSF10	LNCaP, VCaP, LITAD	↑ castration-resistant and cell growth, ↓ apoptosis	[78]
HORAS5	↑	↑ post-transcriptional maintenance of AR mRNA stability	–	LNCaP and C4-2 male, immunocompromised NOD/SCID mice	↑ proliferation and survival	[66]
PCLN16	↑	↑ indirectly by regulating HIP1	HIP1	NCaP and VCaP/tumor tissues and ANC/Ts	↑ proliferation, migration and cell growth	[79]
HOTAIR	↑ in PCa cells after co-culture with HMC-1 cells	↓ at the transcriptional level	PRC2, MMP9	LNCaP, CWR22Rv1, C4-2, C4-2B and HMC-1/male nude mice	recruitment of mast cells; ↑ invasion and stem/progenitor cell population	[80]
PlncRNA-1	↑	↑ by sponging AR-targeting microRNAs	miR-34c and miR-297	RWPE-1, 22RV1, LNCaP, PC3 and DU145/16 PCa tissue samples, 35 biopsy-negative and 37 biopsy-positive blood samples/male nude mice	↑ proliferation, migration and viability, ↓ apoptosis	[69]
PCAL7	↑	↑ indirectly by regulating HIP1	HIP1	104 tumor tissues and ANC/Ts	↑ proliferation, migration	[70]
Malat1	↑	↑ AR-v7 indirectly by interacting with SF2 to splice the AR transcript	SF2	VCaP and ErzR-PCa C4-2/10 CRPC samples before (Pre-Erz) and after (Post-Erz) Erz treatment/nude mice	↑ Erz resistance	[81]
PlncRNA-1	↑	↑	NKX3-1	LNCaP, LNCaP-AI, PC-3, C4-2, RWPE-1 and PWR-1E/16 pairs of PCa tissues and ANC/Ts, 14 pairs of PCa tissues and BPH tissues	↑ proliferation and viability, ↓ apoptosis	[82]
LBCS	↓	↓ 3' UTR	hnRNPK	LNCaP, LNCaP-Bic, and LNCaP-AI/130 PCa tissues and 32 BPH tissues plus 70 PCa tissues and 10 BPH	↓ castration resistance	[83]
PCGEM1	↑	upregulation of PCGEM1 by SAM; ↑ AR3	p54/nrb	LNCaP and CWR22Rv1/male SCID mice	↑ tumor growth and castration resistance, ↓ apoptosis DIM: ↓ PCGEM1-mediated castration resistance	[84]
PCGEM1	↑	facilitating AR binding to some promoters	c-Myc	LNCaP,	↑ glucose uptake and glycolysis, cell-cycle progression, proliferation, and survival	[85]
LOC283070	↑	↑ indirectly by inhibiting PHB2	PHB2	LNCaP and LNCaP-AI	↑ proliferation and migration	[86]
lnc-OPHN1-5	–	↓ 3'UTR	hnRNPA1	C4-2R, C4-2BR, C4-2B/75 PCa samples/male NOD CRISPR Pkdc Il2r Gamma triple-immunodeficient mice	↑ Erz sensitivity	[87]
GAS5	↓	↓ directly by interacting with LBD of AR	–	C4-2, DU145, 293T/GSE6919	↓ proliferation, ↑ apoptosis	[88]
GHSR05	↑	↓	PPP2R2C	PC3, LNCaP, DU145, DUCaP	↑ proliferation, growth, migration, survival, and resistance to the cytotoxic drug docetaxel	[89]
PCA3	↑	PCA3 knock down → ↑ regulation of AR cofactors	–	LNCaP	modulating the expression of EMT markers and AR cofactors Δ PCA3: ↓ cell viability	[90]
PRNCR1	↑	↑	–	LNCaP and C4-2	↑ proliferation and invasion, ↓ apoptosis	[91]

3.3. Effects of lncRNAs on AR in Other Different Cancer Types

The effects of lncRNAs on AR signaling have also been assessed in other types of cancers. In bladder cancer, XIST has been found to be up-regulated parallel with up-regulation of AR. Over-expression of XIST and AR has been correlated with advanced TNM stage in this cancer. XIST silencing has decreased proliferation, invasion, and migratory potential of bladder cancer through modulation of AR signaling. Mechanistically, XIST suppresses expression of miR-124 through direct interaction. Besides, miR-124 has been

shown to target 3'UTR of AR [92]. Another experiment in bladder cancer has shown over-expression of LINC00460. LINC00460 levels have been correlated with poor prognosis of these patients. LINC00460 silencing decreased proliferation of 5637 and T24 bladder cancer cells. Based on the observed down-regulation of AR in bladder urothelial cancer tissues, it has been suggested that LINC00460 might exert its oncogenic roles through modulation of AR expression [93]. LINC00278, SLNCR1, SARCC and HOTAIR are other lncRNAs whose effects on AR have been investigated in different cancer types (Table 4).

Table 4. Effects of lncRNAs on AR in different cancer types (ND: nutrient deprivation, ccRCC: clear cell renal cell carcinoma, ↓: decrease in, ↑: increase in).

Cancer Types	lncRNAs	Expression of lncRNAs in Different Cancer Types	Target Region of AR mRNA/How lncRNAs Affect AR	Molecular Mechanisms	Cell Lines/Samples/Animal Models	Function of lncRNAs in Cancer Cells	References
Bladder cancer	XIST	↑	↑ by sponging AR-targeting microRNA	miR-124	TCC-SUP, EJ, SW780 and UM-UC-3, SV-HUC-1 67 pairs of tumor tissues and ANCTs	↑ proliferation, migration and invasion	[92]
	LINC00460	↑	↓	—	5637, T24, J82, TCCSUP, UM-UC-3 and SV-HUC-1/TCGA database	↑ proliferation and migration	[93]
Esophageal squamous cell carcinoma	LINC00278	↓	indirectly inhibited interaction between YY1 and AR	YY1, eEF2K, YY1BM	DMEM, RPMI1640, FBS, Eca-109, TE-1, and KYSE-30/281 pairs of ESCC tissues and ANCTs	ND treatment: ↑ LINC00278: ↓ survival, ↑ apoptosis	[94]
Melanoma	SLNCR1	↑	SLNCR1 binds to AR-binding motifs 1 and 2	—	A375, HEK293T, WM1976,	↓ binding SLNCR1 to AR: ↓ SLNCR1-mediated invasion	[95]
	SLNCR1	↑	↑ AR binding to the MMP9 promoter	Bm3a, MMP9	A375, HEK293T, CY and WM	↑ invasion	[96]
Renal cell carcinoma	SARCC	↓	destabilizing AR protein	miR-143-3p, AKT, MMP-13, K-RAS and P-ERK	SW839, OSRC-2, A498, 769-P, 786-O, Caki-1, Caki-2, HK2/66 ccRCC tissues and 8 metastatic ccRCC tissues/male athymic nude mice	↓ proliferation, invasion, migration and resistance to Sunitinib	[97]
	SARCC	Differentially expressed by hypoxia in a VHL-dependent manner	↓ binding and destabilizing AR protein	HIF-2α, C-MYC signals	SW839, OSRC-2, A498, 769-P, and 786-O, Caki-1, Caki-2, HK-2 and 293T/16 ccRCC samples/male athymic nude mice	Differentially modulates proliferation under hypoxia	[98]
	HOTAIR	↑	↑	GLI2	HK-2, 786-O, ACHN, 769-P, SW839, OSRC-2, HUVEC/male nude mice	↑ angiogenic phenotype and stemness	[99]

4. Effects of circRNAs on AR

circZMIZ1 has been shown to be over-expressed in plasma samples of patients with prostate cancer compared with those having benign prostatic hyperplasia (BPH). In vitro studies have shown that circZMIZ1 silencing inhibits cell proliferation and arrests cells at G1. Functionally, circZMIZ1 enhances expression of AR and its splice variant 7 (AR-V7) [100].

On the other hand, expression of cir-ITCH has been shown to be decreased in the tissues and cell lines of prostate cancer compared to corresponding controls. Up-regulation of cir-ITCH could suppress proliferation, migratory potential, and invasiveness of human prostate cancer cells. A reciprocal inhibitory effect has been found between this circRNA and miR-17. Several molecules within Wnt/ β -catenin and PI3K/AKT/mTOR cascades have been found to be influenced by cir-ITCH. This circRNA could indirectly reduce expression of AR through regulating the coactivator of this nuclear factor [101]. hsa_circ_0004870 [102] and circRNA17 [103] are two other circRNAs that reduce AR-V7 levels through U2AF65 and miR-181c-5p mediated routes, respectively, thus enhancing efficacy of enzalutamide. Table 5 shows the effects of different circRNAs on AR in prostate cancer.

Table 5. The effects of different circRNAs on AR in prostate cancer (HCs: healthy controls, Enz: enzalutamide, ↓: decrease in, ↑: increase in).

circRNAs	Expression of circRNAs in PCa	Target Region of AR mRNA/How circRNAs Affect AR	Regulated Pathway	Cell Lines/Samples/Animal Models	Function of circRNAs in Cancer Cells	References
circZMZ1	↑	↑ AR and AR-V7	–	DU145, C4-2, LNCaP, 22Rv1, RWPE-1, 14 PCa samples, and 14 HCs	↑ proliferation, ↓ G1 arrest	[100]
circ-ITCH	↓	↓ indirectly by regulating the coactivator of AR	miR-17, Wnt/β-Catenin, and PI3K/AKT/mTOR Signaling Pathways	RWPE-1, LNCaP, PC-3/10 pairs of tumor tissues and ANCIs	↓ migration and invasion	[101]
hsa_circ_0004870	↓	↓ AR-V7 indirectly through U2AF65	RBM39, U2AF65	LNCaP, BPH1, 22Rv1	↓ Enz resistance	[102]
circRNA17	↓	↓ AR-v7 indirectly by regulating miR-181c-5p	miR-181c-5p	C4-2, CWR22Rv1, and 293T/13 BPH samples, and 14 PCa samples/male nude mice	↓ Enz resistance and invasion	[103]

5. Effects of AR on ncRNAs

5.1. AR Responsive miRNA

AR has been found to regulate the expression of several ncRNAs. For instance, activated AR has been shown to increase the expression of miR-203 and decrease the expression of SRC kinase in prostate cancer model systems. MiR-203 has a direct interaction with the 3'UTR of SRC and affects its stability following AR activation. A reduction in AR-induced miR-203 levels has been associated with an increased growth and migration potential of prostate cancer cells. The dysregulation of the AR signaling in prostate cancer cells results in the over-expression of SRC and enhancement of metastatic ability of these cells [104].

Another experiment has shown that AR represses the expression of both miR-221/-222. The derepression of their expression after androgen deprivation has enhanced proliferation of prostate cancer cells via facilitating G1/S phase transition. Although this effect might be transient, it has a possible role in the evolution of CRPC. The restoration of AR activity via AR up-regulation could subsequently down-regulate miR-221/-222 [105]. MiR-182-5p is another AR-regulated miRNA that facilitates the progression of prostate cancer by targeting the ARRDC3/ITGB4 axis [106]. Another study has shown the effects of AR on the down-regulation of miR-1 expression and subsequent suppression of TCF7. This process has been shown to participate in the evolution of resistance to androgen deprivation in this type of cancer [107].

AR has been shown to differentially affect the metastasis of prostate and breast cancers, through distinctively changing vasculogenic mimicry (VM) formation. In fact, AR can enhance miR-525-5p transcription in prostate cancer, while decreasing its transcription in breast cancer by binding to different AREs in the precursor promoter of this miRNA. NFIX and HDAC2 have been identified as co-factors of AR in prostate and breast cancer cells, respectively [108]. Figure 4 shows the impact of AR on miRNAs expressions, in the context of prostate cancer.

5.2. AR Responsive lncRNA and circRNA

AR has also been shown to affect expression of several lncRNAs. For instance, LINC00304 is an androgen-responsive lncRNA that induces cell cycle transition and increases the proliferation of prostate cancer cells, through the regulation of CCNA1 [109]. Moreover, the androgen-associated up-regulation of POTE-AS1 has been shown to affect apoptosis-associated pathways, in favor of prostate cancer cells survival [110]. On the other hand, the expression of PSLNR has been shown to be decreased by androgens. This lncRNA suppresses prostate cancer progression partly through regulation of the p53-dependent axis [111]. PART1, as another androgen-regulated lncRNA, can influence the toll-like receptor pathways in this type of cancer. The expression of PART1 has been induced in prostate cancer cells treated with 5α-dihydrotestosterone, indicating that this lncRNA is directly induced by androgen [112]. Figure 5 shows the effects of AR on lncRNAs in prostate cancer.

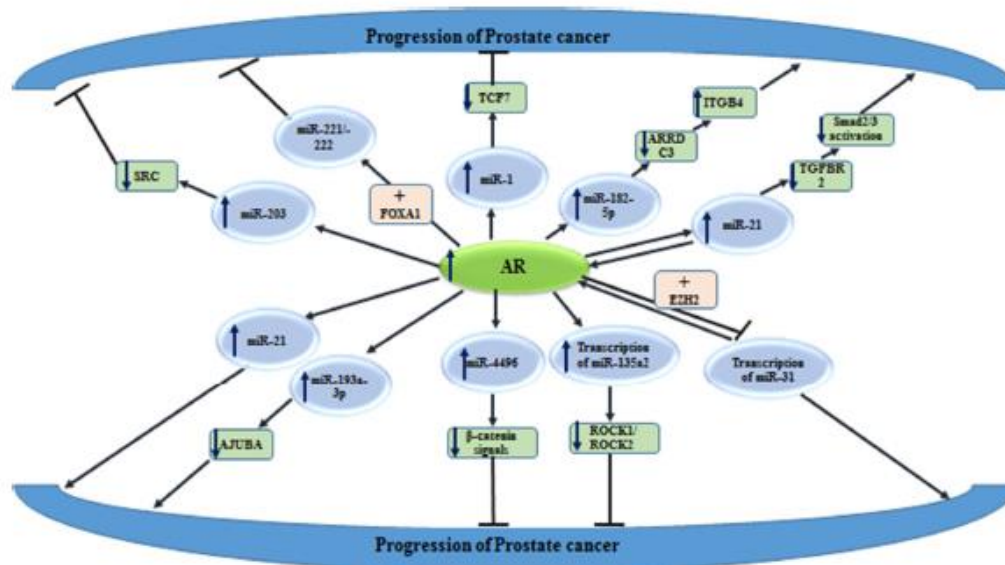


Figure 4. Effects of AR on miRNAs expressions, in the context of prostate cancer. Detailed information about these miRNAs is shown in Table 6 (↓ reduction or inhibition of, ↑ increased levels of, ↓ decreased levels of).

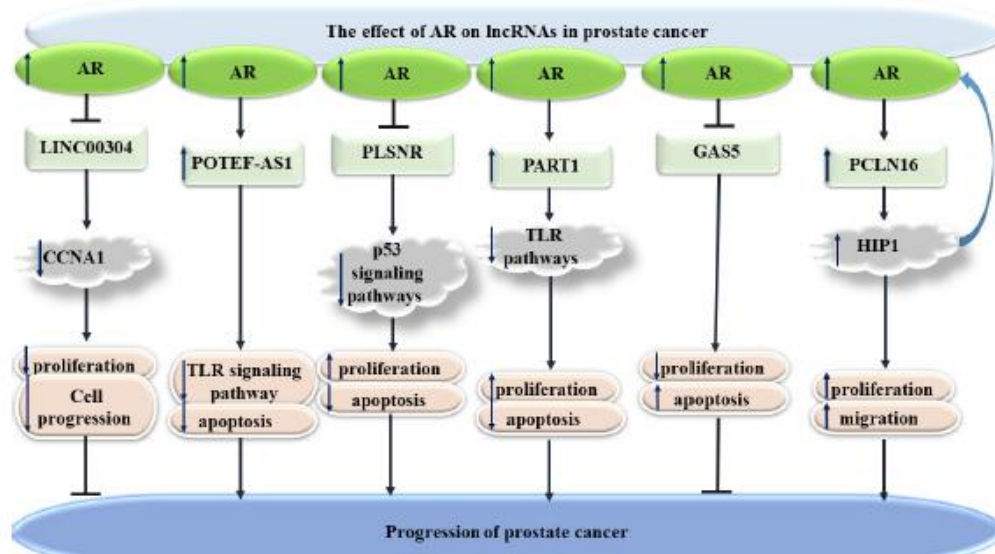


Figure 5. Effects of AR on lncRNAs in prostate cancer (↓ reduction or inhibition of, ↑ increased levels of, ↓ decreased levels of).

Other studies, in the context of prostate cancer, have identified AR-regulated miRNAs and lncRNAs. Moreover, a number of circRNAs, such as circRNA-51217, circRNA-ARC1, and circZMIZ1, have been found to be influenced by AR signaling (Table 6). A recent study has identified more than 3000 androgen-responsive circRNAs, using a microarray technique. Notably, the expression of more than 1000 of these circRNAs has been consistent with the expression of their parent genes, suggesting that AR may modulate their expression at the transcriptional level [113].

Table 6. Effects of AR on different ncRNAs in prostate cancer. (PCa: prostate cancer, CRPC: castration-resistant prostate cancer, HCs: healthy controls, NE: neuroendocrine, BPH: benign prostatic hyperplasia, CRPC: castration-resistant prostate cancer, DOX: doxorubicin, NED: neuroendocrine differentiation, NE: neuroendocrine, Enz: enzalutamide, PRAD: prostate adenocarcinoma, R-2HG: R-2-hydroxyglutarate, ↓: decrease in, ↑: increase in).

ncRNAs	Regulation by AR	Molecular Mechanisms	Cell Line/Samples/Animal Models	Function of ncRNAs in Cancer Cells	References
miR-203	↑	SRC	—	↓ migration, growth, and metastasis	[104]
miR-221/-222	↓	FOXA1	LNCAp and C4-2B/LuCaP 35 and LuCaP 35CR xenografts	↑ proliferation and development of CRPC	[105]
miR-182-5p	↑	ARRDC3, ITGB4	RWPE-1, 22RV1, LNCAp, DU145/65, pairs of tumor tissues and ANCTs, and 18 pairs of tumor tissues and ANCTs/male nude mice	↑ proliferation, invasion, migration and growth, ↓ apoptosis	[106]
miR-1	↑	TCF7	PC3, LNCAp/111 PCa samples/nude mice	↓ proliferation	[107]
miR-525-5p	↑	SLP1, NFIX	—	↓ PCa metastasis	[108]
miR-21	↑	TGFBR2, Smad2/3	RWPE-1, MDA-PCa-2b, 22RV1, PC-3, and LNCAp/male athymic nude mice	↓ tumor-suppressive activity of TGFβ pathway	[18]
miR-21	↑	—	LNCAp, LAPC-4, C4-2, CWR22Rv1/10 PCa samples/male athymic Nu/Nu mice	↑ androgen-dependent and -independent proliferation, tumor growth, and castration resistance	[114]
miR-193a-3p	↑	AJUBA	LNCAp, C4-2B	↑ migration and metastasis	[115]
miR-4496	↑	β-catenin signals	C4-2 and PC3	↓ invasion	[116]
miR-135a	↑	ROCK1 and ROCK2	LNCAp, PC-3/56 pairs of tumor tissues, and ANCTs/chick embryos and adult male mice	↓ invasion	[117]
miR-31	↓	EZH2	RWPE-1, VCaP, LNCAp, 22RV1, PC3, DU145, and HEK293	↓ proliferation, cell growth and colony formation, ↑ cell cycle arrest	[22]
miR-421	↓	NRAS, PRAME, CUL4B, and PFKFB2	LNCAp, 22RV1, PC-3 and DU 145/microarray data: GSE21036, GSE45604, GSE38241, and 13 PCa samples 11 samples without PCa	↓ viability, glycolysis and migration, ↑ cell cycle arrest	[118]
miR-1	↑	SRC	LNCAp, DU145RasV12G37, DU145/RasB1/28 HCs, 98 primary tumor, and 13 distant metastasis samples/male nude mice	↓ proliferation, invasion, and metastasis	[119]
miR-32	↓	NSE	RWPE1, LNCAp, and CWR22Rv1/male nude mice	enzalutamide treatment (most cells) → suppression of AR: ↑ miRNA32: ↑ NE differentiation	[120]
miR-21 promoter	↑	PDCD4	LNCAp and HEK 293, LAPC4/male athymic nu/nu mice	↑ androgen-dependent and -independent growth and castration resistance, ↓ apoptosis	[121]
miR-22	↓	LAMC1	LNCAp, PC3, DU145, VCaP, CWR22RV1, DUCaP, BPH-1, PC3-AR, LAPC-4, RWPE-1, and EPI567/ 41 pairs of tumor tissues and ANCTs, TCGA analysis: 52 pairs of tumor tissues and ANCTs	↓ migration	[122]
miR-29a	↓	MCL1	LNCAp, PC3, DU145, VCaP, CWR22RV1, DUCaP, BPH-1, PC3-AR, LAPC-4, RWPE-1, and EPI567/ 41 pairs of tumor tissues and ANCTs, TCGA analysis: 52 pairs of tumor tissues and ANCTs	↓ migration and viability, ↑ apoptosis	
miR-99a/let7c/125b-2 cluster	↓	IGF1R	LNCAp, C4-2, and PC3	↓ proliferation	[123]
miR-2909	↑	TGFBR2, TGFβ signaling, PSA	PC3 and LNCAp	↑ cell growth	[43]
miR-32	↑	BTG2	LNCAp/ 5 BPH and 28 PCs, plus 7 BPH and 14 CRPCs	↑ cell growth	[124]
miR-148a	↑	PIK3IP1	LNCAp/ 5 BPH and 28 PCs, plus 7 BPH and 14 CRPCs	↑ cell growth and the number of cells in the S phase	
miR-194	↓	FOXA1, ERK Signaling	LNCAp, PC3, and 22RV1	↑ EMT process, migration, invasion and epithelial-neuroendocrine transdifferentiation	[125]
miR-27a (miR-23a/27a/24-2 cluster)	↑	PHB	HeLa, Cos-1, LNCAp, DuCaP, VCaP, C42, DU145, PC3, and PC3wtAR	↑ cell growth	[126]
miR-200b	↑	—	PC3/male athymic mice	↓ proliferation, invasion, cell growth, EMT process and metastasis	[127]

Table 6. Cont.

ncRNAs	Regulation by AR	Molecular Mechanisms	Cell Line/Samples/Animal Models	Function of ncRNAs in Cancer Cells	References
miR-19a	↑	SUZ12, RAB13, SC4MOL, PSAP, and ABCA1	LNCAp	↑ cell viability	[128]
miR-27a	↑	ABCA1 and PDS5B	LNCAp	↑ cell viability	
miR-133b	↑	CDC2L5, PTPRK, RB1CC1, and CPNE3	LNCAp	↑ cell viability	
miR-22	↑ and ↓ during two different mechanisms	IL-6, AR c-MYC, miR-22, PHF8, KDM3A (↑) and AR c-MYC, miR-22, PHF8, KDM3A (↓)	LNCAp-Abl, LNCAp-IL-6, LNCAp/male mice	↑ sensitivity LNCAp-Abl cells to the enzalutamide treatment, ↓ proliferation	[129]
miR-17-92a	↑	ATG7	NCAp, 22Rv1, DU145, and PC-3	↓ autophagy induced by celestrol treatment	[130]
miR-204	↓	XRN1, PSA, miR-34a	LNCAp, 22Rv1, PC-3, and CL1/171, BPH, plus PCa samples/nude mice and rats	↓ growth and colony formation of LNCAp and 22Rv1 cells but ↑ growth and colony formation of CL1 and PC-3 cells	[52]
miR-34	miR-34a ↑ after DOX, but did not change with si-AR, miR-34c ↑ after DOX, but to a small extent changed with si-AR	p53, SPAK, MDC1, and CaMKII	LNCAp, C4-2b, PC3, and DU145	↑ caspase activity and apoptosis	[131]
miR-135a	↑	MMP11, RBAK, PI3K/AKT pathway	LNCAp, 22Rv1, DU145, PC-3, and WPMY-1	↓ proliferation and migration, ↑ cell cycle arrest, and apoptosis	[132]
the miR-200 family, miR-17-92 cluster, and miR-99a/let-7c/miR-125b-2 family	↓	HOXC6 and NKX2-2	RWPE-1 and LNCAP	↓ metastasis and EMT process	[133]
miR-101	↑	–	LNCAp, 22Rv1, DU145, and PC-3	↓ celestrol-induced autophagy	[134]
miR-27a	↓	MAP2K4, PI3K signaling pathways	TCGA database: GSE45604 and GSE21036	↓ proliferation and migration, ↑ apoptosis	[135]
miR-190a	↓	YB-1	LNCAp, C4-2, PC-3, DU-145, 22Rv1/mal nude mice	↓ proliferation and cell growth	[57]
ARLNC1	↑	–	VCAp and LNCAp/11 benign prostate samples, 85 localized prostate cancer samples, and 37 from metastatic PCa samples/athymic nude mice	↑ Proliferation and cell growth, ↓ apoptosis	[74]
PRCAT38	↑	TMPRSS2, FOXA1	LNCAp, DU145, and VCAp/20 samples (HCs and PCa)	↑ cell growth and migration	[136]
H19	↓	–	LNCAp	Enzalutamide treatment: ↑ H19	[137]
GAS5	↓	–	PC3 and 22Rv1	dexamethasone treatment in AR-PCa cell line PC3: ↑ GAS5; ↓ proliferation, ↑ G0/G1 cell arrest	[138]
p21	↓ AR binding to the ARE5, ↑ AR binding to the AGRE	EZH2, STAT3	C4-2, CWR22RV1, NE1.8, NCI-H660, and DU145	Enz treatment: ↓ AR binding to the ARE5 region of p21; ↑ p21: ↑ NED, NE-like structure	[139]
LINC00304	↓	CCNA1	LNCAp, 22Rv1, DU145, PC-3, and WPMY-1/GEO database: GSE38241: 18 PCa samples and 21 HCs	↑ proliferation and cell cycle progression	[109]
POTEF-AS1	↑	TLR signaling pathway	LNCAp, VCAp, LTAD, and VCAp-LTAD	↑ cell growth, ↓ apoptosis	[110]
PLSNR	↓	p53 signaling pathway	LNCAp, 22Rv1, DU145, PC-3, and WPMY-1/GEO database: GSE55909, 3 pairs of tumor tissues and ANCTs, 13 tumor tissues and ANCTs, plus 20 pairs of PCa sample	↑ proliferation and cell-cycle progression, ↓ apoptosis	[111]
PART1	↑	TLR pathways	LNCAp and PC3/30 pairs of tumor tissues and ANCTs	↑ proliferation, ↓ apoptosis	[112]
GAS5	↓	–	LNCAp, 22Rv1, DU145, PC3, WPMY-1 14 tumor tissues, and 11 normal tissues	↑ proliferation, ↓ apoptosis	[140]
PCLN16	↑	HIP1	NCAp and VCAp/tumor tissues and ANCTs	↑ proliferation, migration, and cell growth	[79]

Table 6. Cont.

ncRNAs	Regulation by AR	Molecular Mechanisms	Cell Line/Samples/Animal Models	Function of ncRNAs in Cancer Cells	References
PlncRNA-1	↑	miR-34c and miR-297	RWPE-1, 22RV1, LNCaP, PC3 and DU145/16 PCa tissue samples, 35 biopsy-negative and 37 biopsy-positive blood samples/male nude mice	↑ proliferation, migration and viability, ↓ apoptosis	[69]
PCAL7	↑	HIP1	104 tumor tissues and ANCts	↑ proliferation, migration	[70]
PCGEM1	↑	–	131 primary PCa, 19 metastasized PCa, and 29 normal prostatic tissue samples/intact mice	↑ PCGEM1 in primary PCa Androgen receptor regulated PCGEM1 in vivo.	[141]
DRAIC	↓	–	Vcap, PC3M-luc	↓ transformation of cuboidal epithelial cells to fibroblast-like morphology, migration, and invasion	[142]
PCAT29	↓	–	Vcap, PC3M-luc	↓ migration and metastasis	
a subset of TPCATs, most notably EPCAT	↑	ERG	LNCaP, VCaP, and DuCaP/87 prostatectomy-treated samples	↑ proliferation, migration	[143]
PCAT29	↓	–	VCaP, LNCaP, and DU145/GEO database: GSE58397/male nude athymic BALB/c nu/nu mice	↓ proliferation, migration	[144]
Malat1	↓	–	VCaP and ErzR-PCa C4-2/10 CRPC samples, before (Pre-Erz) and after (Post-Erz) Erz treatment/nude mice	Erz treatment ↑ Malat1	[81]
PlncRNA-1	↑	NKX3-1	LNCaP, LNCaP-A1, PC-3, C4-2, RWPE-1 and PWR-1E/16 pairs of PCa tissues and ANCts, 14 pairs of PCa tissues and BPH tissues	↑ proliferation and viability, ↓ apoptosis	[82]
CtBP1-AS	↑	PSE, CtBP1	VCaP, LNCaP, DU145, RWPE and PrEC/105 PCa samples	↑ castration-resistant tumour growth	[145]
RP11-783K16.13, RP11-228B15.4, and CTD-2228K2.7	↑	–	GEPIA dataset	Higher expression of the lncRNAs were significantly correlated with shorter DFS time in PRAD.	[146]
PCAT1	↑ rs7463708 increases binding ONECUT2 and AR to the PCAT1 promoter	ONECUT2, LSD1, GNM1, and DHCR24	LNCaP, LNCaP/shPCGEM1/TCGA dataset	↑ proliferation and tumor growth	[147]
FAM83H-AS1	↑	miR-15a, CCNE2	LNCaP, LNCaP-A1, and DU145/GEPIA data sets: GSE513217 and GSE55062, plus 20PCa and 8 normal samples	↑ proliferation, migration, and cell cycle progression	[148]
DANCR	↓	TIMP2/3	CWR22Rv1, PC-3, and C4-2B/nude mice	↑ migration and invasion	[149]
GAS5	↓	–	LNCaP/GSE22606	↓ proliferation, ↑ apoptosis	[88]
PCAT18	↑	PES	LNCaP, C4-2, BPH/131 PCa, and 29 normal samples/NOD/SCID mice	↑ proliferation, migration, and invasion	[150]
RP1-4514.2, LINC01138, and SUZ12P1	↓	–	22RV1, DU145, PC-3 and LNCaP, WMPY-1/3 tumor tissues and 11 ANCts, plus 14 tumor tissues, and 11 ANCts and TCGA database	–	[151]
KLKP1	↑	–	22RV1, DU145, PC-3 and LNCaP, WMPY-1/3 tumor tissues, 11 ANCts plus 14 tumor tissues, and 11 ANCts and TCGA database	–	
TMPO-AS1	↓	–	LNCaP, DU145, 22Rv1, PC-3, and WPMY-1/54 pairs of PCa samples and TCGA data	↑ proliferation and migration, ↓ apoptosis	[152]
circRNA-51217	↓	R-2HG, miRNA-646, TGFβ1/p-Smad2/3 signaling, ADAR2	C4-2, PC3, DU145, LNCaP, and HEK293T/TCGA database	IDH1 mutation And R-2HG: ↑ circRNA-51217: ↑ invasion	[153]
circRNA-ARC1	↓	miR-125b-2-3p/miR-4736/PPARγ/MMP-9 signals	CWR22Rv1 and C4-2	Erz treatment: ↑ invasion	[154]
circZME1	↑	–	DU145, C4-2, LNCaP, 22RV1, RWPE-1, 14 PCa samples, and 14 HCs	↑ proliferation, ↓ G1 arrest	[100]

In brief, the effect of AR on the expression of ncRNAs is mainly associated with its role as a transcription factor.

Effects of AR on expression of ncRNAs are also implicated in the pathoetiology of bladder, breast, liver, renal, and gastric cancers (Table 7). Yet, these effects are largely

context-dependent. For instance, AR could reduce the expression of miR-21 in breast cancer [155], while inducing its expression in hepatocellular carcinoma [156].

Table 7. Effects of AR on ncRNAs in different cancer types (Enz: enzalutamide, VM: vasculogenic mimicry, DHEA: dehydroepiandrosterone, RBM: ccRCC bone metastases, ↓: decrease in, ↑: increase in).

Cancer Types	ncRNAs	Regulation by AR	Molecular Mechanisms	Cell Line/Samples/Animal Models	Function of ncRNAs in Cancer Cells	References
Bladder cancer	miR-525-5p	↓	SLPI, HDAC2	–	↑ metastasis	[108]
	circFNTA	↑	ADAR2, miR-370-3p, FNTA pathway, KRAS signaling	SVHUC, T24, J82, 5637, and UMUC3/male athymic BALB/c nude mice	↑ invasion, metastases, and cisplatin chemo-resistance	[157]
	circRNA-ARCI	↑	miR-125b-2-3p/miR-4736/PPARγ/MMP-9 signals	T24 and UMUC3	Enz treatment: ↓ invasion	[154]
Breast cancer	let-7a	↑	CMYC and KRAS	MCF-7, MDA-MB-453, and MDA-MB-231/24 breast cancer samples	↓ proliferation, cell growth	[158]
	miR-21	↓	–	MCF-7, ZR-75-1, MDA-MB-231, SKBR3, and LNCap	↑ proliferation (miboleron: ↓ miR-21: ↓ proliferation)	[155]
Triple-negative breast cancer	ARNILA	↓	miR-204, Sox4	MDA-MB-231 and Hs578T, MDA-MB-436/88 TNBC samples/female BALB/c nude mice	↑ migration, invasion, and EMT process	[159]
Early Hepatocarcinogenesis	miR-216a	↑	TSLC1	HepG2/48tumor tissues and 13 non-tumor tissues/male athymic nude mice	↑ proliferation and migration	[160]
Hepatocellular carcinoma	miR-21	↑	PDCD4, ERβ	HepG2, HBEC2-KT/male C57BL/6 mice	DHEA: ↑ miR-21: ↑ proliferation	[156]
	miR-146a-5p	↓	BRCA1 and BCL2	SK-HEP-1 and HepG2/TCGA database analysis/male nude mice	Enz plus Olaparib treatment: ↑ miR-146a-5p: ↓ proliferation, cell growth, and viability	[161]
	circRNA7	↓	miR-7-5p, VE-Cadherin, Notch4	SKhep1, HA22T	↑ formation of VM	[162]
	circ-LNPEP	↓	miR-532-3p, RAB9A	HA22T, SK-HEP-1, and 293/male nude mice	↑ invasion and metastasis	[163]
	circARSP91	↓	ADAR1	MHCC-97h, LM3 and LO2, HEK-293T/83 pairs of tumor tissues, and ANCTs/nude mice	↓ tumor growth	[164]
Melanoma	miR-539-3p	↑	MITF-AXL signals, USP13	A375 and WM115 and C32/102 melanoma tissue samples/male nude mice	↑ invasion and metastasis	[165]
	SLNCR	SLNCR and AR cooperatively regulate several growth-regulatory genes.	p21, EGR1, MMP9	WM1976 or A375, SK-MEL-28, WM858	↑ proliferation and invasion	[166]
	SLNCR1	↑	Bmi3a, MMP9	A375, HEK293T, CY and WM	↑ invasion	[96]
Cholangiocarcinoma	ZEB1-AS1	↑	miR-133b, HOXB8	HIBEC, QBC939, CCLP-1, RBE, TFK-1/54 pairs of tumor tissues, and ANCTs/female BALB/c nude mice	↑ migration, invasion, EMT process, viability, and stemness	[167]
Gastric cancer	PART1	AR interacts with PART1 to stimulate PLZF expression	PLZF, EZH2, PDGFRβ/P13K/Akt signaling pathway	GES-1, MGC-803, BGC-823, and SCC-7901/ GEO database: GSE27342, GSE33335, and GSE3072, plus 136 GC samples	↓ migration, invasion, and metastasis	[168]
Clear cell renal cell carcinoma	TANAR	↑	TWIST1	786O, SW839, HEK293T/51 ccRCC tissues, and 23 ANCTs/male athymic BALB/c nude mice	↑ VM formation	[169]
	circHIAT1	↓	HIAT1, miR-195-5p/29a-3p/29c-3p, and CDC42	SRC-2, VHL(b), Caki-1, SW-839, and ACHN	↓ migration and invasion	[170]
	circEXOC7	↓	DHX9, miR-149-3p, CSF1	SW839, 786-O, Caki-1, ACHN, HEK293T/4 RCC samples with RBM, and 10 RCC samples without RBM/Balb/c nude mice	↑ RBM and osteolytic formation	[171]

6. Discussion

AR has an essential role in the pathogenesis of human cancers, particularly prostate cancer. Since it is required for the development of prostate cancer, androgen deprivation therapy is regarded as a treatment for this type of cancer. Thus, the identification of the regulatory mechanisms of AR signaling is important in the design of treatment options. The importance of this process is further highlighted by the fact that castration resistance might occur during the course of treatment, as a result of expression of constitutively active AR splice variants [172], whose expressions can be modulated by ncRNAs.

Integrative transcriptomic analyses of diverse cancer cell lines and tissues have resulted in the identification of several AR-interacting ncRNAs. In the current study, we have listed ncRNAs that affect expression of AR, as well as those being affected by AR. Notably, mutual interactions have been identified between AR and some of these non-coding transcripts. For instance, the expression of the lncRNA ARLNC1 has been shown to be enhanced by the AR protein. Conversely, ARLNC1 can increase the stability of the AR mRNA through RNA-RNA interaction [74].

AR-targeting miRNAs have been suggested as potent tumor suppressors in prostate cancer. However, a number of other miRNAs have also been found to induce CRPC, by changing the activity of AR signaling. Moreover, AR signaling can affect the expression of miRNAs through different mechanisms, including feedback loops.

LncRNAs and circRNAs that regulate AR signaling have been found to interact with miRNAs. MALAT1/miR-320, CCAT1/miR-28-5p, PlncRNA-1/miR-34c, PlncRNA-1/miR-297, XIST/miR-24, SARCC/miR-143-3p, circ-ITCH/miR-17, and circRNA17/miR-181c-5p are examples of the cooperation between lncRNAs/circRNAs and miRNAs in the regulation of AR signaling. Similarly, AR-regulated lncRNAs and circRNAs have been shown to influence the expression or bioavailability of miRNAs, adding novel layers of complexity in this interaction network.

Taken together, the data presented above indicates the complexity of the transcriptional regulation of miRNAs by AR and the effects of AR on them. Moreover, the interactions between ncRNAs and AR signaling can be context-dependent.

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3.4 Manuscript IV

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Unveiling four axes ADAMTS9-AS2|MEG3/hsa-miR-150/PRKCA|MMP14 within prostate cancer through establishment of the ceRNA network

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ABSTRACT

Prostate cancer is among the most common cancers in males. Recent application of system biology methods has resulted in identification of key genes in the process of carcinogenesis. In the current study, we selected two datasets related to prostate cancer (PCa) and performed bulk RNA-seq analysis by selecting samples with Gleason scores greater than 7 and combining them. Subsequently, using several systems biology approaches, we constructed the ceRNA network and ultimately identified key axes related to PCa. Our analyses revealed importance of ADAMTS9-AS2/miR-150/PRKCA, ADAMTS9-AS2/miR-150/MMP14, MEG3/miR-150/PRKCA and MEG3/miR-150/MMP14 with miR-150 being a central component. Remarkably, miR-150 exhibited strong statistical significance in survival analyses. Further, analyzing expression levels from TCGA datasets, the expression of the identified genes associates significantly with prostate cancer compared to normal tissue confirming the bioinformatic analyses. Therefore, these genes can be regarded as prognostic markers in prostate cancer and the pathways are potential targets for therapeutic interventions.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in most of assessed countries accounting for 7.3 % of diagnosed cancers and 3.8 % of cancer-related deaths [30]. European Association of Urology recommends evaluation of prostate-specific antigen (PSA) levels for diagnosis of this type of cancer [24]. A number of novel biomarkers, including TMPRSS2-ERG fusion gene and the long non-coding RNA (lncRNA) PCA3 have been reported to enhance sensitivity and specificity PSA leading to reduction of over-diagnosis [11,26]. Meanwhile, the emergence of high throughput sequencing methods has facilitated identification of new biomarkers that contribute to the progression of prostate cancer [3,28]. The publicly available data of such experiments provides a valuable resource for recognition of the functional network between different kinds of transcripts, including mRNAs, lncRNAs and miRNAs in this type of cancer [33]. As proposed by the ceRNA theory, lncRNAs are suggested to function as endogenous sponges that regulate mRNA expression by binding to and sequestering miRNAs [12]. Moreover, gene profiling provides a comprehensive and quantitative measure of these biological features. Such *in silico* analyses would facilitate design of novel therapies that target important molecules or axes in the course of

cancer initiation or progression. Studies with such type of design have also identified promising biomarkers for the early detection of prostate cancer. For example, numerous differentially expressed genes (DEGs), such as KCNK3, AK5, and ARHGEF38, have been found between cancerous and normal tissue of prostate [6]. Moreover, additional functional enrichment assessments have revealed the association between these DEGs and survival of patients [19,34]. A number of DEGs, particularly lncRNAs are also involved in enhancement of carcinogenic events in prostate cancer [36]. Furthermore, in another study, a combination of whole exome sequencing and RNA-seq analysis was employed to identify signature genes associated with metastatic prostate cancer [39].

The sequencing data utilized in this research were sourced from public databases. The identified factors exhibit a differential expression in patient prostate tumor samples compared to normal tissue supporting our ceRNA axes. Analyzing the identification of differentially expressed mRNAs, we employed inverse predictions to determine the associated miRNAs and their respective lncRNAs, constructing a comprehensive ceRNA network. Bioinformatics tools were utilized to scrutinize pivotal pathways, evaluate diagnostic capabilities, and assess the prognostic significance of key genes. The identified lncRNAs, miRNAs, and their

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targeted mRNAs within the ceRNA network hold promise as potential diagnostic biomarkers and therapeutic targets for prostate cancer (PCa) [32]. In overall, the current study utilizes *in silico* tools for re-assessment of RNA sequencing datasets, analysis of DEGs in prostate cancer samples and identification of related pathways and molecular axes. The identified axes were validated using TCGA expression datasets comparing normal prostate with tumor samples.

2. Methods

2.1. Collection of RNA-seq datasets

The human expression profiling by high throughput sequencing of GSE179321 (Illumina NovaSeq 6000 (Homo sapiens)) and GSE89223 (Ion Torrent Proton (Homo sapiens)), which included 6 and 32 samples, respectively, were attained using the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>). GSE179321 dataset contained three tumor samples and three normal samples, while GSE89223 dataset included 17 tumor samples and 15 normal samples (Table 1). Furthermore, for greater homogeneity among the samples, we selected samples with Gleason scores higher than 7 from GSE89223.

The data processing and validation is shown in Fig. 1.

2.2. Processing RNA-seq dataset, gene filtering and assessing data quality

In this study, each dataset was separately analyzed, and its quality was assessed. Firstly, we filtered out genes with a total count of reads less than 10 across all samples for filtering step. Then, we utilized the variance stabilizing transformation (VST) normalization method and *vst* function in the DESeq2 package (Version 1.36.0) [20] in the R programming language to transform the count matrix values of each dataset into logarithmized scale. Using these transformed values, we visualized the sample correlations with a heatmap using the pheatmap package (Version 1.0.12) (<https://cran.r-project.org/package=pheatmap>). Subsequently, utilizing the normalized values, we plotted the Principal Component Analysis (PCA) graph of the datasets using the plotPCA function.

2.3. Analysis of differentially expressed genes (DEGs)

The DESeq2 package was used to screen differentially expressed mRNAs (DEmRNAs) and lncRNAs (DElncRNAs) between prostate cancer and normal samples. DEGs were appraised with the cut-off criteria of false discovery rate (FDR; adjusted p value) < 0.05 and |log2 fold Change (FC)| > 1. Then, we identified DElncRNAs using the HUGO gene nomenclature committee standards (<https://www.genenames.org/>).

2.4. Gene ontology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis

The clusterProfiler R package (Version 4.4.4) [38] was used to implement gene ontology (GO) enrichment analyses to investigate the function of the upregulated and downregulated of the identified DEmRNAs. This analysis involves the examination of biological process (BP), molecular function (MF), cellular component (CC) of DEGs. The functional category criteria were recognized at an adjusted p-value of 0.05 or below. Also, KEGG pathway analysis of noticeably DEGs was performed to discover the potential function of these genes that participate in the pathways based on the KEGG database [17,18].

Additionally, we visualized two of the top significantly enriched signaling pathways using the pathview package (Version 1.36.1) [21].

2.5. Protein-protein interaction (PPI) network construction, hub genes and clusters identification

The STRING database [31] was used to create the PPI network for DEmRNAs. The highest level of confidence was used to establish the interactions parameter (confidence score > 0.9). The Cytoscape software v3.10 [27] was used to visualize the interactions between proteins. In addition, using the cytoHubba [7], we obtained 15 hub genes with the best betweenness centrality (BC), closeness centrality (CC), eccentricity centrality (EC), radiality centrality (RC) and stress centrality (SC) parameters, and we reported the common hub genes among these parameters as the final hub genes. Finally, the identified hub clusters were chosen and functional annotation was carried out using the Cytoscape software plug-in Molecular Complex Detection (MCODE) (Version 2.0.3) [2]. The GO terms and KEGG pathways enrichment analysis of mRNAs in the hub clusters and hub genes were obtained utilizing ClusterProfiler package. A criterion of an adjusted p-value of 0.05 or less was established for the functional category.

2.6. Constructing the ceRNA network, hub genes identification and assessing the axes

We constructed a ceRNA network through the following phases:

- 1) Obtaining those miRNAs associated with prostate cancer through the HMDD (Human microRNA Disease Database) database;
- 2) judging the interactions between lncRNAs and miRNAs based on the prostate cancer-related miRNAs using miRcode (<http://www.mircode.org/>);
- 3) Application of miRTarBase (<https://mirtarbase.cuhk.edu.cn/>) [15], TargetScan (<http://www.targetscan.org/>) [1] and miRWalk (<http://p://129.206.7.150/>) [29] for prediction of miRNAs-targeted mRNAs;
- 4) Identification of common mRNAs between DEmRNAs and target mRNAs of miRNAs, and discarding those mRNAs that were not present in the DEmRNA list;
- 5) Selection of interactions where lncRNAs were oppositely expressed with miRNAs, and at the same time, those mRNAs were also oppositely expressed with miRNAs;
- 6) establishment of lncRNA/mRNA/miRNA ceRNA network using Cytoscape v3.9;
- 7) predicting hub genes using cytohubba plugin based on degree centrality, betweenness centrality, closeness centrality and eccentricity centrality parameters and
- 8) Intersection of hub genes between the parameters.

2.7. Key axes of the ceRNA network identification

Within the ceRNA network, we identified the axes that consisted the final hub genes derived from both the ceRNA and PPI networks.

2.8. Genetic alterations in key axes of prostate cancer patients

We utilized the cBioPortal for Cancer Genomics [5] to explore the genetic changes of the key axes of ceRNA network in prostate cancer patients. Our investigation focused on prostate adenocarcinoma datasets

Table 1
Information about RNA-seq datasets used.

Datasets	Platform	Use	Patient	Control	Tissue	Status
GSE179321 GSE89223	GPL24676	DEmRNA – DElncRNA	3	3	ProstateProstate	Public on Jan 12, 2022
	GPL17303	DEmRNA – DElncRNA	17	15		Public on Mar 27, 2017

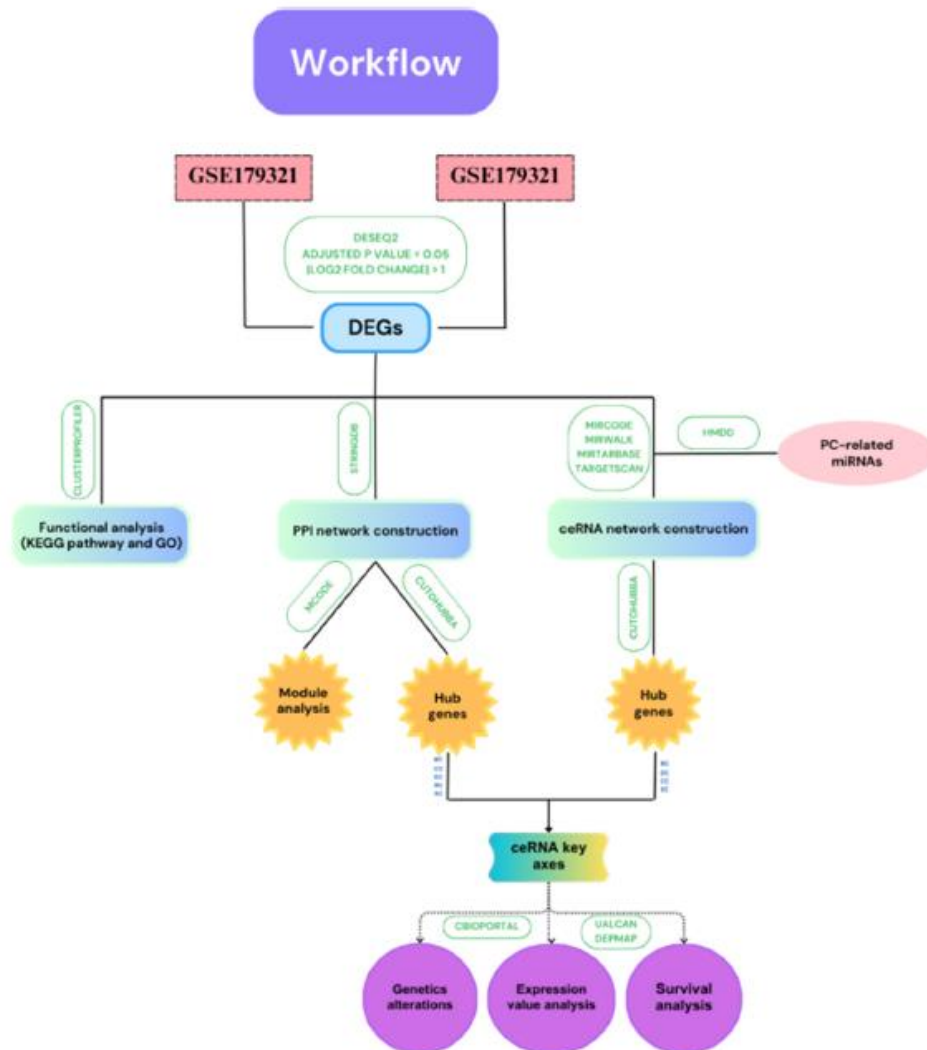


Fig. 1. Flowchart of the research design to identify prostate cancer specific hub genes and ceRNA networks comparing normal and tumor samples. DEGs, differentially expressed genes; PPI, protein-protein interaction; PC-related miRNAs, Prostate cancer-related microRNAs; lncRNAs, long non-coding RNAs; circRNAs, circular RNAs; ceRNA, competing endogenous RNA.

from the TCGA, encompassing 1328 samples from 3 studies. Furthermore, we specifically investigated the hub genes in these axes that displayed significant and co-occurring mutations in the samples from these datasets.

2.9. Validation of hub genes in key axes via expression values and survival analysis

The expression levels and survival analysis of hub genes in key axes were assessed using the UALCAN database [6]. The hub gene expressions in the TCGA-PRAD samples data were examined. Furthermore, we utilized the Dependency Map (DepMap) portal (<https://depmap.org/portal/>) to investigate the expression of the hub genes in prostate cancer cell lines. To assess the significance of expression values between the control

cell line and cancerous cell lines, we conducted a One-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism version 9.

3. Results

3.1. RNA-sequencing data processing and assessment of data quality with strong distinctions between tumor and normal groups

Prior to assessing, for dataset quality control we performed gene filtering and removed genes from each dataset whose total counts across all samples were less than 10 and first visualized the correlation between samples using a heatmap, which was generated using the heatmap package (Fig. 2). Seven samples from GSE89223 were identified as

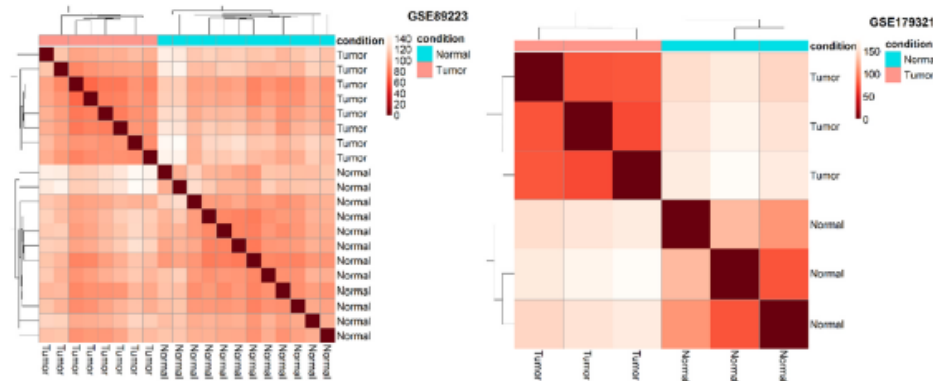


Fig. 2. Hierarchical clustering of the samples within each dataset after filtering the outlier samples indicates good correlation between samples. In these heatmaps, the pink bars indicate tumor samples, and the light blue bars represent normal samples.

outliers and subsequently removed from the study. Those samples with Gleason scores higher than 7, with a total of 8 prostate tumor samples and 12 normal samples remained in this dataset and were used for further analysis. As further quality control, the relationships between samples through principal component analysis based on their first two principal components (PC1 and PC2) using plotPCA function were examined (Fig. 3). After removing the outliers, both plots demonstrated clear distinctions between tumor and normal sample groups, indicating a good sample quality in both datasets. Furthermore, to investigate gene dispersions and their expression distributions, we utilized the Dispersion Estimates Plot (DEP). In this plot, the dispersion of each gene is represented in relation to the normalized mean counts (Figure S1).

3.2. Differential expression analysis identified significant both up- and downregulated DEGs and highly selective tumor-specific clustering

Following data normalization, we applied the DESeq2 package to detect Differentially Expressed Genes (DEGs) between prostate cancer and normal samples. By utilizing a false discovery rate (FDR) threshold < 0.05 and considering an absolute $\log_2$ fold change (FC) > 1 , we identified 2338 downregulated and 1524 upregulated DEGs in GSE179321 dataset. Additionally, GSE89223 dataset displayed 502 downregulated and 231 upregulated DEGs that met the specified criteria. To visually assess the variation in lncRNA and mRNA expression between prostate cancer and normal samples, we employed the EnhancedVolcano package (Version 1.14.0) [16] in R to generate the

volcano plots (Fig. 4). The two-way clustering analysis revealed distinct expression patterns of all DEGs between prostate cancer and normal samples (Fig. 5). In DESeq2, the MA-plot (mean average) function was employed to display the $\log_2$ FC attribution to a given variable over the mean of normalized counts for a two-group comparison in the DESeqDataSet (Fig. 6). This plot highlights that significant calls are obtained only for genes with a large average normalized count, containing sufficient information. Points outside the window are represented as open triangles pointing either up or down, indicating genes with more significant fold changes.

Based on these analyses, we identified the overlapping DEGs between two datasets, resulting in a final set of 255 downregulated and 76 upregulated DEGs (Fig. 7). We also utilized the HUGO database to distinguish DElncRNAs from DEGs, revealing the presence of 6 downregulated and 6 upregulated DElncRNAs among the DEGs. The most significantly upregulated and downregulated DElncRNAs and DElncRNAs are shown in Table S1.

3.3. Gene ontology (GO) enrichment analysis identified terms for biological processes, cellular components and molecular function

To identify GO terms, the clusterProfiler package was utilized. 317 GO entries were found to have an adjusted p-value less than 0.05. Among these entries, the majority belonged to biological process (BP), followed by CC (cellular component) and MF (molecular function). MF delineates the actions of molecules, CC specifies the locations of these

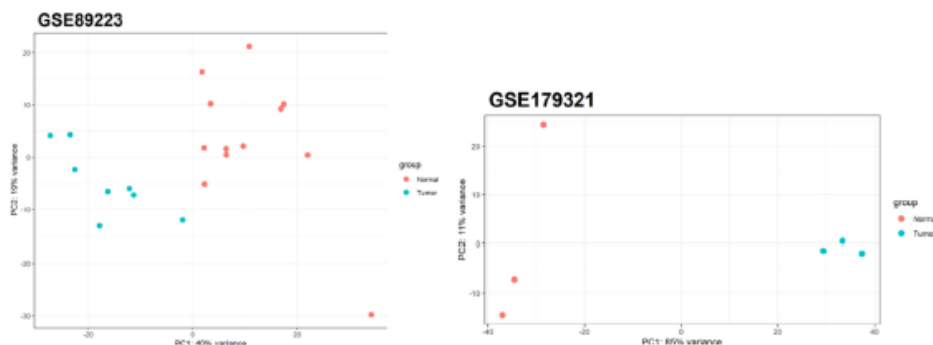


Fig. 3. Principal component analysis (PCA) indicates good sample qualities in both datasets. plots. These plots display the samples in a two-dimensional space with PC1 and PC2 axes. Orange points represent normal samples, and blue points represent tumor samples.

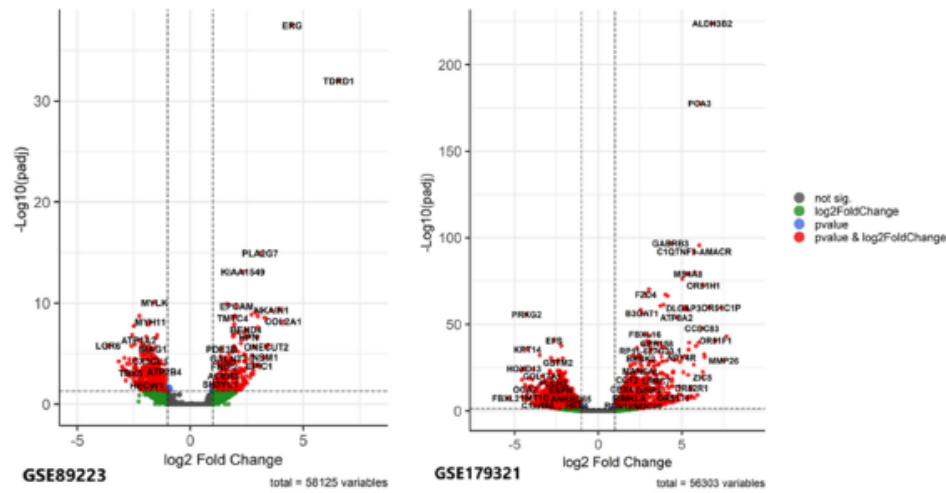


Fig. 4. The volcano plot of DEGs in both datasets indicates significant both up- and downregulated DEGs. Horizontal axis, $\log_2(FC)$; vertical axis, $-\log_{10}(\text{adjusted } P \text{ value})$.

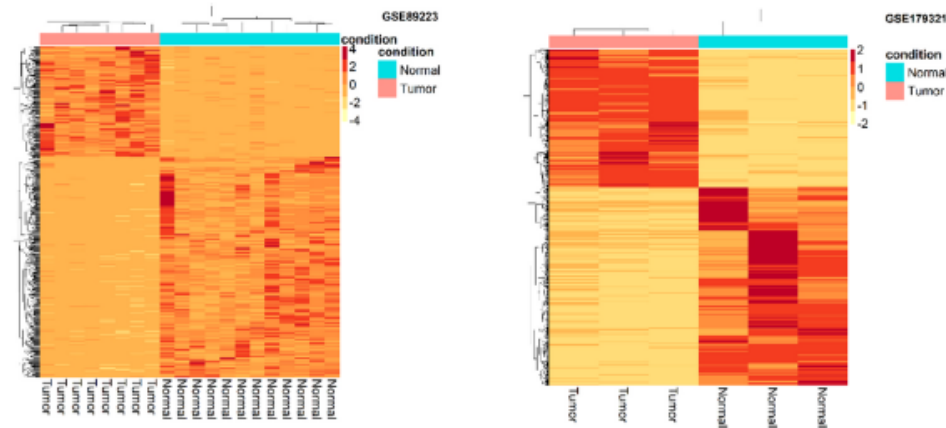


Fig. 5. The two-way clustering of DEGs between prostate and normal samples indicates gene sets with highly selective cluster. Horizontal axis, the samples; vertical axis, DEGs.

molecular actions, and BP outlines organized programs consisting of controlled molecular activities [35]. The top 20 entries encompassed collagen-containing extracellular matrix (CC), sarcolemma (CC), contractile fiber (CC), sarcomere (CC), Z disc (CC), cell-cell junction (CC), I band (CC), myofibril (CC), basement membrane (CC), muscle cell development (BP), muscle cell differentiation (BP), extracellular matrix organization (BP), extracellular structure organization (BP), external encapsulating structure organization (BP), muscle system process (BP), striated muscle cell differentiation (BP), glycosaminoglycan binding (MF), extracellular matrix structural constituent (MF), cell-substrate junction (CC), and muscle tissue development (BP). To visualize these enrichments, Fig. 8A and B illustrate the barplot and dotplot of the top 10 enriched functions, respectively. The gene-concept network of the top 7 GO terms depicts external encapsulating structure organization, extracellular matrix organization, extracellular structure organization, muscle cell development, muscle cell differentiation, muscle system process, and striated muscle cell differentiation (Fig. 9). Notably, a

relationship between most significant categories were identified (Figure S2).

3.4. KEGG pathway analysis identified 12 significantly enriched signaling pathways including Wnt signaling

Using the clusterProfiler package, we conducted KEGG pathway analysis, which identified 12 significantly enriched signaling pathways (adjusted p-value < 0.05). These pathways include focal adhesion, vascular smooth muscle contraction, dilated cardiomyopathy, hypertrophic cardiomyopathy (HCM), salivary secretion, gastric acid secretion, arrhythmogenic right ventricular cardiomyopathy (ARVC), aldosterone-regulated sodium reabsorption, calcium signaling pathway, long-term depression, Wnt signaling pathway, and protein digestion and absorption pathways (Fig. 10A and B). Additionally, the Pathview [22] and gage [23] packages in R were employed to visualize two pathways with the most significant adjusted p-values, as depicted in

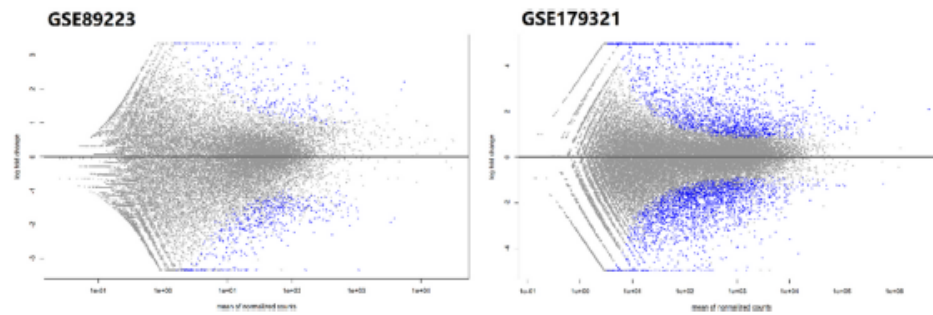


Fig. 6. MA plots indicate the significant calls that are obtained only for genes with a large average normalized count. The presented plots visualize each gene as a dot, where the x-axis corresponds to the average expression across all samples, and the y-axis represents the log2 Fold Change between the normal and tumor groups. Genes with an adjusted p-value below the specified threshold (here set at 0.001) are displayed in blue, allowing for a clear distinction of significantly differentially expressed genes.

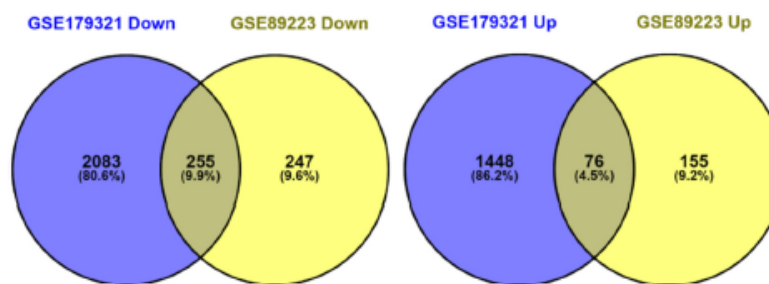


Fig. 7. Venn diagram of overlap of DEGs between two datasets with indicated up- and downregulated expression.

Figure S3.

3.5. PPI network construction and identification of 8 hub genes

Using the STRING database, we constructed a PPI network based on the final set of DE mRNAs. Subsequently, we imported this network into Cytoscape software (Fig. 11A). Through the cytoHubba plugin, we identified 15 hub mRNA coding genes based on Betweenness Centrality (BC), Closeness Centrality (CC), Eccentricity Centrality (EC), Radiality Centrality (RC) and Stress Centrality (SC) (Fig. 11B). To filter these hub genes, we intersected the hub genes of these five centralities, resulting in 8 mRNA coding hub genes, namely PRKCA, MMP14, EPHA2, ANXA2, ADD3, CAV1, FLNC, and ITGB4 (Fig. 11C). The analysis of both Gene Ontology (GO) and KEGG pathway for the identified hub genes was performed using the clusterProfiler package. It was demonstrated that these hub genes are involved in the focal adhesion signaling pathway (Fig. 11D).

3.6. Identification of two hub clusters

By utilizing the MCODE plugin integrated into cytoscape, we conducted a comprehensive analysis to identify significant clusters within the PPI network. Notably, we discovered two clusters with the highest scores (Fig. 12A). The first cluster exhibited a score of 5.6, comprised of LMOD1, ACTG2, MYH11, MYLK, TPM2, and MYL9, while the second cluster, scoring 3.714 consists of FGFR2, FGF2, EPHA2, PGF, ANGPT1, CAV1, FLNC, and FLNA. Subsequently, we performed GO and KEGG enrichment analyses on these hub clusters using the clusterProfiler package in R (Fig. 12B).

3.7. ceRNA network construction identified 6 lncRNAs, 12 miRNAs, and 14 mRNAs as ceRNA network in prostate cancer

In constructing the lncRNA-miRNA-mRNA ceRNA network, in general investigators focus on miRNAs displaying either upregulation or downregulation. Additionally, they consider lncRNAs and mRNAs that exhibit inverse relationships with these miRNAs in the miRNA-mRNA and lncRNA-miRNA interaction pairs [40]. In the search for miRNAs associated with prostate cancer, we utilized the Human microRNA Disease Database version 4.0 (HMDD v4.0). To obtain miRNAs associated with prostate cancer, we downloaded the entire dataset of miRNA-disease association data (version 2023.07) and filtered for miRNAs related to the prostate. Subsequently, miRcode was employed to investigate the connections between lncRNAs and miRNAs.

The analysis revealed that out of the 12 DE lncRNAs, 8 were potentially targeting 72 out of the 322 prostate cancer-specific miRNAs. We proceeded to employ miRWalk, along with miRTarBase and TargetScan filters, using a high confidence interval of 0.95 and a 1 % False Discovery Rate (FDR) stringency. This analysis aimed to identify the mRNAs targeted by the 72 miRNAs associated with prostate cancer with high stringency. From the data analysis, we identified 27 miRNAs linked to prostate cancer that might target 28 of the 319 DE mRNAs.

To enhance the stringency, those miRNA-targeted mRNAs that were not present among the differentially expressed mRNAs were eliminated. Moreover, we eliminated any interactions between lncRNAs and miRNAs, as well as miRNAs and mRNAs that had opposing expression patterns, following the ceRNA network theory. The results suggest a stringent ceRNA network comprised of 6 lncRNAs, 12 miRNAs, and 14 mRNAs, as detailed in Table 2. Notably, the network includes 32 nodes and 37 edges (22 lncRNA-miRNA and 15 miRNA-mRNA edges). Utilizing Cytoscape 3.9, we constructed a comprehensive ceRNA network

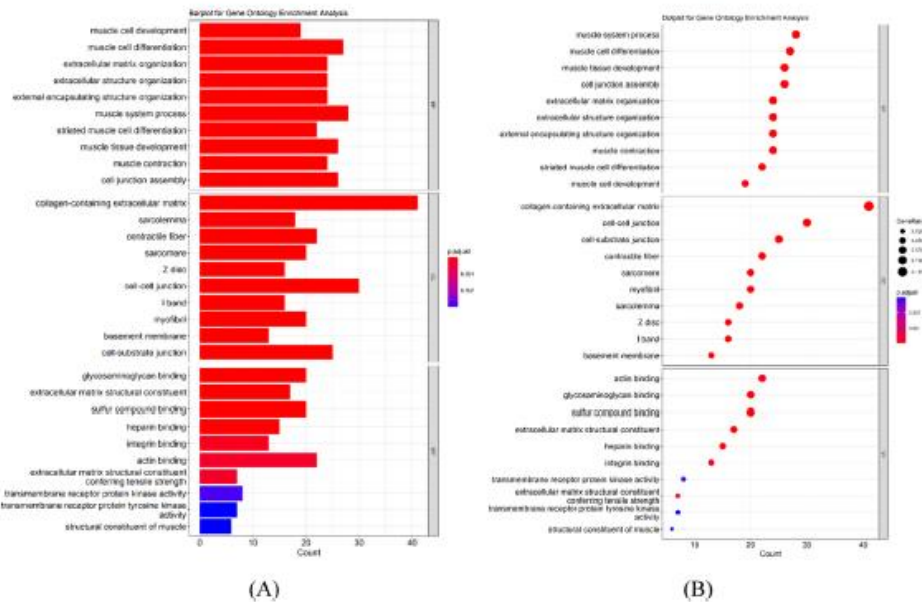


Fig. 8. Barplot and dotplot depict the identified top 10 enriched functions; BP (biological process), CC (cellular component) and MF (molecular function). A) The barplot illustrates the count of gene sets on the x-axis, while the gene set functions are represented on the y-axis. The bar color corresponds to the adjusted p-value, with red indicating the most significant results and blue representing the least significant ones; B) On the dotplot, the x-axis displays the count of genesets, and the y-axis shows the geneset functions. The dot color represents the adjusted p-value, ranging from dark blue (most significant) to red (least significant). Additionally, the dot size indicates the GeneRatio, where larger dots signify higher values of the gene ratio.

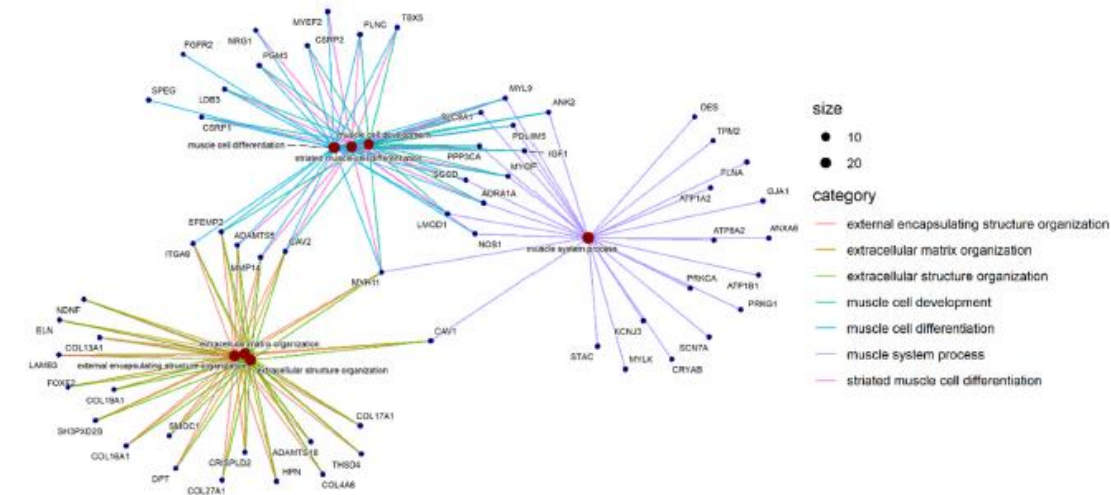


Fig. 9. The identified top 7 GO terms as a network plot indicate association with focal adhesion and other cancer-related pathways. In this graph, the represented GO terms are linked to genes. Each connection of a gene to its corresponding GO term is highlighted with a distinct color. Notably, a larger dot size for a particular GO term indicates the presence of more genes associated with it.

incorporating these 12 miRNAs, 6 lncRNAs, and 14 mRNAs, as illustrated in Fig. 13.

The objective of this network was to investigate the impact of lncRNAs on mRNAs in prostate cancer, specifically through their interactions with miRNAs. In order to illustrate these interactions

comprehensively, we created a Sankey diagram using the ggalluvial R package (version 0.12.3) [4], which is depicted in Fig. 14. This diagram provides a clear and visual representation of the connections and relationships among the different elements, offering a deeper insight into the complex interactions within the ceRNA network. Using the

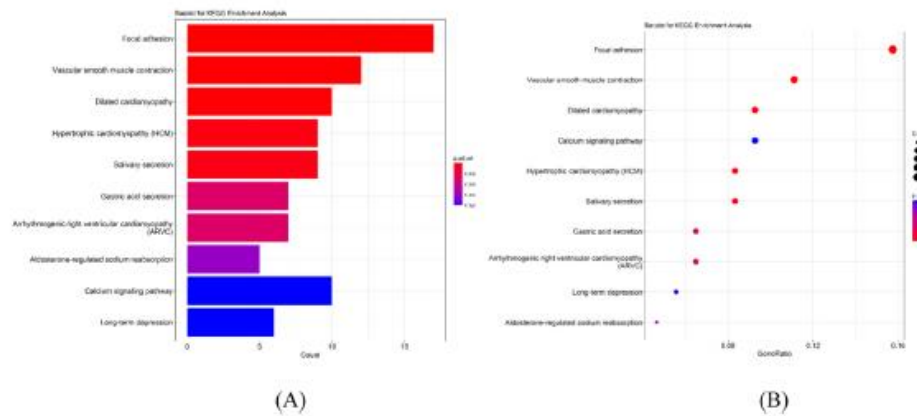


Fig. 10. Boxplot and dotplot of KEGG pathways support the identified GO terms. A) The barplot illustrates the count of genesets on the x-axis, while the pathways are represented on the y-axis. The bar color corresponds to the adjusted p-value, with red indicating the most significant results and blue representing the least significant ones; B) On the dotplot, the x-axis displays the count of genesets, and the y-axis shows the pathways. The dot color represents the adjusted p-value, ranging from dark blue (most significant) to red (least significant). Additionally, the dot size indicates the GeneRatio, where larger dots signify higher values of the gene ratio.

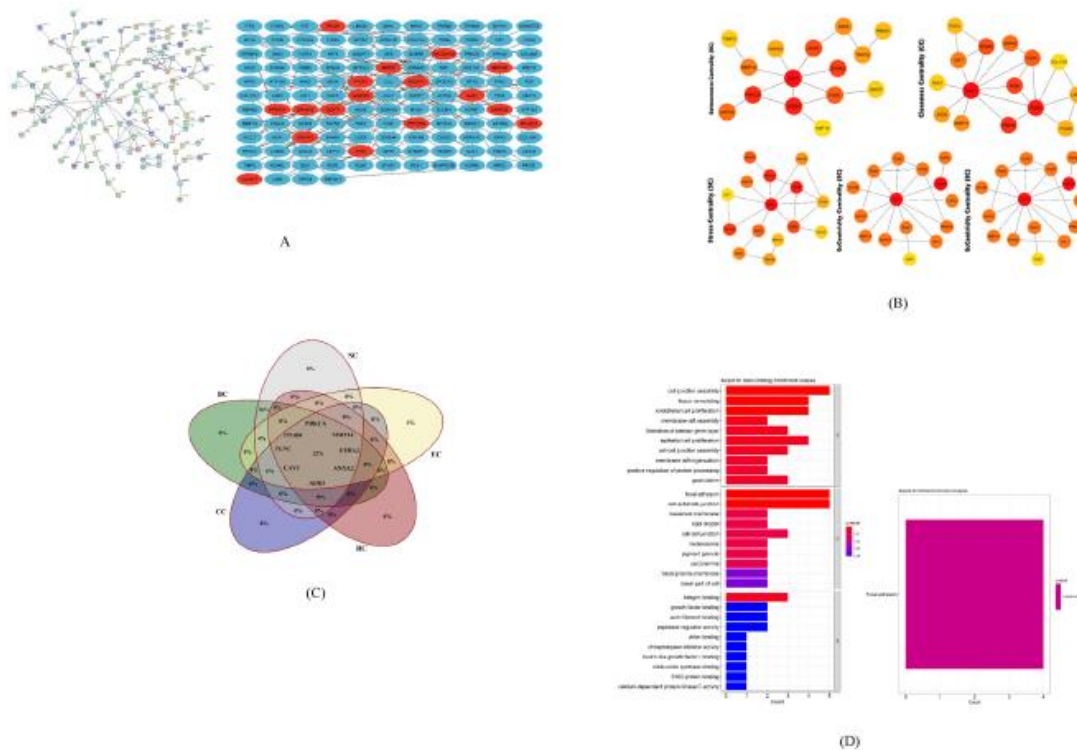


Fig. 11. Protein-protein interaction (PPI) network of DEMRNAs. A) Total PPI network. In the PPI network, blue nodes represent downregulated genes, while red nodes represent upregulated genes; B) Identification of 15 protein coding hub genes based on centrality measures, including betweenness, closeness, radiality, eccentricity, and stress; C) A quintuple Venn diagram illustrating the shared hub genes across all centrality measures; D) barplot of GO and KEGG pathways of protein coding hub genes.

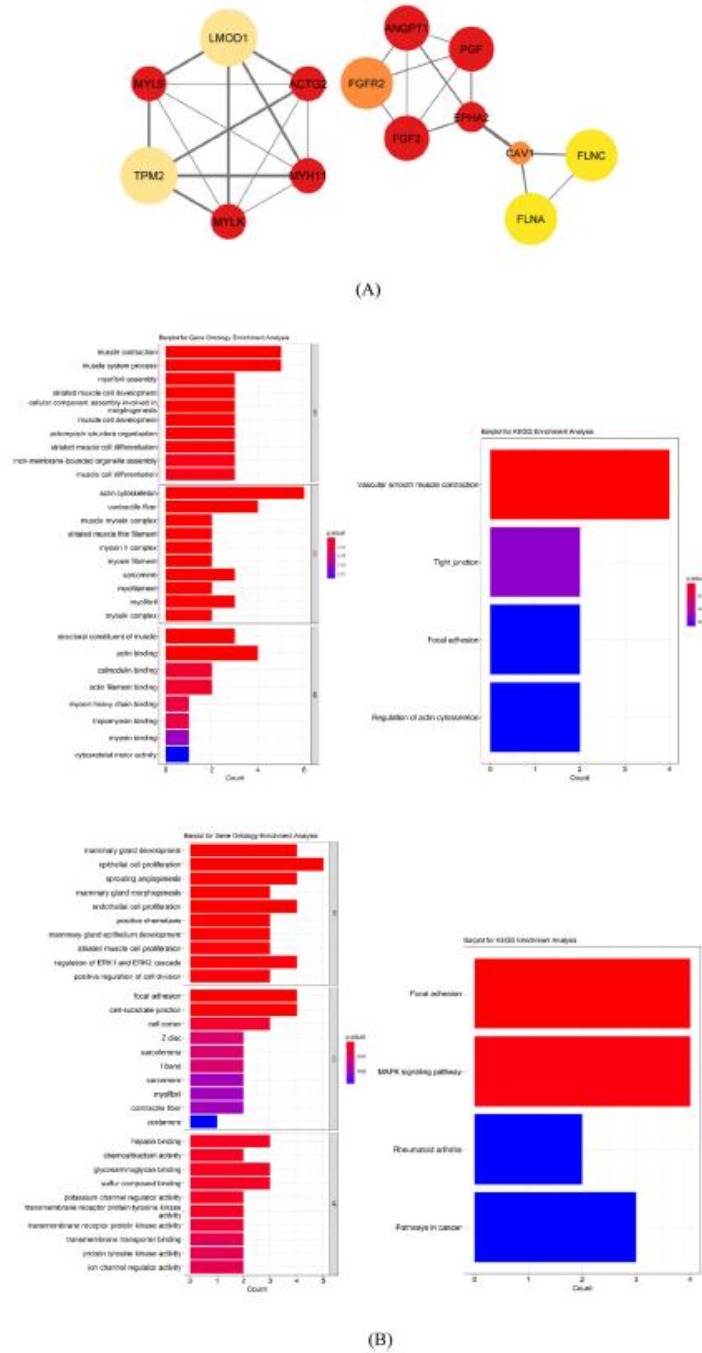


Fig. 12. PPI Network Clusters. A) The first cluster consists of 6 nodes, while the second cluster comprises 8 nodes. As the degree increases, the node sizes also increase, and as edge betweenness increases, the thickness of the edges also increases; B) GO and KEGG pathway analysis of two clusters.

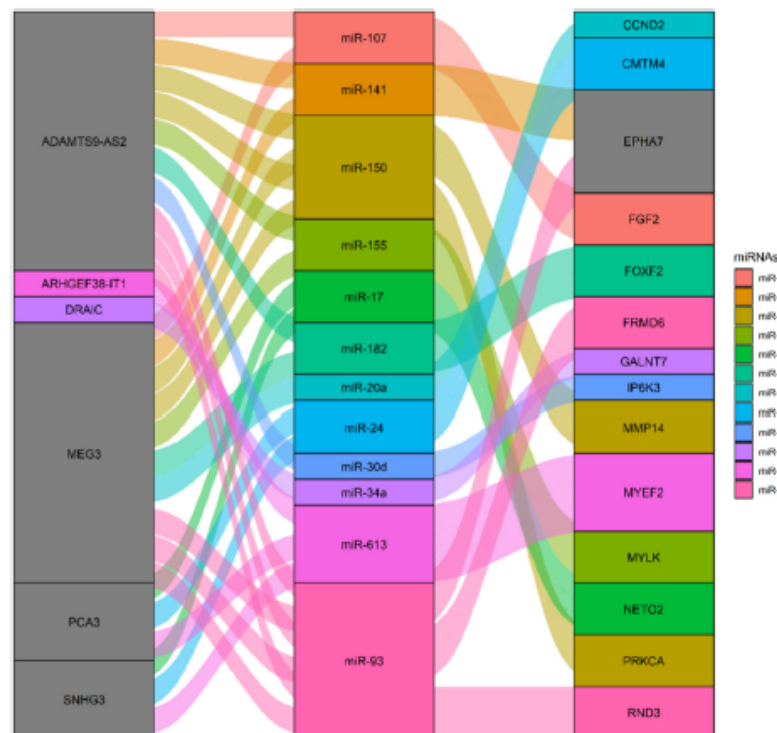


Fig. 14. The ceRNA network Sankey diagram depicts relationships among the identified hub factors and interactions within the prostate cancer ceRNA network. In the diagram, each rectangle symbolizes a gene, and the size of the rectangle reflects the degree of relationship between each gene.

Prism software and conducted One-way ANOVA statistical test to assess the significant differences in the expression of these genes between control cell line and cancer cell lines. As a result, it was revealed that both genes showed significant differential expression between the control cell line and all selected cancer cell lines (Figure S4).

4. Discussion

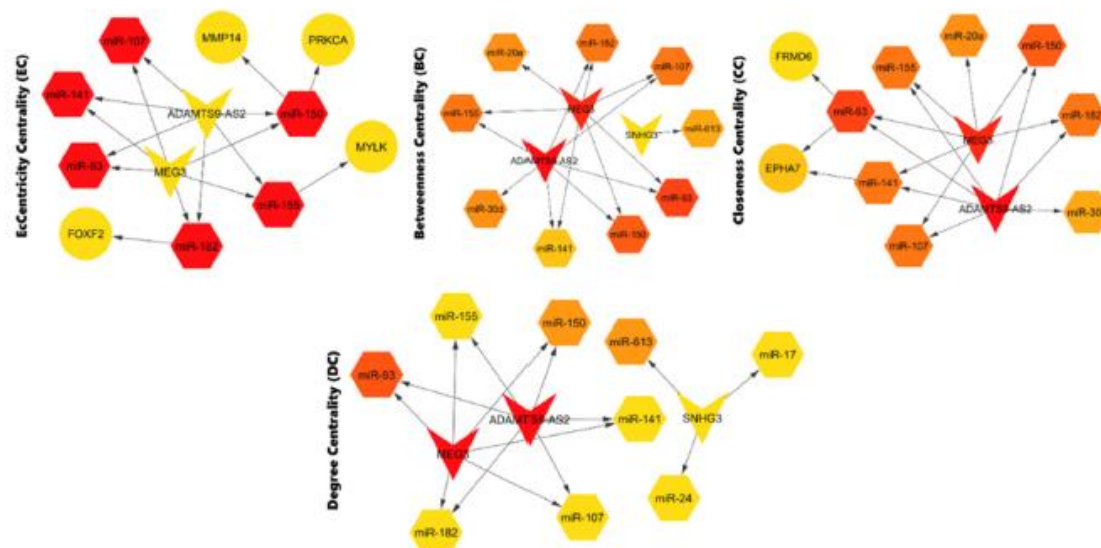
We identified novel hup gene axes of which the expression of included factors associate with prostate tumorigenesis. A central factor identified is the miR-150. Interestingly, the expression of miR-150 is associated with survival of patients. Assessment of expression profile of genes is a practical approach for identification of genes that potentially affect the progression of a disease. However, to be more precise, it is necessary to integrate multiple datasets to control dataset-specific features, alternatively named as batch effects. In the current study, we identified important molecules that affect pathogenesis of prostate cancer through a multi-step approach.

Our analyses indicate an importance of four ceRNA axes composed of ADAMTS9-AS2/miR-150/PRKCA, ADAMTS9-AS2/miR-150/MMP14, MEG3/miR-150/PRKCA and MEG3/miR-150/MMP14. The lncRNA ADAMTS9-AS2 is a non-coding transcript that acts on one hand as a tumor suppressor in most of assessed tissues, but on the other hand it is a possible oncogene in some other tissues [10]. The diagnostic and prognostic values of this lncRNA have been confirmed in a variety of cancers [10] confirming our bioinformatic analysis for prostate cancer. He et al. showed ADAMTS9-AS2 exhibits reduced expression in prostate cancer (PCa), and its decreased levels are associated with an unfavorable disease prognosis [13]. Similarly, the lncRNA MEG3 is regarded as a biomarker with diagnostic and prognostic applications in different types

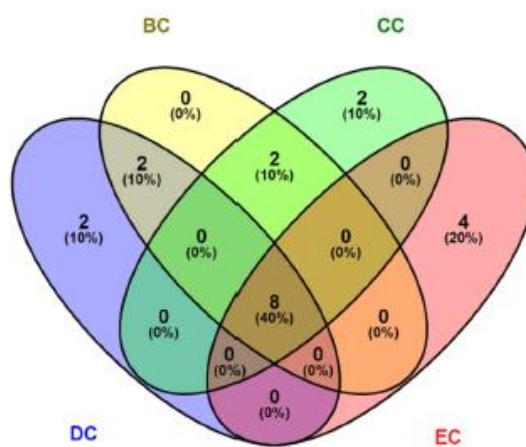
of cancers [14]. Wu et al. discovered that MEG3 is downregulated in prostate cancer, affecting cellular functions such as proliferation, migration, invasion, and apoptosis rate through the action of miR-9-5p and its target gene QKI-5 [37]. In another study, Zhou, Y. et al. demonstrated that MEG3's interaction with EZH2 resulted in the augmentation of H3K27 trimethylation on EN2, ultimately suppressing the progression of prostate cancer [41]. Although MMP14 showed consistent down-regulation in prostate cancer cell lines compared with the control cell line, PRKCA did not follow such consistent pattern among cancer cell lines. This finding may indicate specific roles for PRKCA during the course of carcinogenesis.

The involvement of miR-150 in various molecular axes found in the current study potentiates this miRNA as a therapeutic target for prostate cancer. A previous study has shown association between miR-150-5p plasma and tissue levels in prostate cancer patients suggesting a biomarker role for this miRNA [25]. Moreover, extracellular vesicle-derived miR-150-5p has been recently suggested as a possible diagnostic marker for early-stage metastasis and high-grade Gleason score prostate cancers [8]. In various studies, the decrease in the expression of miR-150-5p has been shown in prostate tumor samples [9]; while our study has shown an increase in the expression of this gene in more endocrine prostate cancer. Since we focused on Gleason Score 7 samples it is unlikely to have neuroendocrine features, which can explain the dual expression.

Moreover, in the current study, it was determined that MEG3 lncRNA and miR-150-5p, as well as ADAMTS9-AS2 lncRNA and miR-150-5p, experienced co-occurrence mutations in several PRAD-TCGA samples, suggesting a potentially crucial role in the progression of prostate cancer. Thus, these analyses discovered novel axes that associate with cancerogenesis and suggest a role in prostate cancer and also with



(A)



(B)

Fig. 15. CeRNA network identified hub genes based on their highest degree centrality. A) Fifteen hub genes were obtained based on the description in text and parameters of BC, CC, EC, and DC; B) The overlap of 15 hub genes for each parameter was visualized using a Venn diagram.

survival.

Although one of our datasets has a relatively small sample size in our study, we used stringent parameters and reinforced our results by incorporating another dataset with a larger sample size. Nevertheless, future studies can focus on transcriptional profiles of prostate cancer samples with different clinical stages to better understand stage-specific events.

Abbreviations

ceRNA: Competing Endogenous RNA; PPI: Protein-Protein Interaction; mRNA: Messenger RNA; lncRNA: Long Non-Coding RNA; miRNA: Micro RNA; BC: Betweenness Centrality; CC: Closeness Centrality; EC: Eccentricity Centrality; DC: Degree Centrality; SC: Stress Centrality; RC: Radiality Centrality

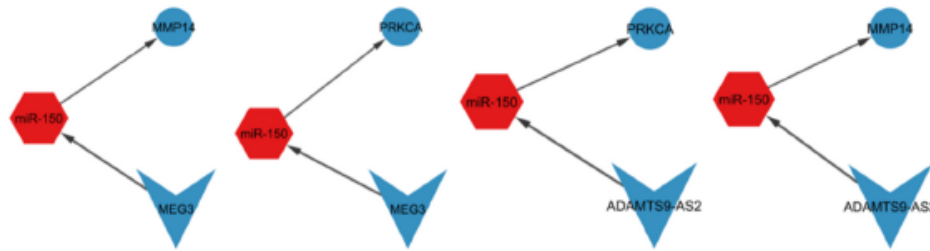


Fig. 16. Identified key axes in the ceRNA network based on hub genes by applying stringent 5 filters to PPI and 4 filters to the ceRNA network.

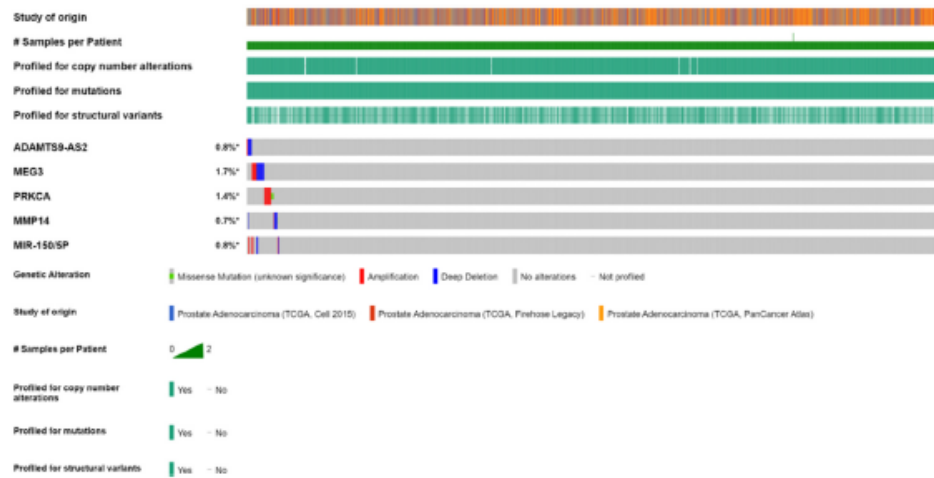


Fig. 17. The genetic alterations of the ceRNA network key axes in samples with prostate adenocarcinoma. Genes marked with an asterisk (*) indicate that they have undergone mutations.

Table 3
Mutually exclusive mutation pattern between gene pairs in ceRNA network key axes.

A	B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	p-Value	q-Value	Tendency
MEG3	MIR-150/5 P	1287	17	4	6	>3	<0.001	<0.001	Co-occurrence
ADAMTS9-AS2	MIR-150/5 P	1296	8	8	2	>3	0.002	0.011	Co-occurrence
ADAMTS9-AS2	MMP14	1296	9	8	1	>3	0.067	0.222	Co-occurrence
MMP14	MIR-150/5 P	1295	9	10	0	<-3	1.000	1.000	Mutual exclusivity
ADAMTS9-AS2	PRKCA	1286	10	18	0	<-3	1.000	1.000	Mutual exclusivity
PRKCA	MIR-150/5 P	1286	18	10	0	<-3	1.000	1.000	Mutual exclusivity

Ethics approval and consent to participate

not applicable.

Consent for publication

Not applicable.

Authors' contributions

MT and AS performed the data analysis. AB designed and supervised the study. MT and AB wrote the draft and revised it. All the authors read the submitted version and confirm it.

Funding

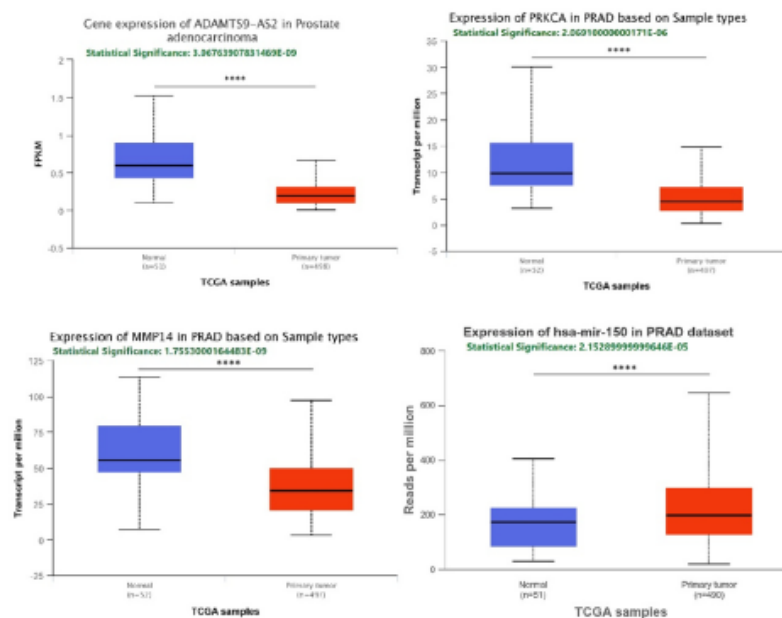
Not applicable.

Ethical statement

none

CRediT authorship contribution statement

Aria Banliahmad: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition. Arash Safarzadeh: Writing – review & editing, Formal analysis. Mohammad Taheri: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis.



(A)



(B)

Fig. 18. Validation of hub genes. A) Box plots of gene expression of hub genes in key axes of the ceRNA network in prostate cancer and normal samples based on TCGA datasets. Red and blue boxes demonstrate expression of hub genes in prostate cancer and normal samples, respectively; B) The overall survival of prostate cancer patients is related to the Kaplan-Meier survival curve of hsa-miR-150.

Declaration of Competing Interest

The authors declare they have nothing to report.

Data availability

The datasets analysed during the current study are available in the GSE179321 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179321>) and GSE89223 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89223>) from gene expression

omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).

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Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2024.155604.

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3.5 Manuscript V

Research Letter



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The lncRNAs *PART1* and *ADAMTS9-AS2* act in an antithetic manner on AR signaling and induction of cellular senescence in prostate cancer cells

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Background: Prostate cancer (PCa) is initially a hormone-dependent disease and the development and spread of PCa are tightly linked to the androgen receptor (AR) signaling activity. Therapy that targets the AR pathway is a standard approach for treating PCa including AR antagonists and supraphysiological androgen levels (SAL) used in bipolar androgen therapy. Here, we identified that the two lncRNAs *PART1* and *ADAMTS9-AS2* mediate in part androgen signaling in PCa cells by examining PCa specimen and response to AR antagonists or SAL as well as functionally by knockdown.

Methods: This study utilized expression analysis of tumor tissues (TT) samples in comparison to their corresponding normal tissue adjacent to the tumor (NTAT) counterparts of 50 patients. RNA-seq and treatment of patient prostatectomy samples ex-vivo confirmed regulation of lncRNAs by SAL. Knockdown of both lncRNAs were used to analyze androgen signaling of AR target genes by qRT-PCR and in PCa senescence pathway by analyzing senescence markers. Correlation analyses of patient tumor samples confirmed co-expression. RNA immunoprecipitation (RIP) used in both LNCaP and C4-2 cells detect AR-lncRNA interaction. Bioinformatic analyses were employed to identify ligand-specific lncRNAs and miRNA interactions with *PART1* and *ADAMTS9-AS2* and confirmed by treatment of patient prostatectomy samples ex-vivo.

Results: *PART1* and *ERVH48-1* were significantly overexpressed in TT samples, *ADAMTS9-AS2* expression is lower in TT samples compared to NTAT samples. SAL treatment indicates opposite regulation in two human PCa cell lines and patient tumor samples. While *ADAMTS9-AS2* is upregulated, *PART1* is repressed by SAL. Furthermore, the obtained data suggest that *ADAMTS9-AS2* may act as a co-activator and *PART1* as a co-repressor of AR signaling. Interestingly, the knockdown indicates that both lncRNAs *ADAMTS9-AS2* and *PART1* regulate AR activity and protein level as well as SAL-mediated induction of cellular senescence. Thus, the data suggest that suggest that *ADAMTS9-AS2* and *PART1* control AR signaling at SAL.

Conclusion: In summary, we identified novel AR signaling pathways that involve lncRNAs oppositely regulated by SAL and in PCa tumorigenesis.

Keywords: ADAMTS9-AS, long non-coding RNAs, *PART1*, prostate cancer

Introduction

On a global scale, prostate cancer (PCa) is one of the most common cancers among men^[1] and developing successful therapy solutions requires a thorough understanding of the molecular mechanisms that underlie its development and progression. The androgen receptor (AR) signaling is essential for-progression of PCa and an important therapeutic target because it

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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HIGHLIGHTS

- The lncRNAs *PART1* and *ADAMTS9-AS2* exert opposite effects on AR signaling in prostate cancer cells. *ADAMTS9-AS2* acts as a co-activator, while *PART1* functions as a co-repressor.
- SAL treatment induces upregulation of *ADAMTS9-AS2* and downregulation of *PART1* in prostate cancer cells, indicating their differential regulation by androgen levels.
- Knockdown of *ADAMTS9-AS2* and *PART1* affects SAL-induced cellular senescence, highlighting their roles in modulating senescence pathways in prostate cancer.
- RIP assays reveal that *PART1* is significantly enriched in AR-immunoprecipitated material, suggesting a direct interaction, whereas *ADAMTS9-AS2* does not show significant enrichment with AR.

controls multiple cellular processes, including proliferation, and cellular senescence^[2].

Similar to supraphysiological androgen level (SAL), second generation AR antagonists induce cellular senescence in PCa cell lines, in 3D tumor spheroids and in prostatectomy samples^[3,4]. Higher, supraphysiological levels of androgens SAL^[5] are

employed in BAT, with PCa patients experiencing cycling supra-physiological and low androgen levels^[6]. Second-generation AR antagonists, such as darolutamide (ODM) and enzalutamide (ENZ), act by reducing AR-mediated transactivation^[7].

In AR signaling pathways a better understanding might lead to the discovery of novel pathways and perhaps biomarkers and to uncover the function of noncoding RNAs such as lncRNAs, miRNAs in PCa^[8]. Among the newly-discovered classes of tumor-suppressor and oncogenic genes are long non-coding RNAs^[9]. Alterations at the transcriptional, post-transcriptional, and epigenetic levels are all potential mechanisms by which lncRNAs influence carcinogenesis^[10,11] and serve as new cancer diagnostic and prognostic biomarkers^[12,13] including PCa^[14,15]. However, the exact roles and molecular pathways within the AR signaling are still not well understood. In this study, we assessed the expression profiles of six lncRNAs selected based on RNA-seq results, which indicated their potential significance in PCa pathogenesis or progression^[16,17] and comparing their expression in PCa with normal adjacent tissue (NTAT) and normal prostate.

While lncRNAs have been implicated in AR signaling, the specific roles of *PART1* and *ADAMTS9-AS2* in regulating this pathway and their impact on cellular senescence in prostate cancer remain poorly understood. This study fills this gap by demonstrating that *ADAMTS9-AS2* promotes AR activity, while *PART1* represses it, with both lncRNAs influencing cellular senescence in distinct ways. Unlike previous research, which primarily focused on lncRNA expression profiles, our work provides mechanistic insights into how these lncRNAs modulate AR signaling and senescence pathways, offering potential therapeutic targets for modulating cancer progression.

The hypothesis is that PCa shows differential expression of these lncRNAs and AR targeted therapies of PCa may influence lncRNAs expression and their activities. Here, we used patient expression data, *ex vivo* treatment of PCa tissue samples, ChIP-seq and functional assays to identify novel androgen-regulated signaling in PCa including SAL controlled cellular senescence.

Methods and material

Study participants and ex-vivo PCa specimen

A total of 50 patients with definite diagnosis of PCa were recruited for the present study. Both tumoral and NTAT were excised during surgery from all patients. Patients did not receive any chemo/radiotherapy before tissue excision. All specimens were examined by clinical pathologists to assess the presence of tumor cells. An additional 20 normal prostate samples were obtained from dead bodies and used as controls. Control subjects had no history of urogenital diseases or malignancies. All patients have signed written informed consent forms as well as the responsible person for autopsy samples.

For *ex-vivo* experiments, nine human prostate tissue samples obtained after the prostatectomy of patients. The Gleason scores ranged from 8 to 9. Specimens were cultured at 5% CO₂ and 37°C for 48 hours with the indicated compounds in RPMI 1640 (treated for 1 nM R1881 and DMSO as solvent control). The medium was supplemented with 10% charcoal stripped serum (CSS), penicillin (100 U/mL), streptomycin (100 U/mL), and 25 mM HEPES (pH 7.8)^[18].

Cell culture and treatments

The LNCaP (lymph node prostate cancer) cell line was used as a model of human androgen-dependent PCa. Cells were cultured in RPMI 1640 medium (Gibco Life Technologies, Carlsbad, California, CA, U.S.) supplemented with 5% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 µg/mL), 1% sodium pyruvate and 25 mM of HEPES pH 7.5 (Carl Roth, Karlsruhe, Germany). The C4-2 cell line is a derivative of LNCaP and was used to represent castrate-resistant PCa (CRPC) cells. Comparisons between these cell lines may reveal common and distinct signaling representing castration sensitivity and resistance. Cells were cultured in DMEM supplemented with 20% F12, 5% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), 1% sodium pyruvate and 25 mM of HEPES pH 7.5. All cells were maintained in a 5% CO₂, humidified atmosphere at 37 °C (Thermo Fisher Scientific, Waltham, Massachusetts, MA, U.S.). Both cell lines were seeded in an appropriate amount for each experiment in cell culture plates. After 48 h of incubation, cells were treated for 72 h with 1 nM R1881 (SAL)^[18], 10 µM darolutamide (ODM), 10 µM enzalutamide (ENZ) or 0.1% Dimethyl sulfoxide (DMSO) (Carl Roth, Karlsruhe, Germany) as solvent control (C) in a 5% CO₂, humidified atmosphere at 37 °C.

3D tumor spheroid generation

3D spheroids of C4-2 and LNCaP cells were generated according to the previous protocol^[4]. Briefly, cells were seeded in 6-well plates and allowed to adhere overnight. The following day, cells were re-seeded in 96-well ultralow attachment plates (PerkinElmer) and incubated at 37 °C with 5% CO₂ to form spheroids. On the subsequent day, treatments with SAL, ENZ, ODM, or DMSO (as a control) were initiated. Treatments were refreshed every three days for a total duration of 13 days.

At the end of the treatment period, RNA was extracted from the spheroids, and qRT-PCR was performed to assess the expression levels of *ADAMTS9-AS2*, *PART1*, and AR direct target genes. This approach facilitated the analysis of androgen receptor signaling and its regulatory components under various treatment conditions.

Expression studies

Total RNA was extracted using RNJia extraction kit (RN983006, Roje Technologies Company, Iran). Afterward, cDNA was produced from these samples using AddScript cDNA Synthesis Kit (ADDBIO Company, South Korea). Expression of *PART1*, *KIAA0087*, *ADAMTS9-AS2*, *CIQTNF3-AMACR*, *ERVH48-1*, *C8orf34-AS1* and *ENO1-AS1* was determined using RealQ Plus 2x Master Mix Green with high ROX purchased (AMPLIQON, Denmark), and primers provided by the METABION Company (Germany). Herein, we used *B2M* and *TBP* as housekeeping genes. Table S1 (available at: <http://links.lww.com/JIS/E34>) depicts information about primer sequences.

miRNA expression assay

The cDNA synthesis was performed using the TaqMan® MicroRNA Reverse Transcription Kit from Invitrogen. The quantification of miRNA transcript levels was conducted using the TaqMan® MicroRNA Assays kit from ThermoFisher

SCIENTIFIC, with the following catalog numbers: A28007 for cDNA synthesis, 4427975 for product number, and the following catalog numbers for miRNA quantification: 00473, 002141, A25576, 00057, and 001973. The RNU6B gene served as the reference for normalization.

Small interfering RNA (siRNA) and transfection

To knockdown the expression of *ADAMTS9-AS2* and *PART1*, cells were subjected to transfection using ON-TARGETplus Human *ADAMTS9-AS2* and *PART1* siRNAs (a group of 4 siRNAs), each at a final concentration of 25 nM per well. As a negative control, ON-TARGETplus nontargeting siRNA was performed in the experiment. All the siRNAs were transfected by the cells using the DharmaFECT reagent from Dharmacon, following the manufacturer's guidelines. This transfection was carried out 24 hours before the administration of R1881 (1 nM) treatment. Subsequently, the cells were incubated for 72 hours in the presence of androgen.

RNA immunoprecipitation (RIP) assay

LNCAp cells underwent a 24-hour treatment with either solvent control (DMSO) or SAL. Antibodies targeting AR (Abcam, ab19066) or IgG (goat IgG, Abcam, ab6885) were incubated with protein G Dynabeads (Invitrogen) and allowed to incubate at 4 °C for 1 h. The beads bound with antibodies were then exposed to cytosolic or nuclear extracts for a 2-hour period at 4 °C. Following three washing steps on a magnetic rack, RNA extraction was carried out using peqGOLD TriFast Glycogen (Invitrogen). Subsequently, cDNA was generated using the High-Capacity cDNA reverse transcriptase kit (Invitrogen).

Senescence-associated beta-galactosidase (SA- β -Gal) staining

For SA- β -Gal staining, 50 000 cells were seeded per well in a 6-well plate. The staining procedure was conducted following the previously established protocol^[18].

Protein extraction and immunoblotting

The day before transfection, 250 000 C4-2 or LNCAp cells were placed in six-well plates. After a 72-hour incubation in the presence of androgen (solvent control (DMSO) or SAL), the cells underwent homogenization in 1x RIPA buffer and halt protease and phosphatase inhibitor cocktail from Thermo Fisher Scientific®. This was followed by centrifugation at 14 000 rpm at 4 °C to isolate total protein lysates. Protein concentration was assessed using the Pierce™ BCA Assay Kit by ThermoFisher Scientific®. The Synergy™ HT plate reader from Agilent Technologies Inc was used for measuring linear absorbance. The separation of total protein in 1 × Laemmli buffer with 10% 2-mercaptoethanol occurred through SDS/PAGE, with subsequent transfer for 15 hours to a PVDF membrane (IPVH00010; MilliporeSigma®) via electroblotting with 20% (vol/vol) methanol and blocked for 1 h in in Intercept (TBS) blocking buffer (LI-COR®). Immunodetection used primary antibodies AR (Biogenex, 256 M), β -Actin (Abcam, ab6276, GR3324554-1), p15 (MyBioSource #MBS821044) and p21 (Cell Signaling, 2946). Secondary antibodies, either horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling, 7076S, lot 32) or anti-rabbit IgG (Cell Signaling, 7074S, lot 28), were

used. Detection was carried out using ImageQuant™ LAS 4000 (GE Healthcare Bio-Sciences AB, Chicago, Illinois, U.S.), and band quantification was conducted through the LabImage D1 program.

ChIP-seq

Analysis Quality control was conducted using the software tools “fastqc” (v0.11.9, Simon Andrews, Babraham Institute, Cambridgeshire, England) and “fastqscreen” (v0.14.0, Steven Wingett, Babraham Institute, Cambridgeshire, England). The fastq files were aligned to the GRCh37/hg19 reference genome using Bowtie (v1.1.2, Ben Langmead, College Park, MD, USA) with specific parameters (“bowtie -q --max/dev/null --chunkmbs 3,200,000 -p 4 -S --sam-nohead -best --strata -m 1”). Subsequently, the resulting SAM files were sorted by genomic coordinates using the command “sort -k 3,3 -k 4,4 n -T”. Reads in the SAM files were deduplicated while retaining their original length of 150 bp.

To generate wiggle files from the sorted SAM files, MACSv1.4 (Yong Zhang, Boston, MA, USA) was employed with parameters (“macs14 --gsize=hg19 -t input_file --format=sam -w -S -p 1e-5 --nolambda --nomodel -n output_file”). These wiggle files were then converted into tdf files using Igvtools (v2.3.97, James T. Robinson, Cambridge, MA, USA).

Peaks were identified in treatment samples compared to paired control samples using MACS (v2.2.6, Yong Zhang, Boston, MA, USA) with parameters (“macs2 callpeak -t treatment_sample -c control_sample -f SAM -g hs -p 1e-5 --slocal 1000 --llocal 10,000 -n output_file”). Subsequently, peaks were annotated for their locations within promoters, exons, introns, and intergenic regions using the script “annotatePeaks.pl” from the software “HOMER” (v4.9.1, Sven Heinz, San Diego, CA, USA).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) v.18.0 (SPSS Inc., Chicago, IL) was used for statistical analyses, and all results were validated to ensure robustness. Graphics were generated using GraphPad Prism version 9.0 for Windows (GraphPad Software, La Jolla, California, USA), and gene expression data were analyzed using Gene Expression software (GenEx SW, Multid Analysis AB, Göteborg, Sweden). For gene expression analysis, the efficiency-adjusted comparative delta Ct method was employed, where expression levels were normalized to the geometric mean of two reference genes, B2M and TBP, with adjustment for amplification efficiency. The normality of data distribution was assessed using the Shapiro–Wilk test. When comparing gene expression between prostate cancer (PCa) tissues, non-tumor adjacent tissues (NTAT), and normal controls, paired t-tests or Wilcoxon signed-rank tests were used for PCa versus NTAT comparisons, while unpaired t-tests or Mann–Whitney tests were applied for comparisons with normal controls, depending on data distribution.

Pearson correlation coefficient was used for correlation analyses of gene expression in patients prostate adenocarcinoma datasets based on UCSC Xena project (<http://xena.ucsc.edu>) retrieved from Gepia. Spearman correlation coefficients were calculated to assess correlations between gene expression levels. Additionally, Mann–Whitney tests and Kruskal–Wallis one-way ANOVA were used to compare gene expression across patient

subgroups. Associations between clinicopathological factors and gene expression in PCa tissues were examined using Chi-square tests. Two-way ANOVA with Tukey's post hoc tests was employed to compare proportions of senescence-associated β -galactosidase (SA- β -gal) positive cells and to identify significant differences in gene expression between siControl and siADAMTS9-AS2, siPART1 treatments. Fold changes in gene expression were calculated relative to DMSO-treated C4-2/ LNCaP cells transfected with non-targeting siRNA. All *P* values were two-sided, and statistical significance was set at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

Results

General information about specimen-derived cases

Table S2 (available at: <http://links.lww.com/JS9/E34>) summarizes the available clinicopathological information of the samples.

General information about selected lncRNAs and the protein coding gene examined in the current study is shown in Table S3 (available at: <http://links.lww.com/JS9/E34>).

Differentially expression in patient PCa samples

Expression of *PART1* was different between tumor tissues (TT) and normal controls (NC) as well as between TT and NTAT (Fig. 1). Expression of *ADAMTS9-AS2* was higher in NTAT samples compared with both TT and NC samples. The expression of *C1QTNF3-AMACR* was not different between three groups of samples. *C8orf34-AS1* was shown to be over-expressed in NC samples compared with the other groups of samples. Expression of *ERVH48-1* was demonstrated to be

different in all three comparisons (TT/NC, TT/NTAT and NC/NTAT). Finally, *KIAA0087* and *ENO1-AS1* were found to have different expression levels between TT and NC, and between NC and NTAT (Fig. 1).

Expression levels of *PART1* and *ERVH48-1* were higher in TT samples compared with NTAT samples (expression ratios = 13.1 and 3.6; *P* < 0.0001 and 0.04, respectively). Notably, expressions of *PART1*, *KIAA0087*, *ERVH48-1*, *C8orf34-AS1*, and *ENO1-AS1* were lower in TT samples versus NC samples (*P* = 0.0002, < 0.0001, < 0.0001, < 0.0001, and = 0.04, respectively). While expressions of *PART1*, *KIAA0087*, *ERVH48-1*, *C8orf34-AS1*, and *ENO1-AS1* were lower in NTAT samples compared with NC samples, expression of *ADAMTS9-AS2* was higher in NTAT samples compared with NC ones [expression ratio (95% CI) = 135 (27.3–670), *P* < 0.0001]. Detailed statistics are shown in Table S4 (available at: <http://links.lww.com/JS9/E34>). These data suggest that the analyzed genes except *C1QTNF3-AMACR* exhibit a significantly distinct expression level in these specimens. Based on significant differences in expression of three genes between NTAT and NC, the data also suggest that NTAT-sample-derived data cannot be always considered as a non-tumor control (Table S4, available at: <http://links.lww.com/JS9/E34>).

Significant pairwise correlations were identified between expression levels of *PART1*, *KIAA0087*, *ADAMTS9-AS2*, *C1QTNF3-AMACR*, *ERVH48-1*, *C8orf34-AS1* and *ENO1-AS1* among TT, NTAT and NC samples (Table S5, available at: <http://links.lww.com/JS9/E34>). The strongest correlations were reported between *ENO1-AS1* and *KIAA0087* (correlation coefficient = 0.95); and between *C8orf34-AS1*, and two other lncRNAs, namely *KIAA0087* and *ERVH48-1* in TT samples

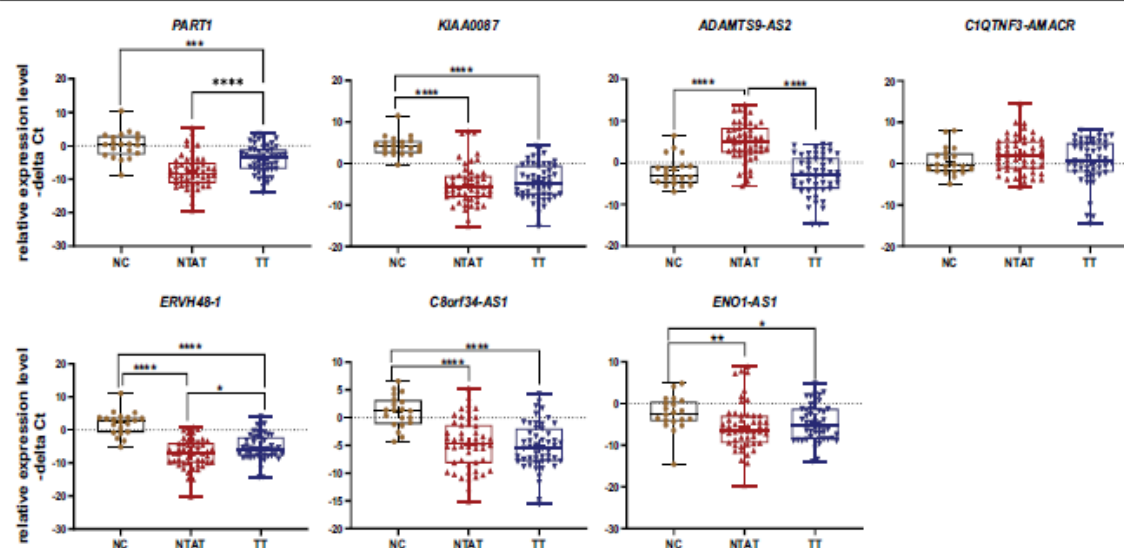


Figure 1. Distinct expression patterns of selected lncRNA genes comparing normal control tissues (NC) with normal tissue adjacent to the tumor (NTAT) and prostate tumor tissues (TT). The relative expression levels of *ERVH48-1* protein coding gene and six lncRNA genes in prostate tumor tissues (TT), Normal Tissue Adjacent to the Tumor (NTAT), and Normal Controls Tissues (NC) (Δ Ct values: Ct Housekeeping gene – Ct Target gene Median [line], mean [cross], interquartile range [box], and minimum and maximum values are shown. The data were analyzed using paired t-test or Wilcoxon rank-sum test between TT and NTAT, and using unpaired t-test or Mann-Whitney test between TT and NC. *P* < 0.05 was considered statistically significant.

(correlation coefficient = 0.95). Similarly, expression levels of *ENO1-AS1* are correlated with those of *PART1* and *C8orf34-AS1* in these samples with correlation coefficient of 0.94.

Then, we classified lncRNAs based on their expression levels using a hierarchical clustering method (Ward's method, Euclidean distances) into four clusters. *C1QTNF3-AMACR* and *ADAMTS9-AS2* were clustered in one group; while *ERVH48-1* and *C8orf34-AS1* were recognized to be clustered in another group. Finally, *PART1* and *KIAA0087* designated another cluster. Notably, most of the patient samples (TT1-TT50 and NTAT1-NTAT50) have a decreased expression levels of the analyzed lncRNAs (Figure S1, available at: <http://links.lww.com/JS9/E35>).

ROC curve analyses revealed efficient performance of *KIAA0087* in separation of TT from NC samples (AUC = 0.96) and NTAT from NC samples (AUC = 0.93) (Figure S2, available at: <http://links.lww.com/JS9/E35> and Table S6, available at: <http://links.lww.com/JS9/E34>). Moreover, *ERVH48-1* has appropriate performance in separations of TT samples from NC samples (AUC = 0.91) and NTAT samples from NC samples (AUC = 0.95). The relative expression levels of *C1QTNF3-AMACR* in TT samples were associated with BMI ($P = 0.02$). Besides, expression levels of *ADAMTS9-AS2* were associated with prostate volume ($P = 0.05$). Table S7 (available at: <http://links.lww.com/JS9/E34>) shows other comparisons.

There was a significant positive association between age of PCa patients and prostate volume ($\chi^2 = 6.41$, $P = 0.01$). There was also a significant positive association between Gleason score and PSA concentration ($\chi^2 = 6.28$, $P = 0.04$) as expected.

SAL induces ADAMTS9-AS2 up-regulation, while PART1 is down-regulated

Experiments in the castration-resistant C4-2 cells revealed significant up-regulation of *ADAMTS9-AS2* following treatment with SAL. Treatments with the second-generation AR antagonists ENZ and ODM did not affect expression of this lncRNA

significantly (Fig. 2). On the other hand, expression of *PART1* was down-regulated following treatment with SAL, and also by the AR antagonists ENZ, or ODM. The downregulation of *PART1* by SAL was confirmed analyzing our previous RNA-seq data^[3] (Figure S3, available at: <http://links.lww.com/JS9/E35>).

This suggests that these two lncRNAs are part of AR signaling and also indicates that AR antagonists may not exhibit always the opposite regulation as androgens and also suggest in case of *PART1* that AR antagonists are not inactivating the AR but may also impose transcriptional activity on AR.

Similar experiments in the androgen-sensitive LNCaP cells revealed a potent induction of the expression of *ADAMTS9-AS2* by SAL treatment. Upregulation of expression was also observed by treating with Enz or ODM treatment (Fig. 3). In contrast, the expression of *PART1* was down-regulated in LNCaP cells following treatment SAL or the AR antagonists. Thus, the data suggest that the expression of *PART1* and *ADAMTS9-AS2* are within AR signaling. Interestingly, the expression of *PART1* is repressed by either SAL or AR antagonists whereas that of *ADAMTS9-AS2* is activated by AR ligands including AR antagonists.

3D tumor spheroid model reveals differential effects of AR-modulating treatments

The 3D tumor spheroid model is regarded as a more accurate representation of tumor complexity compared to traditional 2D adherent cultures^[19]. To better mimic the tumor microenvironment, we employed a 3D tumor spheroid model using LNCaP and C4-2 cells generated from parental C4-2 or LNCaP cells using ultra-low attachment plates, as previously described^[4]. This model provided a more physiologically relevant system to evaluate the impact of androgen receptor (AR)-modulating treatments, including SAL, ENZ, and ODM, in comparison to DMSO control.

Quantitative assessment of spheroid volumes revealed significant reductions in tumor size across both LNCaP and C4-2

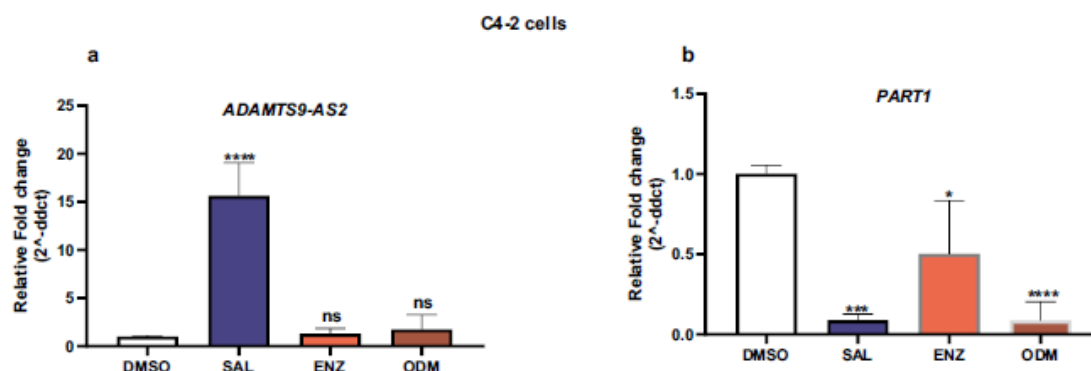


Figure 2. SAL treatment enhanced the expression of the lncRNA *ADAMTS9-AS2* whereas that of the lncRNA *PART1* is repressed by SAL and second-generation AR antagonists in the castration resistant C4-2 cells. Fold change expression ($=2^{\Delta\Delta CT}$) of two lncRNAs including *ADAMTS9-AS2* (A) and *PART1* (B) genes in merged three biological replicates (each biological replicate contained duplicate experiments) of C4-2 cells treated with SAL (1 nM R1881), ENZ (10 μ M) and ODM (10 μ M) relative to DMSO treated prostate cancer cells as control. One-way ANOVA was used to identify differentially expression between treated cancer cells and control cells.

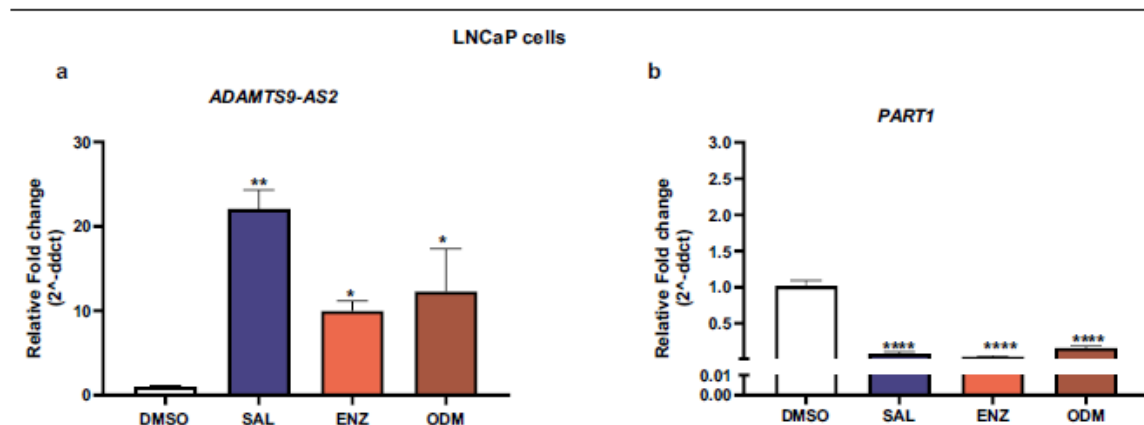


Figure 3. SAL and AR antagonists enhance the expression of the lncRNA *ADAMTS9-AS2* whereas that of the lncRNA *PART1* is repressed by SAL AR antagonists in the androgen-sensitive LNCaP cells. Fold change expression ($2^{-\Delta\Delta CT}$) of two lncRNAs including *ADAMTS9-AS2* (A) and *PART1* (B) genes in merged three biological replicates (each biological replicate contained duplicate experiments) of LNCaP cells treated with SAL, ENZ and ODM relative to DMSO treated prostate cancer cells as control. One-way ANOVA was used to identify differentially expression between treated cancer cells and control cells (* $P < 0.05$, **** $P < 0.0001$; ns: non-significant).

spheroids upon treatment with SAL, ENZ, or ODM (Fig. 4A and B). This reduction was consistent with prior observations in 2D culture systems, emphasizing the effectiveness of these agents in disrupting tumor growth.

Molecular profiling of key AR-regulated genes and lncRNAs demonstrated treatment-specific expression patterns. *ADAMTS9-AS2*, an lncRNA previously implicated in AR signaling modulation, exhibited a marked increase in expression following treatment with SAL, ENZ, and ODM in both LNCaP and C4-2 spheroids (Fig. 4A and B). This pattern supports its role as a potential co-regulator in the AR pathway and suggests that AR antagonists do not completely inactivate AR signaling.

Conversely, expression of AR target genes, including *TPRPS2*, *KLK3*, and *FKBP5*, displayed a distinct dependency

on the type of treatment. These AR target genes showed upregulation by SAL treatment but were downregulated with ENZ and ODM in both cell lines (Fig. 4A and B). The differential responses underscore the contrasting effects of SAL as an AR agonist and ENZ/ODM as AR antagonists.

Interestingly, the lncRNA *PART1* demonstrated consistent downregulation across both LNCaP and C4-2 spheroids treated with SAL, ENZ, or ODM (Fig. 4A and B). This finding aligns with its proposed role as a co-repressor of AR signaling, particularly under conditions of AR pathway modulation.

These results collectively highlight the capacity of the 3D spheroid model to capture the complexity of tumor-specific gene expression responses to AR-targeted therapies. They also provide further evidence of the interplay between AR signaling

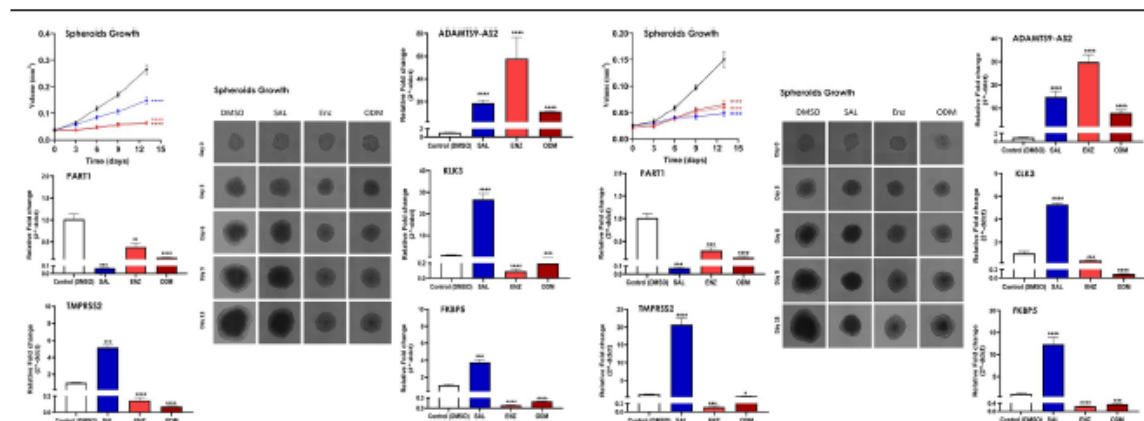


Figure 4. SAL, ENZ, and ODM treatments inhibit spheroid growth in both C4-2 and LNCaP cells and regulate the expression of *ADAMTS9-AS2* and *PART1* in tumor spheroids. Growth analysis of treated spheroids ($n = 18$) after indicated days were analyzed and statistics were calculated with two-way ANOVA. Expression levels of *ADAMTS9-AS2*, *PART1* and AR target genes were analyzed of RNA extracts isolated from spheroids generated from C4-2 and LNCaP cells following treatment with SAL (agonist) and AR antagonists.

and lncRNA regulation, emphasizing their therapeutic relevance in prostate cancer.

Differential recruitment of AR to PART1 and ADAMTS9-AS2 loci in C4-2 cells

ChIP-seq analysis indicates the recruitment of AR to the *PART1* (intron) locus in C4-2 cells by DHT (Figure S4A, available at: <http://links.lww.com/JIS9/E35>). Additionally, the ChIP-seq results demonstrated selective recruitment of AR to the *ADAMTS9-AS2* (intron and exon) locus in C4-2 cells treated with DHT (Figure S4B, available at: <http://links.lww.com/JIS9/E35>).

Thus, the data suggests that *PART1* is a novel AR target gene, however, a non-classical AR target gene with the expression being repressed by SAL treatment^[20].

Ex-vivo analysis reveals significant alterations in ADAMTS9-AS2 and PART1 expression induced by SAL

Non-fixated, native patient PCa specimen derived from prostatectomy was analyzed for changes of expression by SAL treatment. We detected significant difference in the *ex-vivo* expression levels of both *ADAMTS9-AS2* and *PART1* after treatment with SAL (Fig. 5) confirming the SAL-mediated regulation of these lncRNAs in LNCaP and C4-2 cells.

Similar to the inducibility of *ADAMTS9-AS2* expression by SAL the *ex-vivo* expression of *ADAMTS9-AS2* was significantly elevated in SAL-treated PCa tissues [expression ratio (95% CI) = 54.2 (16.9–175), $P < 0.0001$]. In contrast to *ADAMTS9-AS2* and as expected from the cell line data, the *ex-vivo* expression of *PART1* was reduced in SAL-treated PCa tissues [expression ratio (95% CI) = 0.12 (0.02–0.77), $P = 0.03$].

Furthermore, significant correlations were detected between PSA levels and BMI (correlation coefficient = 0.726, $P = 0.027$), *PART1* expression and age (correlation coefficient = -0.644, $P = 0.049$), as well as expression levels of *PART1* and *ADAMTS9-AS2* (correlation coefficient = 0.8, $P = 0.10$).

The lncRNA ADAMTS9-AS2 is an AR co-regulator

The obtained data led to the hypothesis that there could be a cross-talk between the lncRNA *ADAMTS9-AS2* with AR by influencing AR transcriptional activity. For that purpose, each the lncRNAs *ADAMTS9-AS2* and *PART1* were knocked down by small interfering RNA (siRNA) and the expression of *KLK3*, encoding the diagnostic marker prostate specific antigen (PSA), *TMPRSS2* and *FKBP5* known as direct AR target genes were analyzed. The knockdown was validated in both cell lines (Fig. 6A and B) indicating an efficient knockdown. The AR target (*FKBP5(b)*, *TMPRSS2(c)*, and *KLK3(d)*) were upregulated by SAL, as expected. Interestingly, the knockdown of *ADAMTS9-AS2* reduced the SAL-induced expression of the AR target genes *FKBP5*, *TMPRSS2*, and *KLK3*. This observed effect suggests that the lncRNA *ADAMTS9-AS2* may function as a coactivator of the AR. Therefore, the data indicates that lncRNA *ADAMTS9-AS2* is part of the AR signaling.

PART1 lncRNA knockdown elicits overexpression of AR target genes as a co-repressor

Similarly, we performed knockdown for *PART1* in both C4-2 and LNCaP cell lines. In contrast to the knockdown of *ADAMTS9-AS2*, the expression of the lncRNA *PART1* is inhibited by SAL. The knockdown of the lncRNA *PART1* resulted in enhanced expression of AR these target genes (Fig. 7) suggesting that *PART1* is a co-repressor. In line with this observation, the expression of *PART1* is positively correlated significantly in patients prostate adenocarcinoma datasets (GEPIC) with *KLK3* (Figure S5A, available at: <http://links.lww.com/JIS9/E35>) and *FKBP5* (Figure S5B, available at: <http://links.lww.com/JIS9/E35>) and weaker but also significantly with *TMPRSS2* (Figure S5C, available at: <http://links.lww.com/JIS9/E35>). Therefore, the data indicates that lncRNA *PART1* could be involved in regulating AR as a co-regulator.

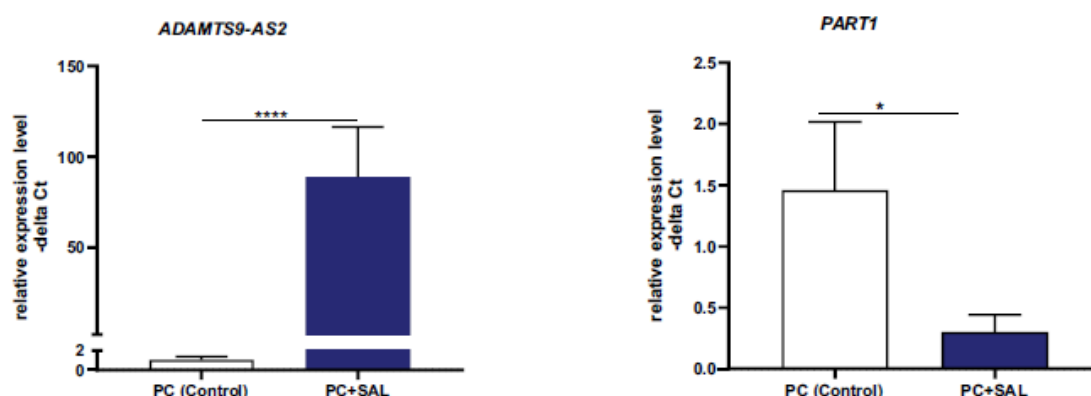


Figure 5. The expression of *ADAMTS9-AS2* and *PART1* lncRNAs in PCa tissues treated with SAL is controlled by SAL in native human PCa tissues. Relative *ex-vivo* expression of *ADAMTS9-AS2* and *PART1* lncRNAs in PCa tissues treated with SAL and untreated PCa tissues as controls Paired t test was used to identify differential expression of lncRNAs between the untreated prostate cancer tissues and prostate cancer tissues treated with SAL.

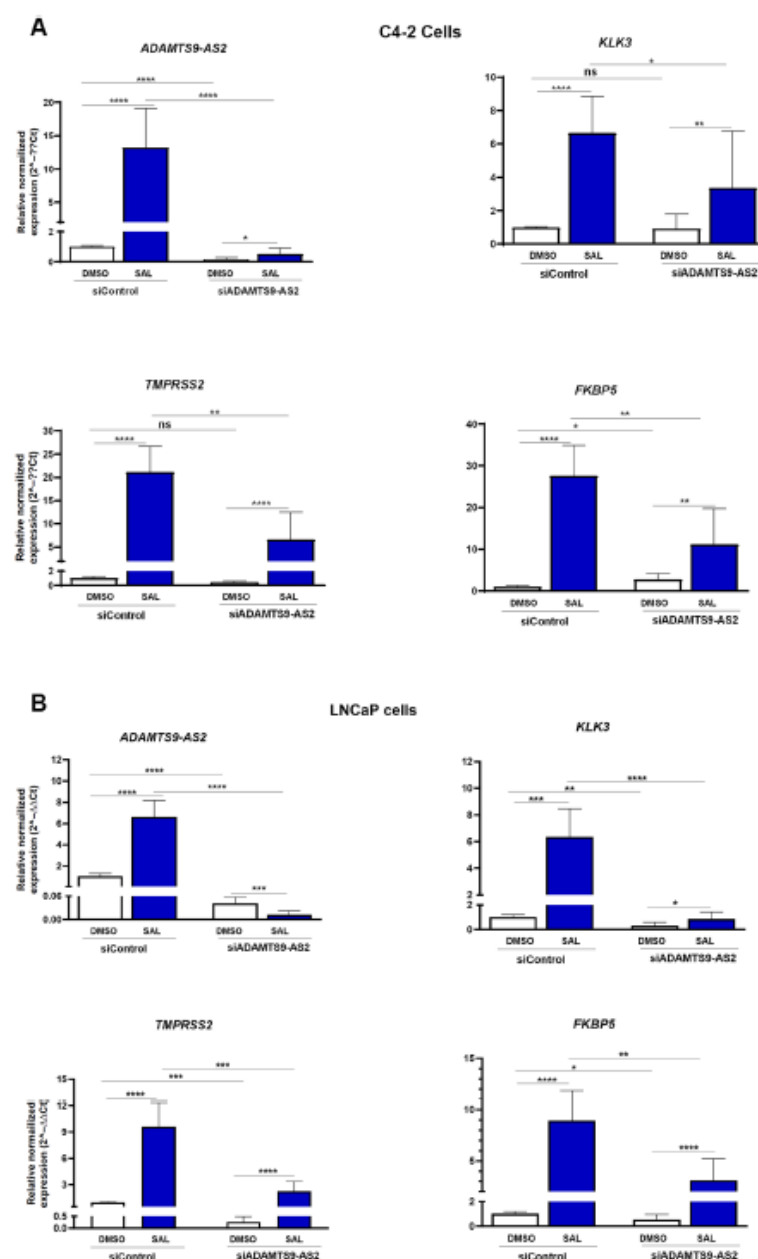


Figure 6. The knockdown of *ADAMTS9-AS2* reduces the expression of AR target genes. The expression ratio ($2^{-\Delta\Delta CT}$) of *ADAMTS9-AS2*, *KLK3*, *TMPRSS2* and *FKBP5* genes in merged three independent replicates ($n = 6$) in C4-2 cells (A) and LNCaP cells (B) transfected with nontargeting siRNA (siControl; as negative control) and *ADAMTS9-AS2* targeting siRNA (siADAMTS9-AS2), treated with DMSO and SAL. The fold changes were calculated relative to DMSO treated C4-2 and LNCaP cells transfected with nontargeting siRNA. Two-way ANOVA and Tukey post hoc tests were used to identify differentially expression between groups.

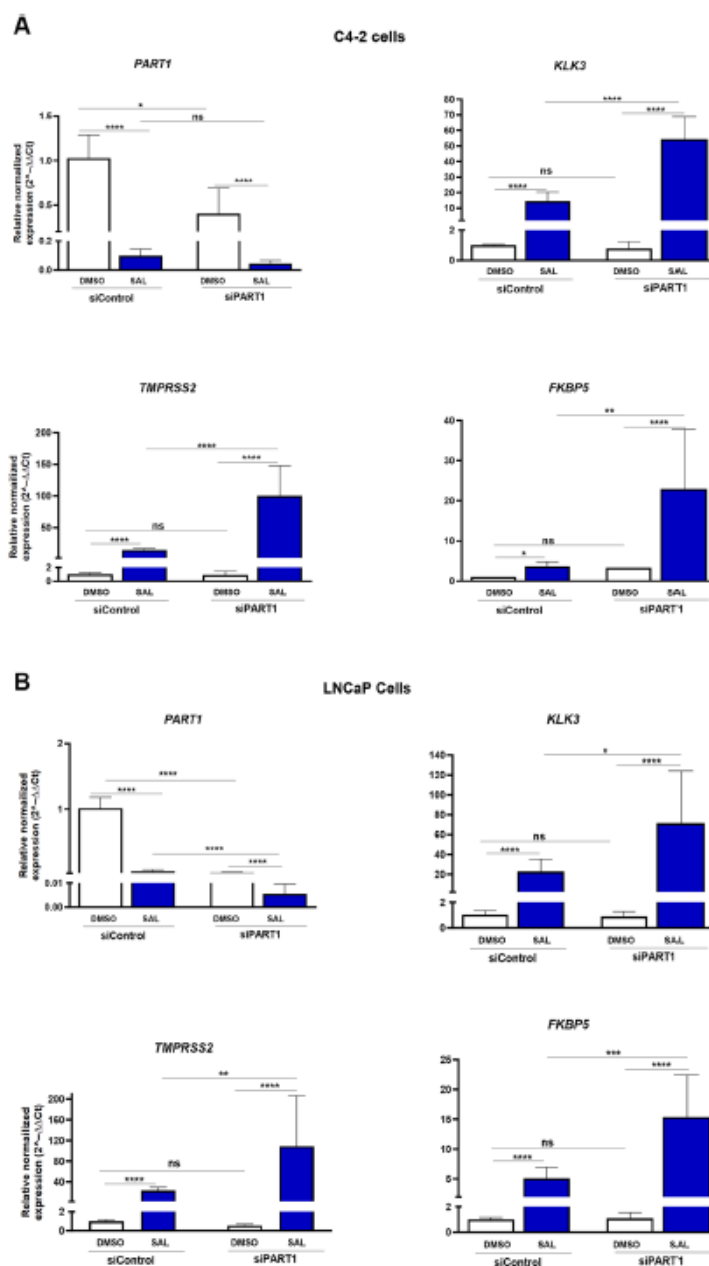


Figure 7. The knockdown of *PART1* enhances the expression of AR target genes in both cell lines. The expression ratio ($2^{-\Delta\Delta CT}$) of *PART1*, *KLK3*, *TMPRSS2* and *FKBP5* genes in merged three independent replicates ($n = 6$) in C4-2 cells (A) and LNCaP cells (B) transfected with nontargeting siRNA (siControl; as negative control) and *PART1* targeting siRNA (siPART1), treated with DMSO and SAL. The fold changes were calculated relative to DMSO treated LNCaP cells transfected with nontargeting siRNA. Two-way ANOVA and Tukey post hoc tests were used to identify differential expression between groups.

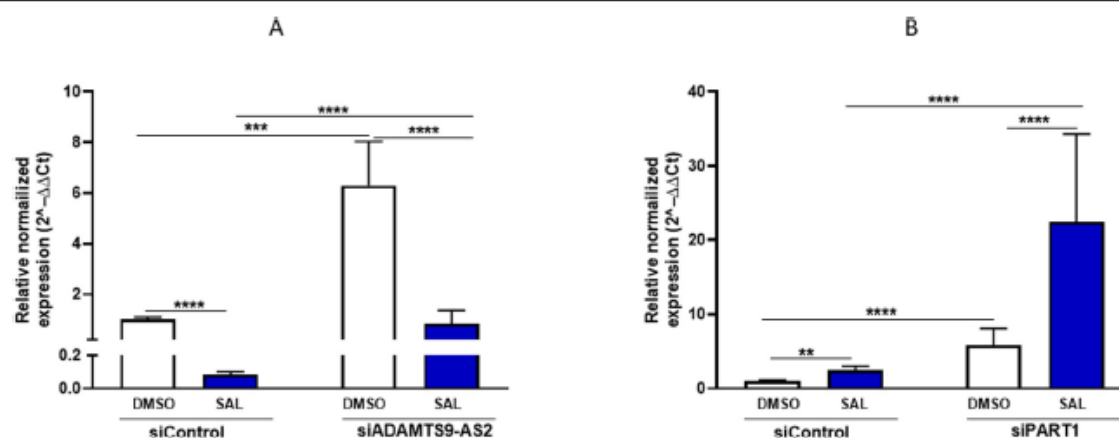


Figure 8. Differential Expression of *PART1* and *ADAMTS9-AS2* lncRNAs in C4-2 Cells Following siRNA-Mediated Knockdown and SAL Treatment. The expression ratio (2^{-ΔΔCt}) of *PART1* (A) and *ADAMTS9-AS2* (B) lncRNAs in three independent biological replicates and merged replicates in C4-2 cells transfected with nontargeting siRNA (siControl; as negative control), *ADAMTS9-AS2* targeting siRNA (siADAMTS9-AS2) (A) as well as *PART1* targeting siRNA (siPART1) (B), treated with DMSO and SAL. The fold changes were calculated relative to DMSO treated C4-2 cells transfected with nontargeting siRNA.

Reciprocal regulation of *PART1* and *ADAMTS9-AS2* in C4-2 cells: impact on AR signaling and cellular senescence

The expression analysis of *PART1* and *ADAMTS9-AS2* in C4-2 cells following siRNA-mediated knockdown revealed a dynamic regulatory interplay influencing AR signaling (Fig. 8). In the absence of SAL, siADAMTS9-AS2 knockdown led to increased *PART1* expression, acting as a co-repressor and suppressing AR target genes. Conversely, siPART1 knockdown enhanced *ADAMTS9-AS2* expression, promoting AR activation. This indicates a crosstalk between these two lncRNAs. Upon SAL treatment, *ADAMTS9-AS2* levels increased while *PART1* decreased, reinforcing a reciprocal crosstalk where the rise in one lncRNA corresponded to the suppression of the other. This interaction suggests that AR becomes “super-activated,” driving cellular senescence.

RIP assays highlight distinct enrichment patterns: *PART1* is significantly enriched by AR antibodies whereas *ADAMTS9-AS2* is not

Our results unveiled a distinctive enrichment pattern in the immunoprecipitates using AR antibodies in LNCaP cells. Notably, the *PART1* transcript exhibited a substantial increase in abundance in the immunoprecipitated material using anti-AR antibody, signifying an interaction with AR. In stark contrast, *ADAMTS9-AS2* demonstrated no significant enrichment, suggesting a lack of direct interaction with the AR (Fig. 9).

siRNA mediated KD of *ADAMTS9-AS2* reduces the ability of SAL to induce cellular senescence

The treatment of PCa cells with SAL induces cellular senescence including patient PCa specimen treated *ex-vivo*^[18], PCa 3D tumor spheroids^[4], and in mouse xenografts^[21]. Since the lncRNAs are regulating AR signaling, we hypothesized that they regulate the SAL-mediated induction of cellular senescence. Employing si-mediated knockdown our results reveal that the targeted reduction of *ADAMTS9-AS2* expression leads to a notable attenuation in the ability of SAL to induce cellular

senescence in both cell lines (Fig. 10). This suggests that *ADAMTS9-AS2*, which is upregulated by SAL, promotes SAL-mediated cellular senescence. Investigating the impact of siRNA-mediated KD on *PART1*, we observed a reduction in the cellular response to SAL-induced level of cellular senescence in both cell lines. Specifically, in both C4-2 and LNCaP cells, the reduction of *PART1* expression significantly reduces the ability of SAL to induce cellular senescence. This heightened sensitivity implies a regulatory role for both *PART1* and *ADAMTS9-AS2* in the AR-mediated senescence process in both androgen-sensitive and castration-resistant PCa cell lines.

Contrasting impact of *ADAMTS9-AS2* and *PART1* knockdown on p15^{INK4B} mRNA and protein levels in C4-2 and LNCaP cells

The levels of *CDKN2B* mRNA encoding p15^{INK4B} protein levels were analyzed in both C4-2 and LNCaP cells, with and without the knockdown of *ADAMTS9-AS2*. The results indicate a reduction in both mRNA and protein levels of p15^{INK4B} upregulated by SAL (Figure 11A-C) and the knockdown of *ADAMTS9-AS2* downregulated the levels of *CDKN2B* mRNA and p15^{INK4B} protein in both cell lines (Fig. 11C). This finding suggests a regulatory role of *ADAMTS9-AS2* in the expression of the senescence inducer p15^{INK4B}. Our data is also consistent with the results obtained from the senescence-associated beta-galactosidase (SA-β-gal) assays, which also showed a decrease in cellular senescence upon *ADAMTS9-AS2* knockdown. Thus, our observations further validate that the knockdown of *ADAMTS9-AS2* reduces the senescence-inducing capability of SAL and indicates that *ADAMTS9-AS2* is mediating in part SAL-induced cellular senescence.

The knockdown of *PART1* in LNCaP and C4-2 PCa cell lines resulted, in contrast to si-*ADAMTS9-AS2*, in a significant induction of p15^{INK4B} mRNA and protein levels. This observation underscores the role of *PART1* in the regulation of p15^{INK4B} expression suggesting that *PART1* may normally act as a suppressor of p15^{INK4B} expression. Another cell cycle inhibitor

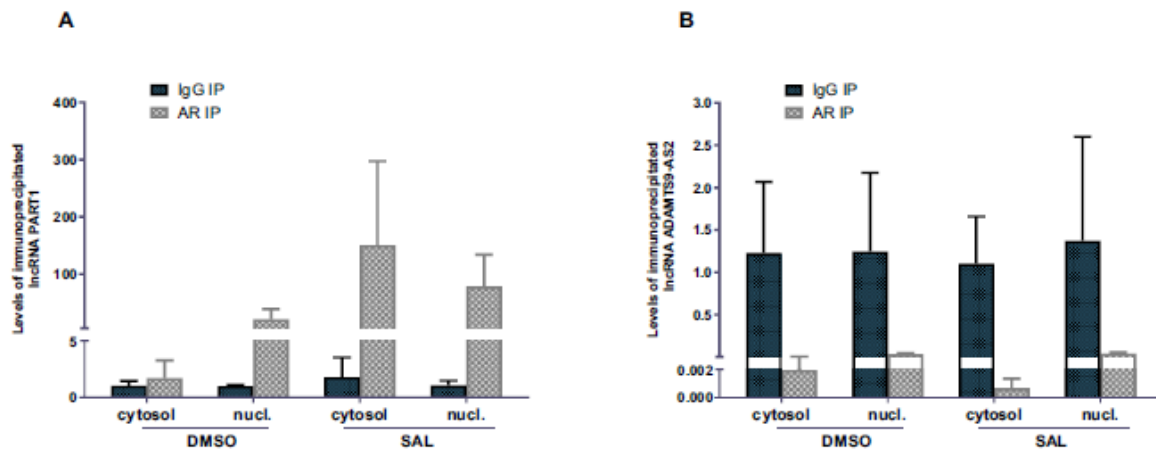


Figure 9. The AR binds to the lncRNA *PART1*. The association of the RNA-binding protein (RBP) AR with *PART1* and *ADAMTS9-AS2* lncRNAs was tested by RIP analysis using anti-AR antibody. Following immunoprecipitation, RNA was extracted and the abundance of *PART1* lncRNA (A) and *ADAMTS9-AS2* lncRNA (B) in AR IP and IgG IP control samples was assessed by RT-qPCR analysis using *PART1* and *ADAMTS9-AS2* lncRNA-specific primers in cytosol and nucleus. These results were normalized to the levels of relevant input RNA in each sample, and then plotted as the enrichment of lncRNAs in the AR IP relative to the lncRNA levels observed in the IgG IP samples (total merged biological replicates, $n = 4$).

p21 was also analyzed and suggest that in contrast to *ADAMTS9-AS2* the knockdown of lncRNA *PART1* enhances p21 protein levels indicating that p21 is also a target of *PART1*. Interestingly, in the presence of SAL and in line with reduced cellular senescence by knockdown of both lncRNAs the p21 levels are reduced (Fig. 11C).

These findings indicate that the SAL-mediated downregulation of *PART1* is part of SAL-mediated induction of cellular in PCa cells. The upregulation of p15^{INK4B} following *PART1* knockdown indicates also a potential mechanism through which *PART1* contributes to the progression and possibly the aggressiveness of PCa. In line with this observation, datasets from patient PCa samples show a significant negative correlation of *PART1* with *CDKN2B* (Figure S5D, available at: <http://links.lww.com/JS9/E35>).

Of note, the knockdown of either *PART1* or *ADAMTS9-AS2* enhances the protein levels of AR in both cell lines (Fig. 11C) indicating that both lncRNAs *PART1* might regulate AR activity by regulating endogenous AR protein levels.

Bioinformatic analysis

Using the STRING database, we constructed a PPI network based on the final set of DE mRNAs. Subsequently, we imported this network into Cytoscape software (Figure S6A, available at: <http://links.lww.com/JS9/E35>). Through the cytoHubba plugin, we identified 20 hub genes based on maximal clique centrality (MCC), degree centrality (DC), closeness centrality (CC), radiality centrality (RC) and stress centrality (SC) (Figure S6B, available at: <http://links.lww.com/JS9/E35>). To filter these hub genes, we intersected the hub genes of these five centralities, resulting in 8 hub genes, namely TAGLN, KRT5, FLNA, FLNC, CAV1, FGF2, EPHA2, and ITGB4 (Figure S6C, available at: <http://links.lww.com/JS9/E35>). The analysis of Gene Ontology (GO) and KEGG pathway for the identified hub genes was performed using the clusterProfiler package. It was demonstrated that these

hub genes are involved in the focal adhesion signaling pathway (Figure S6D, available at: <http://links.lww.com/JS9/E35>).

Identification of hub clusters

By utilizing the MCODE plugin integrated into cytoscape, we conducted a comprehensive analysis to identify significant clusters within the PPI network. Notably, we discovered the cluster with the highest scores (Figure S7A, available at: <http://links.lww.com/JS9/E35>). This cluster exhibited a score of 6.615, comprised LMOD1, ACTG2, MYH11, MYLK, TPM2, MYL9, FLNA, TAGLN, ITGB4, KRT14, KRT5, COL17A1, DST and LAMB3. Subsequently, we performed GO and KEGG enrichment analyses on this hub cluster using the clusterProfiler package in R (Figure S7B, available at: <http://links.lww.com/JS9/E35>).

ceRNA network construction in prostate cancer

While building the ceRNA network involving lncRNA-miRNA-mRNA, researchers concentrated on miRNAs that were either upregulated or downregulated. lncRNAs and mRNAs that demonstrated opposing associations with these miRNAs in both the miRNA-mRNA and lncRNA-miRNA interaction pairs were included^[22]. We employed miRcode to investigate the connections between lncRNAs and miRNAs. The analysis revealed that out of the 9 DE lncRNAs, 6 of them were potentially targeting 17 out of the 55 miRNAs. We utilized miRNameConverter package (version 1.30.0) to convert miRNA names to those available in the miRbase v22 database. Then, using multiMiR package (version 1.24.0), we identified target mRNAs for the miRNAs. To improve our results, we eliminated miRNA-targeted mRNAs that was not present among the differentially expressed mRNAs. From the data analysis, we identified 13 miRNAs linked to prostate cancer that might target 107 of the 266 DE mRNAs. Moreover, we eliminated any interactions between lncRNAs and miRNAs, as well as miRNAs and mRNAs that had opposing expression

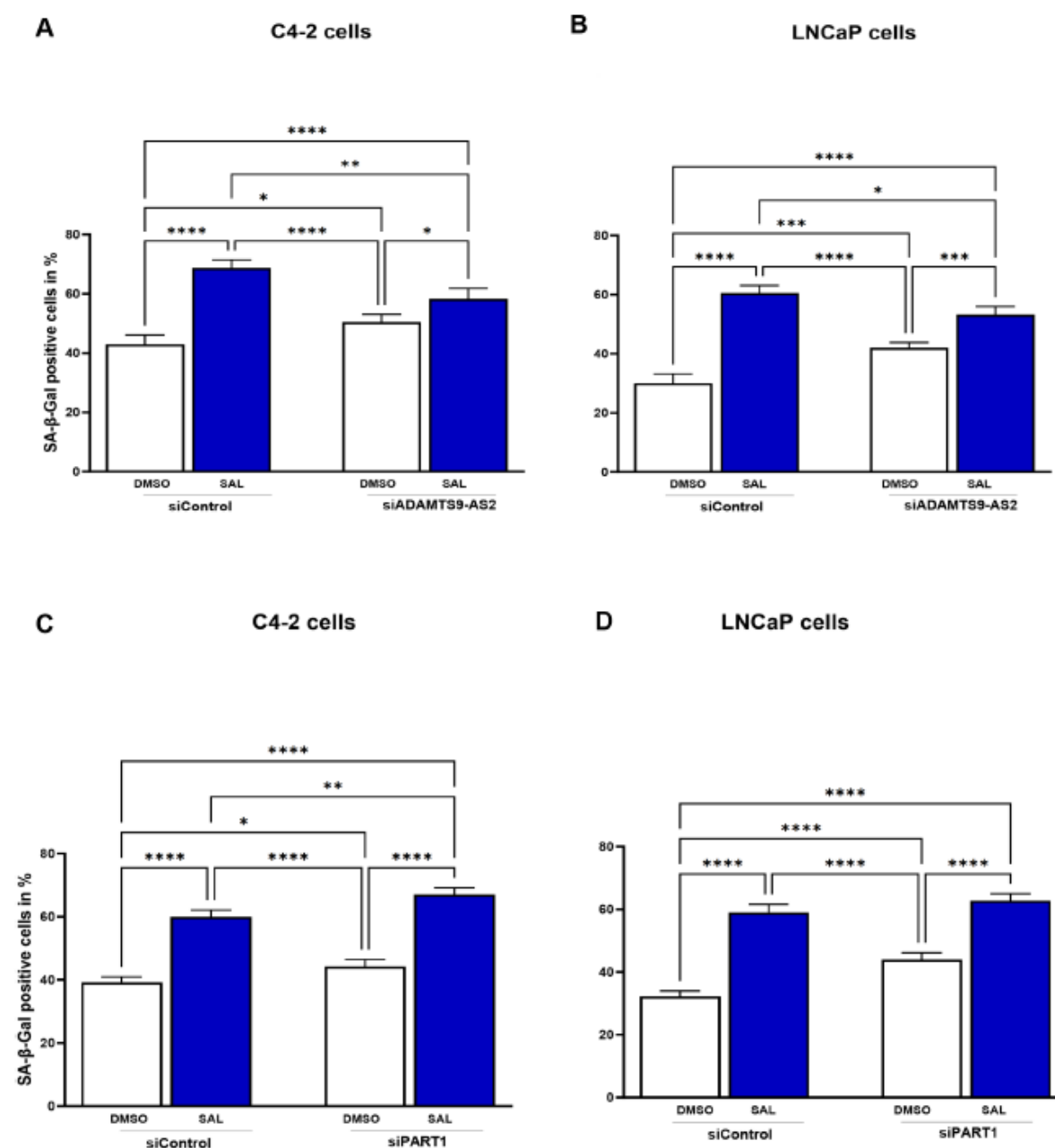


Figure 10. The knockdown of the lncRNA *ADAMTS9-AS2* reduces whereas that of *PART1* enhances SAL-mediated cellular senescence. Proportion of SA-β-gal positive C4-2 (A) and LNCaP (B) cells transfected with nontargeting siRNA (siControl; as negative control) and *ADAMTS9-AS2* targeting siRNA (siADAMTS9-AS2), treated with DMSO and SAL. Two-way ANOVA and Tukey post hoc tests were used to identify different proportion of SA-β-gal positive cells between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; ns: non-significant). Proportion of SA-β-gal positive C4-2 (C) and LNCaP (D) cells transfected with nontargeting siRNA (siControl; as negative control) and *PART1* targeting siRNA (siPART1), treated with DMSO and SAL. Two-way ANOVA and Tukey post hoc tests were used to identify different proportion of SA-β-gal positive cells between groups.

patterns, following the ceRNA network theory. Utilizing Cytoscape 3.9, we constructed a comprehensive ceRNA network incorporating 3 lncRNAs, 13 miRNAs, and 107 mRNAs (Table

S8, available at: <http://links.lww.com/JS9/E34>; Figure S8, available at: <http://links.lww.com/JS9/E35>). Also, the network includes 123 nodes and 124 edges.

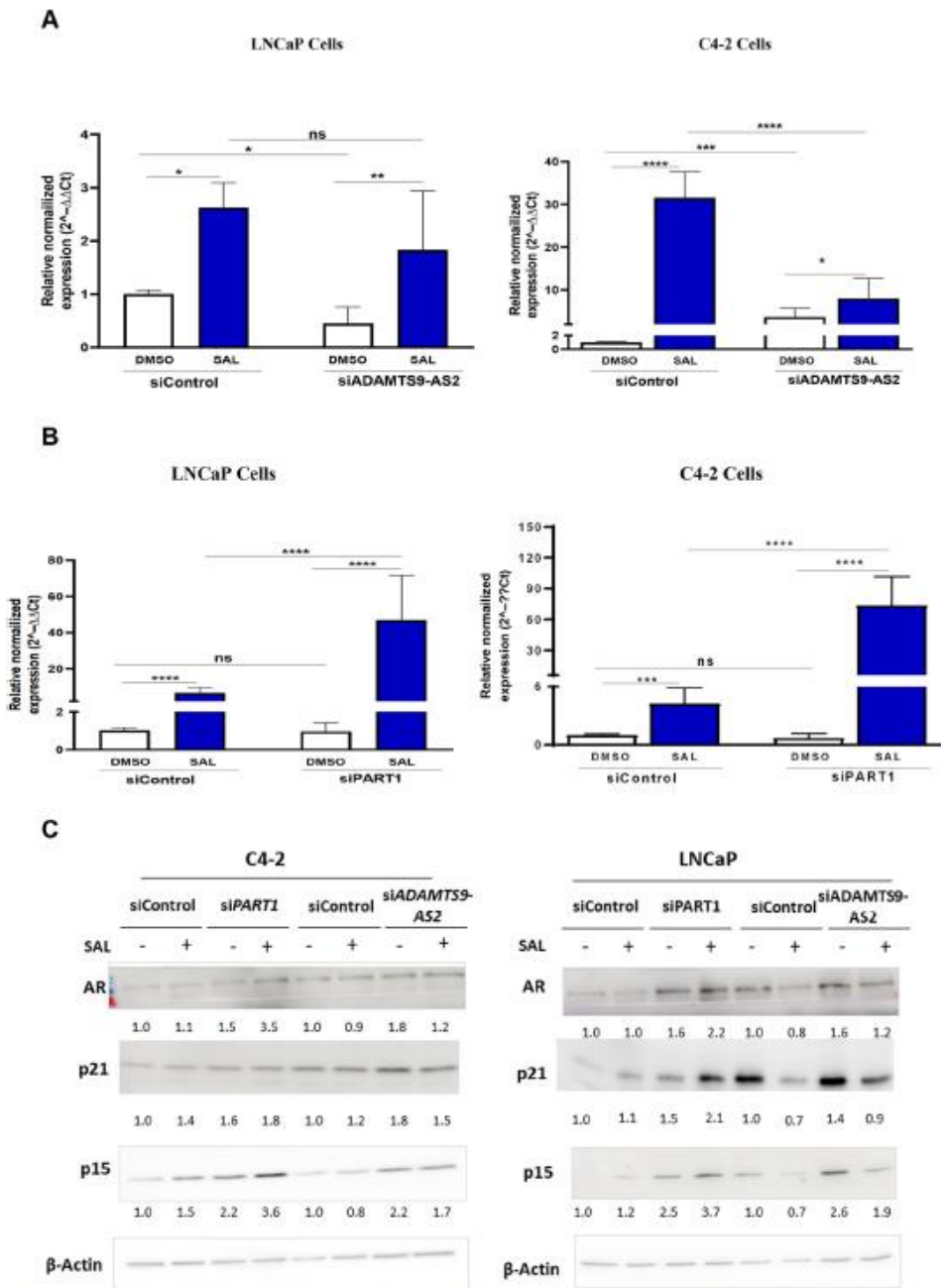


Figure 11. The levels of the cell cycle inhibitor and senescence marker p15 are changed by SAL and knockdown of the lncRNAs ADAMTS9-AS2 and PART1. The expression ratio ($2^{-\Delta\Delta Ct}$) of CDKN2B mRNA in merged data from three independent biological replicates ($n = 6$) in LNcaP (A) and C4-2 cells (B) transfected with nontargeting siRNA (siControl; as negative control) and ADAMTS9-AS2 targeting siRNA (siADAMTS9-AS2), treated with DMSO and SAL. The fold changes were calculated relative to DMSO treated LNcaP cells transfected with nontargeting siRNA. Two-way ANOVA and Tukey post hoc tests were used to identify differentially expression between groups. (C) Western blot of AR, p15INK4B, and p21 proteins in C4-2 and LNcaP cells under various treatment conditions.

Using the cytoHubba plugin, we identified 15 hub genes based on their highest maximal clique centrality, degree centrality, betweenness centrality, radiality centrality and eccentricity centrality (Figure S9A, available at: <http://links.lww.com/JS9/E35>). By further analyzing the intersection of these hub genes, we found a group comprising *ADAMTS9-AS2*, *hsa-miR-10b-5p*, *hsa-miR-148a-3p*, *hsa-miR-183-5p*, *hsa-miR-18b-5p*, *hsa-miR-199b-5p*, *hsa-miR-200a-3p*, *hsa-miR-203a-3p*, *hsa-miR-26b-5p*, *hsa-miR-301a-3p*, *hsa-miR-454-3p*, *hsa-miR-7-5p* and *hsa-miR-98-5p* (Figure S9B, available at: <http://links.lww.com/JS9/E35>). We also constructed a PPI network from the mRNAs present in the ceRNA network, applying centralities to this network (Figure S10, available at: <http://links.lww.com/JS9/E35>). Finally, by sharing interactions among the hub genes of these centralities, we obtained *CAV1* and *FLNA* as hub mRNAs (Fig. 11). These hub genes represent crucial players in the network and are of particular interest for understanding their significance in the studied system.

Analysis of prostate adenocarcinoma datasets indicates that elevated expression levels of both *CAV1* and *FLNA* are positively associated with improved survival probabilities in patients. This finding underscores the potential significance of these two hub genes in the pathophysiology of prostate cancer. The observed correlation aligns closely with the outcomes of our bioinformatic analyses, which identified *CAV1* and *FLNA* as critical components of regulatory networks. These results suggest that the upregulation of these genes may confer a survival advantage, potentially by modulating key molecular pathways involved in tumor progression, cellular structure, or metastatic behavior. Further experimental validation is warranted to elucidate the precise mechanistic roles of *CAV1* and *FLNA* and to explore their potential as prognostic biomarkers or therapeutic targets in prostate cancer management (Figure S11, available at: <http://links.lww.com/JS9/E35>).

Kaplan-Meier survival analyses for *CAV1* and *FLNA* expression in prostate adenocarcinoma patients reveal significant associations with patient outcomes. High *CAV1* expression ($n = 334$) is linked to improved survival probability compared to low expression ($n = 146$), indicating its potential as a favorable prognostic marker (Figure S11, available at: <http://links.lww.com/JS9/E35>). Similarly, elevated *FLNA* expression is associated with better overall survival, suggesting its protective role in prostate cancer progression. These findings highlight the

prognostic relevance of *CAV1* and *FLNA* in prostate adenocarcinoma.

Key axes of the ceRNA network

To select the axes, we obtained genes through the intersection among hub genes. This was accomplished by employing the shared interactions between hub genes in the ceRNA network, specifically for the selection of lncRNAs and miRNAs. For the selection of mRNAs, we also utilized the shared interactions among gene hubs in the PPI network of mRNAs within the ceRNA network. The mRNAs, including *CAV1* and *FLNA*, are located in the final gene hubs of the main PPI network. These axes include *ADAMTS9-AS2*/*hsa-miR-7-5p*/*CAV1* and *ADAMTS9-AS2*/*hsa-miR-98-5p*/*FLNA* (Fig. 12).

LncRNA-miRNA interactions

Furthermore, we explored the interactions between miRNAs and *ADAMTS9-AS2* and *PART1* utilizing the LncBase database. We implemented various filters to refine our analysis including tissue filter to focus, set the miRNA conf filter selected as HIGH to ensure confidence in the miRNA interactions, and used the Validated as filter to only include interactions that have been experimentally validated. Our analysis suggests significant interactions between *PART1* and two miRNAs: *hsa-miR-99a-5p* and *hsa-miR-150-5p*. Additionally, we identified noteworthy interactions between *ADAMTS9-AS2* and two miRNAs: *hsa-miR-7-5p* and *hsa-miR-98-5p*.

LncRNA interactions

Using the RNAinter database, we identified multiple interactions of *ADAMTS9-AS2* and *PART1* with histone modifications, transcription factors (TFs), proteins, and miRNAs, suggesting their regulatory roles in chromatin dynamics and transcriptional control. *ADAMTS9-AS2* shows strong associations with active chromatin markers such as H3K4me3 (score: 0.8377) and H3K27ac (0.7737), as well as repressive markers like H3K27me3 (0.7579), indicating its dual role in activating and repressing gene expression. It also interacts with key TFs, including CTCF (0.6255) and AR (0.4822), and proteins like POLR2A (0.528), highlighting its potential as a chromatin and transcription regulator. Similarly, *PART1* is associated with active histone marks like H3K4me3

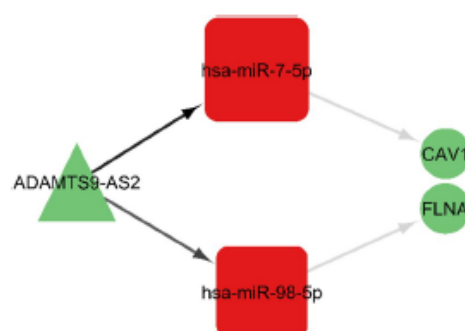


Figure 12. Key axes in the ceRNA network based on hub genes.

(0.6952) and interacts with TFs such as FOXA1 (0.5984) and AR (0.5479), suggesting its involvement in transcriptional regulation, particularly in hormone-driven pathways. These findings highlight the lncRNAs' pivotal roles in modulating gene expression and cellular processes in prostate cancer (Table S9, available at: <http://links.lww.com/JS9/E34>).

KEGG Pathway Enrichment Analysis of PART1 and ADAMTS9-AS2

KEGG Pathway Enrichment Analysis of *PART1* and *ADAMTS9-AS2* were conducted using the ncPath database. The obtained KEGG signaling pathways are related to *PART1* and *ADAMTS9-AS2*, and with the cellular senescence pathway showing the lowest adjusted *P* value. In Figure S12 (available at: <http://links.lww.com/JS9/E35>), the genes influenced by *PART1* in the cellular senescence pathway are outlined.

This suggests that the KEGG pathway enrichment analysis highlights the significant involvement of *PART1* and *ADAMTS9-AS2* in the cellular senescence pathway. Among the enriched pathways, cellular senescence exhibits the lowest adjusted *P* value, indicating its strong association with the target lncRNAs. Detailed examination revealed that *PART1* influences several key genes within this pathway, as depicted in Figure S12 (available at: <http://links.lww.com/JS9/E35>). This finding suggests that *PART1* may play a regulatory role in cellular senescence, which could be critical in the context of androgen receptor signaling and prostate cancer progression.

The KEGG cellular senescence pathway (Figure S12, available at: <http://links.lww.com/JS9/E35>) includes critical genes associated with cell cycle regulation, DNA damage response, and oncogenic stress. Notably, genes such as p53, CDK4/6, Cyclin D/E, and p21 were identified as targets influenced by *PART1*. The enrichment analysis indicates that *PART1* may mediate its effects by modulating these key regulators of cellular senescence, further supporting its proposed function as a co-repressor in AR signaling.

The expression of miR-98-5p exhibited upregulation, demonstrating a noteworthy correlation with ADAMTS9-AS2

For confirmation of the bioinformatic analysis, we examined expression levels of *miR-98-5p* and *miR-7-5p* in clinical samples (Fig. 13A). Both miRNAs were down-regulated in TT samples as compared to NTAT samples [expression ratios (95% CI) = 0.033 ± 0.76 (0.011–0.097) and 29.65 ± 0.6 (12.85–68.4); $P < 0.0001$ and 0.001 for *miR-98-5p* and *miR-7-5p*, respectively]. On the other hands, both *miR-99a-3p* and *miR-150-5p* were downregulated in TT samples compared to NTAT samples [expression ratios (95% CI) = 19.22 ± 0.46 (10.1–36.8) and 0.32 ± 0.56 (0.15–0.71); $P < 0.0001$ and 0.006 for *miR-99a-3p* and *miR-150-5p*, respectively]. The combination of the bioinformatic analyses with the validation with combination of bioinformatic analyses with patient data sets suggest a novel pathway and a novel miRNA network of *ADAMTS9-AS2*.

To further investigate potential interactions, we performed Spearman's correlation analysis. The expression levels of *miR-99a-3p* and *miR-150-5p* showed a weak correlation with *PART1* in tumor tissue samples of prostate cancer patients (Fig. 13B). Likewise, *miR-7-5p* and *miR-99-5p* exhibited a weak correlation with *ADAMTS9-AS2* (Fig. 13D).

While these correlations do not reach statistical significance, the observed trends, in combination with bioinformatic predictions and expression validation, suggest a potential miRNA network involving *ADAMTS9-AS2* that warrants further investigation.

Discussion

The bipolar androgen therapy (BAT) uses intermittent SAL treatment for patients with metastatic PCa. We had identified that SAL induces cellular senescence in both androgen-sensitive and CRPC cells. Cellular senescence was also induced by SAL in 3D spheroids and xenografted mice with the CRPC C4-2 cells^[13,4,18,21]. However, the underlying molecular mechanisms to induce cancer cell senescence by SAL remain not fully understood and may help understanding the advantageous of BAT at molecular level and may help identifying novel pathways used by AR.

Our RNA-seq data suggest the changes of expression of many genes including lncRNAs. lncRNAs have been recognized as important players in the pathogenesis of PCa^[23]. CRPC is recognized for its reliance on AR signaling, even when the cells exhibit resistance to androgen removal, as seen in ADT. Over time, CRPC cells and tumors develop resistance to therapy, even when subjected to complete AR blockade through ADT and co-treatment with second-generation AR antagonists, owing to adaptive signaling mechanisms^[24]. Several lncRNAs have been found to modulate AR signaling in PCa^[25].

ADAMTS9-AS2 has been discerned as a pivotal mediator in various malignancies, including diverse types of malignant tumor cells^[26]. Furthermore, it is noteworthy that *ADAMTS9-AS2* is downregulated in PCa^[27]. *PART1*, as another lncRNA, exhibits dual functionality in different human tissues, being classified as an oncogene in certain tissues while assuming the role of a tumor suppressor gene in others^[28]. Elevated *PART1* expression correlate with advanced cancer stage and poor survival prediction. SAL treatment is suggested to induce a tumor suppressive pathway. In line with this *PART1* expression is reduced by SAL treatment. SAL in cell culture and in ex vivo patient samples mediates a significant up-regulation of *ADAMTS9-AS2* and a concurrent down regulation of *PART1*. This observation suggests a potential role for SAL in modulating the expression levels of these selected lncRNAs in the opposite direction^[29]. Furthermore, an association between higher levels of *ADAMTS9-AS2* and lower prostate volume in PCa patients which is in line with the tumor suppressor role of this lncRNA was observed^[27]. In line with this, SAL induces the expression of *ADAMTS9-AS2* and suggests also a tumor suppressive pathway.

Furthermore, we identified a cross-talk between these two lncRNAs and the AR since the knockdown of each of the two lncRNAs modulate the expression of known direct AR target genes in both cell lines. This is confirmed by correlation analyses of patient tumor samples. Furthermore, the knockdown experiments suggest that the AR signaling to induce cellular senescence is regulated by these two lncRNAs. Interestingly, also AR protein levels are controlled by these lncRNAs suggesting a cross-talk between these two lncRNAs and the AR signaling. Without SAL, siADAM enhances *PART1* as a co-repressor, leading to repression of AR target genes, while siPART1 enhances *ADAMTS9-AS2*, leading to activation of AR target genes. In the presence of SAL, *ADAMTS9-AS2* increases and *PART1*

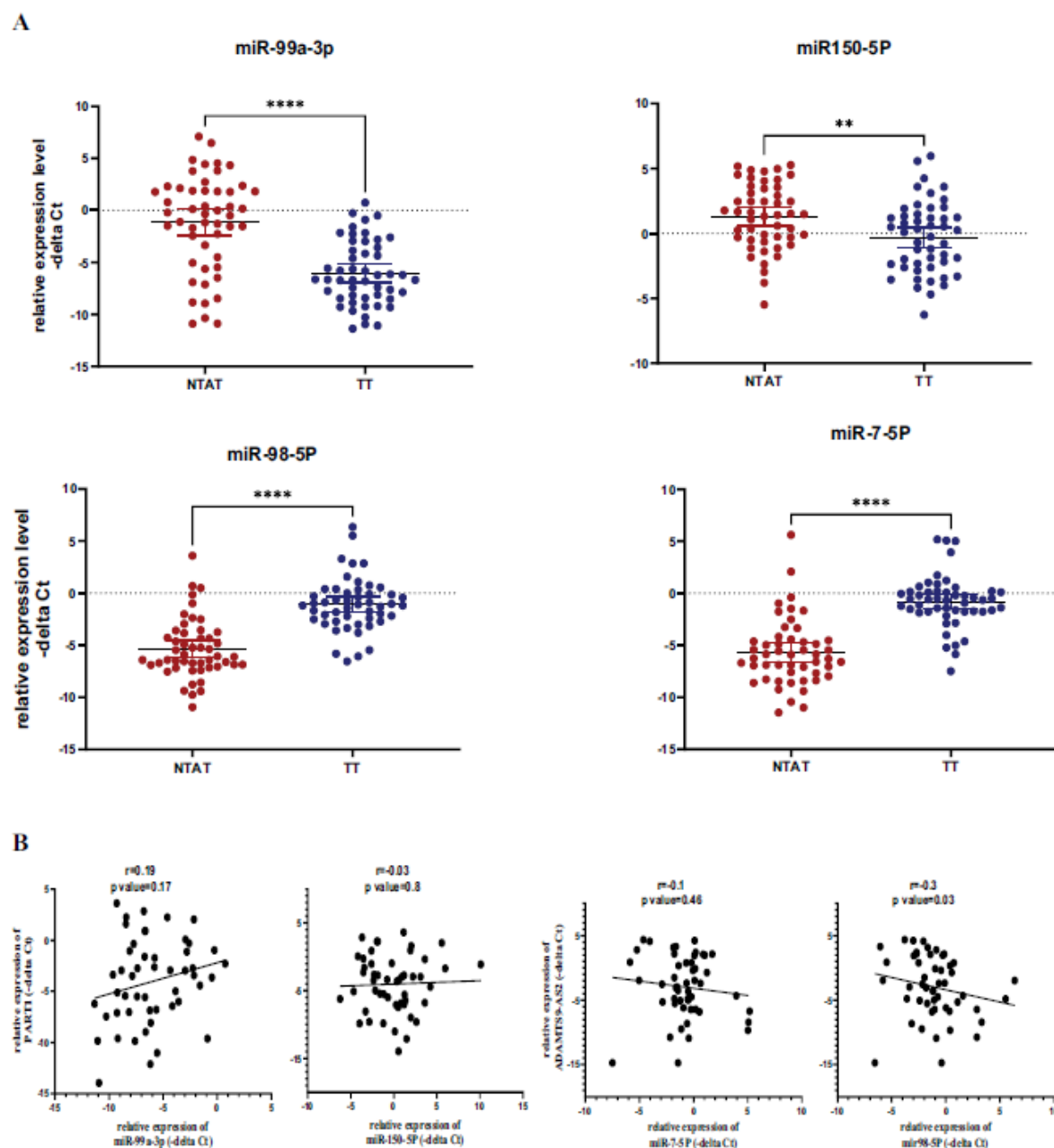


Figure 13. Confirmation of bioinformatic predictions using patient samples. The relative expression levels of four miRNA genes (A–D) in prostate tumor tissues (TT) and Normal Tissue Adjacent to the Tumor (NTAT) as described by $-\Delta\Delta Ct$ values (Ct Housekeeping gene $- Ct$ Target gene). $-\Delta\Delta Ct$ data were plotted as individual values (including the mean with 95% CI). The data were analyzed using Wilcoxon rank-sum test between tumor tissues (TT) and Normal Tissue Adjacent to the Tumor (NTAT). $P < 0.05$ was considered statistically significant. (B) Correlation analysis between miRNAs and lncRNAs in prostate cancer samples. Spearman's correlation analysis between the expression levels of miR-99a-3p and miR-150-5p with *PART1*, and miR-7-5p and miR-98-5p with *ADAMTS9-AS2* in tumor tissue samples of prostate cancer patients. The R values and P values are shown. While the correlations for miR-99a-3p, miR-150-5p and miR-7-5p were weak and not statistically significant, miR-98-5p exhibited upregulation and showed a noteworthy correlation with *ADAMTS9-AS2*. These findings suggest a potential regulatory interaction that requires further validation.

decreases. This reciprocal regulation suggests that AR is 'super' activated, mediating senescence through this dynamic lncRNA crosstalk.

The correlation data between *ADAMTS9-AS2* and *PART1* with key genes such as AR, *CDKN2B*, *FKBP5*, and *KLK3* provide valuable insights into their roles in therapeutic response and cellular senescence (Figure S13, available at: <http://links.lww.com/JS9/E35>). *ADAMTS9-AS2* shows a negative correlation with AR ($R = -0.18$, $p = 3.7 \times 10^{-6}$), suggesting it may down-regulate AR signaling, which could influence the response to therapies targeting androgen pathways, such as bipolar androgen therapy (BAT). Its positive correlation with *CDKN2B* ($R = 0.42$, $p = 0$) implies a role in promoting senescence, as *CDKN2B* is a known cell cycle regulator associated with senescence induction. In contrast, *PART1* shows a negative correlation with AR ($R = -0.14$, $p = 0.0014$) and *CDKN2B* ($R = -0.26$, $p = 9.6 \times 10^{-10}$), suggesting it may inhibit AR signaling while potentially counteracting senescence. These opposing relationships underscore the potential of targeting *ADAMTS9-AS2* and *PART1* in therapeutic strategies to modulate AR activity and enhance the effectiveness of BAT, particularly in regulating senescence and cancer progression.

Chromatin Immunoprecipitation sequencing (ChIP-seq) results substantiated the binding, particularly in the presence of an agonist to introns of *ADAMTS9-AS2* suggesting that *ADAMTS9-AS2* is a novel identified direct AR target gene.

The implications of these results extend to the intricate molecular mechanisms underlying cellular senescence, warranting further exploration to elucidate the specific pathways and interactions influenced by *ADAMTS9-AS2* in the context of androgen responsiveness and cellular aging. On the contrary, the evaluation of miRNA expression revealed the upregulation of both *miR-98-5p* and *miR-7-5p* in PCa samples relative to non-tumor adjacent tissues (NTAT), suggesting a potential interaction of these miRNAs with *ADAMTS9-AS2*. The prevailing body of research underscores the pivotal role of miRNAs in exhibiting anti-apoptotic properties. Empirical investigations reveal that *RHPN1-AS1* acts as a competitive endogenous RNA (ceRNA) for *miR-7-5p*, leading to the upregulation of *EGFR* expression and orchestrating the progression of prostate cancer (PCa) through the *miR-7-5p/EGFR/PI3K/AKT/mTOR* pathway^[30]. Furthermore, corroborated data from validated databases substantiate that *miR-7* assumes a central regulatory role in suppressing anti-apoptotic genes^[31]. Notably, the inherent characteristics observed in the expression profiles of tumor-associated *miR-7-5p* suggest promising utility as potential markers indicative of the aggressiveness of the prostate cancer trajectory^[32]. To confirm our bioinformatic analysis with wet lab experiments, our data supports the theory that these miRNAs may bind to *ADAMTS9-AS2*. Interestingly, SAL enhances the expression of *ADAMTS9-AS2*, which functionally acts as a coactivator of AR, suggesting the presence of a positive feedback loop. In contrast, a different regulatory mechanism is observed with *PART1*, where its expression is repressed by SAL. Knockdown experiments reveal that *PART1* negatively regulates AR target gene expression, indicating its role as a corepressor of AR.

We analyzed the potential cross-talk between the expression of *ADAMTS9-AS2* and *PART1*, which indicated distinct yet interconnected regulatory pathways.

Interestingly, AR recruitment to the *PART1* gene locus in the presence of SAL suggests that androgen-dependent recruitment

might mediate either gene induction, as seen with *ADAMTS9-AS2* and other AR target genes, or gene repression, as observed for *PART1*. This dual regulatory behavior is consistent with previous findings on SAL-mediated downregulation of *TERT* expression^[4], where AR was recruited in a SAL-dependent manner to the *TERT* gene locus, leading to the inhibition of *TERT* expression. Furthermore, treatment with second-generation AR antagonists also suppressed *TERT* expression, highlighting that these antagonists do not completely inhibit AR activity but instead modulate a subset of AR signaling pathways.

In contrast to *PART1*, RIP assays indicate that *ADAMTS9-AS2* directly interacts with AR, forming a complex. This direct interaction suggests that *ADAMTS9-AS2* exerts its effects on AR by binding to it. On the other hand, *PART1* appears to function as a corepressor through an indirect mechanism.

Interestingly, AR binding to the *PART1* gene locus was not detected under our experimental conditions, implying that the SAL-mediated repression of *PART1* expression likely occurs through an indirect pathway. The ChIP data suggest that AR recruitment to specific genomic loci is context-dependent, and further analysis is needed to confirm whether SAL indirectly modulates *PART1* expression through additional transcriptional regulators.

This study differs from our previous work on ceRNA networks in prostate cancer, where we identified four axes involving *ADAMTS9-AS2*, *MEG3*, *miR-150*, *PRKCA*, and *MMP14*^[33]. That analysis highlighted the regulatory role of *miR-150* as a central component in prostate tumorigenesis and its association with patient survival, focusing on ceRNA-mediated interactions as key drivers of prostate cancer progression. In contrast, the current work explores the functional role of *ADAMTS9-AS2* and *PART1* in androgen receptor (AR) signaling and cellular senescence, particularly in response to bipolar androgen therapy (BAT) using SAL treatment. Notably, we demonstrate that SAL differentially regulates *ADAMTS9-AS2* and *PART1*, inducing tumor-suppressive pathways by upregulating *ADAMTS9-AS2* while downregulating *PART1*. This regulation affects AR signaling, with *ADAMTS9-AS2* acting as a direct AR target gene, unlike *PART1*, which modulates AR signaling indirectly. These findings suggest novel AR-associated pathways and potential therapeutic strategies for targeting senescence in prostate cancer cells, expanding on the ceRNA-mediated mechanisms previously explored.

Thus, although antithetically regulated by SAL, both lncRNAs *PART1* and *ADAMTS9-AS2* are not only regulated by androgens but are acting within the AR signaling to control SAL-mediated induction of cellular senescence and suggest the identification of new pathways and networks within AR signaling in PCa and may help understanding the molecular pathways using BAT.

A key limitation of this study is the reliance on cell lines, tumor spheroids and ex vivo samples, which may not fully capture the complexity and heterogeneity of prostate cancer in patients. While these models provide valuable insights into molecular mechanisms, they may not reflect the variability in responses observed in patient-derived tissues. Additionally, controlled experimental models may not account for the influence of the tumor microenvironment or systemic factors. Future studies should focus on in vivo validations and include patient-derived xenografts or organoid models to better understand the clinical relevance of our findings.

Ethical approval

The Ethical Committee of Shahid Beheshti University of Medical Sciences approved this study, and it was conducted under the institution's ethical standards (IR.SBMU.UNRC.REC.1400.009).

Consent

All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent forms were obtained from all study participants. The study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.UNRC.REC.1400.009).

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Author's contribution

M.T. and A.B. wrote and revised the draft, and designed and supervised the study. M.T. analyzed the data and performed the experiment and data collection. K.S. performed the Western Blotting. All the authors read and approved the submitted version.

Conflicts of interest disclosure

The authors declare they have no conflict of interest.

Research registration unique identifying number (UIN)

None.

Guarantor

Mohammad Taheri and Aria Baniahmad.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Data availability statement

All data generated or analyzed during this study are included in this published article [and its supplementary information files]. The datasets analyzed during the current study are available in the GSE179321 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179321>) and GSE89223 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89223>) and GSE179688 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179688>) from gene expression omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>).

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4. Discussion

We identified novel hup gene axes of which the expression of included factors associate with prostate tumorigenesis. A central factor identified is the miR-150. Interestingly, the expression of miR-150 is associated with survival of patients. Assessment of expression profile of genes is a practical approach for identification of genes that potentially affect the progression of a disease. However, to be more precise, it is necessary to integrate multiple datasets to control dataset-specific features, alternatively named as batch effects. In the current study, we identified important molecules that affect pathogenesis of prostate cancer through a multi-step approach.

Our analyses indicate an importance of four ceRNA axes composed of *ADAMTS9-AS2/miR-150/PRKCA*, *ADAMTS9-AS2/miR-150/MMP14*, *MEG3/miR-150/PRKCA* and *MEG3/miR-150/MMP14*. The lncRNA *ADAMTS9-AS2* is a non-coding transcript that acts on one hand as a tumor suppressor in most of assessed tissues, but on the other hand it is a possible oncogene in some other tissues (Ghafouri-Fard et al. 2023b). The diagnostic and prognostic values of this lncRNA have been confirmed in a variety of cancers (Ghafouri-Fard et al. 2023b) confirming our bioinformatic analysis for prostate cancer. He, L. et al. showed *ADAMTS9-AS2* exhibits reduced expression in PCa, and its decreased levels are associated with an unfavorable disease prognosis (He et al. 2021a). Similarly, the lncRNA *MEG3* is regarded as a biomarker with diagnostic and prognostic applications in different types of cancers (He et al. 2017). Wu, M. et al. discovered that *MEG3* is downregulated in PCa, affecting cellular functions such as proliferation, migration, invasion, and apoptosis rate through the action of miR-9-5p and its target gene QKI-5 (Wu et al. 2019). In another study, Zhou, Y. et al. demonstrated that *MEG3*'s interaction with *EZH2* resulted in the augmentation of H3K27 trimethylation on *EN2*, ultimately suppressing the progression of PCa (Zhou et al. 2020). Although *MMP14* showed consistent down-regulation in prostate cancer cell lines compared with the control cell line, *PRKCA* did not follow such consistent pattern among cancer cell lines. This finding may indicate specific roles for *PRKCA* during the course of carcinogenesis.

The involvement of *miR-150* in various molecular axes found in the current study potentiates this miRNA as a therapeutic target for prostate cancer. A previous study has shown association between *miR-150-5p* plasma and tissue levels in prostate cancer patients suggesting a biomarker

role for this miRNA (Paunescu et al. 2019). Moreover, extracellular vesicle-derived miR-150-5p has been recently suggested as a possible diagnostic marker for early-stage metastasis and high-grade Gleason score prostate cancers (Cruz-Burgos et al. 2023b). In various studies, the decrease in the expression of *miR-150-5p* has been shown in PCa samples (Cruz-Burgos et al. 2023a); while our study has shown an increase in the expression of this gene in more endocrine prostate cancer. Since we focused on Gleason Score 7 samples it is unlikely to have neuroendocrine features, which can explain the dual expression.

Moreover, in the current study, it was determined that *MEG3* lncRNA and *miR-150-5p*, as well as *ADAMTS9-AS2* lncRNA and *miR-150-5p*, experienced co-occurrence mutations in several PRAD-TCGA samples, suggesting a potentially crucial role in the progression of PCa. Thus, these analyses discovered novel axes that associate with cancerogenesis and suggest a role in PCa and also with survival.

Although one of our datasets has a relatively small sample size in our study, we used stringent parameters and reinforced our results by incorporating another dataset with a larger sample size. Nevertheless, future studies can focus on transcriptional profiles of prostate cancer samples with different clinical stages to better understand stage-specific events.

The BAT uses intermittent SAL treatment for patients with metastatic PCa. We had identified that SAL induces cellular senescence in both androgen-sensitive and CRPC cells. Cellular senescence was also induced by SAL in 3D spheroids and xenografted mice with the CRPC C4-2 cells (Bartsch et al. 2021, Heidari Horestani et al. 2024, Mirzakhani et al. 2022, Roediger et al. 2014). However, the underlying molecular mechanisms to induce cancer cell senescence by AR ligands particularly using SAL remain not fully understood. The understanding of AR signaling by SAL treatment may help understanding the advantageous of BAT at molecular level and may help identifying novel pathways used by the AR in PCa and BAT.

Our RNA-seq data suggest the changes of expression of many genes including lncRNAs. LncRNAs have been recognized as important players in the pathogenesis of PCa (Taheri et al. 2023b). CRPC is recognized for its reliance on AR signaling, even when the cells exhibit resistance to androgen removal, as seen in ADT. Over time, CRPC cells and tumors develop resistance to therapy, even when subjected to complete AR blockade through ADT and co-treatment with second-generation AR antagonists, owing to adaptive signaling mechanisms (Perner et al. 2015).

Several lncRNAs have been found to exert such effects through modulation of AR expression or function (Taheri et al. 2021). *ADAMTS9-AS2* has been discerned as a pivotal mediator in various malignancies, including diverse types of malignant tumor cells (Taheri et al. 2023c). Furthermore, it is noteworthy that *ADAMTS9-AS2* exhibits downregulation in the context of prostate cancer (PCa) (He et al. 2021b). *PART1*, as another lncRNA, exhibits dual functionality in different human tissues, being classified as an oncogene in certain tissues while assuming the role of a tumor suppressor gene in others (Ran et al. 2022). Elevated *PART1* expression correlates with advanced cancer stage and poor survival prediction. SAL treatment is suggested to induce a tumor suppressive pathway. In line with this *PART1* expression is reduced by SAL treatment.

In line with the outcomes of expression assays, the investigation conducted in C4-2 cells demonstrates a significant up-regulation of *ADAMTS9-AS2* and a concurrent down-regulation of *PART1* following exposure to SAL. This observation suggests a potential role for SAL in modulating the expression levels of these selected lncRNAs in the opposite direction, offering insights into the intricate regulatory mechanisms at play (Merseburger et al. 2015).

Ex vivo experiments revealed significant up-regulation of *ADAMTS9-AS2* in PCa cells following treatment with SAL. On the other hand, this treatment led to down-regulation of *PART1*. Association studies revealed association between higher levels of *ADAMTS9-AS2* and lower prostate volume in PCa patients which is in line with the tumor suppressor role of this lncRNA (He et al. 2021b). In line with this, SAL induces the expression of *ADAMTS9-AS2* and suggests also a tumor suppressive pathway.

Our findings imply that these two lncRNAs are part of AR signaling in PCa. In addition to the expression regulation by SAL both lncRNAs modulate the expression of known direct AR target genes in both cell lines. This is confirmed by correlation analyses of patient tumor samples. Furthermore, the knockdown experiments suggest that the AR signaling to induce cellular senescence is regulated by these two lncRNAs. Interestingly, also AR protein levels are controlled by these lncRNAs suggesting a cross-talk between these two lncRNAs and the AR signaling.

In contrast to *ADAMTS9-AS2*, the RIP-assays suggest that *PART1* is in a complex with AR. Chromatin Immunoprecipitation sequencing (ChIP-seq) results substantiated the binding, particularly in the presence of an agonist to introns of *ADAMTS9-AS2* suggesting that *ADAMTS9-AS2* is a novel identified direct AR target gene. A binding of AR to *PART1* gene locus was not

detected suggesting that the SAL-mediated repression of *PART1* expression is through an indirect mechanism.

The implications of these results extend to the intricate molecular mechanisms underlying cellular senescence, warranting further exploration to elucidate the specific pathways and interactions influenced by *ADAMTS9-AS2* in the context of androgen responsiveness and cellular aging. On the contrary, the evaluation of miRNA expression revealed the upregulation of both *miR-98-5p* and *miR-7-5p* in PCa samples relative to non-tumor adjacent tissues (NTAT), suggesting a potential interaction of these miRNAs with *ADAMTS9-AS2*. The prevailing body of research underscores the pivotal role of miRNAs in exhibiting anti-apoptotic properties. Empirical investigations reveal that *RHPNI-AS1* acts as a competitive endogenous RNA (ceRNA) for *miR-7-5p*, leading to the upregulation of *EGFR* expression and orchestrating the progression of prostate cancer (PCa) through the *miR-7-5p/EGFR/PI3K/AKT/mTOR* pathway (Ma et al. 2022). Furthermore, corroborated data from validated databases substantiate that *miR-7* assumes a central regulatory role in suppressing anti-apoptotic genes (Darvish et al. 2023). Notably, the inherent characteristics observed in the expression profiles of tumor-associated *miR-7-5p* suggest promising utility as potential markers indicative of the aggressiveness of the prostate cancer trajectory (Borikun et al. 2024). To confirm our bioinformatic analysis with wet lab experiments, our data supports the theory that these miRNAs may bind to *ADAMTS9-AS2*.

The outcomes indicate that heightened expression of lncRNA *PART1* correlates with an increase in the expression levels of AR target genes, suggesting a positive correlation and the potential involvement of lncRNA *PART1* in acting as a co-regulator in the regulation of AR. In another study it was shown that the downregulation of *PART1* triggered apoptosis progression in PCa cells and acted to suppress the development of prostate cancer (Sun et al. 2018). Here we show that *PART1* is within AR signaling and mediates in part the tumor suppressive pathway to regulate SAL-mediated cellular senescence.

Thus, although antithetically regulated by SAL, both lncRNAs *PART1* and *ADAMTS9-AS2* are not only regulated by androgens but are acting within the AR signaling to control SAL-mediated induction of cellular senescence and suggest the identification of new pathways and networks within AR signaling in PCa and may help understanding the molecular pathways using BAT (Figure 5).

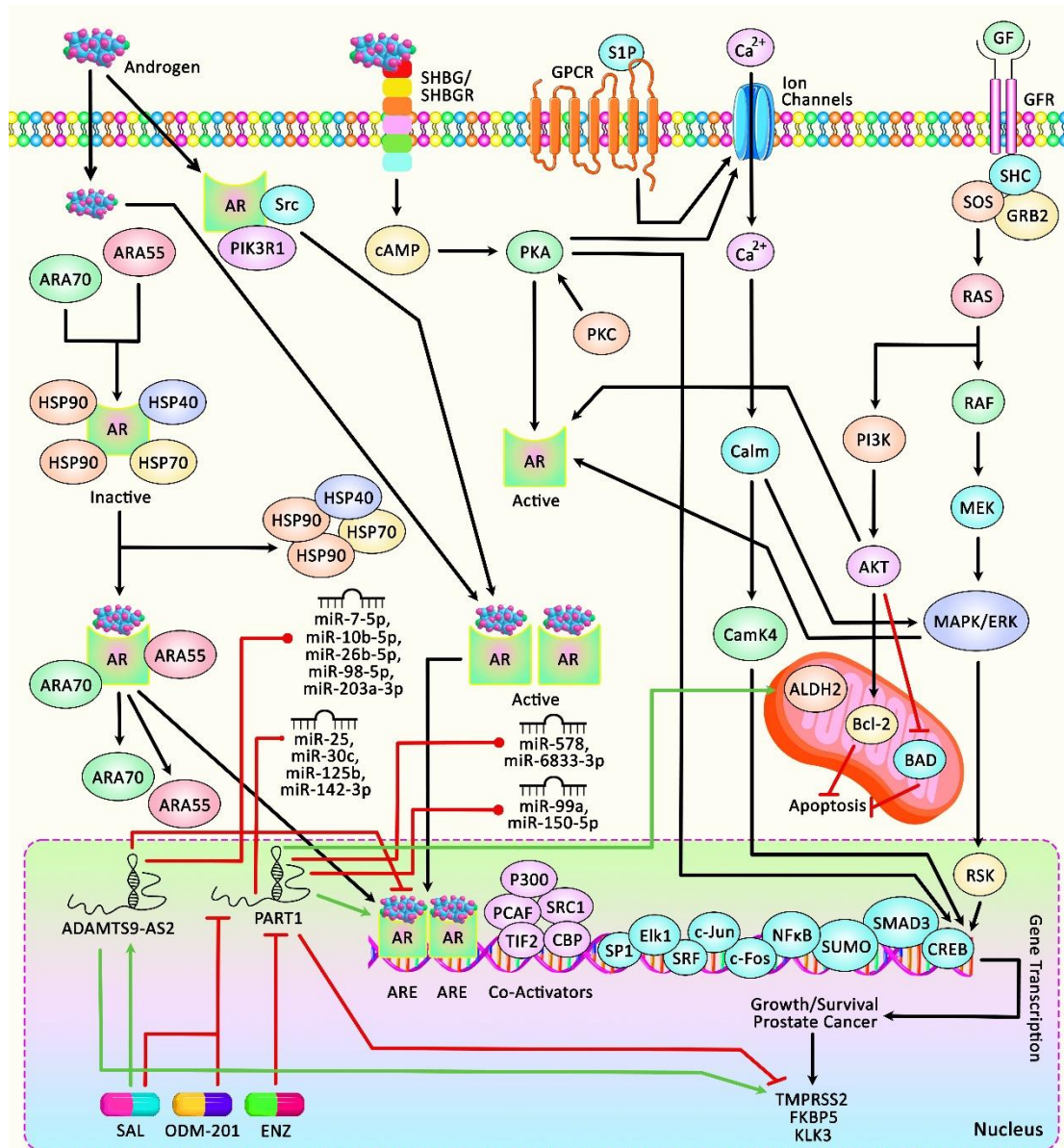


Figure 5. A schematic diagram proposed the roles of *PART1* and *ADAMTS9-AS2* lncRNAs in modulating AR signaling pathway in PCa. In this study, we revealed significant up-regulation of *ADAMTS9-AS2* and down-regulation of *PART1* following treatment with supraphysiological androgen levels (SAL) and AR inhibitors including darolutamide (ODM) and enzalutamide (ENZ) in PCa cells. Therefore, our findings indicate an oncogenic role for *PART1* and a tumor suppressor role for *ADAMTS9-AS2*. In addition, we demonstrated that downregulation of *PART1* and

upregulation of *ADAMTS9-AS2* can lead to the overexpression of androgen receptor regulatory genes (*KLK3*, *FKBP5*, and *TMPRSS2*) levels in both C4-2 and LNCaP cells. Moreover, our research detected the remarkable interactions of miR-99a and miR-150-5p with *PART1* as well as the considerable interactions of *miR-98-5p*, *miR-7-5p*, *miR-10b-5p*, *miR-26b-5p* and *miR-203a-3p* with *ADAMTS9-AS2*. Furthermore, in our previous study we illustrated that *miR-25*, *miR-30c*, *miR-125b* and *miR-142-3p* can be considered as the significant target of *PART1* in prostate cancer cells (Taheri et al. 2022). Another research has also identified that *PART1* can modulate the expression level of ALDH2 through regulating *miR-578* and *miR-6833-3p* in prostate cancer cells (Feng et al. 2022).

5. Conclusion and future perspective

Our study underscores the complex role of lncRNAs *PART1* and *ADAMTS9-AS2* in the modulation of AR signaling in PCa, particularly in the context of SAL treatment. Through a series of detailed experiments, we have demonstrated that SAL treatment induces cellular senescence in both androgen-sensitive and CRPC cells. Notably, SAL treatment results in the upregulation of *ADAMTS9-AS2* and the downregulation of *PART1*, indicating their differential roles in AR signaling and cellular senescence.

Our RNA-seq data suggest that these lncRNAs not only affect the expression of AR target genes but also participate in intricate regulatory networks involving miRNAs, adding layers of complexity to their functions. *ADAMTS9-AS2* appears to act as a tumor suppressor, while *PART1* seems to function as an oncogene, with their expression inversely regulated by SAL treatment. This dualistic regulation suggests that manipulating these lncRNAs could provide therapeutic benefits by enhancing the efficacy of BAT in PCa.

The interaction of *PART1* with AR and its binding to AR target gene loci highlight its direct involvement in AR signaling. Conversely, the repression of *ADAMTS9-AS2* through an indirect mechanism by SAL indicates a more complex regulatory pathway. Our findings suggest that these lncRNAs are integral to the AR signaling cascade and influence the cellular senescence pathway, thus presenting potential targets for therapeutic intervention in PCa.

The elucidation of the molecular mechanisms by which SAL induces cellular senescence through AR signaling and its associated lncRNAs opens new avenues for research and therapeutic strategies in PCa. Further investigation into the molecular pathways and interactions influenced by *ADAMTS9-AS2* and *PART1* is crucial. Understanding how these lncRNAs modulate AR signaling at a granular level will provide deeper insights into their roles in cellular senescence and tumor suppression. Given their differential roles, *ADAMTS9-AS2* and *PART1* could serve as therapeutic targets. Strategies to upregulate *ADAMTS9-AS2* or downregulate *PART1* in combination with BAT could potentially enhance treatment efficacy and overcome resistance mechanisms in CRPC.

The expression profiles of *ADAMTS9-AS2* and *PART1*, along with their associated miRNAs, could be developed as biomarkers for predicting treatment response and disease progression. This could facilitate personalized treatment approaches for PCa patients. Translating these findings into clinical applications will require rigorous testing in clinical trials. Assessing the safety and efficacy of therapies targeting these lncRNAs in combination with BAT will be essential for their potential integration into clinical practice. Expanding the scope of research to include other lncRNAs and miRNAs involved in AR signaling and PCa progression could uncover additional regulatory networks and therapeutic targets.

In conclusion, our research highlights the pivotal roles of lncRNAs in AR signaling and cellular senescence, providing a foundation for future studies aimed at developing novel therapeutic strategies for PCa. By leveraging the regulatory functions of *ADAMTS9-AS2* and *PART1*, there is potential to enhance the effectiveness of current treatments and improve outcomes for patients with metastatic prostate cancer.

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7. Appendix

7.1 Own participation and co-authorship

LncRNA/miRNA/mRNA Network Introduces Novel Biomarkers in Prostate Cancer

Mohammad Taheri, Arash Safarzadeh, Bashdar Mahmud Hussen, Soudeh Ghafouri-Fard, Aria Baniahmad. **Cells**. 2022 Nov 25;11(23):3776.

M.S.c. Taheri. M: Conducting the bioinformatics analysis, writing the first draft of the manuscript, and then revising it to finalize the document.

M.S.c. Safarzadeh. A: Re-analysis of the bioinformatics data, data collection and final revision of the document.

Dr. rer. nat. Hussen BM: Data collection, preparation of figures and final revision of the manuscript.

Prof. Dr. med. Ghafouri-Fard S: Supervising the study and writing the first draft.

Prof. Dr. rer. nat. Baniahmad A: Planning and supervising the study, writing the draft and final revision of the manuscript.

An in-silico Approach for Recognition of Long non-coding RNA-Associated Competing Endogenous RNA Axes in Prostate Cancer

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M.S.c. Taheri. M: Conducting the bioinformatics analysis, writing the first draft of the manuscript, and then revising it to finalize the document.

M.S.c. Safarzadeh. A: Re-analysis of the bioinformatics data, data collection and final revision of the document.

Prof. Dr. med. Ghafouri-Fard S: Supervising the study and writing the first draft.

Prof. Dr. rer. nat. Baniahmad A: Planning and supervising the study, writing the draft and final revision of the manuscript.

Interaction between Non-Coding RNAs and Androgen Receptor with an Especial Focus on Prostate Cancer

Mohammad Taheri, Tayyebah Khoshbakht , Elena Jamali, Julia Kallenbach, Soudeh Ghafouri-Fard, Aria Baniahmad. **Cells**. 2021 Nov 16;10(11):3198.

M.S.c. Taheri. M: Designed the study, collected the data, and wrote the first draft of the manuscript.

M.S.c. Khoshbakht T: Designed the figures and collected the data for the final version of the manuscript.

Dr. med. Jamali E: Designed the tables and collected the data for the final version of the manuscript.

M.S.c. Kallenbach J: Designed the figures and collected the data for the final version of the manuscript.

Prof. Dr. med. Ghafouri-Fard S: Wrote the draft and revised the final version and collected the data.

Prof. Dr. rer. nat. Baniahmad A: Designed and supervised the study, wrote the draft, and revised it several times.

Unveiling four axes ADAMTS9-AS2|MEG3/hsa-miR-150/PRKCA|MMP14 within prostate cancer through establishment of the ceRNA network

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M.S.c. Taheri. M: Conducting the bioinformatics analysis, writing the first draft of the manuscript, and then revising it to finalize the document.

M.S.c. Safarzadeh. A: Re-analysis of the bioinformatics data, data collection and final revision of the document.

Prof. Dr. rer. nat. Baniahmad A: Planning and supervising the study, writing the draft and final revision of the manuscript.

The lncRNAs PART1 and ADAMTS9-AS2 act in an antithetic manner on AR signaling and induction of cellular senescence in prostate cancer cells

Mohammad Taheri, Katrin Schindler, Aria Baniahmad. **International Journal of Surgery**. 2025

Mar 26.

M.S.c. Taheri. M: Performing the experiment, analyzing the data, writing the first draft of the manuscript, and then revising it to finalize the document.

Dr. rer. nat. Schindler K: Performing Western Blotting and analyzing the data. Writing the draft.

Prof. Dr. rer. nat. Baniahmad A: Planning and supervising the study, writing the draft and final revision of the manuscript.

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7.3 Ehrenwörtliche Erklärung

Hiermit erkläre, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller- Universität bekannt ist,

Ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

Mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Professor Dr. rer. nat. Baniahmad A, M.S.c. Safarzadeh A und Professor Dr. med. Ghafouri-Fard S, die bei der Publikation der Artikel geholfen haben.

Die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

Dass ich die Dissertation noch nicht als Prüfungsarbeit für eine Staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

das ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena, 01.04.2025

Mohammad Taheri

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