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AMPKα Is Suppressed in Bladder Cancer through Macrophage-Mediated Mechanisms[1,2]

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Abstract

Bladder cancer presents as either low- or high-grade disease, each with distinct mutational profiles; however, both display prominent mTORC1 activation. One major negative regulator of mTORC1 is AMPK, which is a critical metabolic regulator that suppresses cellular growth in response to metabolic stress by negatively regulating mTORC1. Alterations in the activation and protein levels of AMPK have been reported in breast, gastric, and hepatocellular carcinoma. To investigate whether AMPK suppression is responsible for mTOR activation in bladder cancer, the levels of AMPKα were quantified in a cohort of primary human bladder cancers and adjacent nontumor tissues. The levels of p-AMPKα, AMPKα1, AMPKα2, and total AMPKα were significantly suppressed in both low- and high-grade disease when compared with nontumor tissue. To elucidate the AMPKα suppression mechanism, we focused on inflammation, particularly tumor-infiltrating macrophages, due to their reported role in regulating AMPK expression. Treatment of HTB2 cancer cells with varying doses of differentiated U937 macrophage conditioned medium (CM) demonstrated a dose-dependent reduction of AMPKα protein. Additionally, macrophage CM treatment of HTB2 and HT1376 bladder cells for various times also reduced AMPKα protein but not mRNA levels. Direct TNFα treatment also suppressed AMPKα at the protein but not RNA level. Finally, staining of the human cohort for CD68, a macrophage marker, revealed that CD68+ cell counts correlated with reduced AMPKα levels. In summary, these data demonstrate the potential role for inflammation and inflammatory cytokines in regulating the levels of AMPKα and promoting mTORC1 activation in bladder cancer.

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Introduction

Bladder cancer is currently the fifth most diagnosed cancer and the most expensive to treat due to the need for lifelong surveillance and invasive procedures [1]. Despite many advances in bladder cancer research, there is still a pressing need for new therapies for treating bladder cancer. Although bladder cancer can originate through two distinct pathways which give rise to either low- or high-grade disease, emerging research suggests that both may feed through a common pathway [2]. It has been observed in both low- and high-grade cancer that mammalian target of rapamycin complex 1 (mTORC1), which controls overall protein synthesis, is activated and that treatment with rapamycin, an mTOR inhibitor, reduces bladder cancer growth [3–7]. This suggests that mTOR

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is an important pathway for bladder tumor growth and that determining what mechanisms govern the activation of mTOR may aid in the development of better therapeutic regimens.

A major negative regulator of the mTOR pathway is adenosine monophosphate–activated protein kinase (AMPK). AMPK is a metabolic sensor in the cell activated by a high AMP:ATP ratio and low nutrient availability and signals to shut off anabolic processes such as protein synthesis in favor of catabolic processes such as fatty acid oxidation [8]. Due to its critical role in regulating protein and fatty acid synthesis, AMPK has been implicated as a therapeutic target for controlling cancer cell growth through suppression of mTOR function [9–13]. AMPK is a heterotrimeric protein composed of an α, β, and γ subunits [14]. The α subunit of AMPK consists of two isoforms, AMPKα1 and AMPKα2, which contain the kinase domain of the protein and the β and γ subunits which function as scaffold and regulatory subunits, respectively [15]. AMPK responds to cellular stresses such as low nutrients through changes in the AMP/ATP ratio and undergoes a conformational change allowing upstream kinases such as liver kinase B1 (LKB1) to phosphorylate the protein on threonine 172 [16–19]. When activated, AMPK functions to control the cell cycle process and apoptosis. In mouse models of tumorigenesis, the loss of AMPK in cancers has been implicated in the metabolic shift phenotype displayed during the Warburg effect [9,20,21]. In an myc-driven model of B-cell lymphoma, loss of AMPKα1 synergizes with myc to drive tumorigenesis [21]. Also, it has been demonstrated that AMPKα2−/− mouse embryonic fibroblasts transformed with H-RasV12–formed tumors in a xenograft model, whereas the AMPKα1−/− and wild-type control mouse embryonic fibroblasts did not, further demonstrating the potential for AMPKα to suppress tumorigenesis [22]. In a mouse model of bladder cancer, Shorning et al. demonstrated that loss of LKB1 (upstream kinase of AMPKα) and PTEN synergizes to activate AMPK and mTOR and that rapamycin treatment reduced tumor burden in mice [23]. All these data demonstrate the importance of AMPK signaling in tumorigenesis and how AMPK activation and/or its loss may impact tumor growth.

The phosphorylation status of AMPK has been reported to be downregulated in many cancers including hepatocellular carcinoma and breast cancer through immunohistochemical and/or Western blotting for phospho-AMPK^{Thr172} (p-AMPK^{Thr172}) [24,25]. Furthermore, AMPKα2 protein has been reported to be repressed in hepatocellular carcinoma, and breast and AMPKα2 mRNA has been reported to be suppressed in gastric cancer [26–28]. Although there have been reports of altered AMPK levels in cancer, the exact mechanisms governing AMPK suppression remain elusive. Also, despite the widespread attention that AMPK has received as far as its role as a potential antitumorigenic protein, little is known about the status and/or role for AMPK in bladder cancer. In this study, we sought to determine if the phosphorylation status and/or protein levels of AMPKα1 and AMPKα2 are altered in human bladder cancer and, if so, what are the mechanisms governing AMPK regulation.

Methods

Cell Lines and Reagents

Cell lines were purchased and maintained according to the American Type Culture Collection. Cells obtained from the American Type Culture Collection were frozen within 5 passages, and each stock was not cultured for more than 15 passages. HTB9 cells were cultured in RPMI + 10% fetal bovine serum (FBS), HTB5 and HT1376 cells were cultured in minimum essential medium + 10% FBS, and HTB2 and HTB4 cells were cultured in McCoy's + 10% FBS. Antibodies targeting p-AMPK and tAMPKα1/α2 were obtained from Cell Signaling (Beverly, MA), AMPKα1 and AMPKα2 were obtained from US Biologicals (Salem, MA), CD68 and pan-cytokeratin were obtained from DAKO (Carpinteria, CA), and β-actin was obtained from Abcam (Cambridge, MA). TNFα was obtained from Invitrogen (Carlsbad, CA). 4α-Phorbol 12-myristate 13-acetate (PMA) was obtained from Cayman Chemical (Ann Harbor, MI).

Immunohistochemistry

Paraffin sections were cleared of paraffin and rehydrated. Antigen retrieval was performed on paraffin sections only and following the endogenous peroxidase activity was quenched through treatment of slides in 3% hydrogen peroxide for 10 minutes. Sections were blocked in 3% bovine serum albumin (BSA)/PBS and incubated in primary antibody overnight. Species -specific biotinylated secondary antibody (1:500) was incubated on the sections for 1 hour followed by 30-minute incubation with ABC Elite reagent (Vector Labs, Burlingame, CA) according to the manufacturer recommendations. 3,3′-Diaminobenzidine was utilized at 1% to visualize staining followed by nuclear counterstain with methyl green (Vector Labs, Burlingame, CA).

Histoscore and Pathological Analysis of Tissue

Histoscore was assessed based on a combination of staining intensity and percent coverage. Staining intensity was scored based on a range of 0 to 3, where 0 represents no staining and 3 represents the strongest staining. Percent coverage was assessed by determining what percent of the tissue received each staining intensity score which results in a total range of 0 to 300. Histoscore is represented as the average of the score from three independent reviewers. Only tumor tissue and adjacent nontumor urothelial tissue verified by a pathologist were used for analysis. Additionally, a pathologist also verified bladder tumor grade as low or high grade. Images were taken on a Zeiss microscope with an Axiocam camera and Axiovision software. Each image taken represents 400× magnification.

U937 Conditioned Medium (CM) and Treatment

U937 cells were plated at a concentration of 4×10^6 in a 10-cm tissue culture dish and treated with 40 nM PMA for 24 hours. After 24 hours, the medium was aspirated, and cells were washed once in complete media followed by the addition of 10 ml of complete medium. After 24 hours, CM was collected and used to treat bladder cancer cells which were plated at a density of 8×10^5 in a 6-well dish for the indicated times and doses.

TNFα Treatment

HTB2 and HT1376 cells were plated at a concentration of 8×10^5 in a 6-well dish and treated the next day with 50 ng/ml of TNFα (Invitrogen, Carlsbad CA) for the indicated times.

SDS-PAGE and Immunoblot Analysis

Whole cell lysates were harvested at the indicated time points and analyzed by immunoblot as described previously [22]. Briefly, protein concentration was determined by using a Bio-Rad protein concentration assay (Bio-Rad, Hercules, CA), and lysates were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes.

Membranes were incubated with the desired primary antibody overnight diluted in 5% BSA/TBS-T. Species-specific horseradish peroxidase–conjugated secondary antibodies were incubated with the membranes for 1 hour diluted in 5%BSA/TBS-T. Immunoblots were visualized using ECL reagents (Millipore, Billerica, MA) and developed on a Kodak Multimodal Imager (2000MM).

Quantitative Reverse Transcriptase Polymerase Chain Reaction (PCR)

RNA isolation was performed using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer instructions, and cDNA synthesis was carried out using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer protocol. Primers for quantitative reverse transcriptase PCR are as follows: AMPKα1 forward (5′-AGGAGAGCTATTTGATTATATCTGTAAGAATG-3′), AMPKα1 reverse (5′-ACACCAGAAAGGATCTGTTGGAA-3′); AMPKα2 forward (5′-CGGCTCTTTCAGCAGATTCTGT-3′), AMPKα2 reverse (5′-ATCGGCTATCTTGGCATTCATG-3′); TNFα forward (5′-CCCATGTTGTAGCAAACCCTC-3′), TNFα reverse (5′-TATCTCTCAGCTCCACGCCA-3′); Cyclophilin A forward (5′-CTGGACCCAACACAAATGGTT-3′), Cyclophilin A reverse (5′-CCACAATATTCATGCCTTCTTTCA-3′). Quantitative PCR was then performed with SYBR green fluorescence on a Bio-Rad thermocycler with a MyIQ detection system.

Results

AMPK Activation Is Reduced in Human Bladder Cancer

Reduced AMPK activation has been demonstrated in multiple cancer types, and the known mTOR activation status suggested that AMPK may be inactive in bladder cancer as well. To test this hypothesis, we utilized a cohort of bladder tissue containing 16 adjacent nontumor and 44 tumor samples, of which 15 samples were patient matched. Of these 44 tumor samples, an oncology pathologist confirmed that 11 were low-grade tumors and the remaining 33 were high-grade tumors. The cohort contained tumors from 31 male patients and 13 female patients, representative of the 3:1 male to female ratio for bladder cancer (Table 1). The average age of the patients in the cohort was 68.5 years old (Table 1). AMPK activation in the tissues was performed by immunostaining tissue slides with antibodies for p-AMPK^{Thr172} and pan-cytokeratin to score p-AMPK only in the epithelial cell population in each tissue sample (Figure 1*A*). Adjacent nontumor tissue staining for p-AMPK^{Thr172} revealed that AMPK activation levels were significantly higher (65%) in adjacent nontumor tissue when compared with bladder cancer (Figure 1*B*). Patient-matched samples revealed that the same trend held true at 67% higher in the adjacent nontumor tissue when compared with the tumor samples (Figure 1*C*). Additionally, AMPK activation was significantly higher by 61% and 66% in adjacent nontumor urothelium when compared with low- and high-grade bladder cancer, respectively (Figure 1*D*). Overall, these data demonstrate that AMPK activation is being downregulated in bladder cancer when compared with adjacent nontumor tissue.

Table 1. Patient Demographics

	Total	Low Grade	High Grade
Number of patients	44	11	33
Sex			
Male	31	7	24
Female	13	4	9
Age (average)	68.5	60.3	70.9

AMPKα Isoform Levels Are Suppressed in Bladder Cancer

To understand whether the reduced AMPK activation in bladder cancer was due to limits in upstream activation events or a reduction in total AMPKα protein levels, immunohistochemical staining for total AMPK was performed on the same sample set. All 60 samples were stained with antibodies directed against tAMPKα1/α2 (Figure 2*A*). Additionally, pan-cytokeratin was used for epithelial cell identification. Immunohistochemistry revealed that tAMPKα1/α2 was highly expressed in adjacent nontumor bladder tissue as well as sporadic individual cells within the suburothelial mucosa. The expression of tAMPKα1/α2 was reduced by 43% ($P < .001$) in tumor tissue when compared with adjacent nontumor tissues (Figure 2*B*). In the patient-matched samples, tAMPKα1/α2 protein expression was reduced by 53% ($P < .001$) in tumor compared with nontumor urothelium (Figure 2*C*). Furthermore, tAMPKα1/α2 protein levels were statistically suppressed by 42% ($P < .001$) and 43% ($P < .001$) in both low- and high-grade cancers, respectively (Figure 2*D*). Taken together, these data demonstrate that the downregulation of p-AMPK in bladder cancer can be attributed in some part at least to a reduced amount of total AMPKα protein.

Because AMPKα2 protein is downregulated in breast cancer and hepatocellular carcinoma, we sought to determine if the downregulation of AMPKα is isoform specific. AMPKα1 and AMPKα2 were both highly expressed in adjacent nontumor urothelium (Figure 2*A*). AMPKα1 expression was significantly decreased by 39% in tumor tissue when compared with nontumor (Figure 2*E*). In the patient-matched samples, AMPKα1 expression was significantly reduced by 51% (Figure 2*F*) in bladder tumors and was statistically downregulated by 27% and 43% in low- and high-grade cancers, respectively (Figure 2*G*). In addition to the AMPKα1 isoform reduction observed in the bladder cancers, AMPKα2 protein expression was also significantly reduced by 65% in tumor tissue compared with adjacent nontumor (Figure 2*H*). When the patient-matched cohort was evaluated independently, the AMPKα2 protein expression was significantly suppressed by 68% compared with nontumor (Figure 2*I*) Furthermore, AMPKα2 protein levels were also significantly reduced by 57% and 67% in low- and high-grade cancers, respectively (Figure 2*J*).

To determine if the downregulation of either or both AMPKα1 and AMPKα2 was occurring at the mRNA or the protein level, we evaluated expression within The Cancer Genome Atlas (TCGA) database. Analysis of adjacent nontumor and bladder tumor specimens revealed that AMPKα1 was not regulated at the RNA level in bladder cancer but AMPKα2 did demonstrate reduced mRNA in tumors (Supplemental Figure 1). Together, these data demonstrate that both the AMPKα1 and AMPKα2 catalytic isoforms are significantly suppressed in bladder cancer when compared with adjacent nontumor and that this suppression occurs in both low- and high-grade disease.

Macrophage CM Suppresses AMPKα Isoforms

The observation that both AMPKα1 and AMPKα2 are suppressed in bladder cancer suggests the possibility of a mechanism that may be selective to the bladder because it is uncommon to observe AMPKα1 suppression in tumors. One potential mechanism might be the contribution of chronic or acute local inflammation in bladder that may promote the tumorigenic process through AMPKα suppression.

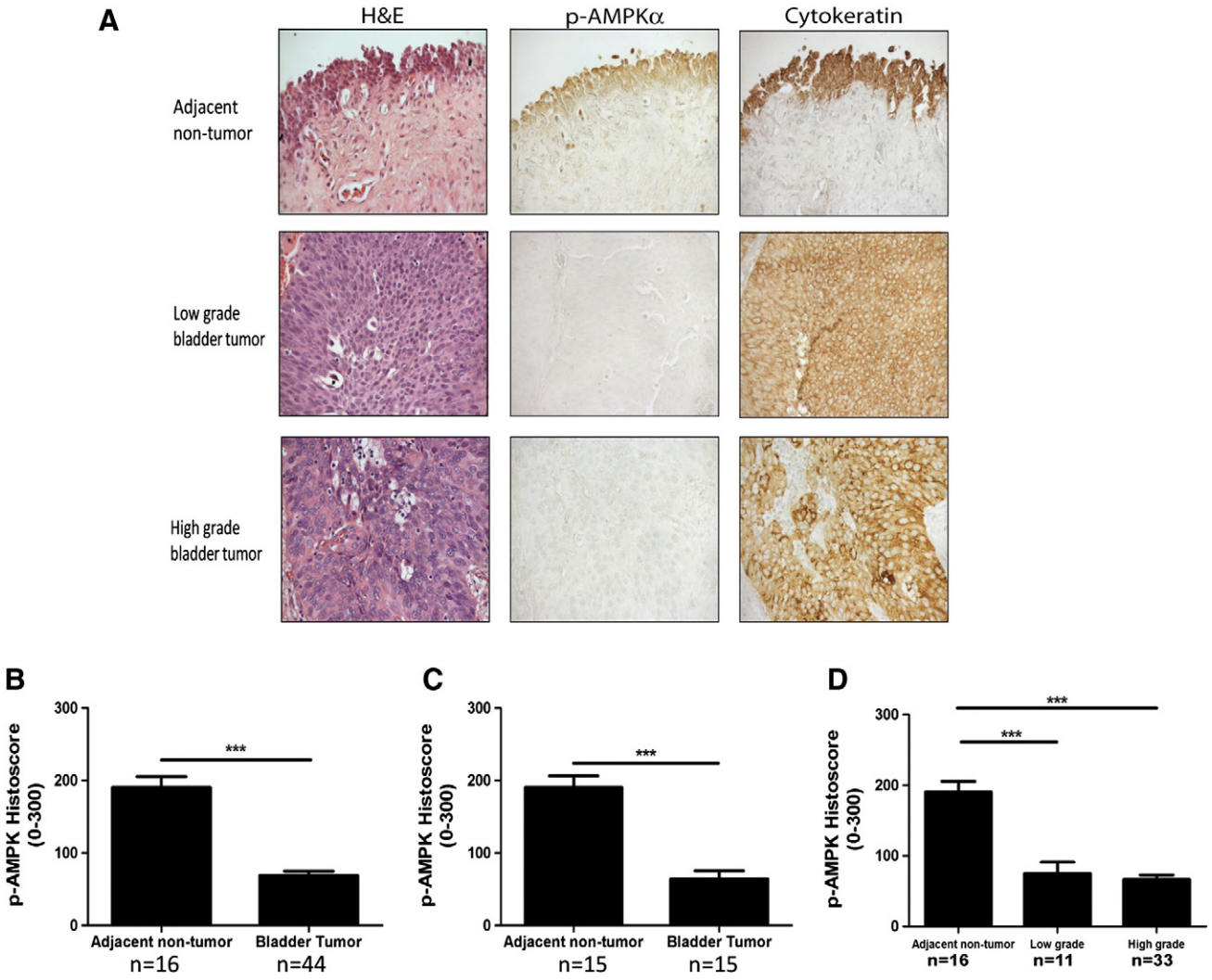

Figure 1. AMPK activation is reduced in human bladder cancer (A) p-AMPK staining in a cohort of 16 adjacent nontumor and 44 tumor samples. Of these 60 samples, 15 were patient matched. Representative 40× images of adjacent nontumor and tumor stained with hematoxylin and eosin (H&E) and immunohistochemical analysis of p-AMPK and cytokeratin. (B) Histoscore quantification of p-AMPK in adjacent nontumor and tumor tissue. (C) Histoscore quantification of patient-matched samples. (D) Quantification stratified by low- and high-grade bladder cancer.

In fact, there have been several reports indicating that inflammation may regulate AMPK protein levels in other systems [29–32]. To test this possibility, human monocytic cells (U937) were differentiated to become macrophage-like cells. CM from these cells was utilized as a model to test the effects of macrophage secretions on bladder cancer. Differentiated U937 cells upregulate cell surface markers such as CD11b, CD14, and CD68, which are similar to cell surface markers of macrophages in bladder cancer and thus are a relevant model of this disease [33–36]. Human HTB2 bladder cancer cells, which express both the AMPKα1 and AMPKα2 isoforms, were treated with varying doses of the macrophage CM starting at a dose of 1 part CM to 1 part complete culture media down to 1 part in 31. The cells were harvested after 24 hours and analyzed by immunoblot for tAMPKα1/α2, AMPKα1, and AMPKα2 levels (Figure 3*A*). CM treatments resulted in a dose-dependent downregulation of tAMPKα1/α2, as well as AMPKα1 and AMPKα2 protein levels, with dilutions greater than 1 part in 15 having no effect. To determine if this phenomenon was broadly applicable to multiple bladder cancer cell lines, HTB2, HTB4, HTB5, HTB9, and HT1376 were treated with a 1:1 dilution for 24 hours. The levels of p-AMPK^{Thr172} and tAMPKα1/α2 were assessed in the control and treated cell extracts (Figure 3*B*). The macrophage CM reduced the levels of phosphorylated AMPKα and total AMPKα in HTB2, HTB5, and HT1376 but did not have a dramatic impact on either the HTB5 or HTB9 cells. To investigate further the dynamics of when AMPK protein levels are reduced in response to macrophage CMs, a time course using the 1:1 dilution of CM was performed on HTB2 and HT1376 bladder cancer cells (Figure 3, *C* and *D*). Neither of the HTB2 and HT1376 cells responded with a reduction in AMPKα protein levels at the 8- and 16-hour time points; however, both cells displayed a marked reduction in tAMPKα1/α2, AMPKα1, and AMPKα2 at the 24-hour time point. Interestingly, neither the HT1376 nor the HTB2 cells demonstrated any change in AMPKα1 or AMPKα2 mRNA levels, suggesting that the regulation of AMPK is occurring at the posttranscriptional level (Figure 3, *E* and *F*). In fact, the AMPKα2 mRNA levels were moderately induced in the HT1376 cells and significantly induced at the 8- and 24-hour periods in the HTB2 cells.

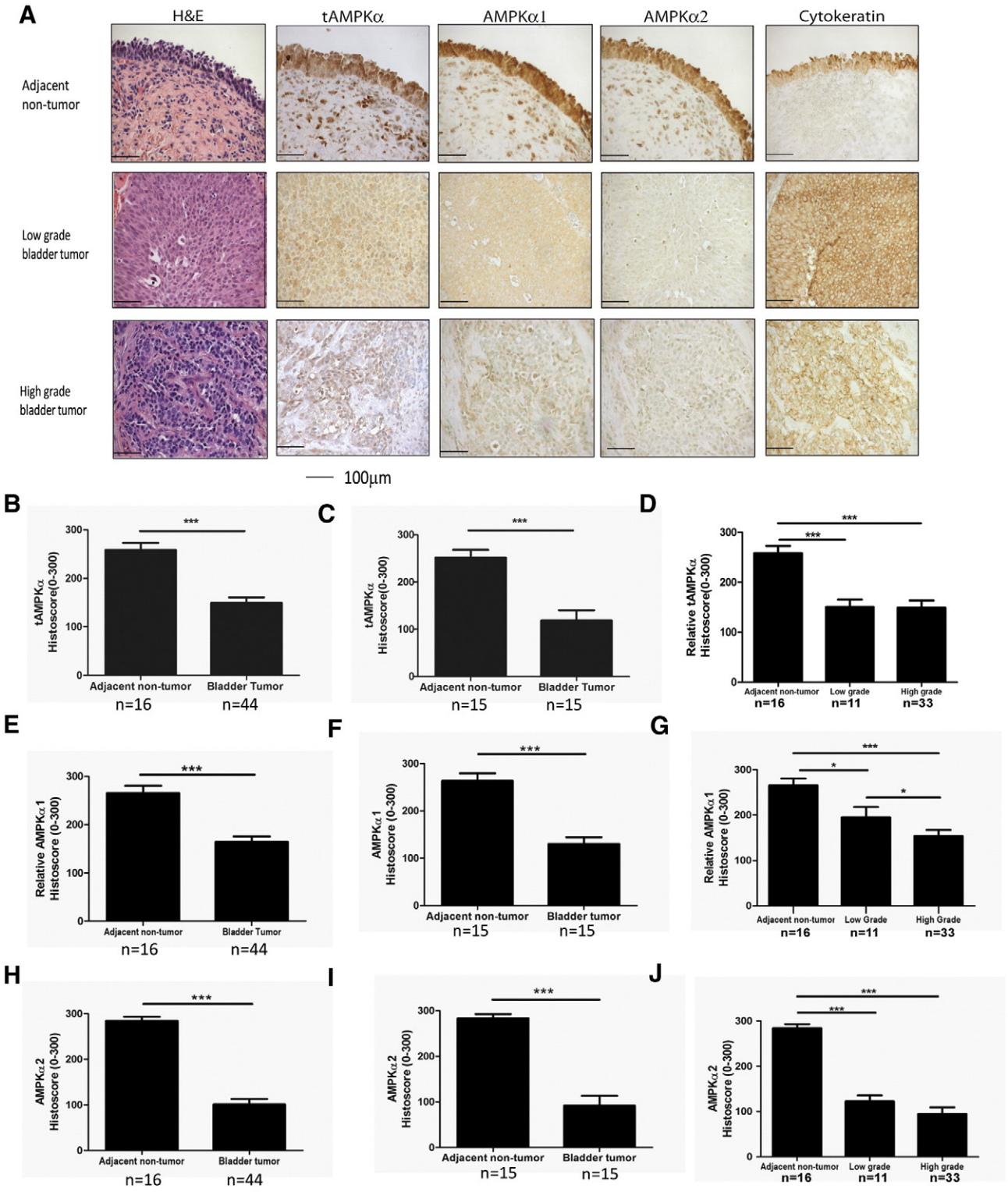

Figure 2. AMPKα isoforms are suppressed in human bladder cancer. (A) Representative 40× images of adjacent nontumor and low- and high-grade bladders tumors stained for AMPKα1, AMPKα2, AMPKα1/α2, and cytokeratin. (B) Quantification of AMPKα1 in adjacent nontumor and bladder tumor samples. (C) Quantification of AMPKα2 in adjacent nontumor and bladder tumor. (D) Quantification of tAMPKα1/α2 in adjacent nontumor and tumor samples. (E) Quantification of AMPKα1 in patient-matched samples. (F) Quantification of AMPKα2 in patient-matched samples. (G) Quantification of tAMPKα1/α2 in patient-matched samples. (H) Quantification of AMPKα1 stratified by low and high grade. (I) Quantification of AMPKα2 stratified by low and high grade. (J) Quantification of tAMPKα1/α2 stratified by low and high grade. Statistical significance indicated as $*P < .05$, $**P < .01$, and $***P < .001$.

To determine if AMPK suppression by macrophage CM requires NF-κb activation as expected, HTB2 cells were pretreated with the NF-κb inhibitor Bay 11-7085 for 1 hour and then stimulated with CM in the presence of the inhibitor for 24 hours (Figure 3*G*). Chemical inhibition of NF-κb abolished the reduction in AMPKα levels induced by U937 macrophage CM, suggesting that this effect

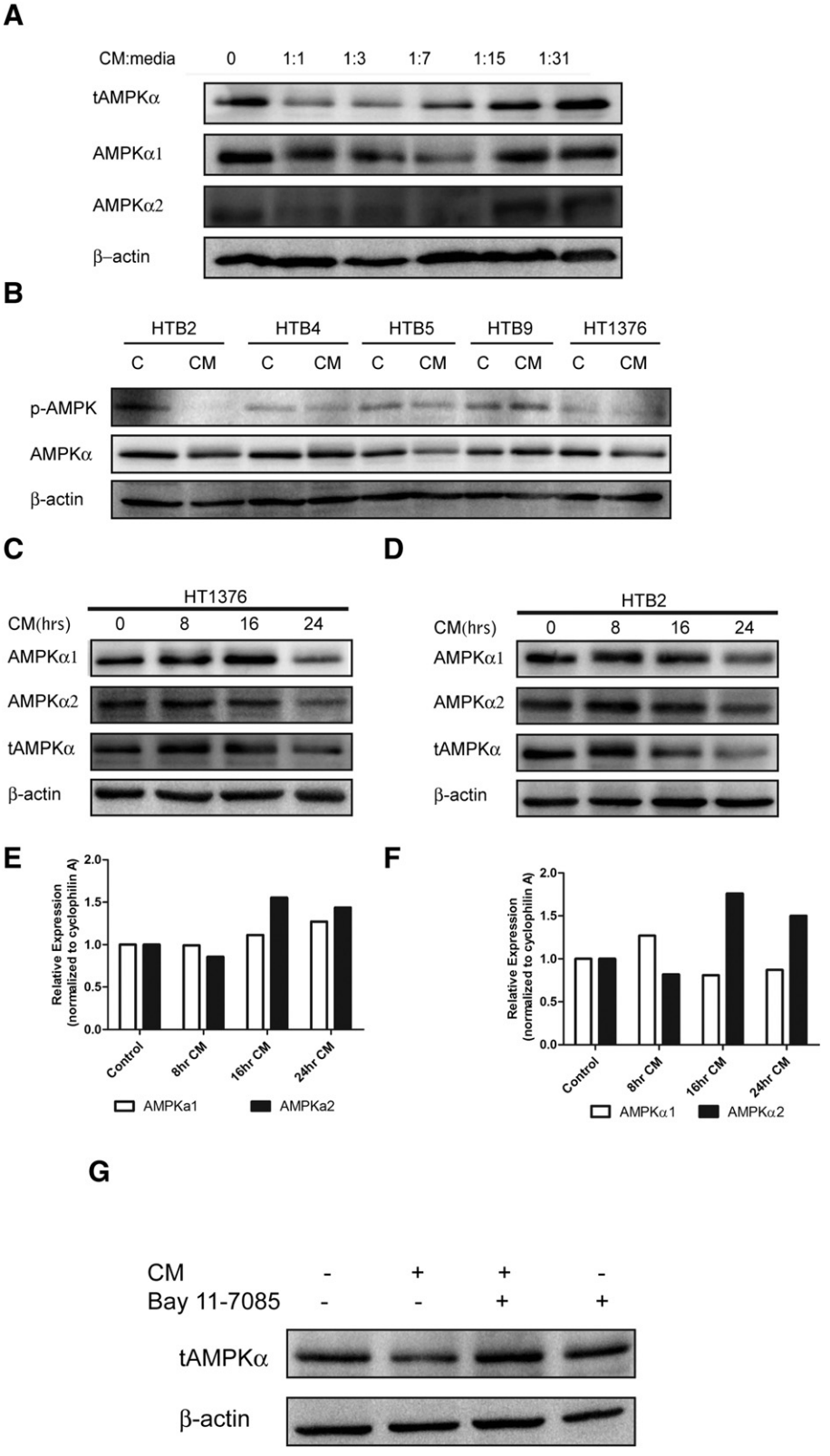

Figure 3. (A) HTB2 cells were treated with the indicated dose of U937 CM for 24 hours and assessed for tAMPKα1/α2, AMPKα1, AMPKα2, and β-actin by immunoblot. (B) HTB2, HTB4, HTB5, HTB9, and HT1376 were treated with 1:1 CM for 24 hours and assessed by immunoblot for p-AMPK, tAMPKα1/α2, and β-actin as a loading control. (C) HT1376 cells were treated with a 1:1 dose of CM for the indicated times and assessed by immunoblot for tAMPKα1/α2, AMPKα1, AMPKα2, and β-actin. (D) HTB2 cells were treated with a 1:1 dose of CM from U937 cells for the indicated times and assessed by immunoblot for tAMPKα1/α2, AMPKα1, AMPKα2, and β-actin. (E) HT1376 cells were treated with a 1:1 dose of CM for the indicated times and assessed for AMPKα1 and AMPKα2 mRNA levels. (F) HTB2 cells were treated with a 1:1 dose of CM for the indicated times and assessed for AMPKα1 and AMPKα2mRNA levels. (G) HTB2 cells were pretreated with 10 μM Bay 11-7085 and then treated with 1:1 CM for 24 hours. Lysates were assessed by immunoblot for tAMPKα1/α2 and β-actin as a loading control.

requires at least initial NF-κb activation. The extended time frame for AMPK suppression however suggests possible secondary events or an indirect mechanism of suppression. Overall, these data suggest that inflammatory mediators in the macrophage CM may signal to suppress AMPKα at the protein level and that this effect is dependent upon NF-κb activation.

TNFα Causes a Reduction of AMPKα Protein Levels in Bladder Cancer

To investigate exactly what may be causing the suppression of AMPKα when treated with CM, we tested TNFα because it is one of the major constituents of differentiated U937 CM and a common inflammatory cytokine present in proinflammatory conditions [37]. HTB2 and HT1376 cells were treated with TNFα over the course of 24 hours, and tAMPKα1/α2, AMPKα1, and AMPKα2 were analyzed by immunoblot (Figure 4, *A* and *B*). TNFα treatment resulted in a reduction in the total and both the AMPKα1 and AMPKα2 isoforms at 16 hours in the HTB2 and HT1376 bladder cancer cell lines. Interestingly, AMPKα1 was not as sensitive to TNFα treatment as AMPKα2 or the tAMPK. Consistent with the CM experiments, neither the HT1376 nor HTB2 cells displayed any regulation of the AMPKα1 or AMPKα2 isoforms at the mRNA level (Figure 4, *C* and *D*); however, there was some trending induction of AMPKα2 mRNA that was not significant. Together, these data demonstrate that TNFα may be one effector cytokine that is capable of suppressing AMPKα at the protein level but has little to no effect on the mRNA levels, further implicating a posttranscriptional or more likely a posttranslational mechanism of AMPKα regulation.

Macrophage Infiltration Is Higher in Areas of Low AMPKα in Human Bladder Cancer

Because it was clear that experimental inflammatory stimuli could affect AMPK expression in human bladder cancer cell lines, it was necessary to determine whether this actually occurs in primary human bladder samples. To this end, we proposed that proinflammatory macrophage and TNFα expression in human bladder cancers could affect the tumor microenvironment such that AMPKα protein expression is suppressed. To investigate this possibility, we cored samples that had been previously stained for AMPKα1 and AMPKα2. RNA was isolated from the samples, and TNFα mRNA was assessed. The RNA levels of TNFα were then correlated with the AMPKα1 or AMPKα2 immunostaining histoscores in the same samples to determine if there was any correlation between TNFα expression and AMPKα expression. This revealed that there was a significant negative correlation between AMPKα1 protein expression and TNFα mRNA expression (Figure 5*A*) ($P < .01$); however, there

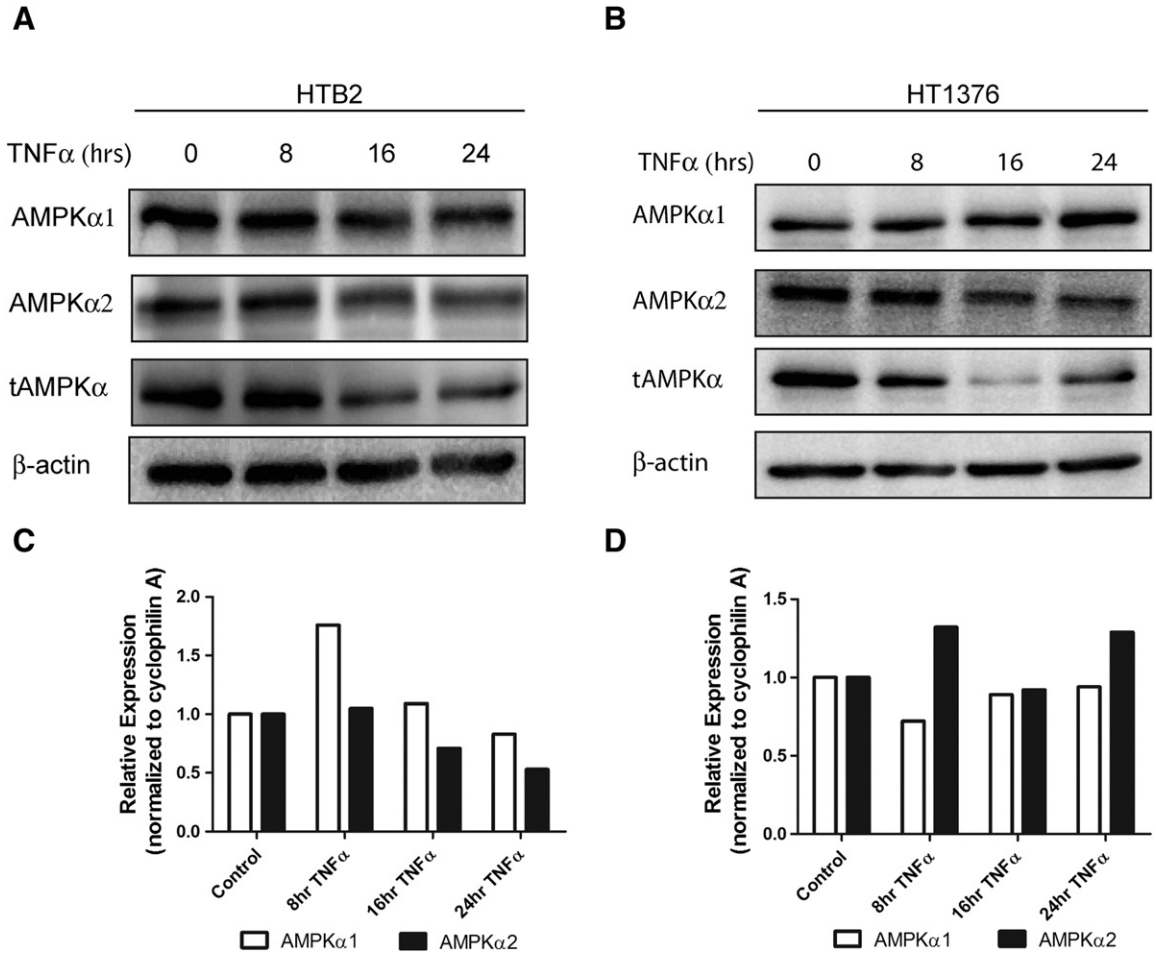

Figure 4. (A) HTB2 bladder cancer cells were treated with 50 ng/ml of TNF-α for the indicated times and assessed for tAMPKα1/α2, AMPKα1, AMPKα2, and β-actin by immunoblot. (B) HT1376 cells were treated with 50 ng/ml of TNF-α for the indicated times and assessed by immunoblot for tAMPKα1/α2, AMPKα1, AMPKα2, and β-actin. (C) HTB2 cells were treated with 50 ng/ml of TNF-α for the indicated times and assessed for AMPKα1 and AMPKα2 mRNA levels. (D) HT1376 cells were treated with 50 ng/ml of TNF-α for the indicated times and assessed for AMPKα1 and AMPKα2 mRNA levels.

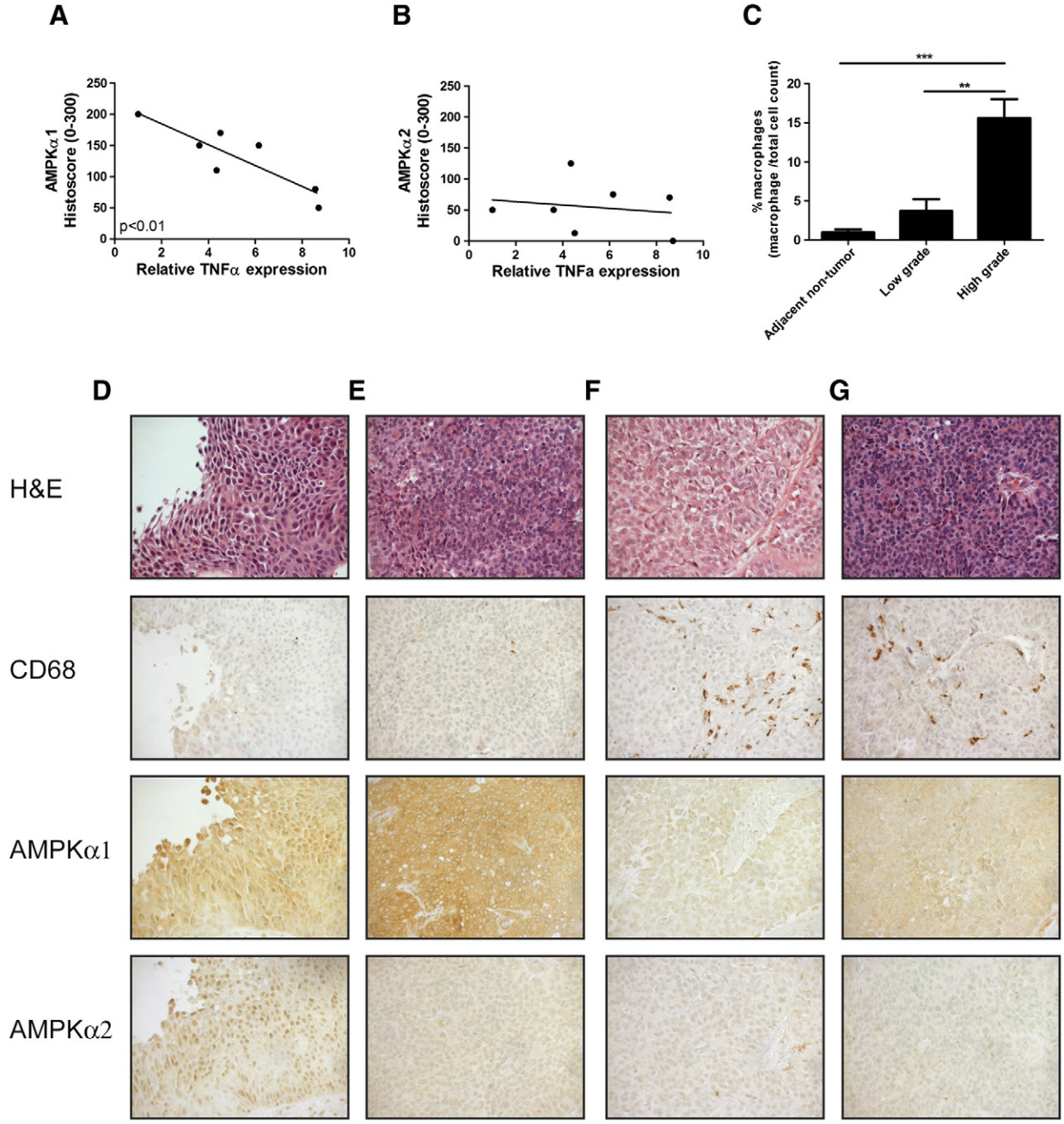

Figure 5. AMPKα1 levels are inversely correlated with TNFα and macrophage number in bladder cancer. (A) Correlation of AMPKα1 histoscore and TNFα mRNA expression. (B) Correlation of AMPKα2 histoscore and TNFα mRNA expression. (C) Graph representing the percent macrophage count per tumor in adjacent nontumor tissue, low-grade bladder cancer, and high-grade bladder cancer. (D and E) Representative 40× images of H&E, CD68, AMPKα1, and AMPKα2 staining in areas of high AMPKα1 expression. (F and G) Representative 40× images of H&E, CD68, AMPKα1, and AMPKα2 staining in areas of low AMPKα1 expression.

was no such correlation observed for AMPKα2 protein (Figure 5*B*). This could be due to the fact that AMPKα2 can be suppressed at the mRNA level by an alternative mechanism as suggested by the TCGA data. Next, the human bladder cancer cohort and nontumor samples were stained for CD68, a macrophage marker. Not surprisingly, there was a significant influx of macrophages in bladder cancer tissue when compared with the adjacent nontumor tissue. This influx followed a stepwise gradient where low-grade bladder cancers showed more macrophages compared with adjacent nontumor, and this was further increased in high-grade bladder cancers (Figure 5*C*).

To determine if the macrophage count was related to total AMPK levels, the macrophage count in each sample was compared with the relative AMPKα1 and AMPKα2 expression in the same tumor. Representative images illustrating CD68 expression and the levels of AMPKα1 and AMPKα2 in the same region show that areas of low CD68 expression have high AMPKα1 (Figure 5, *D* and *E*), whereas areas of high CD68 tend to have lower AMPKα1 protein expression (Figure 5, *F* and *G*). Consistent with the lack of correlation between AMPKα2 protein and TNFα mRNA expression, there was no correlation between CD68 and AMPKα2. Overall, these data

demonstrate a correlation between AMPKα1 suppression and macrophage count as well as TNFα mRNA expression in human bladder cancer. In addition, these data also demonstrate that macrophages may be contributing to the inflammatory microenvironment in bladder tumors which results in reduced AMPKα protein.

Discussion

A number of studies have determined that human cancers have reduced AMPK activity and/or protein expression levels [24–28]. Because AMPK is a central metabolic regulator that can affect the central anabolic and proliferative pathways in cells, loss of activity may facilitate tumor progression under conditions of nutrient restriction. AMPK functions as a heterotrimeric protein to control processes such as fatty acid synthesis and protein synthesis through regulating ACC activation and reducing mTOR activation [14,16,38,39]. Bladder cancer presents as either low- or high-grade disease, each with its own specific mutational profile; however, many studies have reported that mTOR is activated in both grades [3–5,40–42]. Because AMPKα activation and/or protein levels have been reported to be altered in other cancer types, we examined the level of activated AMPKα in primary human bladder cancer samples. We found that AMPKα activation is indeed suppressed in cancer compared with adjacent nontumor urothelium, and this occurred in patient-matched urothelium as well. Diminished AMPK function would explain the mTOR activation observed in bladder cancer because AMPK is the major negative regulator of the pathway. One possibility for reduced AMPK activation is that LKB1, a major upstream kinase activator of AMPK, is mutated in bladder cancers as it has been reported in lung cancer; however, this seems unlikely because the mutation rate of LKB1 is very low in bladder cancer [43,44]. In addition, it has been reported that the AMPKα2 isoform is selectively suppressed in breast cancer, whereas the AMPKα1 isoform is not [26]. Thus, because active AMPKα is reduced in bladder cancers, we sought to determine whether the AMPKα1 and/or AMPKα2 protein levels were altered and by what mechanism. Surprisingly, both AMPKα1 and AMPKα2 protein levels were reduced in bladder cancer when compared with adjacent nontumor urothelium, and this occurred in both low- and high-grade diseases. This suppression was also observed in the patient-matched samples, verifying that this was indeed a tumor-dependent reduction, not a global field effect selective to different individuals. The loss of AMPKα2 has been reported in many other cancers such as breast, hepatocellular carcinoma, and gastric cancer; however, in these cancers, no modulation of AMPKα1 was reported [26–28]. AMPKα1 and AMPKα2 are located on different chromosomes but function to perform many of the same critical roles in the cell and can compensate for each other's function [45]. Our study is the first to report the suppression of both AMPKα1 and AMPKα2 in cancer, and this could provide a rationale for why mTOR is upregulated in bladder cancer. Also, the loss of both isoforms would more severely abolish the activity of the protein than just the loss of AMPKα2 due to the ability of AMPKα1 and AMPKα2 to compensate for each other. Therefore, the loss of both isoforms of AMPKα could facilitate a permissive microenvironment for tumor growth and progression.

To better understand what mechanisms may lead to the suppression of AMPKα in bladder cancer, we focused on the role of inflammation due to the numerous reports suggesting that inflammation, particularly infiltrating macrophages, may cause suppression of AMPKα through posttranslational mechanisms in other disease and tissue types [29–32]. To assess the impact of macrophages on AMPKα, U937 cells which were differentiated with PMA to become macrophage-like cells were utilized because they express similar cell surface markers as the macrophages present in bladder cancer [33,36,46]. Treatment of human bladder cell lines with CM from U937 macrophages elicited downregulation of AMPKα1, AMPKα2, and the total AMPKα1/α2 at the protein level but not at the mRNA level. The U937 CM effect was both dose dependent and time dependent, indicating that some factor or combination of factors in the CM was causing the downregulation of AMPKα. CM-treated cells were treated with an NF-κb inhibitor, and this abolished the suppression of AMPKα, indicating that the CM effect was mediated through NF-κb. U937 cells that are differentiated with PMA represent a suitable model to study inflammatory effects in bladder cancer but also have the capacity to be further skewed to an M1 macrophage or an M2 macrophage through further stimulation with the appropriate cytokines after PMA differentiation. It would be of interest to determine if the suppression of AMPKα in bladder cancer is due to M1 macrophages or M2 macrophages because U937 cells do not clearly represent a model of either subtype.

Although U937 cells that are differentiated to be macrophage-like do not represent an M1 or an M2 phenotype, they do highly upregulate TNFα along with other proinflammatory cytokines upon PMA stimulation, which suggests that they may represent more of an M1 phenotype than an M2 phenotype [37]. Because TNFα is one of the major cytokines secreted by U937 cells into the CM, we sought to determine if it has any effect on the levels of AMPKα protein. Treatment of both bladder cancer cell lines with TNFα resulted in a downregulation of AMPKα1 and AMPKα2 protein and not mRNA; however, the reduction was not quite as potent as the CM, suggesting that other cytokines secreted from macrophages may work in combination with TNFα to cause the suppression of AMPKα in bladder cancer. To this extent, it is important to further determine what other cytokines are upregulated in response to PMA stimulation in U937 cells and determine the impact they have on AMPKα protein levels in bladder cancer cells. Furthermore, it is important to assess if the suppression of AMPKα can be neutralized by treatment of CM with a TNFα neutralizing antibody to determine if this could be a clinically relevant mechanism for treating bladder cancer through the restoration of AMPKα activity. This information would provide valuable information in determining if the suppression of AMPKα is a TNFα-specific signaling event or a broader signaling event which is dependent on NF-κb activation as this may aid in further treating bladder cancer.

Given the implication that inflammation and macrophage-derived cytokines may be responsible or contributing to suppression of AMPKα in bladder cancer, we evaluated the correlation of TNFα or tumor-associated CD68+ macrophage and total AMPK levels in primary tumors. There was a negative correlation between AMPKα1 protein and TNFα mRNA, further suggesting that TNFα may regulate AMPKα1 protein expression. Additionally, there was a negative correlation between AMPKα1 and macrophage count, suggesting that high tumor macrophage counts correspond to lower AMPKα1 expression. Although there was a negative correlation between AMPKα1 protein and TNFα mRNA as well as macrophage count, there was no correlation between AMPKα2 protein expression and TNFα mRNA or macrophage count due to the already low levels of AMPKα2 mRNA and protein in bladder cancer possibly due to methylation of the AMPKα2 promoter which has been reported in hepatocellular carcinoma [28].

The observation that AMPKα is suppressed at the protein level suggests that induced protein degradation may be at work. There have been several reports of E3 ubiquitin ligases that are capable of targeting AMPK which could explain why AMPKα levels are lower in bladder cancer [47–49]. For instance, it has been recently reported that MAGE-A3/6 is a cancer-specific ubiquitin ligase which gets upregulated in cancer and targets AMPK for degradation [48]. Additionally, WWP1 is another protein which is reported to target AMPKα2 for degradation [47]. Although we cannot discount the potential involvement of these proteins in regulating AMPKα protein levels in bladder cancer, they are not induced by inflammatory pathways and are therefore less likely candidates for the mechanism we observe downstream of TNFα and NF-κb activation. However, Ko et al. have reported that, in cardiac tissue, a high-fat diet induces the influx of macrophages into the tissue and results in a reduction in the activation and total protein levels of AMPK. They also demonstrated that the cytokine IL-6 is responsible for this reduction by inducing expression of SOCS3 which in turn can bind to AMPK and target it for degradation [32]. Therefore, it is plausible that SOCS3 could be partially responsible for the reduction in AMPKα protein levels in bladder cancer especially because it has been shown to be induced by inflammatory stimuli, but more research is warranted to determine if this is indeed the case in bladder cancer.

Overall, these data demonstrate that AMPKα activation is reduced in bladder cancer and that this is due to a reduction in the amount of both AMPKα1 and AMPKα2 protein. Although it has been reported previously that AMPKα2 is reduced in breast cancer and hepatocellular carcinoma, this is the first report in which AMPKα1 protein has been demonstrated to be suppressed in cancer. Additionally, these data represent a novel observation that AMPKα1 suppression is mediated though proinflammatory events in cancer, particularly tumor-infiltrating macrophage and TNFα. These data also expand the relevance of AMPKα not only as a critical metabolic regulator of cell proliferation but also as a novel target of prolonged inflammation and how this dynamic relationship can alter mTOR activation to create a permissive environment for tumor growth. Given the function of AMPK as a tumor suppressor-like protein, its reduced expression in bladder cancer likely contributes to enhance tumor growth, demonstrated by mTOR activity in low- and high-grade disease, and a mechanism of tissue response to acute and/or chronic inflammation.

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