JBUON 2019; 24(1): 329-337
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

PI3K/AKT pathway genetic alterations and dysregulation of expression in bladder cancer

Stefanos Kachrilas[1,2], Athanasios Dellis[3,4], Athanasios Papatsoris[1], Socratis Avgeris[2], Dimitra Anastasiou[2], Ariana Gavriil[5], Maria Horti[6], Sofia Tseleni-Balafouta[7], Konstantinos Livadas[1], Dimitrios J. Stravopodis[8], Gerassimos Alivizatos[1], Gerassimos E. Voutsinas[2]*, Charalambos Deliveliotis[1]*

[1]2nd Department of Urology, Sismanogleion General Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [2]Laboratory of Environmental Mutagenesis and Carcinogenesis, Institute of Biosciences and Applications, National Centre for Scientific Research "Demokritos", Athens, Greece; [3]2nd Department of Surgery, Aretaieion Academic Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [4]1st Department of Urology, Laikon General Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [5]Laboratory of Immunology, Center for Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece; [6]Department of Pathology, Sismanogleion General Hospital, Athens, Greece; [7]1st Department of Pathology, Medical School, National and Kapodistrian University of Athens, Athens, Greece; [8]Section of Cell Biology and Biophysics, Department of Biology, National and Kapodistrian University of Athens, Athens, Greece.

*These authors contributed equally to this work.

Summary

Purpose: *To examine the involvement of specific components of the PI3K/AKT pathway in urinary bladder cancer development.*

Methods: *Samples from 65 tumors and 13 normal bladder tissues were collected. Genomic DNA isolation from snap-frozen and paraffin-embedded laser-microdissected tissues was followed by Sanger sequencing, whereas total RNA was purified for use in RT-PCR analyses. Immunohistochemistry was carried out on sections of paraffin-embedded biopsy material.*

Results: *Three pathogenic mutations (two missense and one frameshift) were identified in exon 20 of PIK3CA {c.3140A>G (p.His1047Arg), c.[3172A>T(;)3174C>T] (p.lle1058Phe), c.3203dupA (p.Asn1068Lysfs*5)} after laser capture microdissection, whereas PTEN mRNA expression was found to be downregulated in bladder cancer tissues compared to normal bladder urothelium. Upregulation of cytoplasmic and nuclear p-AKT expression was detected in low grade tumors, whereas in infiltrating carcinomas p-AKT was shown to be downregulated and confined to the cytoplasm. PTEN expression was weak and mainly cytoplasmic in superficial tumors, but stronger and nuclear in the infiltrating tumors.*

Conclusions: *PI3K/AKT pathway activation is crucial for bladder cancer initiation and progression. In this context, PIK3CA, p-AKT and nuclear PTEN could be used along with other biomarkers for prognosis and selection of appropriate therapy in the clinical management of bladder cancer.*

Key words: *bladder cancer, PI3K/AKT pathway, PIK3CA mutation, nuclear PTEN, p-AKT expression*

Introduction

Bladder cancer (BC) is a major cause of morbidity and mortality with about 380,000 new cases arising and 150,000 deaths reported per year, ranking 7th among all types of cancer in men, while being less common in women [1,2]. Approximately 75% of patients present with non- muscle-invasive bladder cancer (NMIBC), a disease confined to the mucosa (stage Ta, carcinoma in situ; CIS) or sub-

Correspondence to: Athanasios Dellis, MD, PhD, FEBU. 2nd Department of Surgery, Aretaieion Academic Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece, and 1st Department of Urology, Laikon General Hospital, Medical School, National and Kapodistrian University of Athens, Athens, Greece.
Tel: +30 2107286136, Fax: +30 2106107289, E-mail: aedellis@gmail.com
Received: 05/01/2018; Accepted: 12/02/2018

epithelial connective tissue (stage T1), with the rest suffering from muscle-invasive bladder cancer (MIBC) [3]. However, about 30% of patients initially diagnosed with superficial disease are estimated to recur to MIBC within two years, whereas radical cystectomy (RC) and pelvic lymphadenectomy (PLND) which is considered as the standard of care for clinically localized MIBC and high-grade recurrent NMIBC still has a 50% 2-year risk of distant metastasis and 60% 5-year risk of death in the setting of muscle-invasive disease [4,5].

Generation and evolution of human urinary bladder cancer has been associated with upregulation of a number of independent or cross-talking growth factor receptor-initiated molecular pathways, such as FGFR3, PDGFR, EGFR, PI3K/AKT, VEGFR, etc., which are known to play a crucial role in cell growth, proliferation and survival [1,6-8]. Because identification of activation of one or more of the above pathways is considered nowadays of significant importance for prognosis and selection of appropriate adjuvant chemotherapy, molecular classification of tumors into specific bladder cancer subtypes is under intense investigation [9-13].

The PI3K/AKT pathway has been the focus of a number of recent studies [14-18]. Genomic alterations have been detected in several genes of this pathway, including the tumor suppressor gene *PTEN*, which was found to be inactivated by mutation and/or deletion in several types of sporadic cancers [19], and the proto-oncogenes PI3KCA, *PIK3R1* and *AKT*, which were demonstrated to be activated by mutation or amplification [20,21]. Furthermore, oncogenic activation of *RAS* genes by point mutation was found to upregulate this pathway through interaction of *RAS* with p110 (*PIK3CA*) [22]. Moreover, increased expression of genes associated with this pathway was also shown to be involved in the onset and progression of bladder carcinogenesis [1,23,24].

In this work, to further investigate the involvement and role of the PI3K/AKT pathway in bladder carcinogenesis, we analyzed PI3KCA for possible existence of mutations, we assessed the expression patterns of *PIK3CA* (p110), *PIK3R1* (p85), and *PTEN* at the mRNA level in tumor versus normal bladder samples, and we studied the levels of expression and cellular localization of p-AKT and *PTEN* in urinary bladder tumors.

Methods

Patients and sample collection

The study protocol and the informed consent forms were approved by the Bioethics Committee of the Sismanogleion General Hospital. The 65 patients included in this work referred to the Sismanogleion General Hospital for diagnosis and therapy. Before having access to tumor material from transurethral resection or surgery, written informed consent forms were signed by all patients, while the study was in agreement with the 2000 revision of Helsinki Declaration. From the above 65 patients, genomic DNA and total RNA were extracted from snap-frozen tissue samples, whereas slices of paraffin-embedded tissue for laser capture microdissection and immunohistochemistry were obtained.

Genomic DNA isolation

After transurethral resection or surgery, snap-frozen bladder cancer tissues from 65 patients that had not received chemotherapy, and slices from 25 paraffin-embedded tumor samples from the same group of patients, followed by laser capture microdissection, were used for extraction of genomic DNA. Additionally, 13 normal bladder samples have been obtained from patients undergoing other type of bladder surgery. In the case of tumor or normal snap-frozen tissue samples, DNA isolation was carried out according to the standard saturated salt-chloroform extraction protocol after using a BioSpec Pulverizer, whereas in the case of laser capture microdissected tissue, DNA was extracted and purified with addition of carrier RNA, using the QIAamp DNA Micro Kit (Qiagen, Venlo, Netherlands cat. no. 56304), according to the Isolation of Genomic DNA from Laser–Microdissected Tissues protocol. In all cases, purity and concentration of isolated DNA were measured using a NanoDrop™ spectrophotometer.

Laser capture microdissection (LCM)

Prior to LCM, 7-μm sections of paraffin-embedded tumor tissue were cut and placed on poly-L-lysine slides. H&E staining was performed according to a standard protocol from Zeiss Labs, Munich, Germany (http://www.zeiss.de/microdissection). The white/opaque AdhesiveCap tubes, size 200 μl, were used and non-contact LCM of selected cells was performed using the PALM MicroBeam (Zeiss, Munich, Germany).

Mutation detection

Exons 9 and 20 of *PIK3CA* were PCR-amplified and directly sequenced using the Sanger method. All primer sequences and PCR conditions used are available upon request. Cycle sequencing reactions were performed using the v3.1 BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and then analyzed on an ABI Prism® Genetic Analyzer. Sequences obtained were aligned against reference sequences from the Genbank, and examined for the presence of variants. Finally, we screened the Catalogue Of Somatic Mutations In Cancer (the COSMIC database, http://cancer.sanger.ac.uk/cosmic), which is the largest and most comprehensive relative resource in the world, in order to possibly detect therein the variants identified in this work and assess their impact as somatic mutations in human bladder cancer.

RNA isolation and RT-PCR

In order to detect and analyze the levels of *PIK3CA*, *PIK3R1*, and *PTEN* gene expression, we performed to-

tal RNA extraction from 65 snap-frozen tumor tissues, plus 13 normal bladder samples as controls, using TRI REAGENT (Molecular Research Center Inc, Cincinnati, OH). Subsequent cDNA synthesis was carried out using M-MLV RT (Life Technologies, Carlsbad, CA).

Densitometry

The band intensity on PCR photographs from agarose-gel-electrophorized RT-PCR samples was quantitated by densitometry using the BandLeader image analysis software. The intensity of the bands representing the *PIK3CA*, *PIK3R1* and *PTEN* RT-PCR products was normalized to the intensity of the band for the *GAPDH* housekeeping gene and is expressed as a ratio of relative band intensities. All RT-PCR experiments were repeated at least three times, to ensure statistical accuracy.

Immunohistochemistry

Immunostaining was performed using monoclonal antibodies against p-AKT and *PTEN* (Cell Signaling, Beverly, MA) at a dilution of 1:100, overnight. For better antigen retrieval, tissue sections were pretreated with microwaving at 750 W, in 0.01 mol/L sodium citrate buffer (pH 6.0) for 30 min. A standard two-step streptavidin peroxidase technique was used. Tumor samples known to overexpress p-AKT or *PTEN* were used as positive controls, whereas negative controls had the primary antibody omitted and replaced by phosphate buffered saline (PBS). Evaluation of the sections was carried out

Table 1. *PIK3CA* (p110), *PIK3R1* (p85) and *PTEN* mRNA expression in normal vs bladder tumor tissues

Normal		*Mean (SD)*	*Tumor*		*Mean (SD)*	*p value*
n=13	p85/GAPDH	0.67 (0.257)	n=60	p85/GAPDH	0.77 (0.096)	0.086
n=13	p110/GAPDH	0.81 (0.097)	n=61	p110/GAPDH	0.56 (0.057)	<0.001
n=13	PTEN/GAPDH	0.81 (0.108)	n=61	PTEN/GAPDH	0.40 (0.038)	<0.001

Table 2. p-AKT expression in relation to p-AKT cellular localization in superficial bladder tumors using immunohistochemistry

	p-AKT expression					
p-AKT cellular localization	*Negative* *n (%)*	*Weak* *n (%)*	*Moderate* *n (%)*	*Strong* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	11 (100)	0 (0)	0 (0)	0 (0)	11	
Cytoplasmic	0 (0)	10 (52.6)	4 (21.1)	5 (26.3)	19	
Nuclear	0 (0)	1 (50)	0	1 (50)	2	p<0.001
Mixed	0 (0)	6 (18.2)	7 (21.2)	20 (60.6)	33	
Total	11 (16.9)	17 (26.2)	11 (16.9)	26 (40.0)	65	

Table 3. p-AKT expression in relation to p-AKT cellular localization in infiltrating bladder tumors using immunohistochemistry

	p-AKT expression					
p-AKT cellular localization	*Negative* *n (%)*	*Weak* *n (%)*	*Moderate* *n (%)*	*Strong* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	5 (100)	0 (0)	0 (0)	0 (0)	5	
Cytoplasmic	0 (0)	0 (0)	0 (0)	1 (100)	1	
Nuclear	0 (0)	0 (0)	0 (0)	0 (0)	0	p=0.002
Mixed	0 (0)	5 (36.4)	5 (36.4)	4 (27.3)	14	
Total	5 (25.0)	5 (25.0)	5 (25.0)	5 (25.0)	20	

Table 4. p-AKT cellular localization in relation to staging in bladder tumors using immunohistochemistry

	Stage			
p-AKT expression	*T1* *n (%)*	*T2* *n (%)*	*Total* *n*	*Fisher's exact*
Weak	8 (40.0)	12 (60.0)	20	
Moderate	1 (6.7)	14 (93.3)	15	p<0.001
Strong	20 (74.1)	7 (25.9)	27	
Total	29 (46.8)	33 (53.2)	62	

by two independent observers without knowledge of the clinical data and similar results were obtained. Semi-quantitative measurement of p-AKT and *PTEN* expression was performed by simultaneous scoring of staining intensity and localization, as previously described [25], on a scale from 0 to +++, corresponding to negative (0), weak (+), moderate (++) and strong (+++) immunoreactivity. The findings were then correlated to tumor characteristics (Tables 2-8). All immunostaining experiments were repeated at least three times.

Statistics

Statistical analyses were performed with the use of SPSS V.16 (IBM, USA). More specifically, statistical significance of comparisons regarding mRNA gene expression was determined using the unpaired Student's t-test, whereas in the statistical evaluation of all immunohistochemically detected levels of protein expression the Fisher's exact test was applied. P<0.05 was considered as statistically significant.

Table 5. *PTEN* expression in relation to grade in superficial bladder tumors using immunohistochemistry

	Grade				
PTEN expression	*G1* *n (%)*	*G2* *n (%)*	*G3* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	0 (0)	1 (20.0)	5 (80.0)	6	
Weak	3 (25.0)	5 (50.0)	3 (25.0)	11	
Moderate	0 (0)	0 (0)	7 (100)	7	p=0.002
Strong	0 (0)	14 (78.6)	4 (21.4)	18	
Total	3 (6.3)	20 (50.0)	19 (43.8)	42	

Table 6. *PTEN* expression in relation to grade in infiltrating bladder tumors using immunohistochemistry

	Grade				
PTEN expression	*G1* *n (%)*	*G2* *n (%)*	*G3* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	3 (6.9)	21 (55.2)	14 (37.9)	38	
Weak	0 (0)	0 (0)	1 (100.0)	1	
Moderate	0 (0)	0 (0)	3 (100.0)	3	p=0.01
Strong	0 (0)	0 (0)	3 (100.0)	3	
Total	3 (5.9)	21 (47.1)	21 (47.1)	45	

Table 7. *PTEN* expression in relation to *PTEN* cellular localization in superficial bladder tumors using immunohistochemistry

	PTEN expression					
PTEN cellular localization	*Negative* *n (%)*	*Weak* *n (%)*	*Moderate* *n (%)*	*Strong* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	0 (0)	0 (0)	0 (0)	0 (0)	0	
Cytoplasmic	0 (0)	8 (22.9)	7 (20.0)	20 (57.1)	35	
Nuclear	0 (0)	0 (0)	0 (0)	2 (100.0)	2	p<0.05
Mixed	0 (0)	7 (25.0)	8 (28.6)	13 (46.4)	28	
Total	0 (0)	15 (23.1)	15 (23.1)	35 (53.8)	65	

Table 8. *PTEN* expression in relation to *PTEN* cellular localization in infiltrating bladder tumors using immunohistochemistry

	PTEN expression					
PTEN cellular localization	*Negative* *n (%)*	*Weak* *n (%)*	*Moderate* *n (%)*	*Strong* *n (%)*	*Total* *n*	*Fisher's exact*
Negative	0 (0)	0 (0)	0 (0)	0 (0)	0	
Cytoplasmic	0 (0)	1 (33)	3 (67)	0 (0)	4	
Nuclear	1 (13)	7 (63)	1 (13)	1 (13)	10	p>0.05
Mixed	0 (0)	7 (56)	3 (22)	3 (22)	13	
Total	1 (5.0)	15 (55.0)	7 (25.0)	4 (15.0)	27	

Results

Identification and characterization of PIK3CA mutations

No mutations were detected within the coding region of exons 9 and 20 of PI3KCA gene using genomic DNA from 65 snap-frozen tumors. Therefore, we decided to look for possible mutations in 25 out of the initial 65 samples using paraffin-embedded laser-capture-microdissected bladder cancer tissues. In these microdissected samples, we were able to identify the following three mutations: 1) c.3140A>G (p.His1047Arg); 2) c.[3172A>T(;)3174C>T] (p.Ile1058Phe); and 3) c.3203dupA (p.Asn1068Lysfs*5) (Figure 1). We have found that all three are already included in the COSMIC database with the following mutation ID numbers: 1) COSM775, 2) COSM30606, and 3) COSM249879. Variant c.3203dupA is a frameshift mutation creating a premature termination codon, and thus it is pathogenic. The remaining two genetic alterations are missense mutations that are predicted as pathogenic in the above database, with the use of the Functional Analysis through Hidden Markov Models (FATHMM) tool (http://fathmm.biocompute.org.uk/).

PIK3CA, PIK3R1 and PTEN mRNA expression in normal vs bladder tumor tissues

In order to assess the possibility of expression dysregulation at the mRNA level of *PIK3CA*, *PIK3R1* and *PTEN* genes in bladder tumors, mRNA expression values of these genes in normal bladder were compared to the ones in malignant tissues with the use of Student's t-test (Table 1). No significant difference or meaningful result was found for *PIK3R1* or *PIK3CA* between the two tissue types (t=1.382, p=0.086; t=8.970), whereas, for *PTEN*, mean values in controls versus malignant tissues were shown to be significantly higher (t=13.510, p<0.001) (Table 1). Therefore, downregulation of *PTEN* mRNA expression is an important parameter in bladder carcinogenesis.

Expression and cellular localization of p-AKT and PTEN

Eighty two percent of the low grade superficial bladder carcinoma cases were found to express p-AKT in the cytoplasm (29%), the nucleus (4%), or both (49%), whereas the mixed cytoplasmic plus nuclear immunohistochemical (IHC) localization pattern of p-AKT expression was detected in almost 93% of the high grade infiltrating tumors (p<0.001) (Tables 2 and 3). Moreover, strong p-AKT expression in bladder cancer samples was found to exhibit significant correlation with T1 tumor stage (74.1%, p<0.001) (Table 4).

PTEN IHC staining was seen more often in low grade superficial tumors (86%) than in high grade infiltrating carcinomas (16%) (Tables 5 and 6), with strong *PTEN* expression being less prominent in superficial G3 samples (21.4%, p<0.001) (Table 5), whereas *PTEN* nuclear expression was more frequent in infiltrating tumors (37%) than in superficial ones (3.5%) (Tables 7 and 8).

Discussion

A number of genes of the PI3K/AKT pathway have been found to be mutated or exhibit alterations in expression pattern at the mRNA or the protein level in a variety of human cancers [26-28], while also associating activation of this pathway with bladder carcinogenesis [1,19,29]. Nevertheless, because the details of the above association are not completely solved yet, we decided to further investigate a number of critical relative parameters, such as PI3KCA mutations, *PIK3CA*, *PIK3R1*, and *PTEN* expression patterns at the mRNA level, as well as expression levels and cellular localization of p-AKT and *PTEN* in a new cohort of urinary bladder tumors from the Greek population.

Using genomic DNA from 65 snap-frozen tumors, we were unsuccessful in our attempt to identify mutations within the coding region of exons 9 and 20 of PI3KCA in our cohort. Since *PIK3CA* structural alterations are considered a relatively frequent event in bladder carcinogenesis, we additionally performed laser capture microdissection in 25 unselected samples within the original set of 65 bladder tumor specimens. This time, we were able to identify 3 mutations (12%). All mutations have been reported previously as pathogenic in the COSMIC database, but two out of the three are encountered here for the first time in the frame of bladder cancer.

Missense mutation c.3140A>G (p.His1047Arg) is quite common, since it has already been found in breast cancer (in 1352 samples), colorectal (355), endometrial (167), ovarian (102), pharyngeal (43), bladder (30), melanoma (6), etc (Figure 1a). In our case, it was detected in a Ta/G2 superficial tumor exhibiting strong p-AKT expression localized both in the cytoplasm and the nucleus, with moderate cytoplasmic (40%) expression of *PTEN*. Missense mutation c.[3172A>T(;)3174C>T] (p.Ile1058Phe) was found in a T2a/G3 tumor, exhibiting no infiltration (Figure 1b). It showed moderate cytoplasmic (40%) p-AKT expression and strong cytoplasmic (60%) expression of *PTEN*. Previously, it has been detected in 4 independent tumor samples (3 endo-

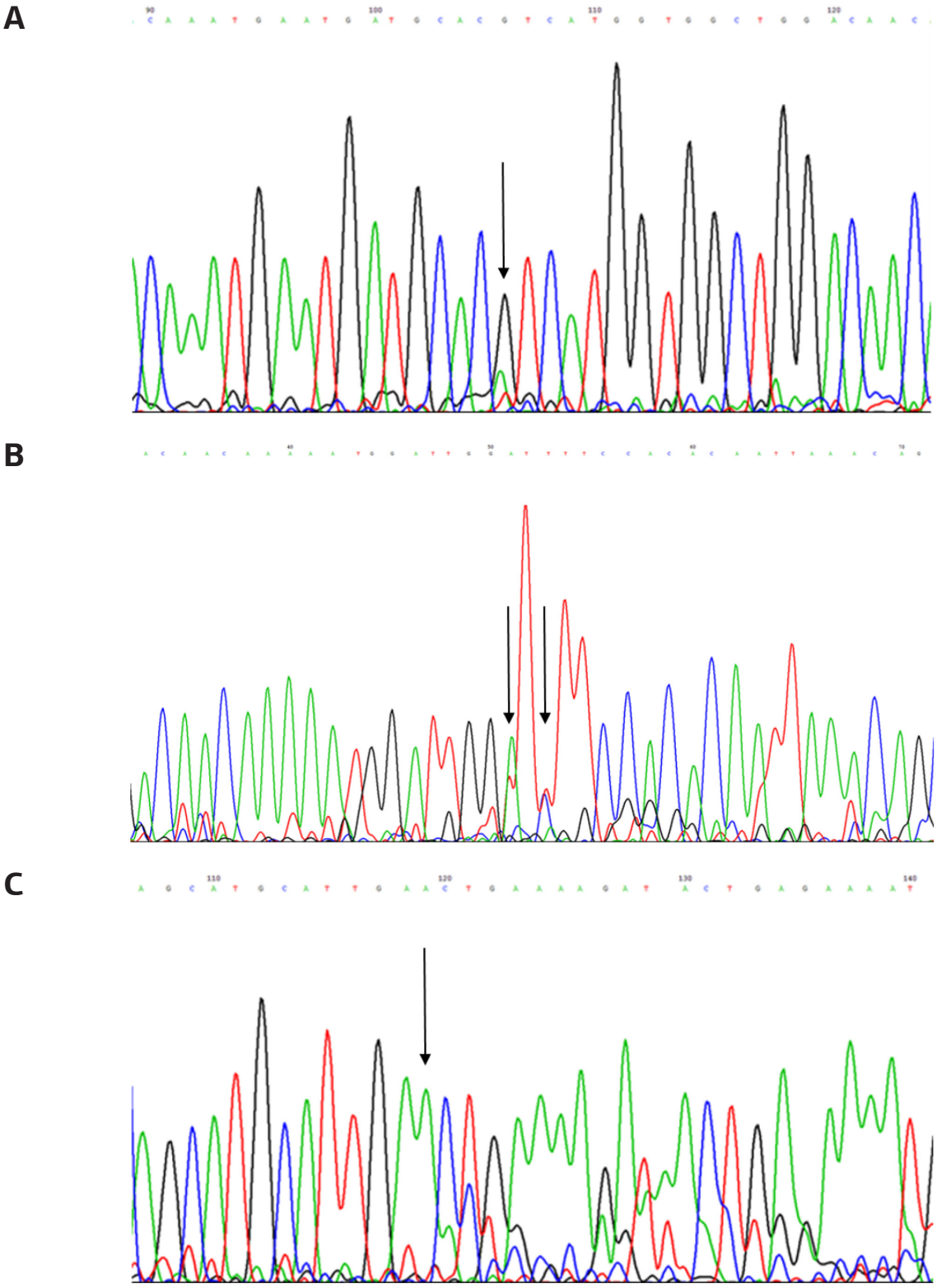

Figure 1. (A) *PIK3CA* missense mutation c.3140A>G (p.His1047Arg) detected in a Tα/G2 superficial bladder tumor, which was found to exhibit strong cytoplasmic and nuclear p-AKT expression with moderate cytoplasmic (40%) expression of *PTEN*. The arrow shows the observed A to G base pair substitution, resulting in a His to Arg amino acid change in the protein. **(B)** *PIK3CA* missense mutation c.[3172A>T(;)3174C>T] (p.Ile1058Phe) found in a T2α/G3 bladder tumor, exhibiting infiltration. This tumor showed moderate cytoplasmic (40%) p-AKT expression and strong cytoplasmic expression (60%) of *PTEN*. The arrows show the observed A to T and C to T base pair substitutions, resulting in an Ile to Phe amino acid change in the protein. This mutation was detected here for the first time in bladder cancer. **(C)** *PIK3CA* frameshift mutation c.3203dupA (p.Asn1068Lysfs*5) in a Tα/G2 superficial bladder tumor exhibiting strong cytoplasmic (70%) p-AKT expression and strong cytoplasmic (40%) plus nuclear (60%) expression of *PTEN*. The arrows show the observed A duplication, resulting in a frameshift which produces a premature stop codon 5 triplets after the mutation. This mutation was detected here for the first time in bladder cancer.

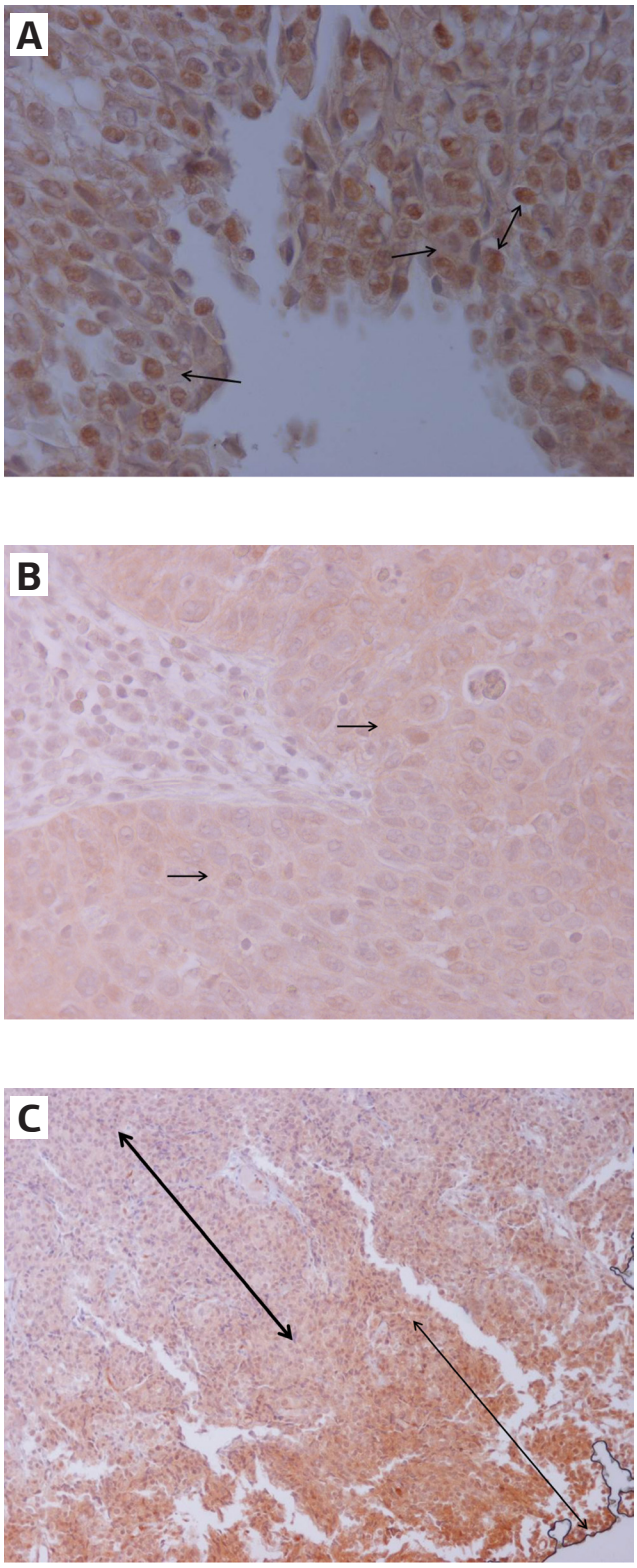

Figure 2. (A) Strong cytoplasmic (simple arrows) and nuclear (double arrow) immunohistochemical staining of p-AKT in superficial low grade carcinoma of the bladder (IHCx400). **(B)** Weak, mainly cytoplasmic p-AKT immunohistochemical staining (arrows) in high grade infiltrating carcinoma of the bladder (IHCx400). **(C)** Strong, mainly cytoplasmic *PTEN* immunohistochemical staining in the superficial part (thin arrow), and mainly nuclear *PTEN* staining (thick arrow) in the deeper, infiltrating part of urothelial carcinoma (IHCx400).

metrial and 1 colorectal). This is the first time to be identified in bladder cancer. Frameshift mutation c.3203dupA (p.Asn1068Lysfs*5) was seen in a Ta/G2 superficial tumor exhibiting strong cytoplasmic (70%) p-AKT expression and strong cytoplasmic (40%) plus nuclear (60%) expression of *PTEN* (Figure 1c). This is also a rather uncommon mutation seen in only 3 tumor samples before this report (2 breast and 1 colorectal), while this is the first time to be reported in a bladder tumor specimen. Based on the above, all three bladder tumor samples with mutant *PIK3CA* were found to possess an upregulated PI3K/AKT pathway.

Next, we analyzed the mRNA expression of *PIK3CA*, *PIK3R1* and *PTEN* genes in bladder tumors compared to normal urothelium. No meaningful statistically significant differences were identified regarding the expression levels of *PIK3CA* and *PIK3R1*. But, *PTEN* was found to be statistically significantly downregulated in tumor samples (Table 1). This coincides with previous relative findings, and fits well to the current model of cytoplasmic and nuclear *PTEN* playing a multitude of roles regarding tumor suppression [30,31].

Once we have identified *PIK3CA* mutation and *PTEN* downregulation in our bladder tumor samples, as a final step, we sought to study protein expression levels and localization patterns for p-AKT and *PTEN* in the same tumor samples. As a general rule, in this work, p-AKT was found to be upregulated in low grade superficial carcinomas, exhibiting a mainly combined cytoplasmic and nuclear expression (Figure 2a). On the contrary, in high grade infiltrating carcinomas expression was downregulated and mainly cytoplasmic (Figure 2b). This fits well to a hypothesis of PI3K/AKT pathway activation earlier during carcinogenesis, whereas in more malignant tumors, when additional oncogenic pathways are becoming activated, this signaling route likely becomes redundant and thus downregulated [17,32,33].

Regarding *PTEN*, in superficial low grade carcinomas we observed weak cytoplasmic expression with nuclear localization being low or absent, whereas a stronger and mainly nuclear expression was detected in high grade infiltrating carcinomas (Figure 2c). This is in accordance with the well-established activity of *PTEN* in the cytoplasm, as well as with the currently conceived multiple roles played by nuclear *PTEN* in genome stability, DNA repair and cell cycle control [31,34].

In this work, we have identified three pathogenic *PIK3CA* mutations, two of which were detected for the first time in bladder tumors, and found that *PTEN* mRNA expression was downregulated in bladder cancer tissues compared to normal bladder

epithelium. More specifically, mutations in *PIK3CA* and *PTEN* mRNA downregulation were detected mainly in superficial tumors. In agreement with this, AKT was found to be phosphorylated in the cytoplasm and the nucleus of low grade tumors, whereas in infiltrating carcinomas p-AKT was shown to be downregulated. Finally, weak, mainly cytoplasmic *PTEN* expression was observed in superficial tumors, with stronger nuclear expression of *PTEN* being mainly confined to the infiltrating tumor areas, in order to possibly control the severe genomic instability of high grade bladder tumors. Taken together, our results suggest that PI3K/AKT pathway activities are crucial for cancer initiation and progression in the bladder urothelium, whereas components of this pathway like *PIK3CA*, p-AKT and nuclear *PTEN* could eventually be used along with other biomarkers for prognosis and selection of appropriate therapy in the clinical management of bladder cancer.

Acknowledgements

Financial support to GEV was provided by the Greek Secretariat for Science and Technology, and the American College of Greece (Deree).

Conflict of interests

The authors declare no conflict of interests.

References

1. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer 2015;15:25-41.
2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013;49:1374-1403.
3. Burger M, Catto JW, Dalbagni G et al. Epidemiology and risk factors of urothelial bladder cancer. Eur Urol 2013;63:234-41.
4. Stenzl A, Cowan NC, De Santis M et al. Treatment of muscle-invasive and metastatic bladder cancer: update of the EAU guidelines. Eur Urol 2011;59:1009-18.
5. Shariat SF, Karakiewicz PI, Palapattu GS et al. Outcomes of radical cystectomy for transitional cell carcinoma of the bladder: a contemporary series from the Bladder Cancer Research Consortium. J Urol 2006;176:2414-22.
6. Ghosh M, Brancato SJ, Agarwal PK, Apolo AB. Targeted therapies in urothelial carcinoma. Curr Opin Oncol 2014;26:305-20.
7. Carpenter RL, Jiang BH. Roles of EGFR, PI3K, AKT, and mTOR in heavy metal-induced cancer. Curr Cancer Drug Targets 2013;13:252-66.
8. Lekas A, Papathomas TG, Papatsoris AG, Deliveliotis C, Lazaris AC. Novel therapeutics in metastatic bladder cancer. Expert Opin Investig Drugs 2008;17:1889-99.
9. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014;507:315-22.
10. Sjödahl G, Lauss M, Lövgren K et al. A molecular taxonomy for urothelial carcinoma. Clin Cancer Res 2012;18:3377-86.
11. Damrauer JS, Hoadley KA, Chism DD et al. Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. Proc Natl Acad Sci U S A 2014;111:3110-5.
12. Choi W, Porten S, Kim S et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014;25:152-65.
13. Aine M, Eriksson P, Liedberg F, Höglund M, Sjödahl G. On Molecular Classification of Bladder Cancer: Out of One, Many. Eur Urol 2015;68:921-3.
14. Saal LH, Johansson P, Holm K et al. Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity. Proc Natl Acad Sci U S A 2007;104:7564-9.
15. Taniguchi CM, Winnay J, Kondo T et al. The phosphoinositide 3-kinase regulatory subunit p85alpha can exert tumor suppressor properties through negative regulation of growth factor signaling. Cancer Res 2010;70:5305-15.
16. Brait M, Munari E, LeBron C et al. Genome-wide methylation profiling and the PI3K-AKT pathway analysis associated with smoking in urothelial cell carcinoma. Cell Cycle 2013;12:1058-70.
17. Calderaro J, Rebouissou S, de Koning L et al. PI3K/AKT pathway activation in bladder carcinogenesis. Int J Cancer 2014;134:1776-84.
18. Karkoulis PK, Stravopodis DJ, Voutsinas GE. 17-DMAG induces heat shock protein 90 functional impairment in human bladder cancer cells: knocking down the hallmark traits of malignancy. Tumour Biol 2016;37:6861-73.
19. Platt FK, Hurst CD, Taylor CF, Gregory WM, Harnden P, Knowles MA. Spectrum of Phosphatidylinositol 3-Kinase Pathway Gene Alterations in Bladder Cancer. Clin Cancer Res 2009;15:6008-17.
20. Samuels Y, Ericsson K. Oncogenic PI3K and its role in cancer. Curr Opin Oncol 2006;18:77-82.
21. Ross RL, Burns JE, Taylor CF, Mellor P, Anderson DH, Knowles MA. Identification of mutations in distinct regions of p85 alpha in urothelial cancer. PLoS One 2013;8(12):e84411.

22. Ramjaun AR, Downward J. Ras and phosphoinositide 3-kinase: partners in development and tumorigenesis. Cell Cycle 2007;6:2902-5.
23. Papatsoris AG, Kachrilas S, Gekas A. Where are we with the treatment of metastatic bladder cancer? Expert Opin Investig Drugs 2007;16:1311-4.
24. Egawa H, Jingushi K, Hirono T et al. The miR-130 family promotes cell migration and invasion in bladder cancer through FAK and Akt phosphorylation by regulating PTEN. Sci Rep 2016;6:20574.
25. Jahkola T, Toivonen T, Von Smitten K, Blomqvist C, Virtanen I. Expression of tenascin in invasion border of early breast cancer correlates with higher risk of distant metastasis. Int J Cancer (Pred Oncol) 1996;69:445-7.
26. Samuels Y, Wang Z, Bardelli A et al. High Frequency of Mutations of the PIK3CA Gene in Human Cancers. Science 2004;304:554.
27. Tanaka Y, Kanai F, Tada M et al. Absence of PIK3CA hotspot mutations in hepatocellu-lar carcinoma in Japanese patients. Oncogene 2006;25:2950-2.
28. Riener MO, Bawohl M, Clavien PA, Jochum W. Rare PIK3CA hotspot mutations in carcinomas of the biliary tract. Genes Chromosomes Cancer 2008;47:363-7.
29. Ross JS, Wang K, Khaira D et al. Comprehensive Genomic Profiling of 295 Cases of Clinically Advanced Urothelial Carcinoma of the Urinary Bladder Reveals a High Frequency of Clinically Relevant Genomic Alterations. Cancer 2016;122:702-11.
30. Milella M, Falcone I, Conciatori F et al. PTEN: multiple functions in human malignant tumors. Front Oncol 2015;5:24.
31. Chaux A, Compérat E, Varinot J et al. High Levels of PTEN Expression Are Associated With Tumor Progression, Tumor Recurrence, and Systemic Metastases in pT1 Urothelial Carcinoma of the Bladder. Urology 2013;81:116-22.
32. Lopez-Knowles E, Hernández S, Malats N et al. PIK3CA Mutations Are an Early Genetic Alteration Associated with FGFR3 Mutations in Superficial Papillary Bladder Tumors. Cancer Res 2006;66:7401-4.
33. Vilas Jain M, Jangamreddy JR, Grabarek J, et al. Nuclear localized Akt enhances breast cancer stem-like cells through counter-regulation of p21Waf/Cip1 and p27kip1. Cell Cycle 2015;14:2110-21.
34. Davis WJ, Lehmann PZ, Li W. Nuclear PI3K signaling in cell growth and tumorigenesis. Front Cell Dev Biol 2015;3:24.