Asian Journal of Andrology (2021) 23, 400–408
www.asiaandro.com; www.ajandrology.com

Open Access

ORIGINAL ARTICLE

Prostate Cancer

Identifying the role of apolipoprotein A-I in prostate cancer

Jing Wang[1,2,*], Ling-Fan Xu[3,*], Cheng Liu[2], Tao Huang[2], Chao-Zhao Liang[3], Yi-Dong Fan[1]

Although localized prostate cancer (PCa) can be cured by prostatectomy and radiotherapy, the development of effective therapeutic approaches for advanced prostate cancer, including castration-resistant PCa (CRPC) and neuroendocrine PCa (NEPC), is lagging far behind. Identifying a novel prognostic and diagnostic biomarker for early diagnosis and intervention is an urgent clinical need. Here, we report that apolipoprotein A-I (ApoA-I), the major component of high-density lipoprotein (HDL), is upregulated in PCa based on both bioinformatics and experimental evidence. The fact that advanced PCa shows strong ApoA-I expression reflects its potential role in driving therapeutic resistance and disease progression by reprogramming the lipid metabolic network of tumor cells. Molecularly, ApoA-I is regulated by *MYC*, a frequently amplified oncogene in late-stage PCa. Altogether, our findings have revealed a novel indicator to predict prognosis and recurrence, which would benefit patients who are prone to progress to metastasis or even NEPC, which is the lethal subtype of PCa.

Asian Journal of Andrology (2021) **23**, 400–408; doi: 10.4103/aja.aja_92_20; published online: 12 February 2021

Keywords: advanced prostate cancer; apolipoprotein A-I; lipid metabolism; *MYC*

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed noncutaneous cancer in men, especially in Western countries, which leads to approximately 30 000 deaths yearly in the USA.[1] Although the androgen receptor (AR) signaling axis plays an extremely critical role in diagnosis and treatment, an increasing number of limitations have been uncovered. For instance, screening of the AR-regulated effector prostate-specific antigen (PSA), which is widely used to determine the likelihood of PCa in the clinic, has been shown to cause aggressive overtreatment in patients who have already been treated sufficiently with active surveillance.[2] Moreover, PSA is not an ideal tool for some extremely lethal subtypes of PCa, such as neuroendocrine PCa (NEPC), which loses its luminal identity (AR and PSA), but displays distinct NE markers (enolase 2 [ENO2] and chromogranin A [CHGA]).[3] Additionally, despite the initial effectiveness, the efficacy of AR-directed therapies is rather limited even with the second generation of anti-androgen drugs, such as abiraterone and enzalutamide.[4] Therefore, identifying a biomarker whose level may be strongly associated with disease aggressiveness and development as well as exploiting a novel therapeutic target to compensate for the insufficiency of AR-targeted therapies is warranted.

The rewiring of metabolism has been regarded as one of the significant hallmarks of cancer for many decades since the Warburg effect was recognized, which describes the distinct preference of cancer cells for generating energy predominantly through glycolysis rather than the much more efficient oxidative phosphorylation (OXPHOS) pathway, regardless of the presence of oxygen.[5] In contrast to a large number of solid cancers that exhibit this reprogrammed glucose metabolic trait, PCa is generally a nonglycolytic cancer type,[6] which is supported by the clinical fact that PCa is not an ideal candidate for F-deoxyglucose (FDG)-positron emission tomography (PET) detection due to the low absorption rate of infused radiolabeled glucose by PCa tumor cells.[7] Instead of glucose, lipids are one of the critical nutrients and energy suppliers in PCa, as shown by the hyperactivity of lipid synthesis.[8] In addition to the utilization of *de novo* lipogenesis, PCa can also consume exogenous lipids such as androgens to support tumor cell growth.[9] The dependency on lipids is even more notable in advanced PCa, manifesting as elevated levels of a panel of rate-limiting enzymes involved in both lipid synthesis and uptake.[9] Therefore, the hypermetabolic rates of lipids represent a promising diagnostic and prognostic indicator of PCa initiation and progression.

In addition to the enzymes that are directly responsible for lipid synthesis and utilization, there is another specialized category of proteins that mediate lipid transport to improve their distribution and usage, that are known as lipoproteins. Apolipoprotein A-I (ApoA-I) is the major component of high-density lipoprotein (HDL).[10] It has been implicated as a pleiotropic factor, as it not only regulates cholesterol trafficking to reduce the risks of cardiovascular disease but also functions in cancer contexts. Given the canonical role of HDL as "good cholesterol" that ameliorates atherogenic processes, it is conceivable that ApoA-I may be protective against cancer.[10] Indeed, reduced protein levels of ApoA-I have been observed in both cancerous tissue and the serum of patients, particularly within those carrying gastrointestinal tract carcinoma.[10] However, other published data demonstrate that

[1]Department of Urology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan 250012, China; [2]Department of Urologic Oncology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230036, China; [3]Department of Urology, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, China.
*These authors contributed equally to this work.
Correspondence: Dr. YD Fan (fanyd@sdu.edu.cn) or Dr. CZ Liang (liang_chaozhao@163.com)
Received: 05 May 2020; Accepted: 18 November 2020

ApoA-I is a potential oncogenic player in recurrent head-and-neck squamous cell carcinoma, retinoblastoma, and bladder cancer and a disease progression driver in breast cancer.[11–14] This background suggests that ApoA-I can be either pro-oncogenic or tumor suppressive, depending on the cancer context. However, its role in PCa remains largely unexplored.

In this study, we identified a new role of ApoA-I as a metabolic biomarker strongly associated with carcinogenesis and tumor evolution from multiple perspectives. We also propose a putative therapeutic regimen that targets ApoA-I to benefit advanced PCa patients who have few treatment choices.

MATERIALS AND METHODS

Cell culture

LNCaP, C4-2, PC3, and DU145 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (Gibco). LAPC4 cells were maintained in Iscove's modified Dulbecco medium (IMDM; Gibco). RWPE-1 cells were maintained in keratinocyte serum-free medium (Gibco). Androgen deprivation therapy was modeled by charcoal-stripped FBS (CS-FBS; Gibco) application. Cells were preserved at 37°C in 5% CO_2.

Gene silencing and overexpression

APOA1 knockdown was achieved by stable transduction of two sets of short hairpin RNA (shRNA) purchased from Sigma (TRCN0000371319 and TRCN0000371327; St. Louis, MO, USA). *APOA1* complementary DNA (cDNA) was purchased from Origene (Rockville, MD, USA). All the shRNA and cDNA vectors were further packaged into lentiviruses. Briefly, each lentiviral vector was co-transfected with packaging vectors (pMDL, pVSVg, and pRev) into 293T cells by using Lipofectamine 3000 reagent (Invitrogen, Waltham, MA, USA). After 48 h, the viral particles were collected and purified with a 0.22-μm filter. Cells were infected by viruses followed by puromycin selection. Knockdown of *MYC* was performed by using two sets of small interfering RNAs (siRNA; Santa Cruz, Dallas, TX, USA). The efficiency of overexpression and knockdown was validated by western blot.

Cell proliferation, colony formation, and transwell assays

Cells were seeded in 96-well plates at an initial density of 1000 cells per well. At each indicated time point, cell viability was determined with the MTS reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. The colony formation assay was performed as described previously.[15] A total of 1×10^4 cells were plated on a 60-mm cell culture dish and allowed to grow for up to 14 days. Colonies were fixed with cold methanol and then stained with 1% crystal violet. To determine the cell migration capability, 1×10^4 cells were plated in an 8-μm pore Boyden chamber (Sigma) with medium supplemented with 10% FBS surrounding the chamber in a 24-well plate. Chambers were coated with basement membrane extract (BME) solution and coating buffer. After 48 h of incubation, crystal violet was used to stain the migrated cells.

β-gal staining senescence measurement

LNCaP cells were treated with CS-FBS. At each indicated time point, cells were fixed and stained to determine the senescence-associated β-gal activity (Cell Signaling, Danvers, MA, USA). Positive cells were counted in several randomly selected fields to obtain the standard deviation.

Metabolite profiling

Cells were seeded in 6-well plates and grown until 80% confluence was reached. Then, the cells were extracted with prechilled 80% methanol. Macromolecules and debris were removed by centrifugation at 20 000*g* (Microfuge 20R, Beckman Coulter, Indianapolis, IN, USA) for 20 min at 4°C. The supernatant was dried by a speed vacuum at room temperature for 3 h. Dry pellets were further analyzed by liquid chromatography-mass spectrometry (LC/MS). The amount of HDL in the cell culture medium was determined by an HDL enzyme-linked immunosorbent assay (ELISA) kit (MYBioSource, San Diego, CA, USA) according to the manufacturer's instructions.

Gene expression and correlation analysis

Datasets were downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo) or cBioPortal (https://www.cbioportal.org). Relative *APOA1* expression was compared between different groups (*e.g.*, normal and primary tumor, primary tumor and metastasis, and primary-adeno and NEPC). Correlation matrices were computed by the Pearson's method.

Western blot

Cell lysates were prepared with RIPA buffer supplemented with protease and phosphatase inhibitors. The protein concentration was determined with the Bradford assay. Equal amounts of protein (20 μg) were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The protein was then transferred to polyvinylidene difluoride (PVDF) membranes followed by blocking with 5% nonfat milk. The following primary antibodies were applied with overnight incubation: anti-ApoA-I (1:200 dilution; Santa Cruz), anti-N-Myc (1:200 dilution; Santa Cruz), anti-c-Myc (1:1000 dilution; Abcam, Cambridge, MA, USA), and anti-actin (1:200 dilution; Santa Cruz).

RNA extraction and quantitative real-time PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) and then reverse-transcribed into cDNA by applying a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was performed by using SYBR Taq Premix (Quanta, Beverly, MA, USA). The primer sequences were as follows: *APOA1*, forward 5'-AAAGCTGCGGTGCTGACCTT-3', reverse 5'-ATCTTGCTGCCAGAAATGCCG-3'; *actin*, forward 5'-AGAGCTACGAGCTGCCTGAC-3', reverse 5'-AGCACTGTGTTGGCGTACAG-3'.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded samples from PCa patients were obtained from prostate biopsy, prostatectomy, and transurethral resection of prostate (TURP) at the Department of Urological Oncology, The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China (Hefei, China). As described previously,[16] 5-μm-thick paraffin sections were further prepared. The tissue slides were sequentially deparaffinized and rehydrated with xylene with graded ethanol. Endogenous peroxidase activity was blocked in 3% H_2O_2 for 10 min, and the tissue was boiled in citrate buffer for heat-induced antigen retrieval. The tissue was then probed with ApoA-I antibody (1:100 dilution), and the nuclei were stained with hematoxylin. The intensity was assessed by the Q-score.

Statistical analyses

Statistically significant differences between two groups were determined by using a two-tailed paired *t*-test. Comparisons between more than two groups were carried out by analysis of variance (ANOVA) with Tukey's multiple-comparison test. Differences were considered statistically significant at $P < 0.05$. The enriched gene signature was evaluated by gene set enrichment analysis (GSEA;

www.gsea-msigdb.org). Metabolic pathway enrichment and impact analyses were performed with MetaboAnalyst online software (www.metaboanalyst.ca). All statistical analyses were performed with GraphPad Prism software (Insightful Science, San Diego, CA, USA), Microsoft Excel (Microsoft, Redmond, WA, USA), and R (Revolution Analytics, Mountain View, CA, USA).

RESULTS

ApoA-I expression is upregulated in PCa

To determine the potential role of ApoA-I in PCa, we compared *APOA1* gene expression in normal prostatic tissues and primary tumors via bioinformatics analyses.[17–19] A panel of datasets from various studies[17–19] showed a consistent expression pattern, in which *APOA1* was upregulated in the primary PCa samples (**Figure 1a**). IHC staining of paraffin tissues using ApoA-I antibody further validated the elevated protein level of ApoA-I in human PCa samples (**Figure 1b** and **1c**). Consistently, *in vitro*, ApoA-I expression was barely observed in the normal epithelial prostatic cell line RWPE-1, while human PCa cells displayed substantial protein levels of ApoA-I, as detected by western blot (**Figure 1d**). Loss of tumor suppressor genes (TSGs) is known to facilitate tumorigenesis.[20] Phosphatase and tensin homolog (*PTEN*) is among the most frequently lost or mutated TSGs in PCa, which is observed in up to 70% of localized PCa.[21] To confirm that the increase in *APOA1* in PCa is universal and can be caused by a genetically altered background, we interrogated a genetically engineered mouse model (GEMM) sequencing dataset[20] and found that primary PCa tumors resulting from *PTEN* deficiency displayed significantly higher levels of *APOA1,* whereas *APOA1* was undetectable in tissues with wild-type *PTEN* (**Figure 1e**). These findings suggest that PCa harbors a high level of ApoA-I, and, thus, *APOA1* might be a potential oncogene involved in PCa initiation.

ApoA-I is associated with disease progression

To test whether ApoA-I has the potential to drive the disease development of PCa, we next focused on the comparisons of ApoA-I expression between primary and advanced tumors. Surprisingly, although *APOA1* was significantly upregulated in primary tumors, its level was even higher in tissues from metastatic lesions obtained by biopsies according to bioinformatics analyses[17,18] (**Figure 2a**). An extremely high level of *APOA1* was also observed in several datasets of NEPC,[22–25] the terminal stage of PCa, which frequently emerges after long-term hormonal therapy treatment[23] (**Figure 2b**). Concordantly, both castration-resistant PCa (CRPC) and NEPC human tissues exhibited an increased ApoA-I IHC staining intensity (**Figure 1b** and **1c**). Additionally, we observed a similar trend in ApoA-I protein levels within PCa cell lines representing different stages of PCa.

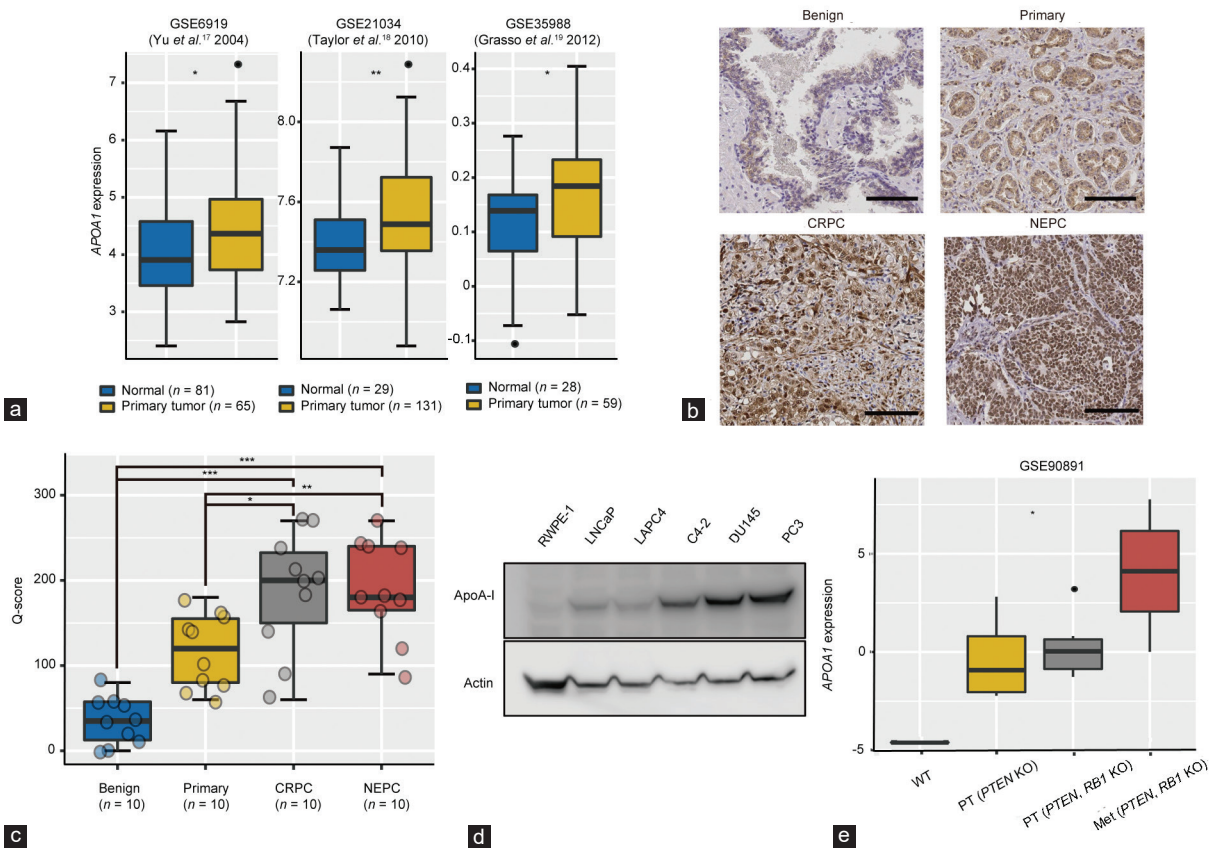


Figure 1: ApoA-I expression is upregulated in PCa. (**a**) *APOA1* mRNA levels in PCa datasets (Yu *et al.*[17] 2004; Taylor *et al.*[18] 2010; and Grasso *et al.*[19] 2012) separated by normal versus primary tumor. (**b**) Representative ApoA-I IHC images of benign prostate, primary PCa, CRPC, and NEPC tissue (scale bars = 100 μm). (**c**) Q-scoring analysis of tissue staining intensity using ApoA-I antibody. (**d**) Western blot showing the protein level of ApoA-I in various human prostate cell lines. Actin was loaded as the internal control. (**e**) Expression levels of *APOA1* mRNA in GEMM samples from WT, PT (*PTEN* KO), PT (*PTEN*, *RB1* KO), and Met (*PTEN*, *RB1* KO) mice. Two-tailed *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ApoA-I: apolipoprotein A-I; PCa: prostate cancer; IHC: immunohistochemistry; PT: primary tumor; Met: metastasis; CRPC: castration-resistant PCa; NEPC: neuroendocrine PCa; GEMM: genetically engineered mouse model; *PTEN*: phosphatase and tensin homolog; KO: knock out; WT: wild-type; *RB1*: RB transcriptional corepressor 1.

ApoA-I experienced a step-wise elevation across the disease spectrum (from androgen dependent to the CRPC and NEPC stages), as shown by the fact that PC3 and DU145, therapy-resistant cells with NE features, were found to display the highest level of ApoA-I in comparison to other hormone-sensitive PCa cell lines (LNCaP and LAPC4) and CRPC cells (C4-2), as shown in **Figure 1d**. In GEMMs of PCa, although *PTEN* deficiency is able to promote tumor formation, its inactivation alone only leads to minimally invasive features after a long latency.[26,27] The additional loss of the TSG retinoblastoma tumor suppressor gene (*RB1*) has been reported to drive metastasis.[20,28] Intriguingly, prostate-specific simultaneous deletion of *PTEN* and *RB1* resulted in an increased level of *APOA1*, and this level was further elevated in double-knockout metastatic mouse tumors (**Figure 1e**). These results suggest the pro-oncogenic role of ApoA-I associated with PCa development. Indeed, pair-wise correlation analyses of two well-established datasets revealed that *APOA1* negatively correlated with TSGs (*PTEN*, *RB1*, and tumor protein P53 [*TP53*])[20,28] and the NEPC repressor gene (RE1 silencing transcription factor [*REST*])[29] but positively associated with NE differentiation genes (enhancer of zeste 2 polycomb repressive complex 2 subunit [*EZH2*], aurora kinase A [*AURKA*], MYCN proto-oncogene [*MYCN*], forkhead box A2 [*FOXA2*], SRY-box transcription factor 2 [*SOX2*], serine/arginine repetitive matrix 4 [*SRRM4*], and one cut homeobox 2 [*ONECUT2*]),[23,28,30–33] NEPC hallmark genes (*CHGA* and synaptophysin [*SYP*]),[22] the CRPC-related gene (ubiquitin conjugating enzyme e2 C [*UBE2C*]),[34] and the epithelial–mesenchymal transition (EMT) marker gene (snail family transcriptional repressor 1 [*SNAI1*])[34], as shown in **Figure 2c**.

ApoA-I increases the survival, proliferation, invasion, and therapy resistance of PCa cells

Because ApoA-I is highly expressed in the late stage of PCa, we next investigated whether ApoA-I is able to enhance the biological aggressiveness of the cellular behaviors of PCa cells. We employed *APOA1* cDNA to ectopically overexpress ApoA-I in hormone-dependent primary LNCaP cells (**Figure 3a**). We observed a significant increase in cell proliferation and survival, as determined by the MTS and colony formation assays, respectively (**Figure 3a–3c**). Conversely, we used a lentivirus-based vector to express either a control shRNA sequence that does not target any known sequence in the mammalian genome (shCont) or independent, nonoverlapping shRNAs targeting ApoA-I (shApoA-I-1 and shApoA-I-2) to inhibit the expression of ApoA-I in CRPC (C4-2) and NEPC cells (PC3), in which ApoA-I is endogenously abundant (**Figure 3d** and **Supplementary Figure 1a**). As expected, the knockdown of *APOA1* attenuated cell viability as well as colony formation ability in

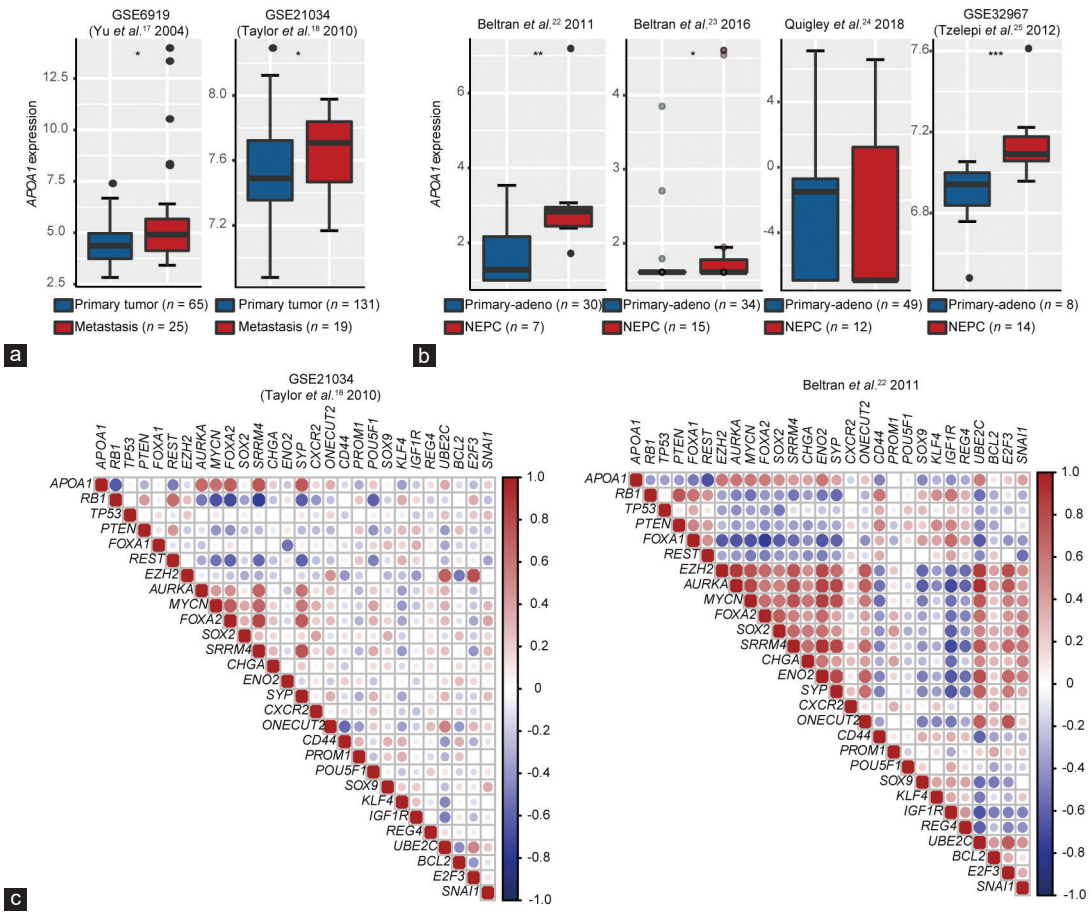


Figure 2: ApoA-I is associated with disease progression. (**a**) *APOA1* mRNA levels in primary versus metastatic PCa from public datasets. (**b**) Expression levels of *APOA1* mRNA in primary-adeno versus NEPC from public datasets. (**c**) Correlogram heatmap displaying correlations between mRNA expression levels of *APOA1* and other canonical genes involved in tumor suppression, NE differentiation, and CRPC-driven and EMT pathways. Two-tailed *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ApoA-I: apolipoprotein A-I; PCa: prostate cancer; NEPC: neuroendocrine prostate cancer; CRPC: castration-resistant prostate cancer; EMT: epithelial-mesenchymal transition; adeno: adenocarcinoma; NE: neuroendocrine; mRNA: messenger RNA. All the full names of the genes in **Figure 2c** are listed in **Supplementary Table 1**.

advanced tumor cells (**Figure 3b–3d** and **Supplementary Figure 1a**). In contrast, knocking down *APOA1* in hormone-sensitive LNCaP cells had minimal effect on cell viability, likely due to its low baseline level (**Supplementary Figure 1b**). To determine the enhanced invasiveness induced by ApoA-I, we performed Boyden chamber assays and found an increase in the invading cell number after ApoA-I overexpression in LNCaP cells (**Figure 3e** and **3f**). In contrast, PC3 cells with *APOA1* knockdown showed diminished ability to transmigrate through the chamber pores in response to FBS-coated stimulation (**Figure 3e** and **3f**).

Hormonal therapy-induced senescence is an early response of primary PCa to androgen-deprivation therapy (ADT), which can be measured by SA-β-gal staining.[15] Evasion of this treatment-induced

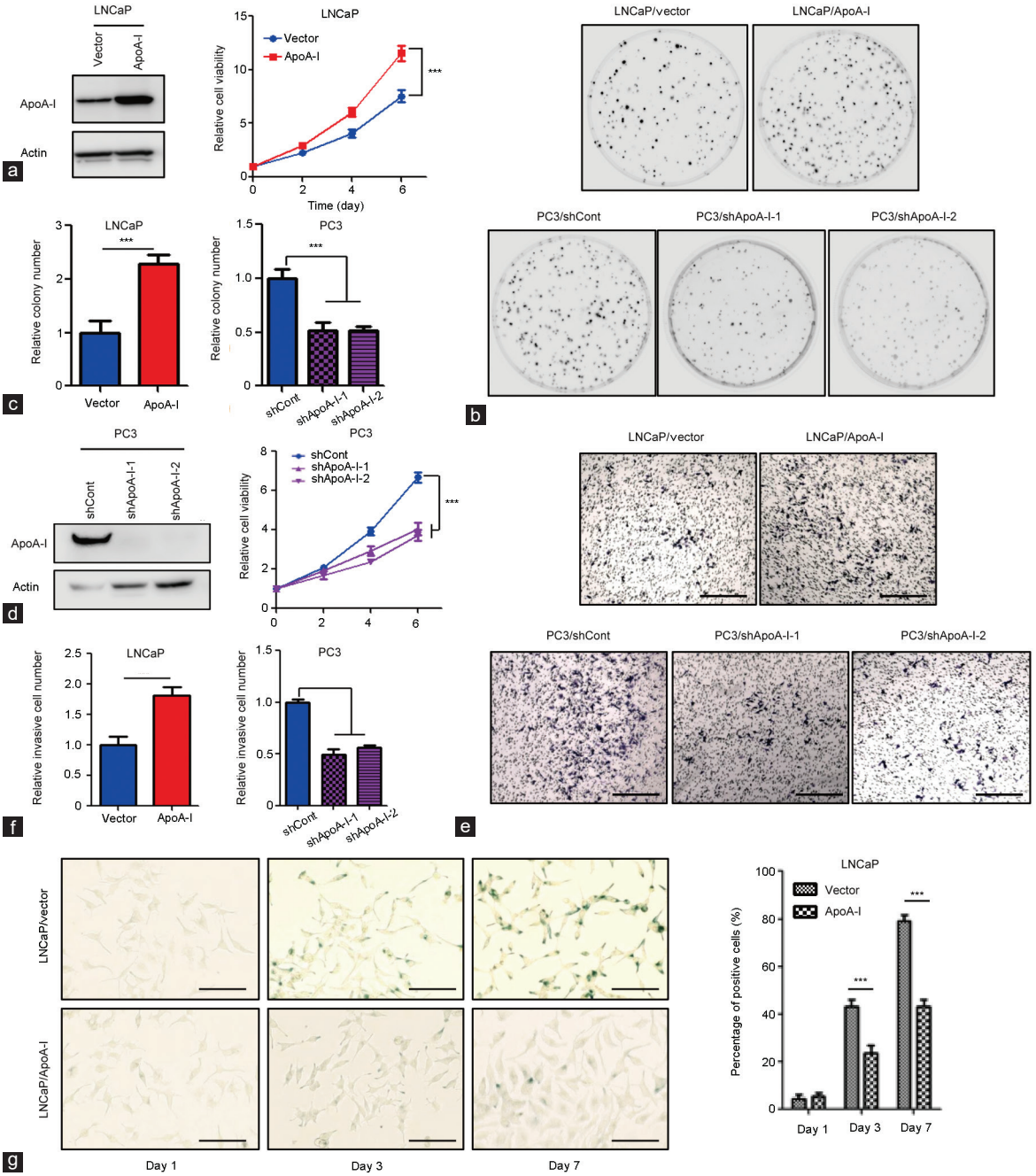


Figure 3: ApoA-I promotes cellular proliferation, clonogenicity, invasiveness, and therapeutic resistance in PCa. (**a**) Western blot showing ApoA-I expression in LNCaP cells by ectopically overexpressing *APOA1* cDNA (left panel). Cell viability was monitored at the indicated time points (right panel). (**b**) Representative colony formation images of LNCaP cells with ApoA-I overexpression and PC3 cells with *APOA1* knockdown. (**c**) Quantification of colony number from **b**. (**d**) Western blot showing the efficiency of shRNA-mediated knockdown of *APOA1* in PC3 cells (left panel). Cell viability was compared between PC3 cells in the presence or absence of ApoA-I (right panel). (**e**) Representative images of the invasion capacity of LNCaP cells with ApoA-I overexpression and PC3 cells with *APOA1* knockdown passing through a transwell barrier. Scale bars = 200 μm. (**f**) Quantification of the relative migrated cell number. (**g**) Representative images of senescent cells after ADT treatment stained at the indicated time points. Scale bars = 20 μm (left panel). Bar plot showing the percentage of β-gal positively stained cells (right panel). Two-tailed *t*-test. $^{***}P < 0.001$. ApoA-I: apolipoprotein A-I; PCa: prostate cancer; cDNA: complementary DNA; shRNA: short hairpin RNA; ADT: androgen deprivation therapy; LNCaP: lymph node carcinoma of the prostate; Cont: control.

senescence has been demonstrated to promote the outgrowth of CRPC.[35] We noticed that ApoA-I overexpression reduced the percentage of SA-β-gal-positive senescent cells (**Figure 3g**), suggesting that ApoA-I leads to resistance to hormonal therapy in tumor cells. Taken together, these results indicate that ApoA-I is a critical factor in accelerating PCa progression that leads to a more proliferative, invasive, and therapy-resistant phenotype.

ApoA-I displays a global effect on lipid metabolism in PCa

Because lipoprotein is a crucial player in coordinating lipid metabolism within organisms, we then wanted to know whether the altered concentrations of ApoA-I, which is an indispensable component of lipoprotein (specifically HDL), might affect reprogramming of lipid metabolism in PCa. Concomitant with the elevation in expression, the ApoA-I-involved lipoprotein metabolism pathway was significantly activated in metastatic PCa and NEPC in comparison to primary adenocarcinoma, as revealed by GSEA (**Figure 4a** and **4b**). Because HDL mainly comprises ApoA-I, next, we measured the concentration of HDL in cell culture medium and found that medium derived from PC3 and DU145 cells had higher levels of HDL than the medium used to culture LNCaP and LAPC4 cells (**Figure 4c**), indicating that the increase in ApoA-I in advanced PCa contributes to increased synthesis of additional HDL products.

Lipid metabolism involves a complex network that includes *de novo* biosynthesis and oxidative catabolism. Products and intermediates within numerous metabolic pathways contribute to lipogenesis. For instance, glucose-derived pyruvate fluxes into the tricarboxylic acid (TCA) cycle to generate citrate, which is a critical intermediate linking fatty acid synthesis and glycolysis and an important precursor for lipogenesis.[8] Alternatively, in cancer cells with malfunctioning mitochondria or that are undergoing hypoxia, citrate is produced from the reductive carboxylation of glutamine-derived α-ketoglutarate.[36] Thus, both glucose and glutamine are crucial carbon providers for the anabolism of fatty acids. To determine the potential rewiring of metabolic features during disease progression, we performed metabolite profiling experiments to obtain an overview of the altered metabolic phenotypes. Compared to the primary LNCaP cells, the advanced PC3 cells exhibited the enrichment of pathways more closely associated with lipid-related metabolism processes, such as the Warburg effect (aerobic glycolysis), TCA cycle, fatty acid metabolism, fatty acid biosynthesis, and oxidation of fatty acids (**Figure 4d**). To further investigate the impact of ApoA-I on metabolic reprogramming, we conducted two more metabolite profiling assays by employing ApoA-I-overexpressing LNCaP and ApoA-I-suppressed PC3 cell models. Both genetic modifications had significant effects on the global metabolic system (**Figure 4e** and **4f**). Specifically, the most remarkably affected pathways were involved in fatty acid metabolism, such as fatty acid elongation and fatty acid degradation (**Figure 4e** and **4f**). These metabolic investigations corroborate that ApoA-I has a profound effect on lipid metabolism and results in a shift in PCa to a lipid-producing phenotype, which is a key turning point in disease progression.

ApoA-I is upregulated by MYC in PCa

MYC is a well-established oncogenic protein family that includes c-Myc (encoded by *MYC*), N-Myc, and L-Myc.[37] Among them, c-Myc and N-Myc have been implicated to be pro-tumorigenic and metastasis driven in PCa, and their amplification is seen in up to 40% of tumor

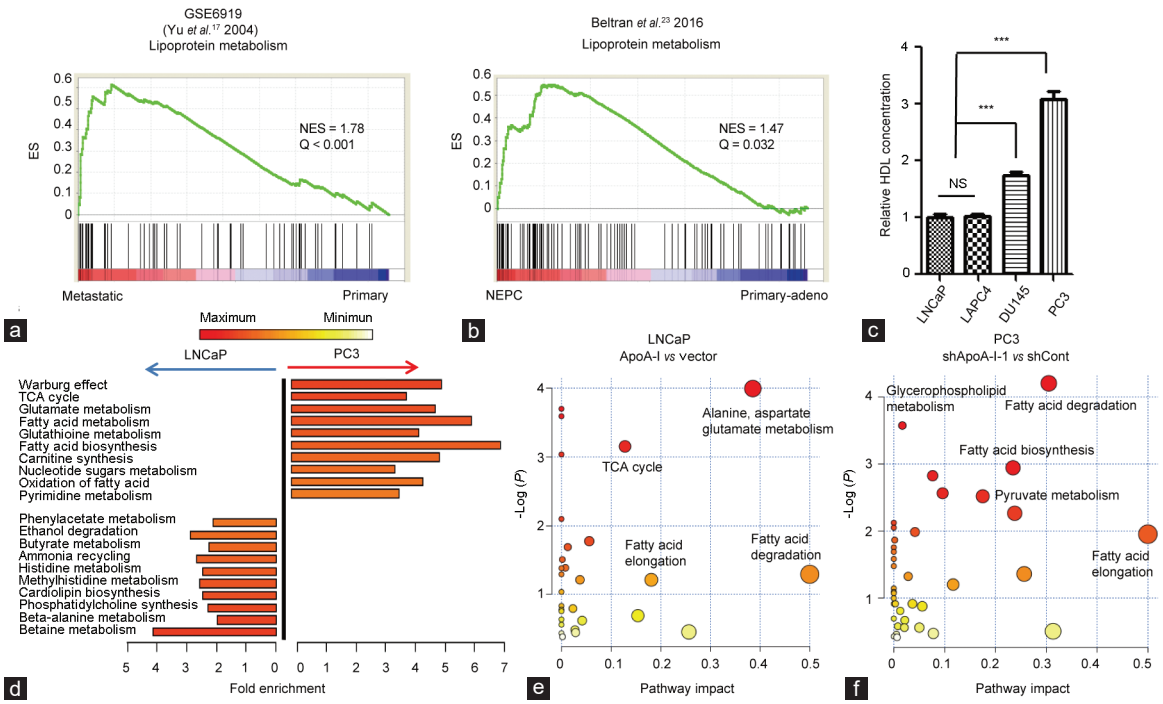


Figure 4: ApoA-I affects lipid metabolism in PCa. (**a**) GSEA of lipoprotein metabolism signatures in metastatic versus primary PCa from Yu *et al.*[17] 2004. (**b**) GSEA of lipoprotein metabolism signatures in NEPC versus primary-adeno from Beltran *et al.*[23] 2016. (**c**) Relative HDL concentration in LNCaP, LAPC4, DU145, and PC3 cell culture medium. (**d**) Pathway enrichment analysis showing the enriched metabolic pathways in LNCaP and PC3 cells. (**e**) Pathway impact analysis showing the effect of overexpression of ApoA-I in LNCaP cells on global metabolic pathways. (**f**) Pathway impact analysis showing the effect of loss of ApoA-I in PC3 cells on global metabolic pathways. Two-tailed *t*-test. $^{***}P < 0.001$. ApoA-I: apolipoprotein A-I; PCa: prostate cancer; GSEA: gene set enrichment analysis; NEPC: neuroendocrine prostate cancer; HDL: high-density lipoprotein; adeno: adenocarcinoma; ES: enrichment score; NES: normalized enrichment score; LNCaP: lymph node carcinoma of the prostate; NS: not significant; Cont: control.

foci.[23] Interestingly, they are believed to govern different directions of histological differentiation, as c-Myc amplification has been highly detected in CRPC tumors that maintain adenocarcinoma characteristics, while N-Myc appears to be exclusively overexpressed in NEPC.[37] Because ApoA-I is correlated with increased disease severity, we hypothesized that *MYC* might account for the overexpression of ApoA-I in advanced PCa. Indeed, interrogation of various advanced PCa publicly available datasets revealed that *APOA1* showed a positive correlation with either *MYCN* or *MYC* in a histology-variant setting[17,19,22,25] (**Figure 5a** and **5b**). GSEA analysis showed that genes involved in the lipoprotein metabolism pathway were significantly enriched in N-Myc-overexpressing LNCaP cells (**Figure 5c**).[38] Moreover, in *in vitro* studies, both c-Myc and N-Myc overexpression in LNCaP cells caused upregulation of *APOA1* transcript levels (**Figure 5d**). Conversely, knocking down *MYC* by two sets of siRNAs significantly reduced *APOA1* mRNA levels in PC3 cells (**Figure 5e**). These results suggest that ApoA-I is regulated by the *MYC* family (both c-Myc and N-Myc) in PCa.

DISCUSSION

Proliferating cancer cells comprehensively rewire their metabolism to sustain growth and survival, particularly in harsh conditions, such as hypoxia, nutrition scarcity, and drug treatment.[39] Unlike many other solid tumors, PCa is less glycolytic but highly lipogenic.[7] In highlighting the importance of lipids to PCa, one established piece of evidence is that obesity significantly increases the mortality of PCa.[40] Furthermore, nonmetastatic PCa mouse models elucidate that a high-fat diet is sufficient to trigger metastasis by inducing lipid accumulation.[41] In a metabolic molecular setting, it is mainly because PCa is more reliant on OXPHOS, driven by either glucose or glutamine, to generate citrate, which is the precursor for *de novo* fatty acid biosynthesis.[9] Consistently, our findings identify a highly enriched lipid metabolism-related signature in the advanced PCa cell line, which strengthens the accumulating evidence that PCa can be facilitated by reprogrammed lipid homeostasis.

Hypermetabolic rates are usually reflected by increased expression of enzymes. For instance, the most extensively studied lipogenic enzyme, fatty acid synthase (FASN), has been reported to be overexpressed in a variety of cancers, including PCa. It serves as a biomarker of PCa progression, with increased association with a more aggressive phenotype.[7] Additionally, carnitine palmitoyltransferase I (CPT1) and alpha-methylacyl-CoA racemase (AMACR), the two enzymes that are responsible for fatty acid β-oxidation, have also been implicated as being elevated in PCa.[9] In contrast to the direct functions in lipid synthesis and utilization, ApoA-I and its assembled lipoprotein are more responsible for modulating lipid movement for reasonable distribution. This process is essential because in cancer cells, lipid metabolism is often dysregulated.[9] Appropriate rearrangement is required to satisfy increased demand of tumor cells for these building blocks and energy sources, which are critical for the proliferation and evasion of apoptosis. Our analyses from both public datasets and our own experimental results uncover that ApoA-I-mediated lipoprotein metabolism is highly activated in advanced PCa, including the lethal form of PCa, NEPC, and its alteration is able to cause global metabolic reprogramming, particularly lipid metabolism. Our observations fill a conceptual gap in the understanding of metabolic underpinnings of PCa, suggesting that in addition to the well-studied lipogenesis and β-oxidation, ApoA-I regulation of lipid transporters enables metabolic flux to be better arranged to desired sites, which further gives rise to the increased lipogenic characteristic of PCa. Future efforts will be aimed at more specific details about how ApoA-I coordinates lipids in cancer.

Our current work reveals the unexpected step-wise elevation of ApoA-I expression across the spectrum of PCa progression from

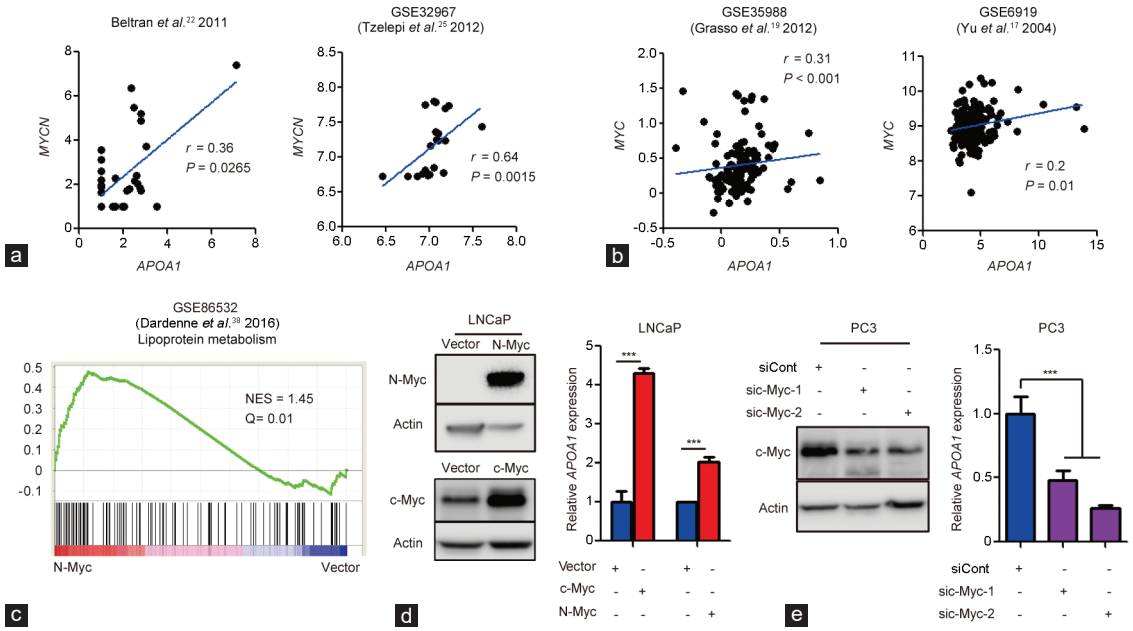


Figure 5: ApoA-I is regulated by the MYC family. Correlation analyses of *APOA1* with either (**a**) *MYCN* or (**b**) *MYC* from public datasets. (**c**) GSEA of lipoprotein metabolism signatures in the LNCaP parental cell line and LNCaP with N-Myc overexpression cell line. (**d**) Overexpression of N-Myc or c-Myc upregulating *APOA1* mRNA levels. Left panel showing the efficiency of N-Myc or c-Myc in LNCaP cells determined by western blot. Right panel showing *APOA1* transcript levels measured by qPCR. (**e**) Repression of c-Myc downregulating *APOA1* mRNA expression. Left panel showing immunoblot validation of *MYC* knockdown by two sets of siRNAs. Right panel showing *APOA1* transcript levels measured by qPCR. Two-tailed *t*-test. $^{***}P < 0.001$. ApoA-I: apolipoprotein A-I; GSEA: gene set enrichment analysis; qPCR: quantitative polymerase chain reaction; siRNA: small interfering RNA; Cont: control; NES: normalized enrichment score.

multiple perspectives. This finding is somewhat contradictory with the canonical role of ApoA-I as a protector in cancer prevention, which indicates that ApoA-I might be a versatile player in a tissue-dependent manner. In the cardiovascular and gastrointestinal systems, ApoA-I and HDL decrease lipid accumulation to reduce the risks of diseases such as atherosclerosis and even prevent normal tissue from malignancy.[10] However, in other organs, especially those who are hormone-dependent, such as the breast, ApoA-I-related lipids become crucial energy suppliers for tumor-promoting processes.[42] The high expression of ApoA-I in PCa and the positive association between ApoA-I and disease progression support ApoA-I as an ideal diagnostic and prognostic indicator for early diagnosis (**Figure 1** and **2**). Consistently, its resultant product, HDL, has also been epidemiologically and experimentally reported to be correlated with increased risks of both overall and high-grade PCa by many other publications.[43–48] This positive linkage is found more dramatic in the disease-recurred population.[45] This is likely because ADT leads to elevated HDL levels to beneficially feed androgen-independent PCa cells, such as DU145 and PC3.[47,48] Interestingly, HDL has few impacts on androgen-sensitive LNCaP cells,[46] which together with our current findings, further highlights that HDL and ApoA-I are much more important in advanced PCa than primary tumors.

The oncogenic role of ApoA-I in PCa, which regulates cellular behavior and mediates drug resistance, also suggests the potential of ApoA-I to be a putative therapeutic target, compensating for the limitations of the currently used anti-AR therapies. However, given the physiologically wide distribution of ApoA-I within organs, avoiding severe side effects is a major challenge. Drugs with super affinity and organ specificity are required. Future studies to employ GEMM with prostate-specific deletion of the *APOA1* gene might address this concern.

Although metabolic rewiring has long been recognized in cancer, more recently, altered molecular drivers are also known to affect cancer metabolism, and the discovery of cross-talk between metabolism and cancer genetics as well as epigenetics broadens the contributions to carcinogenesis and tumor maintenance.[49] We and others have found that MYC, which is frequently amplified in advanced PCa, displays a striking impact on cancer metabolism.[16,50] MYC has been implicated in promoting glycolysis by upregulating lactate dehydrogenase A activity.[16] In glutaminolysis, *MYC* increases glutaminase to fuel the TCA cycle through a posttranscriptional repression mechanism.[50] In the current study, our data reveal the additional regulatory effect of *MYC* on lipid metabolism by increasing ApoA-I expression. Future molecular studies will be aimed at obtaining insights into specific aspects of mechanistic regulation.

CONCLUSION

In summary, our current study uncovers the novel role of ApoA-I in PCa. Overexpression of ApoA-I allows PCa to initiate, survive, and proliferate after hormonal therapies. Incorporating ApoA-I into clinical practice might aid risk stratification of PCa cases that are likely to recur and progress.

AUTHOR CONTRIBUTIONS

JW, LFX, CZL, and YDF conceived the study and participated in its design. LFX, JW, and CL participated in data acquisition. LFX, JW, and TH participated in the data analysis and interpretation. JW drafted the original manuscript. LFX reviewed and helped to edit the manuscript. CZL and YDF revised and supervised the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

ACKNOWLEDGMENTS

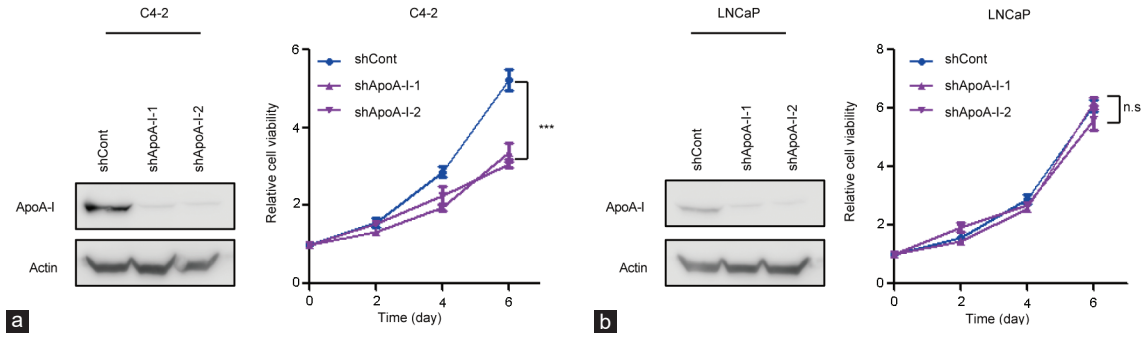
This work is supported by the National Natural Science Foundation of China (No. 81630019, 81902611, and 81672522) and Anhui Natural Science Foundation (1908085QH337).

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

REFERENCES

1 Rawla P. Epidemiology of prostate cancer. *World J Oncol* 2019; 10: 63–89.
2 Carlsson SV, Kattan MW. Prostate cancer: personalized risk-stratified screening or abandoning it altogether? *Nat Rev Clin Oncol* 2016; 13: 140–2.
3 Davies AH, Beltran H, Zoubeidi A. Cellular plasticity and the neuroendocrine phenotype in prostate cancer. *Nat Rev Urol* 2018; 15: 271–86.
4 Carceles-Cordon M, Kelly WK, Gomella L, Knudsen KE, Rodriguez-Bravo V, *et al*. Cellular rewiring in lethal prostate cancer: the architect of drug resistance. *Nat Rev Urol* 2020; 17: 292–307.
5 Kim J, DeBerardinis RJ. Mechanisms and implications of metabolic heterogeneity in cancer. *Cell Metab* 2019; 30: 434–46.
6 Bader DA, Hartig SM, Putluri V, Foley C, Hamilton MP, *et al*. Mitochondrial pyruvate import is a metabolic vulnerability in androgen receptor-driven prostate cancer. *Nat Metab* 2019; 1: 70–85.
7 Eidelman E, Twum-Ampofo J, Ansari J, Siddiqui MM. The metabolic phenotype of prostate cancer. *Front Oncol* 2017; 7: 131.
8 Snaebjornsson MT, Janaki-Raman S, Schulze A. Greasing the wheels of the cancer machine: the role of lipid metabolism in cancer. *Cell Metab* 2020; 31: 62–76.
9 Galbraith L, Leung HY, Ahmad I. Lipid pathway deregulation in advanced prostate cancer. *Pharmacol Res* 2018; 131: 177–84.
10 Georgila K, Vyrla D, Drakos E. Apolipoprotein A-I (ApoA-I), immunity, inflammation and cancer. *Cancers (Basel)* 2019; 11: 1097.
11 Gourin CG, Zhi W, Adam BL. Proteomic identification of serum biomarkers for head and neck cancer surveillance. *Laryngoscope* 2009; 119: 1291–302.
12 Martin LJ, Melnichouk O, Huszti E, Connelly PW, Greenberg CV, *et al*. Serum lipids, lipoproteins, and risk of breast cancer: a nested case-control study using multiple time points. *J Natl Cancer Inst* 2015; 107: djv032.
13 Chen CL, Lin TS, Tsai CH, Wu CC, Chung T, *et al*. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. *J Proteomics* 2013; 85: 28–43.
14 Naru J, Aggarwal R, Mohanty AK, Singh U, Bansal D, *et al*. Identification of differentially expressed proteins in retinoblastoma tumors using mass spectrometry-based comparative proteomic approach. *J Proteomics* 2017; 159: 77–91.
15 Yin Y, Xu L, Chang Y, Zeng T, Chen X, *et al*. N-Myc promotes therapeutic resistance development of neuroendocrine prostate cancer by differentially regulating miR-421/ATM pathway. *Mol Cancer* 2019; 18: 11.
16 Xu L, Ma E, Zeng T, Zhao R, Tao Y, *et al*. ATM deficiency promotes progression of CRPC by enhancing Warburg effect. *Endocr Relat Cancer* 2019; 26: 59–71.
17 Yu YP, Landsittel D, Jing L, Nelson J, Ren B, *et al*. Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J Clin Oncol* 2004; 22: 2790–9.
18 Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, *et al*. Integrative genomic profiling of human prostate cancer. *Cancer Cell* 2010; 18: 11–22.
19 Grasso CS, Wu YM, Robinson DR, Cao X, Dhanasekaran SM, *et al*. The mutational landscape of lethal castration-resistant prostate cancer. *Nature* 2012; 487: 239–43.
20 Ku SY, Rosario S, Wang Y, Mu P, Seshadri M, *et al*. Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and antiandrogen resistance. *Science* 2017; 355: 78–83.
21 Jamaspishvili T, Berman DM, Ross AE, Scher HI, De Marzo AM, *et al*. Clinical implications of PTEN loss in prostate cancer. *Nat Rev Urol* 2018; 15: 222–34.
22 Beltran H, Rickman DS, Park K, Chae SS, Sboner A, *et al*. Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer Discov* 2011; 1: 487–95.
23 Beltran H, Prandi D, Mosquera JM, Benelli M, Puca L, *et al*. Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nat Med* 2016; 22: 298–305.
24 Quigley DA, Dang HX, Zhao SG, Lloyd P, Aggarwal R, *et al*. Genomic hallmarks and structural variation in metastatic prostate cancer. *Cell* 2018; 174: 758–69.e9.
25 Tzelepi V, Zhang J, Lu JF, Kleb B, Wu G, *et al*. Modeling a lethal prostate cancer variant with small-cell carcinoma features. *Clin Cancer Res* 2012; 18: 666–77.
26 Svensson RU, Haverkamp JM, Thedens DR, Cohen MB, Ratliff TL, *et al*. Slow disease progression in a C57BL/6 *pten*-deficient mouse model of prostate cancer. *Am J Pathol* 2011; 179: 502–12.
27 Ma X, Ziel-van der Made AC, Autar B, van der Korput HA, Vermeij M, *et al*. Targeted biallelic inactivation of *Pten* in the mouse prostate leads to prostate cancer

accompanied by increased epithelial cell proliferation but not by reduced apoptosis. *Cancer Res* 2005; 65: 5730–9.
28 Mu P, Zhang Z, Benelli M, Karthaus WR, Hoover E, *et al. SOX2* promotes lineage plasticity and antiandrogen resistance in *TP53*- and *RB1*-deficient prostate cancer. *Science* 2017; 355: 84–8.
29 Zhang X, Coleman IM, Brown LG, True LD, Kollath L, *et al.* SRRM4 expression and the loss of rest activity may promote the emergence of the neuroendocrine phenotype in castration-resistant prostate cancer. *Clin Cancer Res* 2015; 21: 4698–708.
30 Li Y, Donmez N, Sahinalp C, Xie N, Wang Y, *et al.* SRRM4 drives neuroendocrine transdifferentiation of prostate adenocarcinoma under androgen receptor pathway inhibition. *Eur Urol* 2017; 71: 68–78.
31 Puca L, Bareja R, Prandi D, Shaw R, Benelli M, *et al.* Patient derived organoids to model rare prostate cancer phenotypes. *Nat Commun* 2018; 9: 2404.
32 Park JW, Lee JK, Witte ON, Huang J. FOXA2 is a sensitive and specific marker for small cell neuroendocrine carcinoma of the prostate. *Mod Pathol* 2017; 30: 1262–72.
33 Guo H, Ci X, Ahmed M, Hua JT, Soares F, *et al.* ONECUT2 is a driver of neuroendocrine prostate cancer. *Nat Commun* 2019; 10: 278.
34 Li Y, He Y, Butler W, Xu L, Chang Y, *et al.* Targeting cellular heterogeneity with CXCR2 blockade for the treatment of therapy-resistant prostate cancer. *Sci Transl Med* 2019; 11: eaax0428.
35 Ewald JA, Desotelle JA, Church DR, Yang B, Huang W, *et al.* Androgen deprivation induces senescence characteristics in prostate cancer cells *in vitro* and *in vivo*. *Prostate* 2013; 73: 337–45.
36 Chen M, Huang J. The expanded role of fatty acid metabolism in cancer: new aspects and targets. *Precis Clin Med* 2019; 2: 183–91.
37 Rebello RJ, Pearson RB, Hannan RD, Furic L. Therapeutic approaches targeting MYC-driven prostate cancer. *Genes (Basel)* 2017; 8: 71.
38 Dardenne E, Beltran H, Benelli M, Gayvert K, Berger A, *et al.* N-Myc induces an EZH2-mediated transcriptional program driving neuroendocrine prostate cancer. *Cancer Cell* 2016; 30: 563–77.
39 Nakazawa MS, Keith B, Simon MC. Oxygen availability and metabolic adaptations. *Nat Rev Cancer* 2016; 16: 663–73.
40 Santoni M, Cimadamore A, Massari F, Piva F, Aurilio G, *et al.* Key role of obesity in genitourinary tumors with emphasis on urothelial and prostate cancers. *Cancers (Basel)* 2019; 11: 1225.
41 Chen M, Zhang J, Sampieri K, Clohessy JG, Mendez L, *et al.* An aberrant srebp-dependent lipogenic program promotes metastatic prostate cancer. *Nat Genet* 2018; 50: 206–18.
42 Gutierrez-Pajares JL, Ben Hassen C, Chevalier S, Frank PG. SR-BI: linking cholesterol and lipoprotein metabolism with breast and prostate cancer. *Front Pharmacol* 2016; 7: 338.
43 Jamnagerwalla J, Howard LE, Allott EH, Vidal AC, Moreira DM, *et al.* Serum cholesterol and risk of high-grade prostate cancer: results from the reduce study. *Prostate Cancer Prostatic Dis* 2018; 21: 252–9.
44 Murtola TJ, Kasurinen TV, Talala K, Taari K, Tammela TL, *et al.* Serum cholesterol and prostate cancer risk in the Finnish randomized study of screening for prostate cancer. *Prostate Cancer Prostatic Dis* 2019; 22: 66–76.
45 Allott EH, Howard LE, Cooperberg MR, Kane CJ, Aronson WJ, *et al.* Serum lipid profile and risk of prostate cancer recurrence: results from the search database. *Cancer Epidemiol Biomarkers Prev* 2014; 23: 2349–56.
46 Sekine Y, Demosky SJ, Stonik JA, Furuya Y, Koike H, *et al.* High-density lipoprotein induces proliferation and migration of human prostate androgen-independent cancer cells by an ABCA1-dependent mechanism. *Mol Cancer Res* 2010; 8: 1284–94.
47 Sekine Y, Suzuki K, Remaley AT. Hdl and sphingosine-1-phosphate activate STAT3 in prostate cancer DU145 cells via ERK1/2 and S1P receptors, and promote cell migration and invasion. *Prostate* 2011; 71: 690–9.
48 Litvinov IV, Antony L, Dalrymple SL, Becker R, Cheng L, *et al.* PC3, but not DU145, human prostate cancer cells retain the coregulators required for tumor suppressor ability of androgen receptor. *Prostate* 2006; 66: 1329–38.
49 Wang X, Yang K, Wu Q, Kim LJY, Morton AR, *et al.* Targeting pyrimidine synthesis accentuates molecular therapy response in glioblastoma stem cells. *Sci Transl Med* 2019; 11: eaau4972.
50 Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, *et al.* C-MYC suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 2009; 458: 762–5.



Supplementary Figure 1: Loss of ApoA-I shows different effects between advanced and primary tumor cells. Western blot showing the efficiency of shRNA-mediated knockdown of ApoA-I in (**a**) C4-2 and (**b**) LNCaP cells. Cell viability was compared between the indicated cells with the presence or absence of ApoA-I in (**a**) C4-2 and (**b**) LNCaP cells. ApoA-I: apolipoprotein A-I; n.s: not significant; LNCaP: lymph node carcinoma of the prostate; shRNA: short hairpin RNA; Cont: control.

Supplementary Table 1: All the full names of the genes in Figure 2c

Gene symbol	*Official full name*
APOA1	Apolipoprotein A1
RB1	RB Transcriptional Corepressor 1
TP53	Tumor Protein P53
PTEN	Phosphatase and Tensin Homolog
FOXA1	Forkhead Box A1
REST	RE1 Silencing Transcription Factor
EZH2	Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit
AURKA	Aurora Kinase A
MYCN	MYCN Proto-Oncogene
FOXA2	Forkhead Box A2
SOX2	SRY-Box Transcription Factor 2
SRRM4	Serine/Arginine Repetitive Matrix 4
CHGA	Chromogranin A
ENO2	Enolase 2
SYP	Synaptophysin
CXCR2	C-X-C Motif Chemokine Receptor 2
ONECUT2	One Cut Homeobox 2
CD44	CD44 Molecule
PROM1	Prominin 1
POU5F1	POU Class 5 Homeobox 1
SOX9	SRY-Box Transcription Factor 9
KLF4	Kruppel-Like Factor 4
IGF1R	Insulin-Like Growth Factor 1 Receptor
REG4	Regenerating Family Member 4
UBE2C	Ubiquitin Conjugating Enzyme E2 C
BCL2	BCL2 Apoptosis Regulator
E2F3	E2F Transcription Factor 3
SNAI1	Snail Family Transcriptional Repressor 1