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Original Article

Phosphorylated AMP-activated protein kinase expression is significantly associated with poor clinical outcomes in bladder carcinoma patients

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Abstract: The phenotype of p-AMPK has been suggested as a possible marker for diagnosis and/or prognosis in tumors located in different organs. Nonetheless, there are conflicts among the outcomes found in several tumors, and little is proven concerning the correlation between the phenotype of p-AMPK and its clinical significance in urinary bladder carcinomas. Therefore, this research will define the p-AMPK expression patterns, and study the relationship between this pattern of expression, in a panel of urinary bladder carcinomas compared to normal tissues, and clinicopathological features to determine the clinical relevance and the function of p-AMPK in the evolution of bladder cancer. Furthermore, this study will evaluate p-AMPK expression as a diagnostic marker and prognosticator of long term overall survival in bladder cancer patients. This study will utilize the p-AMPK monoclonal antibody using the immunohistochemistry staining standard protocol to identify the location and expression pattern of p-AMPK, which will be graded with respect to the estimated percentage of tumor cells with positive and relative intense stain. 128 cases of urinary bladder carcinoma and 24 non-cancerous bladder tissue samples were employed for the determination of p-AMPK phenotypes applying immunohistochemical staining on tissue microarrays slides. A high score of nuclear p-AMPK immunoexpression has been found in 104 (81.3%) bladder cancer cases, while 24 (100%) control cases showed p-AMPK immunoreactivity. Strong p-AMPK immunohistochemical staining in both epithelial cells and stromal cells has been significantly linked with vascular invasion (p-value = 0.002 and p-value = 0.011 respectively). Lymph node metastasis showed significant association with p-AMPK expression in tumor epithelial cells (p-value = 0.030). The odds of low expression in epithelial cells for a positive lymph node are 3.21 times as great as the odds of low expression in epithelial cells for a negative lymph node. Our findings recommend p-AMPK as a useful biomarker in determining the prognosis of bladder cancer. These preliminary findings suggest that p-AMPK may be a valuable tissue biomarker for predicting a poor prognosis in bladder cancer.

Keywords: p-AMPK, phosphorylated AMP-activated protein kinase, urinary bladder cancer, immunohistochemistry

Introduction

Urinary bladder malignancies are devastating diseases and are major sources of mortality universally [1]. As reported by recent published information, bladder cancer is the sixth most common among the most prevalent tumors with almost 79,000 newly reported cases and more than 15,000 deaths from bladder cancer in the United States of America [1]. Despite substantial advances in neoplasm therapy, bladder malignancy continues to pose a giant challenge to doctors due to its huge relapse rate. For example, about 70% of newly registered bladder cancers recur within 5 years, along with a high chance to progress to muscle infiltrative and metastatic tumors [2, 3]. The type of bladder cancer therapy differs extremely based on some clinical issues such as stage and grade of tumor as signals of mild or terrible prognosis. Nevertheless, these clinicopathological factors are not sufficient to assess the disease aftereffects and worse still, they cause notable conflicts within alike grades or stages. It is mainly due to neoplastic cells' heterogeneity [4]. So, it is an essential requisite to obtain

Table 1. The distribution of various clinicopathological variables with p-AMPK immunostaining in urinary bladder cancer

		p-AMPK expression in epithelial cells					p-AMPK expression in stromal cells				
		Low		High			Low		High		
		n	%	n	%	*P*-Value	n	%	n	%	*P*-value
Tissues	Normal Bladder Tissues	0	0.0%	24	100.0%	0.015[a]	0	0.0%	24	100.0%	0.003[b]
	Urinary Bladder Carcinoma	24	18.8%	104	81.3%		36	28.1%	92	71.9%	
Age at Diagnosis (Years)	<50	2	12.5%	14	87.5%	0.425[a]	4	25.0%	12	75.0%	0.448[a]
	50-60	7	16.7%	35	83.3%		10	23.8%	32	76.2%	
	>60	15	21.4%	55	78.6%		22	31.4%	48	68.6%	
Muscular Invasion	MIBC	19	29.7%	45	70.3%	0.002[a]	25	39.1%	39	60.9%	0.019[b]
	NMIBC	2	4.3%	44	95.7%		7	15.2%	39	84.8%	
	Undecided	3	16.7%	15	83.3%		4	22.2%	14	77.8%	
Histotype of Cancer	Squamous	1	16.7%	5	83.3%	0.323[a]	3	50.0%	3	50.0%	0.371[a]
	Transitional	17	16.8%	84	83.2%		26	25.7%	75	74.3%	
	Transitional/CIS	2	50.0%	2	50.0%		2	50.0%	2	50.0%	
	Transitional/Squamous	4	23.5%	13	76.5%		5	29.4%	12	70.6%	
Stage	0a	1	5.6%	17	94.4%	0.058[a]	3	16.7%	15	83.3%	0.334[a]
	0is	0	0.0%	3	100.0%		0	0.0%	3	100.0%	
	I	2	6.5%	29	93.5%		6	19.4%	25	80.6%	
	II	9	25.0%	27	75.0%		14	38.9%	22	61.1%	
	III	3	42.9%	4	57.1%		3	42.9%	4	57.1%	
	IV	7	30.4%	16	69.6%		8	34.8%	15	65.2%	
	Undecided	2	20.0%	8	80.0%		2	20.0%	8	80.0%	
Grade	High Grade	18	27.3%	48	72.7%	0.023[a]	24	36.4%	42	63.6%	0.041[a]
	Low Grade	4	8.0%	46	92.0%		8	16.0%	42	84.0%	
	NA	2	16.7%	10	83.3%		4	33.3%	8	66.7%	
Lymph Node	Negative	16	15.1%	90	84.9%	0.030[a]	27	25.5%	79	74.5%	0.143[b]
	Positive	8	36.4%	14	63.6%		9	40.9%	13	59.1%	
Vascular Invasion	Negative	15	13.9%	94	86.1%	0.002[a]	26	24.1%	83	75.9%	0.010[b]
	Positive	9	47.4%	10	52.6%		10	52.6%	9	47.4%	
Deceased	Alive	14	15.9%	76	84.1%	0.154	24	26.1%	66	73.9%	0.572[b]
	Dead	10	26.3%	28	73.7%		12	31.6%	26	68.4%	

% are within row percentages, [a]Fisher's Exact two-sided *p*-value, [b]Chi-Square two-sided *p*-value.

an innovative probing and helpful modalities to satisfy doctors' needs for the management of bladder malignancy. Excessive efforts have been made to discover novel biomarkers to facilitate the tumor diagnostic process, improve case stratification, and manage tumors [5, 6]. Up to now these biomarkers were inefficient, so this necessitates the finding of other probing tools which could be more sensitive and specific to be better predictive of clinical outcomes.

A complex enzyme 5' AMP-activated protein kinase (AMPK) is composed of 3 subunits. Alpha is a catalyst, β and γ are regulative ones. There are 12 likely isoforms of AMPK in humans since every single subunit is encrypted by two or three different genes. AMPK is a cellular sensor of energy. AMPK activation is switched on by adding the phosphate group; this activity is initiated by several kinase enzymes, for example CaMKK and LKB1, as a consequence of metabolic stress that enhances ATP depletion (contraction of muscle) or prevents ATP assembly (glucose deficiency, ischemia and hypoxia) and so increases the AMP: ATP ratio [7-9]. It is known that AMP-activated protein kinase inhibits mostly all anabolic metabolic activities which help cells proliferate such as fatty acid, phospholipid and protein production [9, 10]. Consequently, it is anticipated that this kinase inhibits tumor growth and development. AMPK controls cellular proliferation and assesses the checkpoints of the cell cycle of tumor cells after energy resources depletion. Once motivated, phosphorylated AMP-activated protein kinase (p-AMPK) controls energy-diminishing processes such as cellular proliferation and stimulates

energy-producing catabolic pathways such as the oxidation of fatty acid, glucose uptake, and glycolysis [9-12]. Other anti-tumor powers of AMPK might engage growing DNA repair and autophagy following ultraviolet damage [13]. This view was supported and confirmed by some studies which showed that AMP-activated protein kinase activates phosphorylate p21 [14] and p53 [15] and consequently distracts the cell cycle and promotes cell survival. AMPK restrains mTOR (mammalian target of rapamycin) and reduces the synthesis of proteins. Moreover, phosphorylated AMPK, in neoplastic cells, inhibits the enzymes of lipogenesis, such as acetyl CoA carboxylase (ACC) and fatty acid synthase (FA). These enzymes were found in tumors as a result of rising demands for fatty acids to be incorporated in the cytoplasmic membranes of dividing cells [9, 10, 16].

Many reports have reviewed phosphorylated AMPK role's in tumor growth and development [9, 10, 17-19], and other research has allied this kinase with a good prognosis in tumors of various organs including the stomach [20], the colon and rectum [21], the head and neck, and in [22], renal cell carcinoma [23], bronchogenic cancer [24], and hepatocellular carcinoma [25].

Still, little has been identified concerning the clinical importance of the p-AMPK phenotype in urinary bladder carcinomas. Therefore, this research aims to describe the p-AMPK phenotype in urinary bladder carcinomas compared to normal bladder tissues. It also investigates the correlation between the phenotype of p-AMPK and the clinical data of urinary bladder cancer to establish the clinical relevance and the function of the p-AMPK phenotype in the development of urinary bladder carcinomas. Furthermore, this study will evaluate p-AMPK phenotype as a marker for the diagnosis and prognostication of the overall survival in patients with urinary bladder carcinomas.

Material and methods

The protocol of current research has been accepted by the Biomedical Ethical Committee at King Abdulaziz University. All urinary bladder cancer tissue samples (128 cases; 104 males and 24 females) and the control group (24 non-cancerous bladder tissue samples) which have been used in this study were paraffin embedded specimens and were obtained from the archives of the pathology department in King Abdulaziz University. Paraffin blocks were cut into 4 μm thick sections, hematoxylin and eosin stained and reevaluated. Tumor characteristics and clinical data such as gender, age, cancer type, tumor volume, stage, grade, and metastasis) have been collected from King Abdulaziz University Hospital medical records (**Table 1**). All tissues of the control group were selected from people who were sampled for non-cancerous disorders. Recommendations of the World Health Organization were applied in bladder cancer grade and stage processes. All employed tissue samples of bladder cancer and the control cases were utilized to make the tissue microarrays.

Tissue microarray construction

The tissue microarray (TMA) was constructed as it has been illustrated in our earlier papers [26, 27]. All 128 bladder cancer cases and 24 control tissue samples were utilized in the tissue microarray construction. TMA blocks were sliced into 4 μm sections and sited on slides coated with amino silane. Later, the blocks were employed in a p-AMPK immunohistochemistry staining protocol.

Immunohistochemistry staining method

Immunohistochemical analysis was applied using immunohistochemistry autostainer (BenchMark ULTRA, Ventana, Arizona, USA) as previously reported [27]. Anti-p-AMPK rabbit polyclonal antibody with dilution ratio of 1 to 100 (catalog code: sc-101630, Santa Cruz Biotechnology, USA), and ULTRAVIEW TM DAB visualizing protocol were used in immunohistochemistry staining. Every staining run contained a slide treated with tris buffer in place of the anti-p-AMPK antibody as a negative control. A positive control slide containing Hep G2 cell lysate (Santa Cruz Biotechnology, USA) was used. Cases with brown granular nuclear staining in more than 5% of the tumor cells were counted as positive.

Phosphorylated AMP-activated protein kinase immunoreactivity has been scored, by two pathologists, for staining intensity and for the percentage of positively stained cells. The frequency of positive cells was evaluated applying a semiquantitative method in 3 fields with lenses

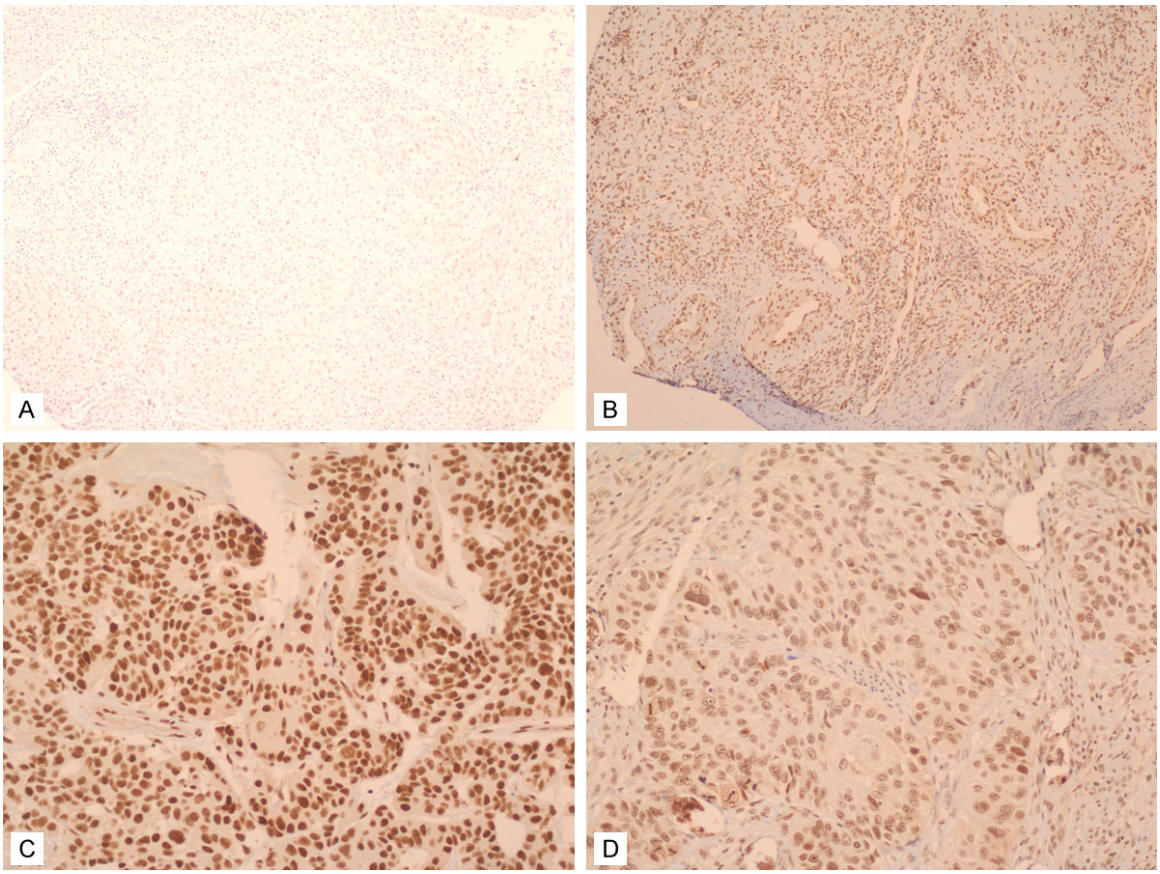

Figure 1. p-AMPK immunostaining pattern in bladder cancer. A. Negative staining in bladder cancer (10×); B. Strong positive staining in normal bladder tissue (10×); C. Strong positive staining in epithelial cells of bladder cancer (20×); D. Moderate positive staining in epithelial cells of bladder cancer (20×).

of 40 amplification power. Staining intensity has been given scores 0, 1, 2, 3 and 4 representing negative, weak, moderate and strong staining respectively. Scores of staining intensity have been presented as low-level immunoreactivity (0 and 1) and high level (2 and 3). When a disparity between the two pathologists' staining scores occurred, the lowest score value was reported.

Statistical analysis

All data were assessed statistically by IBM-SPSS software (version 21). All data values were presented as percentages and incidences. The association between clinicopathological factors of bladder cancer and p-AMPK expression was explored statistically by chi-square and Fisher's exact tests. The comparison of survival distributions for various p-AMPK immunohistochemistry staining intensity levels was assessed by applying the Log Rank test. The level of significance was counted when $P<0.05$.

Results

Both male and female bladder cancer cases shared the same distribution of p-AMPK immunophenotype. **Table 1** shows all clinicopathological parameters of these cases. Urothelial carcinoma was the most common type (78.9%) among bladder tumors, followed by squamous differentiation variant (13.3%), squamous cell carcinoma (4.7%), and carcinoma in situ (3.1%) (**Table 1**). The median age in the present report was 62.4 years (ranged 31-93 yrs). One hundred and eighteen cases of bladder cancer were staged and 116 cases graded (**Table 1**). The total number of deaths from bladder cancer was 38 (29.7%). Sixty-three cases showed muscularis propria invasion, eighteen cases with vascular invasion, nineteen cases with lymph node involvement and twenty-two cases with distant metastases (**Table 1**).

Nuclear immunohistochemical staining of phosphorylated AMPK was found in 123 urinary bladder neoplasms of which 104 (81.3%)

cases showed moderate to strong positive immunostaining. More than 86.9% of the positive cases showed immunostaining in more than sixty percent of the transformed cells. Twenty-four (100%) control cases showed moderate to strong p-AMPK immunostaining. All bladder tissues of control cases showed a high level of p-AMPK expression in both epithelial and stromal cells (**Figure 1A-D**). Phosphorylated AMPK phenotypes of bladder cancer epithelial cells showed significant variation between the tumor cases and the control group (P = 0.015) and similarly stromal cells (P = 0.003). Substantial heterogeneity in p-AMPK immunohistochemical staining patterns between urinary bladder tumors was identified regarding cells, glands and type of cells For example, some tumors showed positive staining in epithelial cells only, but others revealed immunostaining in both epithelial and stromal cells (**Figure 1D**).

Phosphorylated AMPK immunohistochemical staining was found significantly correlated with bladder cancer grade, muscularis propria penetration, lymph node infiltration, and vascular invasion. The grade of disease was correlated with phosphorylated AMPK expression in epithelial cells (*p*-value = 0.023) and stromal cells (*p*-value = 0.041). High scores of p-AMPK immunohistochemical staining was relatively more frequent in low grade tumors (**Table 1**). Muscularis propria invasion was considerably correlated with p-AMPK immunostaining in epithelial and stromal cells (*p*-value = 0.002 and *p*-value = 0.019 respectively). Non-muscle invasive bladder cancer (NMIBC) is significantly more prevalent with high p-AMPK expressions. Immunohistochemical staining of p-AMPK in the epithelial cells of bladder cancer was also correlated with metastasis in lymph nodes (P = 0.030), while no significant relationship between lymph node metastasis and p-AMPK phenotype was observed in stromal cells (P = 0.143). The odds of low level p-AMPK expression in epithelial cells for positive lymph nodes is 3.21 times as great as the odds of low score p-AMPK immunostaining in epithelial cells for negative lymph nodes. Furthermore, vascular invasion is considerably correlated with phosphorylated AMPK immunostaining in epithelial cells and stromal cells (*P*-value = 0.002 and *P*-value = 0.010 respectively). No significant associations were observed with the gender, stage, tumor histotype, or the patient's alive/deceased status. There are no important changes in survival behavior that have been assessed using the Log Rank test. This test was used to liken the survival distribution among the cases of low and high phosphorylated AMPK staining scores in epithelial and stromal cells.

Discussion

Perception of p-AMPK's role in cancer biology has increased in the last decade. This made p-AMPK a promising biomarker in cancer prevention and a possible medicinal target in several tumors. A number of reports have defined the p-AMPK immunohistochemical phenotype and described its important correlation with clinical parameters in several human neoplasms such as gastric cancer [20], colorectal carcinoma [21], head and neck squamous cell carcinoma [22], renal cell carcinoma [23], lung cancer [24], hepatocellular carcinoma [25] and breast cancer [28, 29]. Regarding urinary bladder cancer, to the best of our knowledge, unfortunately, there has been only one study that assessed the p-AMPK expression pattern, but it did not associate this pattern of expression with the tumors' clinicopathological factors [30]. This study found the expression level of p-AMPK was considerably downregulated by 66% and 61% in both high and low-grade neoplasms respectively when compared with adjacent non-cancerous tissue. Consistently the findings of the current study reached a similar conclusion that there is a reduction in the expression of activated AMPK when compared with normal tissue especially in high grade tumors and muscle invasive bladder cancer (MIBC). Similarly, the AMPK phosphorylation activity was described as suppressed in several tumors, such as hepatocellular carcinoma, gastric cancer, and breast cancer [20, 25, 28]. Suppressed activation of AMPK may induce tumor growth through deactivation of a tumor suppressing axis LKB1/AMPK signaling route [31]. Phosphorylated AMPK can efficiently stop the mTOR pathway, which is commonly stimulated in several tumors [32]. Thus, reduced activation of AMPK, a frequent outcome in cancers, decreased the capacity of preventing the mTOR pathway [33]. Otherwise, the suppressed p-AMPK expression in various tumors, including

bladder cancer, can be accredited partially to a decreased AMPK activation, which could be due to a decreased amount of total AMPK protein.

Regarding urinary bladder cancer, the present study is the first to describe a relationship between phosphorylated AMPK expressions and clinicopathological factors. The present investigation showed a statistically substantial association between the expression of p-AMPK and grade, muscular penetration, vascular invasion and lymph node metastasis. These findings agree with many other studies which reported a prognostic value for p-AMPK immunohistochemical expression in several tumors including breast cancer [28, 29], head and neck squamous cell carcinoma [22], lung cancer [24], hepatocellular carcinoma [25], gastric cancer [20], renal cell carcinoma [23], and colorectal carcinoma [21].

However, in the current analysis, the notable p-AMPK expression in high grade tumor cases (72%) might be of clinical importance and may help the function of phosphorylated AMPK in urinary bladder cancer development and tumor cell survival. Moreover, it suggests that, in certain environments, the commonly recognized role of phosphorylated AMPK as a cancer suppressing molecule could be weakened by tumor cells by appropriating p-AMPK to stimulate metabolic modifications to support cell proliferation and survival [34]. Otherwise, AMPK could work as a binary molecule in cancer progression and development based on some factors such as AMPK isoform, level of activation and other motivated compensatory cellular activities. It is possible that medium AMPK phosphorylation that is stimulated by modest stress might apply protective powers and make oncogenic-like activity, while severe stress might motivate AMPK to show suppressing behaviors and initiate neoplastic cell death. Thus, more investigations about the function of p-AMPK in urinary bladder cancer would clarify the processes that underline the function of AMPK in guarding tumor cells and evading stress injury.

Conclusion

Our study suggests that p-AMPK is a useful biomarker in determining the prognosis of bladder cancer. The association of phosphorylated AMPK with several clinical factors proposes the participation of this enzyme in urinary bladder cancer development and cell survival. These preliminary findings recommend that p-AMPK maybe a valuable tissue biomarker for predicting poor prognosis in bladder cancer.

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Disclosure of conflict of interest

None.

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