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Abiraterone Contributed To Androgen-Independent Prostate Cancer Cell Proliferation By Triggering Smad3-Mediated Tumor Warburg

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Androgen-independent prostate cancer cell; Abiraterone; Glycolysis; Cell proliferation

1. Abstract

Androgen-independent, castration-resistant prostate cancer (CRPC) invariably occurs and the outcome is poor. The specific effect and mechanism of abiraterone on the cell proliferation of androgen-independent prostate cancer remains elusive. A number of studies have already reported glycolysis driving prostate tumorigenesis. To address the specific role of abiraterone on glycolysis in androgen-independent prostate cancer, metabolic studies combined with gene expression analysis were performed in vitro. We demonstrated abiraterone increases cell proliferation via promoting glycolysis (the Warburg effect) in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell. Abiraterone-induced cell proliferation and glycolysis in Smad3-dependent. We also found that abiraterone can enhance the interaction between Smad3 and HIF-1α, activate Smad3, and induce the expression of HIF-1α. This study indicated that abiraterone may promote positive feedback loop of Smad3/HIF-1α transcriptional complex \p-Smad3\ HIF-1α, which implies the contribution of tumor glycolysis and proliferation, which may lead to a breakthrough in the treatment of prostate cancer (Figure A).

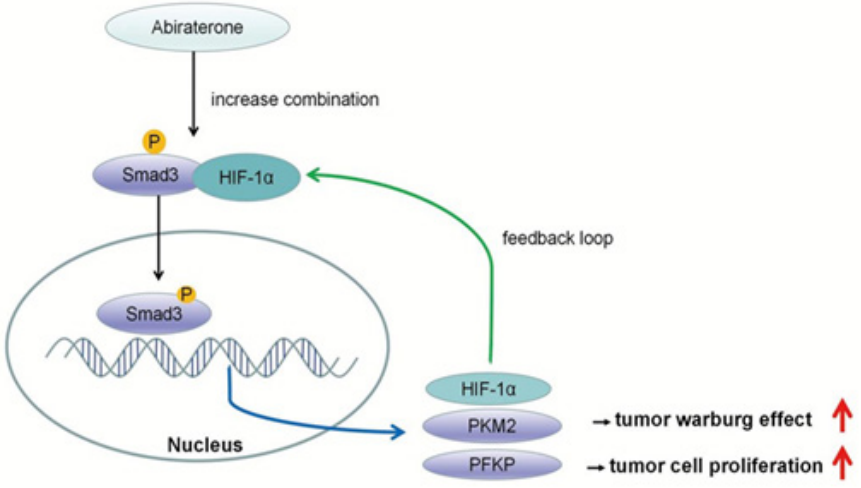

Figure A:

2. Introduction

Prostate cancer is the second most frequent cancer in men worldwide [1]. Although patients with localized prostate cancer generally have a good prognosis, the 5-year relative survival rate is significantly reduced for patients that present with metastatic prostate cancer at diagnosis [2]. ADT and/or radiotherapy remains the mainstay treatment for patients that relapse post-surgery. Although prostate tumors respond initially to ADT, the emergence of androgen-independent, castration-resistant prostate cancer (CRPC) invariably occurs and the outcome is poor [3-5]. In recent years, several new drugs have been approved that prolong CRPC overall survival, abiraterone a new-generation hormonal therapy. However, despite the demonstrated benefit abiraterone, not all patients with CRPC are responsive to it. Approximately one third of patients treated with abiraterone show primary resistance to these agents [6]. Previous researches shown that abiraterone have an anti-proliferative efficacy in androgen-independent prostate cancer cell at extremely high dose in vitro [7, 8]. The specific effect and mechanism of abiraterone on the cell proliferation of androgen-independent prostate cancer still unclear.

From the previous literature, we know that tumorigenesis is dependent on the reprogramming of cellular metabolism. A cancerous cell generates energy mainly through the process of aerobic glycolysis, better known as the Warburg effect. The Warburg effect describes how cancer cells alter their method of metabolism to an increase in glycolysis followed by lactic acid fermentation even in the presence of excess oxygen [9]. Rate-limiting enzymes of glycolysis are hexokinase 2 (HK2), phosphofructokinase-1 (PFK1), and lactate dehydrogenase A (LDHA) [10]. HIF-1α is a crucial transcriptional factor responsible for the Warburg effect and plays key roles in driving prostate tumorigenesis [11]. PFKP (platelet) one isoform of PFK-1 contributes to metabolic pathways in favor of tumor growth and survival. Many studies revealed that PFKP the HIF-1α target gene play an very important role in cancer cell survival and proliferation in muscle-invasive bladder cancer (MIBC) and clear cell renal cell carcinoma (ccRCC) [12, 13]. Although researchers have already reported glycolysis driving prostate tumorigenesis [11, 14, 15], the exact impacts of abiraterone on glycolysis in androgen-independent prostate cancer are far from fully elucidated.

In our study, we found abiraterone can increases cell proliferation in androgen-independent prostate cancer cell via promoting glycolysis. And we revealed that abiraterone can enhanced interaction between Smad3 and HIF-1α to activate Smad3. In summary, we testified that abiraterone may promote positive feedback loop of Smad3/HIF-1α transcriptional complex \p-Smad3\HIF-1α, which implies the contribution of tumor glycolysis and proliferation.

3. Materials and Methods

3.1. Cell Culture, Cell Proliferation Assay

Prostate cancer cell lines PC-3, PC-3M 1E8 were maintained in RPMI1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics at 37 °C in a humidified atmosphere containing 5% CO2 (cell culture incubator, Thermo). The cells proliferation was detected by cell counting kit-8 (CCK-8, Dojindo, Japan). Prostate cancer cells were seeded in 96-well plates (all at 3 × 104 cells/well) within 100 μl culture medium. To evaluate the effect of Abiraterone on cells, Abiraterone was diluted with culture medium to various concentrations (0,2.5,5,10uM) and added to the cells. After 24, 36 and 48h of treatment, 10 μl of CCK-8 reagent (Keygen Biotech, Jiangshu, China) was added to each well and incubated for 1.5 h at 37 °C. The cell viability was measured at 450 nm using a microplate reader (BioTek Instrument Inc., Winooski, VT, USA).

3.2. Immunoblotting and Immunoprecipitation

Cells were lysed in cold cell lysis buffer (HEPES pH 7.4 50 mmol/L, NaCl 150 mmol/L, Triton X-100 1% and glycerol 10%) supplemented with phosphatase and protease inhibitors (B14011 and B15001, Bimake), sonicated and centrifuged for 15 min at 15,000 rpm at 4 °C. Total protein (20 μg) from each lysate was separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with the indicated antibodies. For immunoprecipitation, control IgG or antibody was incubated overnight at 4 °C with supernatant. Antibodies were purchased as follows: Smad3, pSmad3 (Ser213), PKM2, and HIF-1α all from Abcam. α-tubulin (Cell Signaling Technology) used as an internal control.

3.3. Chromatin Immunoprecipitation Assay (ChIP)

Chromatin immunoprecipitation assay was performed as described previously by using Magna ChIP™ Kit (Millipore). ChIP was carried out using an anti-Smad3 antibody (Santa Cruz Biotechnology) or mouse IgG (Millipore, Billerica, MA, USA) and chromatin extracts equivalent to 2 × 10 cells. ChIP samples were quantified by qPCR (SYBR Green Master Mix; Applied Biosystems) and the ChIP qPCR data were normalized using the percent input method. The oligonucleotide primer sequences of HIF-1A forward: 5'-GGTCACTTCCTCCCACCTAAT-3' and reverse: 5'-CAGGCTCACGCTACGGAATC-3'.

3.4. Quantitative Real-Time Polymerase Chain Reaction

Each polymerase chain reaction (PCR) was carried out in triplicate in a 25 μL volume using SYBR Green Mastermix according to the description. The process was performed for 10 min at 95 °C for initial denaturing, followed by 40 cycles of 95 °C for 30 s (denaturing), 60 °C for 1 min (annealing) and 72 °C for 30 s (extension), ended by a final extension step at 95 °C for 1 min, 60 °C for 30 s

and 95 °C for 30 s, using the Stratagene Mx3000 Real-time PCR System. Values were normalized by internal control of β-actin. cDNA was prepared by using SuperscripT™ III First Strand Synthesis SuperMix for qRT-PCR, according to the manufacturer's protocol. Total RNA was extracted using RNeasy Mini kit, and 0.8 μg of RNA was used to synthesize cDNA.

The specific primers for HIF-1A forward: 5'-AAGGTATTGCACTGCACAGG-3' and reverse: 5'-AATGGGTTCACAAATCAGCA-3'; PFKP forward: 5'-GGAGTGGAGTGGGCTGCTGGAG-3' and reverse: 5'-CATGTCGGTGCCGCAGAAATCA-3'; PKM2: forward: 5' -GAGTACCATGCGGAGACCAT-3' and reverse: 5'- GCGTTATCCAGCGTGATTTT-3'; β-actin forward: 5-ATCTCCTTCTGCATCCTGTC-3' and reverse:5' -ACTCTTCCAGCCTTCCTTC-3.

3.5. Lactate and Glucose Measurements

Glucose and lactate concentrations were measured in subconfluent cell culture growing in 12-well plate. Lactate and glucose concentration in the culture media was measured for 24 h using Lactate assay kit from sigma (St. Louis, MO, USA) and Nova Biomedical BioProfile 100 plus machine, respectively, then finally normalized against the number of cells in each well.

3.6. Statistical Analysis

Data are presented as means ± SEM from at least three independent experiments. Protein band densities were quantified using Image J software and the ratio to the control was calculated after normalization to the β-actin band density in the corresponding lane. The band density of the control was set at 1.0 for each comparison. The final density data were plotted using GraphPad Prism software. Differences of multiple groups were examined by oneway ANOVA analysis. Statistical significance between two groups was analyzed by unpaired Student's t test.

4. Results

4.1. Abiraterone increases cell proliferation in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell

To explore the effect of Abiraterone on human androgen-independent PC-3 and PC-3M 1E8 prostate cancer cell [16, 17], CCK-8 assay was performed to detect cell viability treated with Abiraterone in different concentrations (0,2.5,5,10uM) for 48h. As shown in Figure 1A-B, the result and statistical analysis revealed abiraterone significantly promotes cell proliferation in androgen-independent PC-3 and PC-3M 1E8 cell lines compared with control group in a dose-dependent way, respectively. Further results demonstrated abiraterone also induces cell facilitation in a time-dependent manner (Figure 1C-D). Taken together, these results suggested abiraterone enhances cell proliferation in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell.

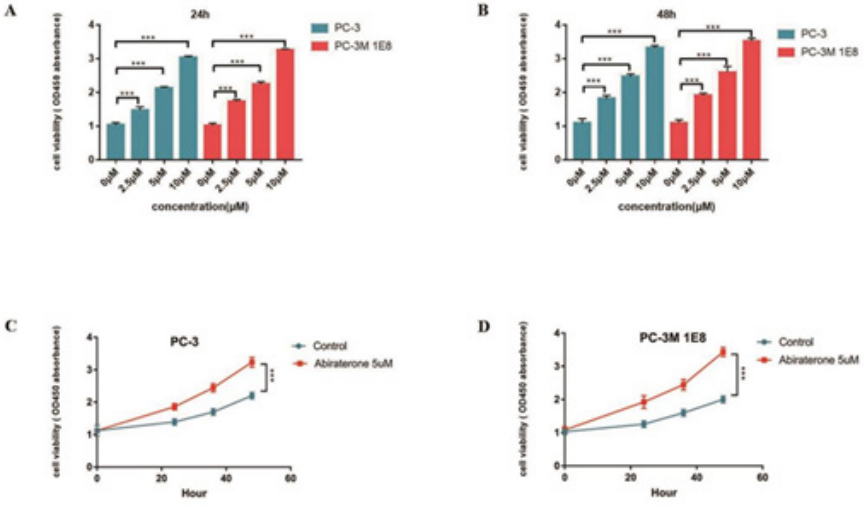

Figure 1: Abiraterone increases cell proliferation in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell. (A-B) The dose course of abiraterone on cell proliferation was evaluated by CCK-8 assay in PC-3 and PC-3M 1E8 cells, respectively. (C-D) The time course of abiraterone on cell proliferation was evaluated by CCK-8 assay in PC-3 and PC-3M 1E8 cells, respectively. Data were presented as mean (± s.d.). n= 3 in each group, *P＜0.05; **P＜0.01; ***P＜0.001 versus control using unpaired Student's t-test

4.2. Abiraterone regulates glycolysis in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell

Tumor glycolysis has been reported to be critical for cellular proliferation [18], we try to examine whether Abiraterone could regulate the high glycolytic phenotype in androgen-independent prostate cancer cell by measure glucose uptake and lactate production. As shown in Figure 2A-B, glucose uptake was significantly enhanced in PC-3 and PC-3M 1E8 cell in response to abiraterone stimulation (5uM) for 24 and 48 h compared with control group(0uM). In line with this, lactate production was obviously increased in a dose-dependent manner in PC-3 and PC-3M 1E8 cell compared with the control group (Figure 2C-D). These results clearly indicated that Abiraterone leads to increased glycolytic activity in androgen-independent PC-3 and PC-3M 1E8 cell lines. PFKP, PKM2, and

HK2 are tumor glycolysis rate-limiting enzymes which are highly expressed in tumors and regulated by HIF-1α [11, 19].

To elucidate the mechanism accounting for abiraterone induced glycolysis activation, Q-PCR were used to analyze abiraterone-induced glycolytic genes expression in 6-phosphofructokinase (PFKP), PKM2 and HIF-1α. We found that abiraterone resulted in a significant upregulation of PFKP, PKM2 and HIF-1α (Figure 2E-F). Consisted with this, protein level of PFKP and HIF-1α were increase in PC-3 and PC-3M 1E8 cell by abiraterone treatment (Figure 2G).

Hence, the present data implied that abiraterone mediate glycolytic protein to promote cell proliferation.

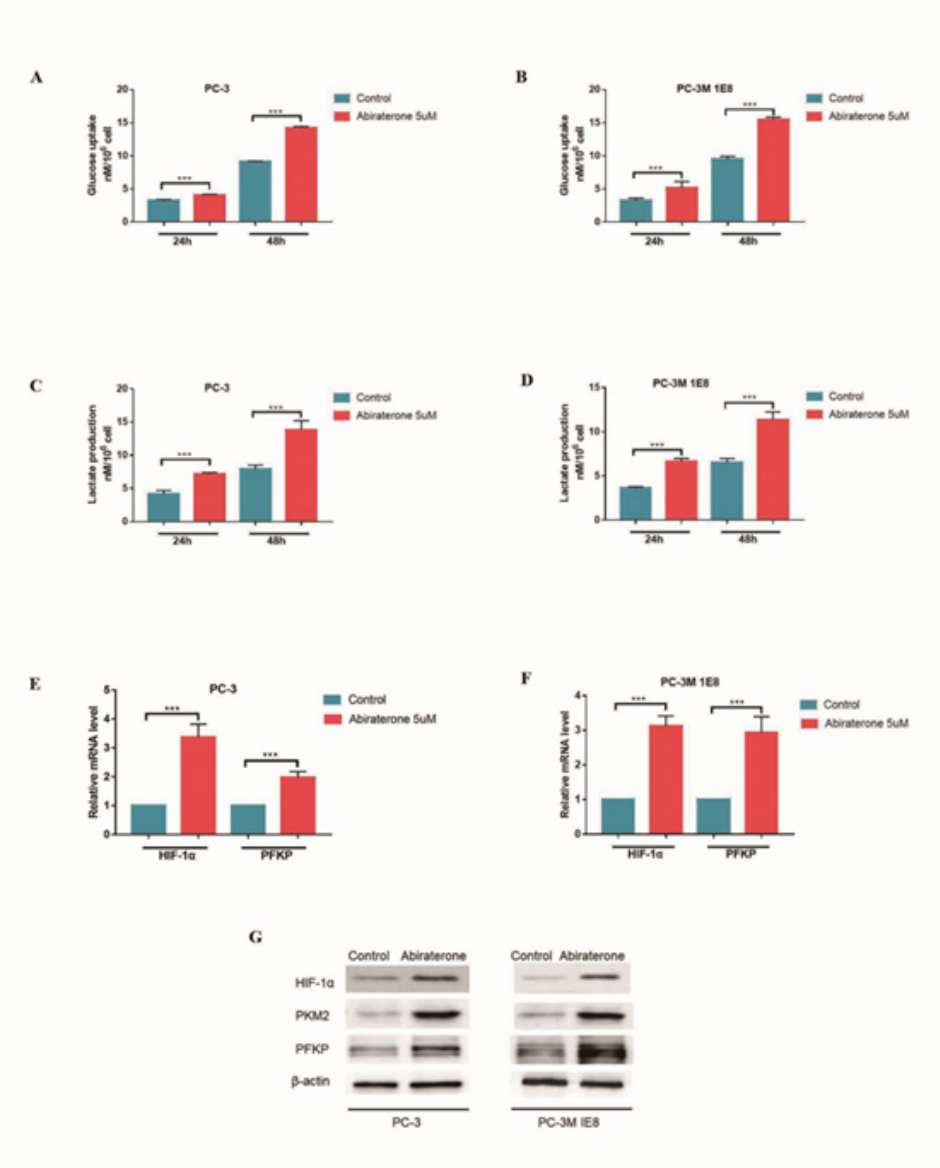

Figure 2: Abiraterone regulates glycolysis in PC-3 and PC-3M 1E8 of androgen-independent prostate cancer cell. (A-D). The effect of abiraterone on the concentrate of glucose consumption and lactate production in PC-3 and PC-3M 1E8 cells was measured as described in Material and Methods between group analyzed by using unpaired Student's t-test, n=3,***P＜0.001. (E-F). Indicated gene mRNA levels was measured by Real-time PCR inPC-3 and PC-3M 1E8 cells. Data were presented as mean (± s.d.). n= 3 in each group. ***P＜0.001 (One sample t-test). G. Protein levels of metabolic enzymes in the Warburg effect were determined by western blot in PC-3 and PC-3M 1E8 cells, β-actin served as loading control.

4.3. Abiraterone promotes proliferation and glycolysis by activation of Smad3

Interestingly, previous studies showed that Smad3 is a repressor of PGC-1a expression and a critical regulator in PKC epsilon-mediated aerobic glycolysis [20, 21]. To investigate tumor glycolysis abiraterone resulting from abiraterone was attributed to Smad3 phosphorylation, SIS3, a Smad3 phosphorylation inhibitor [22], could overcome these phenomena caused by abiraterone. As shown in Figure 3A-B, the cell viability was decreased after co-treatment with abiraterone+SIS3 (5, 10, and 20 uM) for 48 h compared with abiraterone group. As for tumour glycolysis, including glucose uptake and lactate production, was obviously inhibited in androgen-independent PC-3 and PC-3M 1E8 cells compared with the abiraterone treated group (Figure 3C-F). The above finding sug-

gested that abiraterone-induced cell proliferation and glycolysis in Smad3-dependent.

Next, we sought the effect of abiraterone phosphorylation of Smad3. As shown in Figure 3G, western blots revealed that Abiraterone significantly increased phosphorylation of Smad3. Taken together, these results indicated abiraterone modulated cell proliferation and glycolysis through phosphorylation of Smad3 in androgen-independent prostate cancer cell lines.

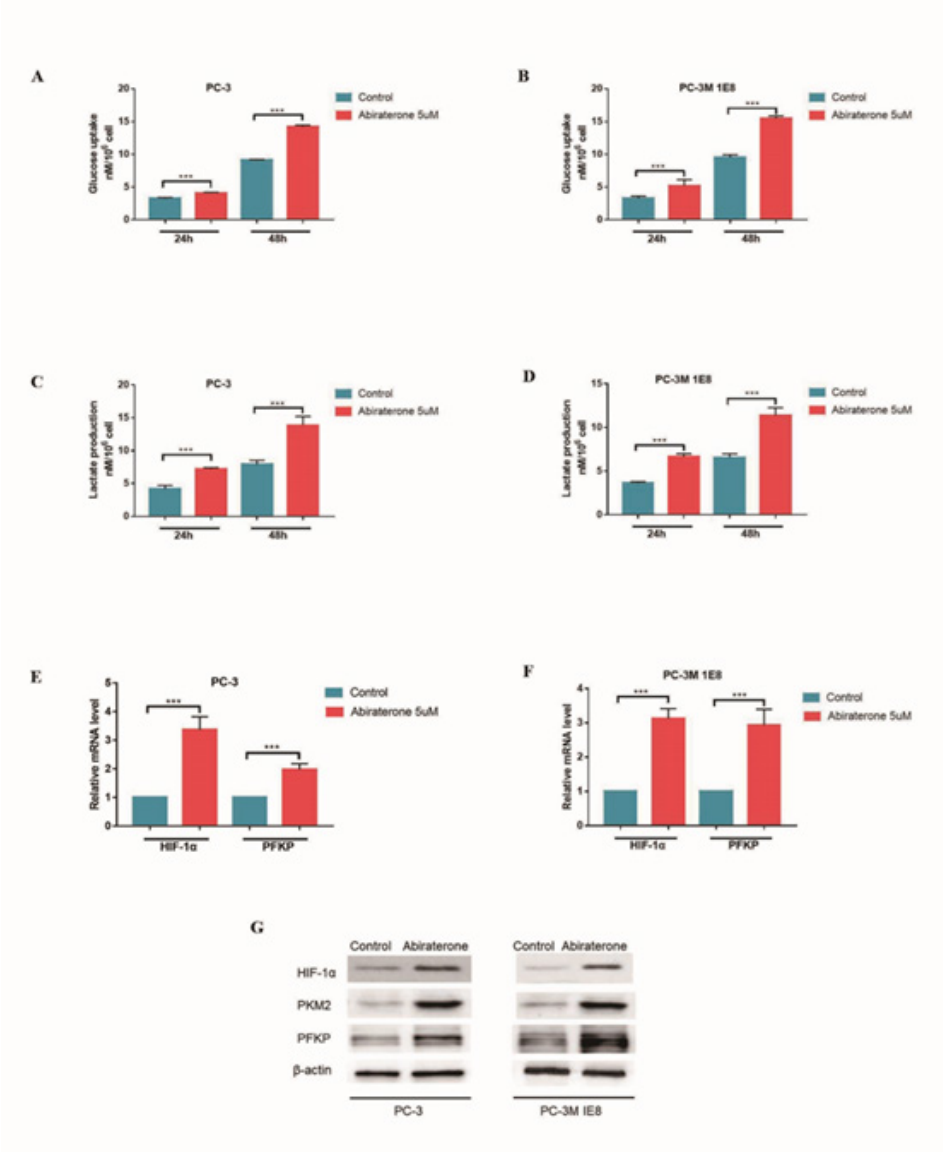

Figure 3: Abiraterone promotes proliferation and glycolysis by activation of Smad3. (A-B) The effect of abiraterone and SIS3 on cell proliferation was evaluated by CCK-8 assay in PC-3 and PC-3M 1E8 cells, respectively. (C-F). The effect of abiraterone and SIS3 on the concentrate of glucose consumption and lactate production in PC-3 and PC-3M 1E8 cells was measured as described in Material and Methods between group analyzed by using unpaired Student's t-test, n=3,***P＜0.001. G. After treated with abiraterone and SIS3, protein levels of PFKP, Smad3 and p-Smad3 were determined by western blot in PC-3 and PC-3M 1E8 cells, β-actin served as loading control.

4.4. Abiraterone enhanced the interaction and regulation between Smad3 and HIF-1α

The above results may imply that Abiraterone regulated HIF-1α expression not only at mRNA transcription level but also at protein expression level; however, it is still unknown the specific mechanism in effect. Interestingly, previous studies showed that endogenous Smad3 regulated and interacted with HIF-1α, resulting in increasing activity of Smad3 [23]. And another study shown that the formation of a Smad3/HIF-1α transcriptional complex led to increase phosphorylation of Smad3, which is consistent with previous reports by Shi X et al, Sanchez-Elsner T et al and Baumann B et al [24-26].

Therefore, we focus our attention on the relationship between HIF-1α and Smad3. Importantly, as shown in Figures 4A-B, immunoprecipitation showed that Smad3 interacts with HIF-1α in PC-3 and PC-3M 1E8 cell, and this interaction was enhanced by abiraterone treatment. In addition, ChIP assays were used to further confirm the possibility that upregulation of HIF-1α genes trans-

activation resulting from abiraterone led to promote of binding of Smad3 to the HIF-1α promoter sequence. The results shown that the baseline of Smad3 binding to HIF-1α promoter sequence were increased in PC-3 and PC-3M 1E8 cells treated with abiraterone (Figures 4C).

These results showed that Smad3 is required for abiraterone induced HIF-1α transactivation.

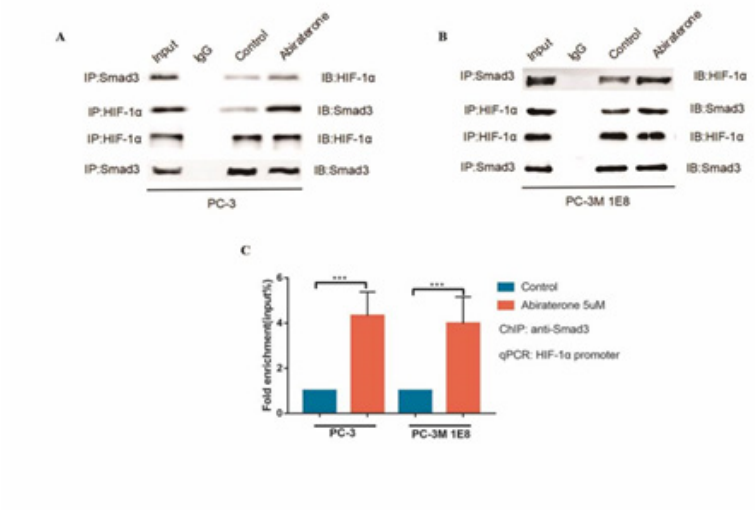

Figure 4: Abiraterone enhanced the interaction and regulation between Smad3 and HIF-1α. (A-B) The lysis of indicated group were immunoprecipitated with antibody targeting endogenous Smad3 and coprecipitated with HIF-1α by immunoblotting. (C) ChIP analysis of binding of Smad3 protein to HIF-1α promoter. Soluble chromatin PC-3 and PC-3M 1E8 cells was precipitated with anti-Smad3 and then analyzed for HIF-1α promoter sequences. Data were presented as mean (± s.d.). n= 3 in each group, ***P＜0.001 versus control using unpaired Student's t-test.

4. Discussion

prostate cancer is the second most frequent cancer and the fifth leading cause of cancer death among men in 2020 [1]. Androgen Deprivation Therapy (ADT) represents the mainstay of treatment of patients with relapsed or metastatic hormone-sensitive disease [27]. Most prostate cancer patients will eventually progress to castration resistant prostate cancer (CRPC) despite receiving ADT treatment. In recent years, several new drugs have been approved that prolong CRPC overall survival, abiraterone a new-generation hormonal therapy. However, despite the demonstrated benefit abiraterone, not all patients with CRPC are responsive to it.

Approximately one third of patients treated with abiraterone show primary resistance to these agents [6]. It is also worth noting that abiraterone doesn't have an anti-proliferative efficacy in androgen-independent prostate cancer cell at clinically achievable concentrations of <1000 nM in vitro [8]. The anti-proliferation effect in androgen-independent prostate cancer cell will not appear until the concentration of abiraterone is extremely increased to 30uM which is three times higher than the androgen-dependent cell inhibitory concentration [7]. These phenomena suggest the specific effect and mechanism of abiraterone on the cell proliferation of androgen-independent prostate cancer remains to be further studied which may lead to a breakthrough in the treatment of prostate cancer.

Glycolysis (Warburg effect) is the Hallmarks of tumor cells. The increase of Warburg effect in cancer cells is essential for tumor growth and indicates a poor prognosis for cancer patients. Our results indicate that abiraterone can increase the cell proliferation of androgen-independent prostate cancer cells by promoting glycolysis. Previous studies have shown that lactic acid is related to tumor resistance such as tumor immune escape [28]. We found that abiraterone is accompanied by an increase in the production of lactic acid in the process of promoting glycolysis. Whether the excessive accumulation of lactic acid in the cellular microenvironment can adversely promote the resistance of androgen-independent prostate cancer cells to abiraterone remains to be explored. Further work is required to explore the other metabolic reprogramming, including De novo lipogenesis, fructose metabolism and glutamine metabolism, involved in the function of abiraterone.

HIF-1α is a crucial transcriptional factor responsible for the Warburg effect and plays key roles in driving prostate tumorigenesis [11]. In the hypoxic tumor microenvironment, HIF-1α protein escapes degradation and translocate into the nucleus, where it initiates a gene expression program that leads to a switch from oxidative phosphorylation to glycolysis [29, 30]. HIF-1α target genes include glucose transporters that increase glucose uptake and pyruvate dehydrogenase kinases (PDK1-3) that shunt pyruvate away from mitochondria through the inhibition of pyruvate dehydrogenase [31]. In this study, we found that abiraterone can enhance the interaction between Smad3 and HIF-1α, activate Smad3, and induce the expression of HIF-1α. Based upon these finding, abiraterone may promote positive feedback loop of Smad3/ HIF-1α transcriptional complex \p-Smad3\ HIF-1α, which implies the con-

tribution of tumor glycolysis and proliferation. Further research is needed to determine this speculation in our work. The interaction between the critical domain of Smad3, response to abiraterone stimulation, and HIF-1α is still remain to be revealed. what more, whether the phosphorylation of the link region of Smad3 involved in the expression of HIF-1α is regulated by abiraterone, in addition, whether abiraterone promotes the stability of HIF-1α, including ubiquitination, sumo modification, etc., needs to be further clarified in next research.

Limited reports were available about abiraterone in metabolic reprogramming in androgen-independent prostate cancer cell proliferation. Our work partly accounts for the therapy frustration of abiraterone in CRPC. However, there were a number of limitations in our study. For example, follow-up data of the prostate cancer patients are absent in this study, in vivo experiment were further required to confirm the function of abiraterone in PC-3 and PC-3M 1E8 cells. Moreover, our current work could not completely rule out the speculation that abiraterone directly regulated SMAD3, including activity and expression, which could be address in our next work. Taken together, this study demonstrated that alteration of glycolysis pathway is a critical event in response to abiraterone-mediated proliferation in PC-3 and PC-3M 1E8, targeting glycolysis pathway co-operated with abiraterone could be an effective treatment in CRPC.

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