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Ser-486/491 phosphorylation and inhibition of AMPK α activity is positively associated with Gleason score, metastasis, and castration-resistance in prostate cancer: A retrospective clinical study

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Abstract

Background: We previously demonstrated that adenosine monophosphate-activated protein kinase (AMPK α) activity is significantly inhibited by Ser-486/491 phosphorylation in cell culture and *in vivo* models of metastatic and castration-resistant prostate cancer, and hypothesized these findings may translate to clinical specimens.

Methods: In this retrospective, single-institution pilot study, forty-five metastatic prostate cancer cases were identified within the University Hospitals Cleveland Medical Center Pathology Archive with both metastasis and matched primary prostate tumor specimens in formalin-fixed, paraffin-embedded blocks and complete electronic medical records. Thirty non-metastatic, hormone-dependent prostate cancer controls, who were progression-free as defined by undetectable prostate specific antigen for at least 79.6 months (range 79.6 – 136.0 months), and matched metastatic cases based on age, race, and year of diagnosis. All specimens were collected from 1991–2014;

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primary tumor specimens were obtained via diagnostic biopsy or prostatectomy, and metastasis specimens obtained via surgery or perimortem. 5-micron sequential slides were processed for phospho-Ser-486/491 AMPK α_1/α_2 , phospho-Thr-172 AMPK α , AMPK α_1/α_2 , phospho-Ser-792 Raptor, phospho-Ser-79 acetyl-CoA carboxylase, and phospho-Ser-872, 3-hydroxy-3-methylglutaryl-CoA reductase immunohistochemistry to determine expression, phosphorylation pattern, and activity of AMPK α .

Results: Increased inhibitory Ser-486/491 AMPK α_1/α_2 phosphorylation, increased AMPK α protein expression, decreased AMPK α activity, and loss of nuclear AMPK α and p-AMPK α are associated with prostate cancer progression to metastasis. Increased p-Ser-486/491 AMPK α_1/α_2 was also positively correlated with higher Gleason grade and progression to castration-resistance.

Conclusions: p-Ser-486/491 AMPK α_1/α_2 is a novel marker of prostate cancer metastasis and castration-resistance. Ser-486/491 phosphokinases should be pursued as targets for metastatic and castration-resistant prostate cancer chemotherapy.

Keywords

prostatic neoplasms; AMP-activated protein kinase; metastasis; castration-resistant prostate cancer; pathology; clinical specimens

Introduction

AMP-activated protein kinase (AMPK) is a critical regulator of cellular metabolism and plays important role in the development and progression of several diseases, including cancer. AMPK is a heterotrimeric protein consisting of a catalytic α subunit and two regulatory β and γ subunits; activation involves increased AMP/ADP binding to the γ subunit and subsequent Thr-183/172 phosphorylation of AMPK α_1/α_2 by upstream kinases serine/threonine kinase 11/liver kinase B1 (STK11/LKB1) or calcium/calmodulin-dependent protein kinase kinase beta (CaMKK β) [1]. When active, AMPK α directly phosphorylates downstream metabolic and transcriptional targets, causing inhibition of cellular anabolism (for example, AMPK α phosphorylates acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), inhibiting fatty acid and cholesterol synthesis, respectively), increase in cellular catabolism, negative regulation of cell growth and proliferation by phosphorylation of the Raptor component of the mammalian target of rapamycin complex 1 (mTORC1) complex, and promotes autophagy [2].

Several phosphothreonine and phosphoserine sites can modulate AMPK α activity (NCBI BLAST Q13131 and P54646, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>, date of accession 02/02/2018); an important inhibitory site is p-Ser-486/491 on the α_1/α_2 subunit. Several upstream effectors have been identified that increase Ser-486/491 phosphorylation, irrespective of Thr-183/172 phosphorylation, including cAMP-dependent protein kinase/protein kinase A (PKA) [3, 4], protein kinase B (Akt/PKB1) [5], P70-S6 kinase (S6K) [6], and extracellular signal-regulated kinase (ERK) [7], all of which play important roles in prostate cancer progression. In post-sprint muscle, high glycolytic rate, marked lactate accumulation, and hypoxia were associated with increased p-Ser-486/491 AMPK α_1/α_2 [8]; a similar environment can be found within the prostate tumor and is associated with

metastasis and poor prognosis [9]. Chemical agents that activate AMPK α lead to death of prostate cancer cells [10, 11]. Elevated fatty acid and cholesterol biomarkers were identified in the serum of prostate cancer patients who progressed to castration-resistant prostate cancer (CRPC) within one year of endocrine therapy, compared to those who did not progress [12]. Metabolomic analysis identified significantly elevated levels of cholesterol, fatty acids, and amino acids within prostate cancer bone metastases [13], indicative of reduced AMPK α activity in bone metastatic disease. To date, no studies have directly investigated changes in AMPK α phosphorylation status, protein expression, or activity in association with metastasis and castration-resistance in clinical specimens, despite evidence suggesting that inactivation of AMPK α may be an important step in the progression of prostate cancer and a potential therapeutic target.

We have previously shown that AMPK α activity is significantly inhibited by Ser-486/491 phosphorylation in cell culture and in vivo models of metastatic and castration-resistant prostate cancer [14]. We hypothesize that these findings may translate to clinical specimens and that progression to metastasis and castration-resistance in prostate cancer will be associated with decreased AMPK α activity and increased Ser-486/491 AMPK α 1/ α 2 phosphorylation.

Materials and Methods

Patient cohort.

All procedures within this retrospective cohort study were in compliance with human studies protocols approved by the Institutional Review Board of Case Western Reserve University & University Hospitals Cleveland Medical Center (UHCMC) (IRB 02-14-36). Within the UHCMC Pathology Archive, 45 metastatic prostate cancer cases were identified with metastasis and matched primary prostate tumor specimens in formalin-fixed, paraffin-embedded (FFPE) blocks and complete electronic medical records (EMR) were accessed. Thirty non-metastatic, hormone-dependent prostate cancer controls were also identified in the database that were retrospectively-matched with metastatic prostate cancer cases on the basis of age, race, and year of initial diagnosis and had primary prostate tumor FFPE specimens within the Pathology Archive. Per EMR, all non-metastatic prostate cancer patients were progression-free post-prostatectomy and hormone therapy, as defined by undetectable prostate specific antigen (PSA) <0.1 ng/mL for at least 79.6 months (range 79.6–136.0 months). All specimens were collected at the UHCMC from 1991–2014; primary prostate tumor specimens for all cases were obtained via diagnosing biopsy or prostatectomy, and metastasis specimens obtained via surgery or perimortem. Pathological reports were issued at the time of biopsy or surgery, and an expert genitourinary pathologist (G.T.M.) extracted pathological characteristics from these reports retrospectively when constructing the cohort and evaluated specimens to determine suitability for the study. Clinical and demographic data, excluding patient identifiers, were obtained from EMR database associated with each anonymous specimen. CRPC in metastatic subject group was defined as cases from patients who became refractory and progressed despite 1 year of hormone therapy.

Immunohistochemistry.

Reagents and equipment for immunohistochemical staining were purchased from Biocare Medical (Pacheco, CA), unless noted otherwise, and were used according to manufacturer instructions. Slides were prepared using 5-micron sequential sections of each FFPE block, and one slide per block was stained with CAT hematoxylin and eosin (H&E). After deparaffinization in xylene, slides were rehydrated in decreasing concentrations of ethanol, followed by distilled water rinses. For antigen retrieval, slides were heated with Diva Decloaker using the Decloaking Chamber Pro (first stage 125°C for 30 seconds, second stage 90°C for 10 minutes, third stage 70°C for 40 minutes). Endogenous peroxidase was blocked using Peroxidized 1, and nonspecific binding blocked with Background Sniper. Slides were incubated overnight with monoclonal antibodies for phospho-Ser-486/491 AMPK α 1/ α 2 (#4185, 1:100), phospho-Thr-172 AMPK α (#2535, 1:50), phospho-Ser-792 Raptor (#2083, 1:100), phospho-Ser-79 ACC (#3661, 1:800), (Cell Signaling Technology, Danvers, MA); phospho-Ser-872 HMG-CoAR (orb6191, 1:100, Biorbyt LLC, San Francisco, CA); or AMPK α 1/ α 2 (sc-25792, 1:250, Santa Cruz Biotechnology, Dallas, TX) (optimum determined by chessboard analysis of 1:50 to 1:500 dilutions). After three 5 minute washes in distilled water, sections were incubated with HRP-conjugated goat anti-rabbit IgG (sc-2004, 1:250, Santa Cruz Biotechnology) secondary antibody for 2 hours at room temperature. Betazoid DAB was used as a chromogen. Sections were counterstained using CAT hematoxylin. Coverslips attached using Cytoseal XYL mounting media (ThermoFisher Scientific, Waltham, MA). For negative controls, primary antibody was omitted.

Pathologic evaluation.

One pathologist (M.A.) performed blinded evaluation of all tissue slides for expression and cellular localization. We used the German Semi-quantitative Scoring System in considering the staining intensity and area extent. Every tissue slide was given a score according to intensity of nuclear and/or cytoplasmic staining (no staining=0; weak=1; moderate=2; strong=3; very strong=4) and extent of stained cells (0%=0; 1–20%=1; 21–50%=2; 51–80%=3; 81–100%=4). The final immunoreactive score was determined by multiplying the intensity and extent of positivity scores of stained cells, with the minimum score of 0 and a maximum score of 16.

Statistical analysis.

The association between two categorical variables was estimated using Fisher's exact test or Pearson's Chi-square test. The difference of continuous measurements among groups was examined by Kruskal-Wallis test. Comparisons between primary and metastatic tumor measurements in the metastatic patient group was made using signed rank test. AMPK localization and expression in relation to disease progression or Gleason grade was examined using Cochran-Armitage test for trend. All tests were two-sided and p-values <0.05 were considered significant.

Results

Demographics, modifiable and genetic risk factors, and comorbidities of the patient cohort.

Table 1 lists demographics of the 30 non-metastatic and 45 metastatic patients. Patients in the metastatic prostate cancer group had a significantly higher median BMI than those in the non-metastatic group, and were more likely to be obese. Tobacco use, alcohol use, blood glucose, cholesterol, and triglycerides were not significantly different amongst metastatic and non-metastatic groups; however, patients in the metastatic prostate cancer group were significantly more likely to have used tobacco for a time period of >20 pack years. Patients in the non-metastatic group were significantly more likely to self-report a first- or second-generation relative with cancer (this data is missing in 10% of non-metastatic and 26.7% of metastatic patient records) (Table 2). Surprisingly, this significance dematerialized when solely analyzing self-reported first- or second- generation relatives diagnosed with prostate, breast, or ovarian cancers. Comorbidities significantly associated with non-metastatic prostate cancer included prostatic intraepithelial neoplasia (PIN) and colon polyps; whereas Type 2 diabetes and hypertension were more significantly associated with metastatic disease.

Pathological characteristics of prostate tumors.

The most common sites of prostate cancer metastasis were bone (77.8%), lung (40.0%), and liver (26.7%); but also included soft-tissue (24.4%) and the central nervous system (13.3%). Of the 45 metastatic prostate cancer patients, 53.3% (24/45) presented with distant metastases at time of diagnosis and 46.7% (21/45) developed metastases despite treatment; 31.1% (14/45) of these patients had documented post-androgen deprivation therapy failure (CRPC). As expected, patients in metastatic group had significantly higher pre-biopsy PSA (Table 3). We hypothesized that this significant difference was due to inclusion of data from the 53.3% of metastatic patients who already had distant metastases at the time of prostate cancer diagnosis (median 426.1, range 35.3–3200), so we compared the pre-diagnostic PSA of only those patients in the metastatic group who did not yet have known metastatic disease at the time of prostate cancer diagnosis with those patients in the non-metastatic group, and found there was no longer a statistically significant difference in pre-diagnostic PSA amongst the two patient populations (median 14.9 [range 1.8–57.8] verses median 5.1 [range 1.2–25.3], $p = 0.072$). The majority of non-metastatic patients had Stage II organ-confined disease; whereas metastatic patients had significantly higher incidence (84.4%) of Stage III and IV non-organ confined disease (Stage III: extraprostatic extension with or without seminal vesicle invasion, Stage IV: invasion of external sphincter, rectum, bladder, levator muscles, and/or pelvic wall). All non-metastatic patients were Gleason 6 or 7 on biopsy; whereas metastatic patients ranged from Gleason 6–10 on initial biopsy, demonstrating a double Gaussian distribution with Gleason 7 and 9 being most common, which corresponded with biopsy Gleason differences amongst patients in the metastatic group without and with distant metastases at the time of prostate cancer diagnosis (mean 7.8 verses 8.7, $p = 0.038$). Percentage of tumor volume in the most positive biopsy core was significantly higher in metastatic group, although laterality was not significantly different amongst the two groups. All patients in the non-metastatic group underwent radical prostatectomy (RP) verses only 26.7% (12/45) of metastatic patients. Gleason scores of RP

specimens in non-metastatic group ranged from 5–8, with Gleason 6 and 7 still most common. Gleason scores of RP specimens in metastatic group were significantly higher, ranging from 7–10, with Gleason 9 most common. Extraprostatic extension, perineural invasion, and positive margins were significantly more frequent in metastatic than in non-metastatic RP specimens; seminal vesicle invasion was also more frequent in metastatic RP specimens, although not significantly so.

AMPK phosphorylation, expression, and activity in primary prostate tumors and metastases.

Mean intensity of p-Ser-486/491 AMPK α 1/ α 2 immunohistochemical staining was significantly greater in the metastasis specimens from the metastatic patient cohort when compared to the primary prostate tumor specimens from both metastatic and non-metastatic patient groups, with a statistically significant trend in increase of p-Ser-486/491 AMPK α 1/ α 2 from non-metastatic primary tumor to metastatic primary tumor to metastatic secondary (metastatic cohort primary tumor staining 3.1x and metastatic cohort metastasis staining 4.7x greater than non-metastatic cohort primary tumor staining) (Table 4 and Figure 1). p-Thr-172 AMPK α did not vary significantly amongst the experimental groups. Significantly stronger AMPK α protein expression was observed in metastatic specimens than in the metastatic group and non-metastatic group primary prostate tumor specimens. Both p-Ser-79 ACC and p-Ser-872 HMG-CoAR staining decreased significantly from non-metastatic group primary tumor to metastatic group primary tumor to metastatic group metastasis; a decreasing trend in p-Ser-792 Raptor staining with progression to metastasis was also seen, but was not significant. p-Ser-486/491 AMPK α 1/ α 2 staining was significantly more intense (2.5x) in castration-resistant specimens than in hormone-dependent specimens (Table 5). p-Ser-486/491 AMPK α / α staining was significantly associated with increasing biopsy Gleason score, whereas p-Ser-872 HMG-CoAR was significantly inversely correlated with increasing biopsy Gleason score in primary prostate cancer specimens (Table 6). p-Ser-79 ACC also demonstrated a decreasing trend with increasing Gleason grade, but did not reach statistical significance. In non-metastatic primary prostate cancer specimens, AMPK α protein expression and phosphorylation are found in both the cytoplasm and nucleus; whereas, staining becomes significantly more cytoplasmic localized and nuclear staining lost in metastasis specimens (Table 7 and Figure 2).

Discussion

To determine translatability of our previous in vitro and in vivo findings, we investigated AMPK α phosphorylation status, protein expression, and activity in clinical prostate cancer specimens. Using primary prostate tumor and metastasis specimens from patients with non-metastatic and metastatic prostate cancer stored within the UHCMC Pathology Archive, we discovered that p-Ser-486/491 AMPK α 1/ α 2 immunohistochemical staining was significantly greater in metastatic cohort primary and secondary site specimens, in castration-resistant metastatic specimens, and in specimens of higher Gleason score. AMPK α protein expression was also significantly increased in metastatic cohort primary tumor and metastasis specimens. Additionally, AMPK α activity, as determined by change in p-Ser-79 ACC, p-Ser-HMG-CoAR, and p-Ser-792 Raptor staining, was diminished in

metastatic subject specimens compared to specimens from patients in the non-metastatic group. These clinical observations confirmed our in vitro data, which had compared AMPK α phosphorylation status, protein expression, and activity amongst hormone-dependent and hormone-refractory, metastasis-derived cell lines [14]. Potential biases exist in this work due to the inter-relationship between high Gleason grade and metastasis and between castration-resistance and metastasis: biopsy specimens of those patients who progressed to metastasis were on average of higher Gleason score, and an association between p-Ser-486/491 AMPK α 1/ α 2 staining and both progression to metastasis and higher Gleason score was established; additionally, all CRPC specimens were metastasis specimens. Yet, importantly, this study has established Ser-486/491 AMPK α 1/ α 2 phosphorylation and inhibition of AMPK α activity as clinical biomarkers of poorer outcome (metastasis and castration-resistance). P-Ser-486/491 AMPK α 1/ α 2 even emerges in primary prostate tumor specimens as potentially predictive of metastasis, when conventional diagnostics were not (PSA at diagnosis was not statistically different amongst the non-metastatic cohort and the portion of the metastatic cohort who had not yet progressed to metastasis). The patient population battling CRPC and metastatic prostate cancer could use more options in their treatment toolbox; identification of drugs which reduce the inhibitory Ser-486/491 phosphorylation by suppression of upstream kinases and/or increase AMPK α activity may be effective treatment for metastatic or CRPC.

Interestingly, we observed that AMPK α protein expression and phosphorylation pattern became significantly cytoplasmic in metastatic group primary and secondary site specimens than in non-metastatic subject primary prostate tumor specimens. Our results suggest that with prostate cancer progression to metastasis, AMPK α loses nuclear localization; one consequence of this is loss of control over the glucose-sensitive G1/S cell cycle checkpoint regulated by AMPK α . When activated, nuclear AMPK α phosphorylates p53 on Ser-15 and initiates AMPK-dependent cell cycle arrest; disruption of this checkpoint permits unchecked proliferation, even with limiting nutrients [15]. In our previous work, we observed that metastatic CRPC cell lines arrested in G1 phase and underwent death by necroptosis following re-activation of AMPK α with combination statin and metformin [16], akin to the observation of Jones et al. (2005) that activation of AMPK with AICAR increased nuclear AMPK α activity and resulted in phosphorylation of p53 and cell cycle arrest [15].

Patients within the non-metastatic prostate cancer cohort were more likely to report a first- or second- generation relative with cancer. Our study relied upon self-report family history data, and data was missing for 10% of the non-metastatic and 26.7% of the metastatic cohort, so accuracy can be questioned with respect to recall bias or completeness. Yet, Kotsis et al. (2002) also previously noted men with either a first-degree relative or any relative with cancer were nearly twice as likely to be diagnosed with well-differentiated (Gleason 6) prostate cancer, whereas men without a family history were diagnosed with higher grade tumors [17]. However, a second study by Spangler et al. (2005) found having a first- or second- degree relative with cancer was associated with increased risk of biochemical failure [18]. Men with a family history of prostate cancer may perceive themselves at greater risk and may be more proactive about screening behavior to detect prostate cancer at an earlier stage [19]. Patients within the non-metastatic prostate cancer cohort were also more likely to have comorbidities of high-grade PIN and colon polyps.

Prior diagnosis of high-grade PIN is highly predictive of prostate cancer diagnosis [20]. Familial cancer link, inflammation, and mutations leading to reduction in phosphatase and tensin homolog (PTEN) expression have been implicated in the development of benign and malignant gastrointestinal lesions and in prostate cancer development [21–23]. Subjects in the metastatic prostate cancer cohort had higher BMI, were more likely to be obese, and more likely to have comorbidities of Type 2 diabetes and hypertension. Obesity, Type 2 diabetes, and hypertension are symptoms of metabolic syndrome, which has been positively correlated in several studies to high Gleason grade prostate cancer and more advanced disease [24, 25].

Conclusions

Increased inhibitory Ser-486/491 AMPK α 1/ α 2 phosphorylation, increased AMPK α protein expression, decreased AMPK α activity, and loss of nuclear AMPK α and p-AMPK α are associated with prostate cancer progression to metastasis. Increased p-Ser-486/491 AMPK α 1/ α 2 was also positively correlated with higher Gleason grade and progression to castration-resistance. This is the first study to correlate alterations in AMPK α protein expression, phosphorylation pattern, and activity with prostate cancer progression in clinical specimens. The current study was limited in that it was a small pilot study involving 75 patients at one academic hospital; yet, it identified Ser-486/491 AMPK α 1/ α 2 phosphorylation as a novel predictive marker and AMPK α activity as a potential target for treatment of metastatic and CRPC. This study yielded compelling results which warrant a larger clinical investigation. Metastatic and CRPC patients are a population in desperate need for development of effective treatment options; AMPK α activators and Ser-486/491 phosphokinase inhibitors are a potential lead that could reduce prostate cancer-related morbidity and mortality in a patient population of particularly high risk of prostate cancer-related death.

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Abbreviations:

ACC	acetyl-CoA carboxylase
ADP	adenosine diphosphate
Akt/PKB	protein kinase B
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
BMI	body mass index

BPH	benign prostatic hyperplasia
CaMKKβ	calcium/calmodulin-dependent protein kinase kinase beta
CRPC	castration-resistant prostate cancer
EMR	electronic medical records
ERK	extracellular signal-regulated kinase
FFPE	formalin-fixed, paraffin-embedded
H&E	hematoxylin and eosin
HMG-CoAR	3-hydroxy-3-methylglutaryl-CoA reductase
mTORC1	mammalian target of rapamycin complex 1
PIN	prostatic intraepithelial neoplasia
PKA	protein kinase A
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
RP	radical prostatectomy
S6K	P70-S6 kinase
STK11/LKB1	serine/threonine kinase 11/liver kinase B1

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Figure 1. Ser-486/491 AMPK α phosphorylation, AMPK α protein expression, and inhibition of AMPK α activity associated with prostate cancer metastasis.

Immunohistochemical staining for p-Ser-486/491 AMPK α_1/α_2 , p-Thr-172 AMPK α , AMPK α , p-Ser-79 ACC, p-Ser-872 HMG-CoAR, and p-Ser-792 Raptor in representative specimens of non-metastatic cohort primary prostate tumor and metastatic cohort primary prostate tumor and metastases. Decrease in Ser-79 ACC, Ser-872 HMG-CoAR, and Ser-792 Raptor phosphorylation demonstrates decreased AMPK α activity.

Figure 2. Prostate cancer metastasis is associated with loss of nuclear AMPK α and p-AMPK α expression.

Immunohistochemical staining for p-Ser-486/491 AMPK α_1/α_2 , p-Thr-172 AMPK α , and AMPK α in samples representative of non-metastatic cohort primary prostate tumor and metastatic cohort primary prostate tumor and metastasis specimens analyzed. AMPK α and p-AMPK α becomes cytoplasmic localized with progression to metastasis.

Table 1.

Demographics and health risk factors of non-metastatic and metastatic prostate cancer patients.

	Non-metastatic (n=30)	Metastatic (n=45)	p-value
Age in years, median (range)	61.3 (45.3–71.8)	63.4 (36.4–86.0)	0.426 [‡]
Race			0.319 [‡]
African-American	9/30 (30.0)	19/45 (42.2)	
Caucasian	19/30 (63.4)	24/45 (53.4)	
Hispanic	1/30 (3.3)	1/45 (2.2)	
Asian	1/30 (3.3)	1/45 (2.2)	
BMI, median (range)	27.5 (22.3–58.0)	29.5 (19.5–39.8)	0.044 [*] [‡]
BMI Category (%)			0.009 ^{**} [‡]
Underweight (< 18.4 kg/m ²)	0/30 (0)	0/45 (0)	
Normal (18.5–24.9 kg/m ²)	2/30 (6.7)	6/45 (13.3)	
Overweight (25–29.9 kg/m ²)	23/30 (76.7)	19/45 (42.2)	
Obese (30–39.9 kg/m ²)	4/30 (13.3)	20/45 (44.5)	
Morbidly Obese (≥ 40 kg/m ²)	1/30 (3.3)	0/45 (0)	
Tobacco Use			0.102 [‡]
Never	19/30 (63.3)	15/45 (33.3)	
Past	6/30 (20.0)	19/45 (42.2)	
Current	4/30 (13.3)	6/45 (13.3)	
Unknown	1/30 (3.3)	5/45 (11.1)	
20 pack years	7/10 (70.0)	6/25 (24.0)	0.038 [*] [‡]
> 20 pack years	2/10 (20.0)	11/25 (44.0)	
Unknown	1/10 (10.0)	7/25 (28.0)	
Alcohol Use			0.928 [‡]
Never	9/30 (30.0)	10/45 (22.2)	
Past	0/30 (0)	5/45 (11.1)	
Current	20/30 (66.7)	24/45 (53.3)	
Unknown	1/30 (3.3)	6/45 (13.3)	
< 1 drink/day	13/20 (65.0)	14/29 (48.3)	0.105 [‡]
1–2 drinks/day	6/20 (30.0)	4/29 (13.8)	
>2 drinks/day	1/20 (5.0)	8/29 (27.6)	
Unknown	0/20 (0)	3/29 (10.3)	
Random (non-fasting) blood glucose, median (range)	100 (79–193)	104 (85–265)	0.367 [‡]
Blood total cholesterol, median (range)	188 (128–250)	173 (81–353)	0.360 [‡]
Blood total triglyceride, median (range)	111 (35–258)	88 (51–293)	0.714 [‡]

BMI, body mass index. Self-reported tobacco use; pack years data from past and current tobacco users. Self-reported alcohol use; usage from past and current consumers. Normal random blood glucose <140 mg/dL. Desirable blood total cholesterol <200 mg/dL. Normal blood triglyceride <150 mg/dL.

^{*} $p < 0.05$,

^{**}
 $p < 0.01$.

[‡], Kruskal-Wallis test;

[†], Fisher's exact test.

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Table 2.

Familial incidence and comorbidities in non-metastatic and metastatic prostate cancer patients.

	Non-metastatic	Metastatic	p-Value
Self-reported first- or second-generation relative with any cancer (%)			0.0137 [*]
None	3/27 (11.1)	13/33 (39.4)	
1	24/27 (88.9)	20/33 (60.6)	
Data missing	3	12	
Self-reported first- or second-generation relative with prostate, breast, or ovarian cancer (%)			0.228
None	13/27 (48.2)	21/33 (63.6)	
1	14/27 (51.8)	12/33 (36.4)	
Data missing	3	12	
Comorbidities:			
Other cancer	7/30 (23.3)	9/45 (20.0)	0.891
Prostatic intraepithelial neoplasia (PIN)	6/30 (20.0)	0/45 (0)	0.0041 ^{**}
Benign prostatic hyperplasia (BPH)	6/30 (20.0)	6/45 (13.3)	0.551
Prostatitis	2/30 (6.7)	0/45 (0)	0.175
Type II Diabetes	2/30 (6.7)	10/45 (22.2)	0.049 [*]
Hyperlipidemia	17/30 (56.7)	17/45 (37.8)	0.113
Hypertension	16/30 (53.3)	32/45 (71.1)	0.028 [*]
Colon polyps	12/30 (40.0)	5/45 (11.1)	0.0067 ^{**}

Colon polyps as a notable comorbidity was not a pre-analysis hypothesis, and instead was observed during data analysis.

^{*}
 $p < 0.05$,

^{**}
 $p < 0.01$, by Chi-square test.

Table 3.

Pre-biopsy PSA and pathological characteristics of primary prostate tumors in non-metastatic and metastatic prostate cancer patients.

	Non-metastatic	Metastatic	<i>p</i> -value
Pre-biopsy PSA (ng/mL), median (range)	5.1 (1.2–25.3)	140.9 (1.8–3200)	<0.0001 *** †
Stage			<0.0001 *** †
I	0/30 (0)	1/45 (2.2)	
II	22/30 (73.3)	6/45 (13.3)	
III	8/30 (26.7)	14/45 (31.1)	
IV	0/30 (0)	24/45 (53.3)	
Biopsy Gleason Score (%)			<0.0001 *** †
6	15/30 (50.0)	4/45 (8.9)	
7	15/30 (50.0)	11/45 (24.4)	
8	0/30 (0)	6/45 (13.3)	
9	0/30 (0)	20/45 (44.5)	
10	0/30 (0)	4/45 (8.9)	
Percent tumor volume in most positive core, median (range)	10 (5–80)	80 (5–100)	<0.0001 *** †
Laterality (%)			0.265 €
Unilateral	8/30 (26.7)	7/45 (15.6)	
Bilateral	22/30 (73.3)	38/45 (84.4)	
RP Gleason Score (%)			<0.0001 *** †
5	1/30 (3.3)	0/12 (0)	
6	11/30 (36.7)	0/12 (0)	
7	15/30 (50.0)	2/12 (16.7)	
8	3/30 (10.0)	2/12 (16.7)	
9	0/30 (0)	6/12 (50.0)	
10	0/30 (0)	2/12 (16.7)	
Extraprostatic extension (%)	8/30 (26.7)	10/12 (83.3)	0.00046 ** €
Seminal vesicle invasion (%)	1/30 (3.3)	4/12 (33.3)	0.0619 †
Perineural invasion (%)	8/30 (26.7)	12/12 (100)	<0.0001 *** †
Margins (%)			<0.0001 *** †
Negative	16/30 (53.3)	0/30 (0)	
Immediately adjacent	2/30 (6.7)	1/12 (8.3)	
Positive	12/30 (40.0)	11/12 (91.7)	

PSA, prostate-specific antigen; normal PSA < 4.0 ng/mL. RP, radical prostatectomy; all 30 non-metastatic cases underwent RP, whereas only 12 metastatic cases underwent RP.

**
 $p < 0.01$,

 $p < 0.0001$.

†, Kruskal-Wallis test;

χ^2 , Fisher's exact test;

χ^2 , Chi-square test.

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Table 4.

Mean German semi-quantitative scoring of immunohistochemical staining in association with progression to metastasis.

Stain	Non-metastatic primary	Metastatic primary	Metastatic secondary	<i>p</i> -value
p-Ser-486/491 AMPKα	1.41	4.41	6.61	<0.0001 ***
p-Thr-172 AMPKα	1.53	2.24	2.44	0.537
AMPKα	6.03	7.83	9.91	0.007 **
p-Ser-79 ACC	3.15	1.21	0.35	<0.0001 ***
p-Ser-872 HMG-CoAR	11.06	7.21	7.94	0.005 **
p-Ser-792 Raptor	3.41	3.21	2.47	0.336

Analysis of immunohistochemical staining of primary prostate tumors and metastases. AMPK α , adenosine monophosphate (AMP)-activated protein kinase, alpha catalytic subunit; ACC, acetyl-CoA carboxylase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

**
 $p < 0.01$,

 $p < 0.0001$ determined by Cochran-Armitage test for trend.

Table 5.

Mean German semi-quantitative scoring of immunohistochemical staining in association with progression to castration-resistance.

Stain	Hormone-Dependent (n=102)	Castration-Resistant (n=18)	p-value
p-Ser-486/491 AMPK α	3.05	7.64	0.0087 **
p-Thr-172 AMPK α	1.95	2.21	0.824
AMPK α	7.92	8.00	0.960
p-Ser-79 ACC	2.37	2.29	0.928
p-Ser-872 HMG-CoAR	8.99	7.69	0.179
p-Ser-792 Raptor	3.22	2.07	0.097

Analysis of immunohistochemical staining comparing hormone-dependent and castration-resistant specimens. AMPK α , adenosine monophosphate (AMP)-activated protein kinase, alpha catalytic subunit; ACC, acetyl-CoA carboxylase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

**
 $p < 0.01$ determined by Kruskal-Wallis test.

Table 6.

Mean German semi-quantitative scoring of immunohistochemical staining of primary tumor phosphorylation and AMPK α protein expression in association with biopsy total Gleason score.

	Biopsy Total Gleason Score					<i>p</i> -value
	6 (n=17)	7 (n=29)	8 (n=10)	9 (n=17)	10 (n=2)	
p-Ser-486/491 AMPKα	1.94	2.50	4.00	5.54	9.00	0.002 **
p-Thr-172 AMPKα	1.65	2.09	2.00	2.62	3.00	0.870
AMPKα	5.71	7.27	8.25	7.46	7.50	0.843
p-Ser-79 ACC	2.06	3.27	0.00	1.46	0.00	0.078
p-Ser-872 HMG-CoAR	10.53	11.27	10.25	5.62	3.50	0.014 *
p-Ser-792 Raptor	3.88	3.91	4.50	1.77	8.00	0.211

AMPK α , adenosine monophosphate (AMP)-activated protein kinase, alpha catalytic subunit; ACC, acetyl-CoA carboxylase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase. No statistical distinction was found between Gleason 3+4=7 and 4+3=7 prostate cancer.

* $p < 0.05$;

** $p < 0.01$ determined by Cochran-Armitage test for trend.

Table 7.AMPK α cellular localization and prostate cancer progression to metastasis.

	Non-metastatic primary	Metastatic primary	Metastatic secondary	<i>p</i> -value
AMPKα (%)				0.009 **
Cytoplasmic only	21/30 (70.0)	33/45 (73.3)	42/45 (93.3)	
Cytoplasmic and nuclear	9/30 (30.0)	12/45 (26.7)	3/45 (6.7)	
p-Ser-486/491 AMPKα (%)				<0.0001 ***
Cytoplasmic only	0/21 (0.0)	4/42 (9.5)	22/41 (53.7)	
Cytoplasmic and nuclear	21/21 (100.0)	38/42 (90.5)	19/41 (46.3)	
p-Thr-172 AMPKα (%)				0.010 *
Cytoplasmic only	11/23 (47.8)	20/37 (54.1)	32/41 (78.0)	
Cytoplasmic and nuclear	12/23 (52.2)	17/37 (45.9)	9/41 (22.0)	

Cytoplasmic localization with progression to metastasis. Only slides with positive staining analyzed.

*
 $p < 0.05$,**
 $p < 0.01$,***
 $p < 0.0001$ determined by Cochran Armitage test for trend.