PIK3CA MUTATIONAL ANALYSIS IN FORMALIN-FIXED, PARA N-EMBEDDED ARCHIVAL TISSUES OF UROTHELIAL CARCINOMA OF URINARY BLADDER

Abstract—Objective: Urothelial carcinoma of the urinary bladder is the fourth most common cancer in males in the United States. In addition to mutations in FGFR3, TP53, AKT1, TSC1, and PTEN genes, mutations in PIK3CA have been also described in urothelial carcinomas, preferentially in low-grade tumors. Herein, we evaluated the presence of PIK3CA mutations in exons 1, 9, and 20 in 21 urothelial carcinomas of the urinary bladder. Methods: Patients were treated by radical cystectomy without neoadjuvant chemotherapy. Representative tissue blocks (1 for each case) were selected. We used a pinpoint DNA extraction technique from formalin- xed, para n-embedded and mutational analysis using the polymerase chain reaction (PCR) assay coupled with sequencing of targeted exons. Patients included 15 men and 6 women, with a median age of 68 years (range, 42 to 76 years), with 3 noninvasive and 18 invasive urothelial carcinomas. Noninvasive carcinomas included 1 case each of low-grade papillary urothelial carcinoma, high-grade papillary urothelial carcinoma, and urothelial carcinoma in situ (CIS). Invasive tumors included 3 pT1, 5 pT2, 6 pT3, and 4 pT4 urothelial carcinomas. Results: We did not nd mutations in the analyzed exons of the PIK3CA gene, in any of the 21 urothelial carcinomas. The preponderance of invasive high-grade and high-stage tumors could explain the absence of identi able mutations in our cohort. Conclusions: PIK3CA mutations as prognosti-cators of outcome or predictors of therapeutic response await further evaluation.

Keywords—Urothelial carcinoma, PIK3CA; Bladder cancer, Mutational analysis, PCR.

1. Introduction

Urothelial carcinoma of the urinary bladder is the fourth most common cancer in males in the United States, with an estimated 74,000 new cases and 16,000 deaths for 2015 [1]. The majority of the tumors are low grade, nonmuscle invasive tumors, as-sociated with a good prognosis. However, approxi-mately one-quarter of patients with bladder cancer are diagnosed with muscle-invasive tumors, with a signi cant risk of progression and a shortened sur-vival [2]. It has been suggested that these two phe-notypes of tumors progress through di erent path-ways, which accounts for the di erences in biological behavior. Low-grade, noninvasive tumors show high frequency in FGFR3 mutation, whereas TP53 muta-tions are associated with muscle-invasive tumors [3].

In addition to mutations in AKT1, TSC1, and PTEN [4, 5], mutations in PIK3CA have been also described in urothelial carcinomas, preferentially in low-grade tumors [6, 7, 8, 9, 10, 11, 12, 13, 14, 15].

Herein, we evaluate the presence of PIK3CA mu-tations in exons 1, 9, and 20 in 21 patients with urothelial carcinomas of the urinary bladder. For this purpose, we use the polymerase chain reaction (PCR) coupled with sequencing of the targeted exons in formalin- xed, para n-embedded tumor samples.

2.   Material and Methods

The current study was approved by the Institu-tional Review Board at the Johns Hopkins School of Medicine (Baltimore, MD). The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

2.1   Tissue Selection

Formalin- xed, para n-embedded tissue samples of 21 patients with urothelial carcinoma of urinary bladder were selected from the pathology les of the Johns Hopkins Medical Institutions (BaltimoreMD). Patients were treated by radical cystectomy without neoadjuvant chemotherapy. Representative tissue blocks (1 for each case) were selected for mi-crodissection and DNA extraction.

2.2   DNA Extraction from Formalin-Fixed, Para n-Embedded Tissue

Tumor areas were identi ed in routine sections stained with hematoxylin and eosin and 10 unstained sections (10 m thick) from each para n-embedded specimen were obtained. DNA isolation of the tar-geted tissue area on tissue sections was done using DNA Isolation System.

A drop of pinpoint solution (Pinpoint Slide DNA Isolation System; Zymo Research, Orange, CA) was applied to the mapped area of the tumor (approxi-mately 5 x 5 mm^2). Next, the targeted tumor tissue was microdissected with a scalpel and placed in a PCR tube. The excised tissues were digested in pro-teinase K bu er solution at 55° C for 8 hours, then at 97° C for 10 minutes.

2.3   PIK3CA Mutation Screening

PCR reactions were prepared with 1X PCR Bu er, 1.5 mM MgCl2, 500 M dNTPs (Applied Biosys-tems; Foster City, CA), 1.5 U AmpliTaq Gold (Ap-plied Biosystems; Foster City, CA), and 500 nM each primer (Table 1) in a 50 l reaction. Six PCR reac-tions were performed to span the target regions: 2 covering exon 1; 1 covering exon 9; 3 covering exon 20 (Table 1).

Reactions were heated to 95° C for 9 min followed by 35 cycles of 95° C for 30 sec, 53° C for 30 sec and 72° C for 1 min, followed by a nal extension at 72° C for 7 min. Eight microliters of ampli cation product were separated by agarose (2%) gel electrophoresis to verify product. Ampli cation products were puri ed using QIAquick Spin Columns according to manu-facturer's protocol (Qiagen; Valencia, CA). Ampli - cation products

were cycle sequenced at an outside facility using Big Dye v3.1 reagents (Applied Biosys-tems; Foster City, CA).

Sequencing was performed in the forward and re-verse directions using the PCR primers as sequenc-ing primers (see Table 1). Sequencing products were puri ed using CleanSEQ Dye Terminator Removal reagents (Agencourt; Beverly, MA) and automated sequencing performed by capillary electrophoresis (CE) on an ABI3700 Avant genetic analyzer (Ap-plied Biosystems; Foster City, CA). Sequence was analyzed with Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI). A cell line with E545K mutation was used as a positive control.

3 Results

3.1 Clinicopathologic Data

The group of patients was composed of 15 men and 6 women, with a median age of 68 years (range 42{76 years). Cases included 3 noninvasive and 18 inva-sive urothelial carcinomas. Noninvasive carcinomas corresponded to 1 case each of low-grade papillary urothelial carcinoma, high-grade papillary urothelial carcinoma, and \ at" in situ urothelial carcinoma. Invasive tumors included 3 pT1, 5 pT2, 6 pT3, and 4 pT4 urothelial carcinomas.

3.2 PIK3CA Mutation Analysis of exons 1, 9, and 20

In 2 cases (1 pT2 and 1 pT4), PCR reactions were not informative for any of the analyzed exons. In 3 additional cases (1 pT1, 1 pT3, and 1 pT4), the PCR reaction was not informative for exon 9. In all the remaining cases and exons, no evidence of PIK3CA mutations was found.

4. Discussion

Herein, we evaluated the presence of PIK3CA mu-tations in urothelial carcinoma of the bladder us-ing formalin- xed, para n-embedded archival tis-sues. Entire exons 1, 9, and 20 were covered using the PCR assay coupled with sequencing of the targeted exons. No PIK3CA mutations were identi ed in any of the 21 patients under examination. Our results di er from previous studies, in which PIK3CA muta-tions were identi ed in 13% to 27% of bladder tumors [6, 7, 11, 12, 8]. Since most of our cases were high-grade urothelial carcinomas, this discrepancy might be due to the association between PIK3CA muta-tion and lower tumor grade, as suggested by previous studies [6, 7, 8] and discussed below.

PIK3CA mutations have been previously char-acterized in bladder cancer. Lopez-Knowles et al sought for mutations in exons 9 and 20 of PIK3CA using para n-embedded tissue from 87 patients with bladder cancer [6]. Eleven (13%) tumors harbored PIK3CA mutations, and the prevalence was signi -cantly higher in low-grade tumors. In the same study, authors reported PIK3CA mutations in 26% of 43 patients with papillary urothelial neoplasm of low

Exon	PCR reaction	Primer	Sequence('5-'3)	Product size
1	1	Ex1-1F	GTTTCTGCTTTGGGACAACCAT	308 bp
		Ex1-1R	CGTAAGTGTTACTCAAGAAGCAG	
	2	Ex1-3F	CCCCCTCCATCAACTTCTTC	281 bp
		Ex1-2R	CTGTCTAAACCAATACCTTCGTAA	
9	3	Ex9F-new	TTGCTTTTTCTGTAAATCATCTGTG	243 bp
		Ex9R-new	CTAAAATGGAGATTCTCTGTTTCTTTTT	
20	4	Ex20-1F	TGGGGTAAAGGGAATCAAAAG	299 bp
		Ex20-1R-new	CTTTTGATGACATTGCATACATTCG	
	5	Ex20-2F-new	TGCCAATCTCTTCATAAATCTTTTC	312 bp
		Ex20-1R	GCAAAGACCGATTGCATAGG	
	6	Ex20-3F-new	GATTCCACACTGCACTGTTAATAAC	285 bp
		Ex20-2R	CAAAACAAAACAAAAATCCCC	

Table 1: PCR Primers for PIK3CA Mutation Analysis

malignant potential (PUNLMP). Similar results were found by Platt et al who identi ed PIK3CA muta-tions in 27% of 92 tissues samples [7]. They also observed a signi cant relationship with tumor grade but not with tumor stage.

Kompier et al evaluated 257 patients with blad-der tumor and found PIK3CA mutations in 24% of the tissue samples [8]. Although the prevalence of PIK3CA mutations was higher in low grade tumors, there was no statistical signi cance regarding tumor grade. In another recent study, Sj•odahl et al investi-gated the role of several genes in urothelial carcino-mas, including PIK3CA and PIK3R1 [10]. PIK3CA was mutated in 37 of 218 patients (17%), and was associated with low grade tumors. In contrast to pre-vious studies, higher proportion of tumors harboring PIK3CA mutation was seen in pTa tumors compared to pT1 tumors. However, the di erence did not hold between pT1 and muscle-invasive (>pT1) tumors. Our results con rm the feasibility of PIK3CA muta-tional analysis in formalin- xed, para n-embedded tissue samples and the rarity of PIK3CA mutations in high-grade and muscle-invasive urothelial carcino-mas.

The usefulness of PIK3CA mutations as prognos-tic factors has been also explored. Lindgren et al

classi ed urothelial carcinomas in 2 groups de ned by gene expression, and found that PIK3CA muta-tions are signi cantly more frequent in the subtype of tumor that is related to a better prognosis [9]. Due~nas et al found that the presence of PIK3CA mutations is signi cantly associated with reduced re-currence in patients with non-muscle invasive blad-der cancer [13]. Kim et al found that PIK3CA mu-tations are associated with improved recurrence-free survival and improved cancer-speci c survival in patients with high-grade urothelial carcinoma of uri-nary bladder treated by radical cystectomy [16]. In spite of these aforementioned studies, Kompier et al found that PIK3CA mutations are not indepen-dent predictors of tumor recurrence, tumor progres-sion, or disease speci c survival [8]. Nevertheless, in the study by Kompier et al, patients with re-currence showed a 100% concordance in the type of PIK3CA mutation between tumor samples. This nding suggests a potential utility for PIK3CA in the screening and follow-up of patients with bladder tumors harboring such mutation. Another direction for future studies could be the putative impact of PIK3CA mutations in the therapeutic response of patients with super cially-invasive urothelial carci-nomas treated locally.

In summary, we analyzed 21 formalin- xed, para n-embedded samples from patients with urothelial carcinomas of the urinary bladder, seeking to identify PIK3CA mutations. Exons 1, 9, and 20 were fully covered using a pinpoint DNA extraction technique and PCR assay. We did not nd any mu-tations in the PIK3CA gene. Most of our cases were high-grade and/or high-stage tumors, which could explain the absence of identi able mutations. The role of PIK3CA mutations as prognosticators of out-come or predictors of therapeutic response awaits fur-ther evaluation.

5   Financial Disclosure

5

REFERENCES

[1] Rebecca L Siegel, Kimberly D Miller, and Ahmedin Jemal. Cancer statistics, 2015. CA Cancer J. Clin., 65(1):5{29, January 2015.

[2] George J Netto. Molecular diagnostics in uro-logic malignancies a work in progress. Arch. Pathol. Lab. Med., 135(5):610{621, 2011.

[3] George J Netto and Jonathan I Epstein. Ther-anostic and prognostic biomarkers: genomic ap-plications in urological malignancies. Pathology, 42(4):384{394, 2010.

[4] Margaret A Knowles, Fiona M Platt, Rebecca L Ross, and Carolyn D Hurst. Phosphatidylinosi-tol 3-kinase (PI3K) pathway activation in blad-der cancer. Cancer Metastasis Rev., 28(3):305{ 316, 2009.

[5] T L Yuan and L C Cantley. PI3K pathway alter-ations in cancer: variations on a theme. Onco-gene, 27(41):5497{5510, 2008.

[6] Elena Lopez-Knowles, Silvia Hernandez, Nuria Malats, Manolis Kogevinas, Josep Lloreta, Al-fredo Carrato, Adonina Tardon, Consol Serra, and Francisco X Real. PIK3CA mutations are an early genetic alteration associated with FGFR3 mutations in super cial papillary blad-der tumors. Cancer Res., 66(15):7401{7404, 2006.

[7] Fiona M Platt, Carolyn D Hurst, Claire F Taylor, Walter M Gregory, Patricia Harnden, and Margaret A Knowles. Spectrum of phos-phatidylinositol 3-kinase pathway gene alter-ations in bladder cancer. Clin. Cancer Res., 15(19):6008{6017, 2009.

[8] Lucie C Kompier, Irene Lurkin, Madelon N M van der Aa, Bas W G van Rhijn, Theo H van der Kwast, and Ellen C Zwartho . FGFR3, HRAS, KRAS, NRAS AND PIK3CA mutations in blad-der cancer and their potential as biomarkers for surveillance and therapy. PLoS One, 5(11), 2010.

[9] David Lindgren, Attila Frigyesi, Sigurdur Gud-jonsson, Gottfrid Sj•odahl, Christer Hallden, Gu-nilla Chebil, Srinivas Veerla, Tobias Ryden, Wiking Ma nsson, Fredrik Liedberg, and Mat-tias H•oglund. Combined gene expression and genomic pro ling de ne two intrinsic molecular subtypes of urothelial carcinoma and gene signa-tures for molecular grading and outcome. Can-cer Res., 70(9):3463{3472, 2010.

[10] Gottfrid Sj•odahl, Martin Lauss, Sigurdur Gud-jonsson, Fredrik Liedberg, Christer Hallden, Gunilla Chebil, Wiking Ma nsson, Mattias H•oglund, and David Lindgren. A systematic study of gene mutations in urothelial carcinoma; inactivating mutations in tsc2 and pik3r1. PLoS One, 6(4), 2011.

[11] Chao Nan Qian, Kyle A Furge, Jared Knol, Huang Dan, Chen Jindong, Karl J Dykema, Eric J Kort, Aaron Massie, Kean Khoo Sok, Kristin Vanden Beldt, James H Resau, John Anema, Richard J Kahnoski, Hans Morreau, Philippe Camparo, Eva Comperat, Mathilde Si-bony, Yves Denoux, Vincent Molinie, Annick Vieillefond, Charis Eng, Bart O Williams, and Tean Teh Bin. Activation of the PI3K/AKT pathway induces urothelial carcinoma of the re-nal pelvis: Identi cation in human tumors and con rmation in animal models. Cancer Res., 69(21):8256{8264, 2009.

[12] Reza R Serizawa, Ulrik Ralfki r, Kenneth Steven, Gitte W Lam, Sven Schmiedel, Joachim Sch•uz, Alastair B Hansen, Thomas Horn, and Per Guldberg. Integrated genetic and epigenetic analysis of bladder cancer reveals an additive di-agnostic value of FGFR3 mutations and hyper-methylation events. Int. J. Cancer, 129(1):78{ 87, 2011.

[13] Marta Due~nas, Monica Mart nez-Fernandez, Ramon Garc a-Escudero, Felipe Villacampa, Miriam Marques, Cristina Saiz-Ladera, Jose Duarte, Victor Mart nez, Mª Jose Gomez, Mª Luisa Mart n, Manoli Fernandez, Daniel Castellano, Francisco X. Real, Jose L. Rodriguez-Peralto, Federico De La Rosa, and Jesus M. Paramio. PIK3CA gene alterations in bladder cancer are frequent and associate with reduced recurrence in non-muscle inva-sive tumors. Mol. Carcinog., pages n/a{n/a, December 2013.

[14] The Cancer Genome Atlas Research Net-work. Comprehensive molecular characteriza-tion of urothelial bladder carcinoma. Nature, 507(7492):315{22, March 2014.

[15] Sherri Z Millis, David Bryant, Gargi Basu, Ryan Bender, Semir Vranic, Zoran Gatalica, and Nicholas J Vogelzang. Molecular pro ling of in ltrating urothelial carcinoma of bladder and nonbladder origin. Clin. Genitourin. Can-cer, 13(1):e37{49, February 2015.

[16] Philip H Kim, Eugene K Cha, John P Sfakianos, Gopa Iyer, Emily C Zabor, Sasinya N Scott, Irina Ostrovnaya, Ricardo Ramirez, Arony Sun, Ronak Shah, Alyssa M Yee, Victor E Reuter, Dean F Bajorin, Jonathan E Rosenberg, Niko-laus Schultz, Michael F Berger, Hikmat A Al-Ahmadie, David B Solit, and Bernard H Bochner. Genomic Predictors of Survival in Pa-tients with High-grade Urothelial Carcinoma of the Bladder. Eur. Urol., August 2014.