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RESEARCH

Implication of RAF and RKIP Genes in Urinary Bladder Cancer

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Abstract RKIP has been shown to regulate the RAS-RAF-MEK-ERK kinase cascade acting as modulator of apoptosis and metastasis in prostate cancer. Our goal was to examine the expression of the RAF (*A-RAF*, *B-RAF* and *RAF-1*) and RKIP genes in urinary bladder cancer. Microarray analysis and qPCR was employed to investigate the expression of *RAF* and *RKIP*, in 30 patients with transitional cell carcinoma (TCC) of the urinary bladder vs. the corresponding levels of adjacent normal tissue. Computational analysis was also performed on Gene Expression Omnibus (GEO) datasets, to unravel differences in the expression of *RAF* or *RKIP* between tumor and control samples, and between superficial and muscle invasive tumors. Microarray analysis revealed >2-fold expression of *BRAF* and *RKIP* in T2, T3, grade III tumors vs. controls. *B-RAF* over-expression was verified by qPCR in pT1, grade III tumors vs. their normal counterparts ($p=0.016$). qPCR revealed a significant *RKIP* reduction in TCC vs. normal tissue ($p=0.002$ and $p<0.001$ for T1, grade II and Ta-T1, grade III, respectively); All RAF genes were positively correlated among each other (*A-RAF*/*B-RAF*, $p=0.003$; *A-RAF*/*RAF-1*, $p<0.001$; *B-RAF*/*RAF-1*, $p=0.050$), whereas *B-RAF* was negatively correlated with *RKIP* in TCC ($p=0.050$). Further computational analysis revealed different expression profiles for the genes of interest, among muscle invasive carcinomas, superficial TCCs, cystectomy specimens and normal tissue. The reduced *RKIP* mRNA levels in TCC and the elevated levels of *B-RAF* in pT1, grade III tumors vs. normal tissue, corroborate that these genes are involved in the pathogenesis of urinary bladder cancer.

Keywords RAF family genes · RKIP · Microarrays · qPCR · Computational analysis · Transitional cell carcinoma of the urinary bladder

Statement The reduced *RKIP* mRNA levels in TCC of the bladder and the elevated levels of *B-RAF* in pT1, grade III tumors vs. normal tissue, provide evidence for the first time, that these genes are involved in the pathogenesis of urinary bladder cancer.

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Introduction

Bladder cancer is the fifth most commonly diagnosed non-cutaneous solid malignancy, and the second most commonly diagnosed genitourinary malignancy in the United States with an annual incidence of approximately eighteen cases per 100,000 population [1]. In Europe, Age-standardised incidence rates (ASR) higher than 40 per 100,000 for males have been reported. Approximately 90% of malignant tumors arising in the urinary bladder are of epithelial origin, the majority being transitional cell carcinomas. Nearly 80% of patients who initially present with bladder urothelial carcinoma have tumors confined to the mucosa or submucosa, so-called superficial non-muscle-invasive bladder cancers; the rest initially present as invasive disease or muscle-

invasive carcinomas. Bladder tumors represent a heterogeneous group of cancers, which includes those that are papillary in nature and limited to the mucosa (non-invasive, stage Ta), high grade and flat confined to the epithelium (non-invasive, stage Tis), invasive into the lamina propria or submucosa (early invasive, stage T1), invasive into the muscularis propria or beyond (invasive, stage T2-T4) [2].

The RAS-RAF-MEK-ERK pathway is an evolutionary conserved signalling mode that regulates many fundamental cellular processes, such as differentiation, proliferation, survival, motility, and transformation [3]. The RAF proteins are highly conserved serine/threonine protein kinases that activate mitogen-activated protein kinase kinase (MEK), which in turn activates the mitogen-activated protein kinase (MAPK) pathway. Inappropriate and/or continuous activation of this pathway provides a potent promitogenic force resulting in abnormal proliferation and differentiation in many human cancers [4].

The mammalian RAF family consists of three genes: A-RAF, B-RAF, and RAF-1 (also known as C-RAF), which have similar but non-overlapping cellular functions [5]. As serine/threonine kinases, RAF proteins phosphorylate serine and threonine residues on essential modulatory proteins downstream of RAS. Each RAF species has a distinct expression profile in tissues, which suggests that individual RAF isoforms perform clearly defined functions [5]. In previous studies on animal tissues, RAF-1 exhibited the highest levels in striated muscle, cerebellum and fetal brain [6], B-RAF was predominantly found in neural tissues [7] while A-RAF was expressed at high levels not only in the epididymis and ovary [6] but also in the bladder, kidney, intestine, heart, spleen, thymus and cerebellum [8].

RKIP, also known as Phosphatidylethanolamine-binding protein 1 (PEBP-1) or Prostatic binding protein (PBP), was initially characterized to be involved in many different physiologic activities, including reproduction and neurophysiology [9]. Recent findings, however, have identified RKIP as a modulator of apoptosis and metastasis through regulation of important signalling cascades, i.e., the RAF-MEK-ERK kinase cascade, G protein-coupled receptors, and the NF-κβ pathway [10–12]. RKIP blocks RAF-induced phosphorylation of MEK, via direct interaction with RAF-1 kinase, and consequently the activation of ERK [11]. RKIP also has weak binding affinity to MEK-1 and ERK-2, interfering with downstream phosphorylation events. In addition to its modulation of RAF signalling, RKIP inhibits NF-κβ activity by interacting with upstream NF-κβ activators such as the NF-κβ-inducing kinase (NIK) and TGF-β-activated kinase 1 (TAK1). RKIP expression has been shown to be down-regulated in metastatic prostate cancers, and it was suggested that the loss of RKIP levels promotes the metastatic potential of prostate cancer cells [13]. Furthermore, a recent study showed a decrease in RKIP expression in malignant melanoma and the absence of RKIP expression in melanoma metastases [14].

In the present study, microarray analysis and qPCR were employed in order to detect and verify the expression levels of the RAF oncogene family (*A-RAF, B-RAF* and *RAF-1*) and *RKIP*, in patients with urinary bladder cancer.

Materials and Methods

Study Design and Clinicopathological Data

Paired tumor and normal tissue samples from a consecutive series of 30 patients with newly diagnosed bladder cancer (BC) undergoing transurethral bladder tumor resection at the Department of Urology of the "Asklipieio" General Hospital in Athens were prospectively studied for RAF and RKIP gene expression analysis. The patients studied were of advanced age (72.2±10.6 years). The majority (26/30, 87%) were smokers or former smokers, while 19 (63%) were characterized by some level of occupational exposure to agents associated with BC such as paints and chemicals (Table 1).

Tumor specimens were classified and graded by the same pathologist. Histological grading was performed according to the 2004 WHO/International Society of Urologic Pathology (ISUP) classifications [2]. Tumor stage was assessed according to the 2002 American Joint Committee on Cancer staging system.

Written informed consent was obtained from the patients included in this study. The study protocol was approved by the Ethics Committee of the University of Crete. Eligibility criteria were: electively resected primary BC and the availability of DNA from normal and tumor tissue for biomolecular analyses. Exclusion criteria were: a history of previous neoplasms and chemotherapy or radiation therapy prior to surgery.

Tissue samples were obtained at surgery from the tumor and the three grossly normal selected sites (cold cup biopsies) were: posterior wall, trigone and area adjacent to the tumor. Parts of the resected normal samples were sent for histopathological analysis. Tumor and normal tissues were frozen immediately in liquid nitrogen, transported and stored at −80°C until DNA extraction.

Patients with non-muscle-invasive BC were followed-up with periodical cystoscopic examinations and intravesical treatment as indicated. Patients with invasive BC were offered radical cystectomy with or without systemic chemotherapy. After a mean follow-up of 24±3 months, 8 (26.6%) patients had recurrent tumors. In Ta/T1 tumors the frequency of recurrence was 29.4% (5/17) compared with 23% (3/13) of T2-T3 tumors. In patients with non-muscle-invasive BC, the progression rate was 11.1 and 22.2% for grade II and III tumors, respectively. All recurrences were confirmed by biopsy.

Table 1 Clinicopathological characteristics of the patients

Subjects (n)	30[a]
Gender	
Male	27
Female	3
Age (years)	
mean	72.2
range	44–86
Smoking status[b]	
NS	4
FS	8
S	18
Occupational exposure[c]	
yes	19
no	11
Tumor stage	
pTa	1
pT1	12
pT1a	4
pT1b	1
pT2	2
pT2a	1
pT2b	5
pT2+in situ	3
pT3a	1
Tumor grade (WHO 1973)	
I	0
II	10
III	20
Tumor grade (WHO/ISUP 2004)	
Low	8
High	22

[a] 30 TCCs and 30 adjacent normal tissue

[b] *NS* non-smoker, *FS* former smoker, *S* smoker

[c] Exposure to chemicals, paints, pesticides, petroleum, ink, etc

Microarray Analysis

Samples from 10 human BCs and 5 normal tissues were selected for microarray analysis. Three T1 grade II, three T1 grade III and four T2, T3 grade III samples were compared with five randomly chosen adjacent normal tissues. RNA extraction was performed with TRIzol® reagent (Invitrogen, Carlsbad, CA), as described by the manufacturer. RNA from the samples was further processed to cRNA (antisense amplified RNA) as previously described [15]. Microarray experiments were performed on the CodeLink Human Whole Genome Platform (AppliedMicroarrays Inc., Tempe, AZ) [16]. For the analysis we used a reference experimental setup as previously reported [17]. Raw data were further analyzed with different algorithms including Global Median, Loess and Rank Invariant. Among those the Global Median was selected for further analysis. Clustering was performed by hierarchical clustering, *k*-Means, Self-Organizing-Maps (SOMs) using the Genesis software (TU, Gratz, Austria) [18].

RNA Extraction and Reverse Transcription

Thirty bladder cancer-normal paired tissue specimens were homogenized in TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer followed by chloroform addition and centrifugation. Total-RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μl DEPC-treated water. RNA concentration was calculated using the NanoDrop™ 1000 Spectrophotometer. Reverse transcription reactions for the preparation of first-strand cDNA from 1 μg of total RNA were performed using the RETROscript® Kit, according to the manufacturer's protocol (Ambion, Austin, TX). Random hexamers were used as amplification primers.

Validation of Differential Gene Expression

Validation of the RAF and RKIP differential gene expression was performed using qPCR. All samples were treated collectively from the mRNA extraction and its conversion to cDNA, to the qPCR analysis. Moreover, all the qPCR primer sets were optimized for their concentration and optimal annealing temperature, so as to give the smallest Cts with the highest ΔRns. Transcribed products were subjected to qPCR assay with the Maxima SYBR green qPCR Mastermix (2X), according to the manufacturer's instructions (Fermentas, Glen Burnie, MD) in a Mx3000P programmable thermal controller apparatus (Stratagene, La Jolla, CA). Two genes were used as internal controls: GAPDH, and B-Actin. However, comparing the expression stability measure *M* for each gene with the geNorm software, GAPDH was determined to be the most stable housekeeping gene with which to normalize RAF and RKIP mRNA expression levels. All primer pairs were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA along with cDNA (Fig. 1). Primer sequences and their amplicon lengths were as follows: *A-RAF*, 5′-CGTCAAAG TATACCTGCCCAACA-3′ (sense) and 5′-GATTTAGAC CCCGCACCTTCA-3′ (antisense), (107 bp); *B-RAF*, 5′-AG AAAGCACTGATGATGAGAGG-3′ (sense) and 5′-GG AAATATCAGTGTCCCAACCA-3′ (antisense) (101 bp); *RAF-1*, 5′-ACTGCCTTATGAAAGCACTCAAG-3′ (sense) and 5′-ACGCAGCATCAGTATTCCAATC-3′ (antisense) (120 bp); *RKIP*, 5′-AGACCCACCAGCATTTCGTG-3′ (sense) and 5′-GCTGATGTCATTGCCCTTCA-3′ (antisense) (150 bp); *GAPDH*, 5′-GGAAGGTGAAGGTCGG AGTCA-3′ (sense) and 5′-GTCATTGATGGCAACAA

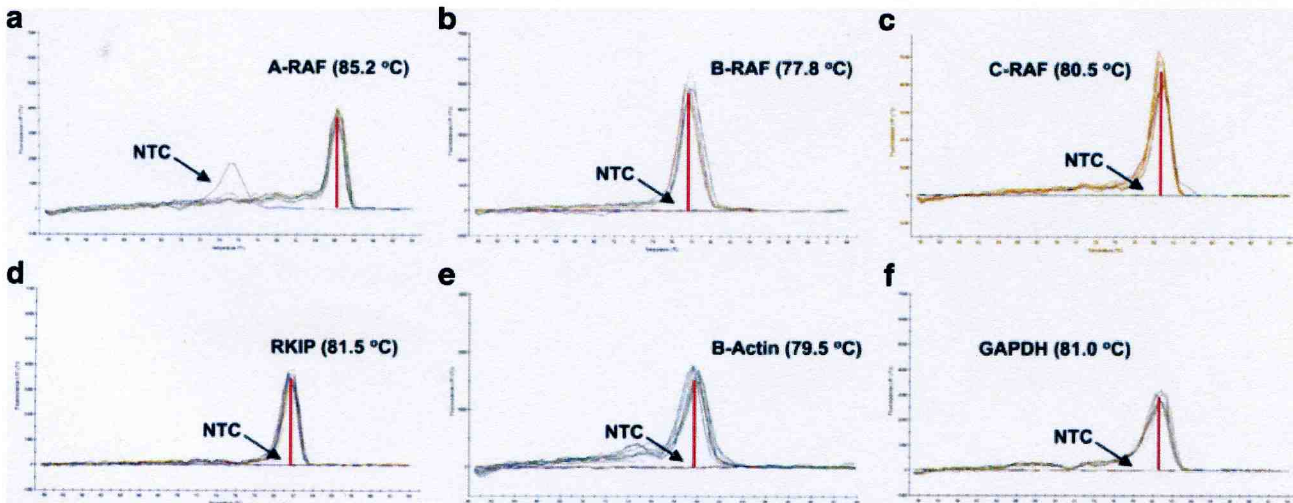

Fig. 1 Melt Curve analysis for the RAF family and RKIP genes. Single peaks for all genes and absence of peaks for the non-template controls (NTC) demonstrates the exclusion of any genomic DNA contamination and the specificity of the PCR products

TATCCACT-3′ (antisense) (101 bp); *B-Actin*, 5′-CGGC ATCGTCACCAACTG-3′ (sense) and 5′-GGCACACG CAGCTCATTG-3′ (antisense) (70 bp). The optimized annealing temperatures for each primer set were: 56°C for *A-RAF*, 53°C for *B-RAF*, 57°C for *RAF-1*, 55°C for *RKIP*, 60°C for *GAPDH* and 60°C for *B-Actin*. All reactions were performed in triplicates. PCR products were further confirmed by analysis on 2% ethidium bromide-stained agarose gels. Normalized values to GAPDH, ΔCts, were initially calculated using the equation: $\Delta Ct_{sample} = Ct_{GOI} - Ct_{GAPDH}$ (where $_{GOI}$ is the gene of interest). The ΔΔCt values and the expression of the normalized to GAPDH RAF and RKIP genes in TCC vs. the normal tissue, was calculated as previously described [19]. A two-fold increased or decreased expression was considered significant.

Computational Analysis

Computational analysis was also performed in order to further understand whether differences of *RAF* and *RKIP* transcript levels occur among Ta-T1 stage [superficial transitional cell carcinomas (sTCC) with or without surrounding in situ lesions (CIS)], T2-T4 stage [muscle invasive carcinomas (mTCC) and cystectomy specimens] and normal tissue; as well as among tumors of grade I (low grade), grade II-III (high grade) and normal tissue. Two publicly available Gene Expression Omnibus (GEO) datasets were analyzed [20, 21]. The gene expression patterns of *RAF* and *RKIP* were extracted from the normalized datasets, the results were expressed as mean levels of the $\log_2$ intensity and were statistically compared by the Mann-Whitney *U* test (Fig. 2).

Statistical Analysis

The non-parametric Pearson's correlation was used to examine pair-wise correlations between the GOI mRNA levels and their association with continuous variables (age, smoking, histologic type and tumor stage). The Mann-Whitney *U* test was used to examine the differences between the expression levels of the genes among TCCs of different stage and grade, and normal tissue. All statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level ($p<0.05$).

Results

Unsupervised cluster analysis of DNA microarray data showed a clear distinction between BC and control samples. Genes with at least 2-fold differential expression in BC vs. control (~57,000 transcripts) and in low *vs.* high grade tumors were identified and ranked (unpublished data). Emphasis was given to the GOI: *A-RAF*, *B-RAF*, *RAF-1* and *RKIP*. x-fold expression of the GOI microarray data is presented on Fig. 3. Microarray analysis revealed >2-fold expression of *BRAF* and *RKIP* in T2, T3, grade III tumors vs. controls (*B-RAF*, 2.97±3.70; *RKIP*, 2.90±2.04). T1 grade III tumors also exhibited increased *RKIP* levels compared to the controls (2.29±0.35). *A-RAF* mRNA levels were 2.28-fold±1.72 vs the controls, in T1 grade II tumors. T1 grade II and III tumors did not reveal more than 2-fold or less than 0.5-fold expression, compared to the controls, either for *RAF* or for *RKIP*.

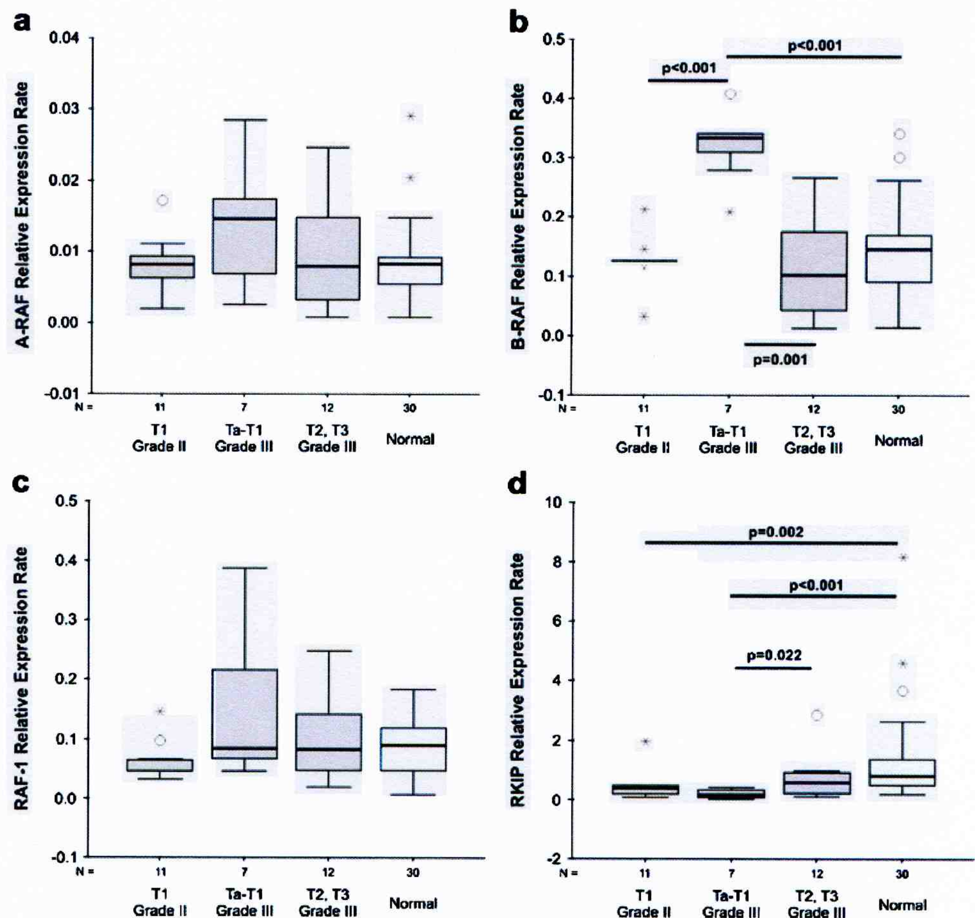

Fig. 2 *A-RAF* and *RAF-1* presented equal expression levels in TCC and controls. pTa-pT1 grade III tumors exhibited significantly higher *B-RAF* expression vs. the normal tissue ($p<0.001$; Mann-Whitney *U* test), as well as compared to pT1 grade II ($p<0.001$; Mann-Whitney *U* test) or pT2, pT3 grade III tumors ($p=0.001$; Mann-Whitney *U* test). The adjacent normal tissue presented higher RKIP mRNA levels compared to the TCC. Boxplots show the 25th, 50th (median), and 75th percentile values. Whiskers show the minimum and maximum values. TCC, transitional cell carcinoma

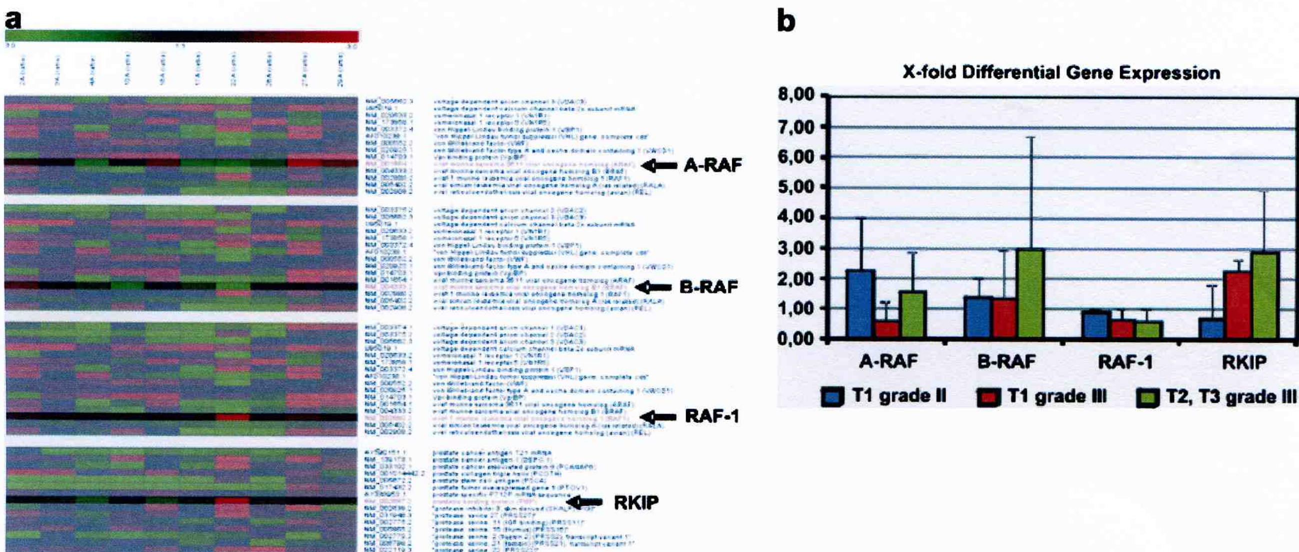

Fig. 3 A. A total of ~57,000 transcripts were analyzed using the *CodeLink Human Whole Genome* Platform (*Applied Microarrays* Inc.). Red: Under-expressed in tumors (<3-fold); green: over-expressed in tumors (>3-fold); black: not differentially expressed. B. x-fold differential gene expression for the genes of interest: *A-RAF*, *B-RAF*, *RAF-1* and *RKIP* (*PBP*)

Table 2 The expression status of the RKIP and RAF genes in TCC, compared to adjacent normal tissue (N), stated as x-fold difference. Red color denotes over-expression, green denotes equal expression and blue denotes under-expression between TCC and the adjacent normal tissue

						x-fold difference (TCC/N)			
Patient No	Age/Sex	Smoke	Occup. Exposure	Stage	Grade	A-RAF	B-RAF	RAF-1	RKIP
1	68/M	FS	no	pT1	III	0.84	1.02	1.35	0.24
2	86/M	NS	no	pT1	III	24.08	11.00	6.02	0.01
3	69/M	S	no	pT2b	III	5.15	2.83	40.68	0.05
4	81/M	NS	yes	pT1	II	0.97	1.02	0.38	0.09
5	83/M	FS	yes	pT1	II	6.45	7.62	0.66	0.05
6	50/M	S	no	pT1	II	1.26	0.88	0.81	0.24
7	78/M	S	yes	pT1	III	0.28	0.70	0.44	0.02
8	86/F	S	yes	pT2b	III	0.65	0.37	0.78	0.09
9	78/F	FS	yes	pT1	II	2.04	1.46	1.69	0.13
10	70/M	S	yes	pT1b	III	3.34	2.16	2.45	0.53
11	80/M	S	yes	pT1	II	0.23	0.54	1.06	3.78
12	68/M	FS	yes	pT1a	II	1.58	0.68	1.49	0.36
13	69/M	S	yes	pT1a	II	1.22	1.02	3.04	0.17
14	69/F	S	no	pT1a	II	4.64	1.02	6.48	0.16
15	52/M	S	yes	pT2b	III	3.81	1.72	0.95	0.08
16	85/M	FS	yes	pT1a	II	0.17	0.10	0.28	0.38
17	80/M	NS	no	pT1	II	1.87	1.65	6.48	0.32
18	80/M	FS	yes	pT1	III	1.78	4.47	1.21	0.20
19	76/M	S	no	pT1	III	0.88	1.39	0.73	1.15
20	56/M	S	no	pT2 + in situ	III	3.97	3.27	3.78	2.07
21	70/M	S	yes	pT2b	III	0.14	0.12	6.96	1.54
22	77/M	NS	yes	pT3a	III	0.21	0.12	0.26	0.51
23	63/M	FS	no	pT2a	III	0.62	0.52	0.31	0.63
24	80/M	S	yes	pT2 + in situ	III	4.96	1.49	4.03	0.06
25	78/M	S	yes	pT2b	III	0.52	0.27	0.31	0.97
26	75/M	S	no	pT2 + in situ	III	0.40	0.78	0.85	2.10
27	44/M	S	no	pT1	III	0.75	1.00	0.40	0.93
28	76/M	FS	yes	pTa	III	2.45	1.43	0.97	0.58
29	72/M	S	yes	pT2	III	1.39	1.54	0.85	2.68
30	68/M	S	yes	pT2	III	0.47	1.02	0.94	0.32

Regarding the validation of the GOI differential expression by qPCR, *RAF* and *RKIP* were expressed in all TCCs and adjacent normal tissues. Similarly to the microarray experiments, three groups of expression were defined according to each gene level of expression in TCC vs. the adjacent normal tissue (<0.5, under-expression; 0.5–2.0, equal expression; >2.0, over-expression) (Table 2). Seven TCCs (23%) presented over-expression of only one RAF gene, and further seven (23%) exhibited over-expression in two and/or all three RAF genes.

A-RAF and *RAF-1* presented a similar expression pattern, exhibiting over-expression in 33% (mean±SD, 6.09±6.46) and 30% (mean±SD, 3.39±7.61) of the TCC samples, respectively. The majority of the TCC samples exhibited equal *A-RAF* and *RAF-1* mRNA levels vs. the normal tissue (43%; mean±SD, 1.10±0.45 and 47%; mean± SD, 1.02±0.31, respectively); thus, bearing no statistically significant difference between TCCs and normal tissues (Fig. 2). Nineteen TCCs (63%) presented equal *B-RAF* transcript levels with the adjacent normal tissue (mean±SD, 1.10±0.37), whereas six TCCs exhibited over two-fold *B-RAF* expression vs. their normal counterparts (mean±SD, 5.23±3.43, respectively). Interestingly, pTa-pT1 grade III tumors showed significantly higher *B-RAF* expression vs. the normal tissue ($p<0.001$; Mann-Whitney U test), as well as compared to pT1 grade II ($p<0.001$; Mann-Whitney U test) or pT2, pT3 grade III tumors ($p=0.001$; Mann-Whitney U test) (Fig. 2).

The majority of the TCC samples (18/30, 60%), exhibited less than 0.5-fold *RKIP* mRNA levels (mean± SD, 0.17±0.12) vs. the controls, and the difference was statistically significant ($p=0.002$ for pT1 grade II and $p<0.001$ for pTa-pT1 grade III vs. controls; Mann-Whitney U test). Moreover, tumors of pT2, pT3 grade III exhibited significantly higher *RKIP* expression levels compared to tumors of pTa-pT1 grade III ($p=0.022$; Mann-Whitney U test) (Fig. 2). The corresponding x-fold values of each gene's expression in every TCC sample vs. its adjacent normal tissue, are provided in Table 2.

The Pearson's correlation test was used in order to evaluate the co-expression patterns of *RKIP* and *RAF*, separately in TCC and normal tissue groups. Positive

correlations among all three RAF genes were detected, both in TCC and the adjacent normal tissue. Specifically in TCC, *A-RAF* was positively correlated with *B-RAF* ($p=0.003$, Correlation Coefficient=0.520) and *RAF-1* ($p<0.001$, Correlation Coefficient=0.650). Moreover, *B-RAF* was positively correlated with *RAF-1* ($p=0.050$, Correlation Coefficient=0.360), and negatively correlated with *RKIP* ($p=0.050$, Correlation Coefficient=−0.360) in urinary bladder cancer. In the normal tissue, *A-RAF* was also positively correlated both with *B-RAF* ($p<0.001$, Correlation Coefficient=0.834) and *RAF-1* ($p=0.001$, Correlation Coefficient=0.591), whereas *B-RAF* was further positively correlated with *RAF-1* ($p<0.001$, Correlation Coefficient=0.614). No negative correlation could be detected between RKIP and the RAF genes in normal tissue, apart from a marginally negative correlation detected between *A-RAF* and *RKIP* ($p=0.054$, Correlation Coefficient=−0.355).

Computational analysis of expression profile of the RAF and RKIP genes in urinary bladder cancer, was also performed in microarray data extracted from the GEO Datasets GSE3167 [20] and GSE7476 [21]. Dyrskjøt et al. performed microarray analysis in bladder biopsies of superficial transitional cell carcinomas with or without surrounding carcinoma in situ (CIS) lesions and muscle invasive carcinomas (mTCC). Their results provide insight into which tumors in early stage bladder cancer are likely to progress [20]. The results of our computational analysis, including all the statistically significant differences detected among sTCC with/without CIS, mTCC, cystectomy specimens and normal tissue, are depicted in Fig. 4. *A-RAF* mRNA levels were higher in sTCC without CIS vs. normal tissue, whereas *RAF-1* exhibited the reverse pattern of expression. Computational analysis of the GSE3167 dataset, also suggested lower *B-RAF* expression levels in sTCC with CIS vs. normal tissue, and in mTCC vs. normal tissue, whereas *RKIP* exhibited higher rates of expression both in sTCC and mTCC vs. their normal counterparts. Mengual et al. analyzed the gene expression profiles of the most important pathological categories of bladder cancer in order to detect potential marker genes (GSE7476) [21]. Analysis of their dataset revealed quite similar results with our microarray and qPCR data, for *A-RAF, B-RAF* and *RAF-1.* However, pT1 high grade tumors exhibited higher *RKIP* mRNA levels vs. normal tissue. Analysis of our microarray data revealed a similar *RKIP* expression profile. However, due to the small number of samples analyzed in that dataset, and given the contradictory results provided by the qPCR analysis, *RKIP* over-expression should be carefully taken into consideration. All the statistically significant differences in the mRNA levels of *A-RAF, B-RAF, RAF-1* and *RKIP* between urinary bladder cancer and control samples, as well as between superficial and muscle invasive tumors, are depicted in Fig. 4.

Discussion

The signalling pathway RAS-RAF-MEK-ERK has a pivotal role in the regulation of the cell processes. Any abnormality in the regulation of the pathway can be caused by primary molecular defects occurring in malignant cell proliferation [4].

This study evaluated the mRNA levels of the three RAF and RKIP genes, in TCC of the bladder and the adjacent normal tissue. We detected significantly reduced *RKIP* mRNA levels in the former, compared with the latter. Moreover, *B-RAF* mRNA levels were significantly increased in pT1 grade III tumors vs. normal tissues. The majority of TCCs exhibited equal *A-RAF* and *RAF-1* expression levels vs. the normal tissues.

A-RAF over-expression has been detected in the past in murine bladder cancer. Increased expression levels have also been found in other urogenital tissues, such as kidney, ovary, prostate and epididymis [8]. In the present study we did not detect significantly increased *A-RAF* mRNA levels in TCC of the urinary bladder vs. normal tissue by qPCR; however our computational analysis performed in two GEO datasets corroborates its important role in human bladder cancer, as previously stated [8].

The vast majority of the studies investigating *B-RAF* mutation frequency in different types of cancer, including melanoma and colorectal adenocarcinoma [22–29], focus mainly at the thymine (T) 1799 nucleotide. This is the first study measuring the expression status of *B-RAF* in TCC of the urinary bladder. We and others, have previously reported either a non-significant percentage, or complete absence of *B-RAF* mutations in urothelial bladder carcinoma or renal cell carcinoma [30, 31]. Apart from the elevated *B-RAF* levels in the pTa-pT1 grade III tumors (detected both in our qPCR data and in the computational analysis of the GSE7476 dataset), we did not find any statistically significant difference between normal tissue and the rest of the TCCs. On the contrary, computational analysis of the GSE3167 dataset, revealed lower *B-RAF* levels in the pTa-pT1 and in the pT2-pT4 tumors vs. controls. The present data, along with those reporting lack of *B-RAF* mutations, can only suggest that *B-RAF* alterations either in DNA or RNA level, do not seem to be a very frequent event in TCC of the urinary bladder. In contrast, significant spotlight has been thrown on *H-RAS,* another significant activator of the MAP kinase pathway, since a significant proportion of TCCs has been found to harbour *H-RAS* codon 12 mutations [19].

In a recent study by Mhawech-Fauceglia a et al. [32], RAF-1 protein expression was found to be significantly associated with tumor grade. However, in the present study we did not detect such a correlation. *RAF-1* is ubiquitously expressed in most tissues, but it has exhibited highest levels

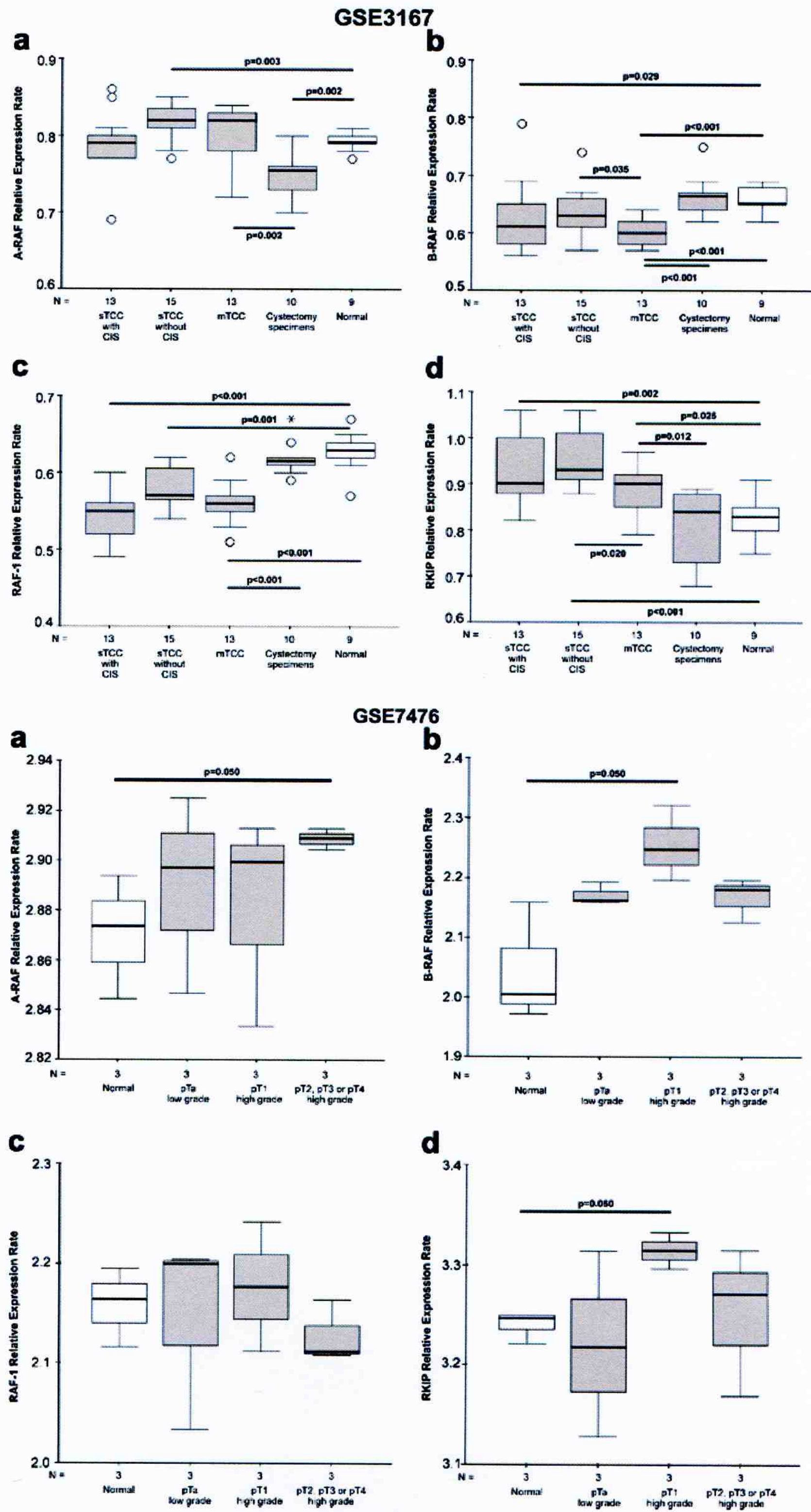

Fig. 4 Computational analysis of expression profile of the RAF and RKIP genes in urinary bladder cancer, was performed in microarray data extracted from the GEO Datasets GSE3167 and GSE7476

in striated muscle, cerebellum and fetal brain [7]. These data further support the findings of Simon et al., who revealed that the chromosomal region 3p25 (*RAF-1*) is amplified in 4% of human bladder tumors. Thus it seems that *RAF-1* over-expression could be linked to amplification. Moreover, *RAF-1* was recently characterized as the predominant RAF isoform responsible for regulating cellular growth in ovarian cancer cells [33], further enhancing the possibility that its activation plays a role in bladder cancer development. The study of Simon et al. [34] also showed that the RAF-1 gene was lost in 2.2% of human tumors and this loss correlated with tumor grade and stage. Notably, RAF-1 has been implicated in other signalling pathways, especially survival, where it has been shown to bind to apoptosis-stimulating kinases such as mammalian sterile 20-like kinase 2 (MST2) and apoptosis-signal-regulating kinase 1 (ASK1) [35], and to kinases such as Roka [36], which controls cytoskeletal rearrangements. RAF-1 is also implicated in survival mediated by Bcl-2 and NF-κβ activation, although the mechanisms of regulation are unclear.

Another important finding of the present study was the significant correlations detected among *A-RAF*, *B-RAF* and *RAF-1*, in urinary bladder cancer. The correlations between *RAF-1* and *B-RAF* might probably derive from the activation of the first through the latter. The exact mechanism implies a direct binding and trans-phosphorylation of *RAF-1* by *B-RAF* [37]. The correlation between *A-* and *B-RAF*, could probably be explained by a similar mechanism of *A-RAF* activation by *B-RAF*. In this model, all RAF proteins could hypothetically signal MEK. Similar correlations among the three RAF genes were also found in the adjacent normal tissue. However, the importance of a possible RAF cross-talk network in normal cell signalling is still unclear.

RKIP expression has been reported to be diminished in many tumors and completely absent in metastases [13, 38, 39]. As regards bladder carcinogenesis, recent array CGH analysis from Blaveri et al., showed significant increases in copy number alterations and genomic instability with increasing stage and with outcome, as well as they revealed higher expression in the superficial tumors than the invasive cancers [40]. Our present results are in good agreement with these studies, since we detected a statistically significant decrease in the RKIP expression levels between TCC and normal tissue. However, further investigation including immunostaining of tissues with RAF and RKIP specific antibodies should provide validation of our results. RKIP has been well-studied in prostate cancer cells, in which loss of RKIP expression confers a metastatic phenotype. Keller et al. concluded that RKIP expression inhibits prostate cancer metastasis [41]. Woods et al. recently reported that the loss of RKIP, as seen in primary prostate cancer tumors and metastases, confers protection against radiation-induced apoptosis [42]. The results of Li et al., indicate that RKIP is also a metastasis suppressor gene of human epithelial ovarian cancer [43].

RKIP has been shown to bind and inhibit B-RAF, apart from RAF-1, thus disrupting the interaction between RAF and its substrate MEK [12]. In good accord with these data, we detected a negative correlation between the expression of *RKIP* and *B-RAF* in TCC. Apart from the MEK cascade, RKIP blocks the activation of several signalling pathways, G-proteins and NF-κβ [38].

Given the high proportion of cancers with constitutively activated RAF, RAS mutations, or growth factor hyperactivity, which result in increased signalling through RAF, RAF is a good target for therapeutic development. Although there have been many attempts to develop therapeutics against RAF, most efforts have been directed at RAF-1 rather than B-RAF [44]. However, the most recent published studies regarding RKIP indicate that this molecule seems to be a novel, promising target for therapeutic development. Chaterjee et al. [45] reported that RKIP may represent a novel effector of signal transduction pathways leading to apoptosis and a prognostic marker of the pathogenesis of human cancer cells and tumors after treatment with clinically relevant chemotherapeutic drugs.

The lower mRNA levels of *RKIP* in TCC vs. normal tissue, as well as the higher levels of *B-RAF* in pT1, grade III carcinomas compared to controls, provide evidence that these factors seem to be reversely involved in the pathogenesis of urinary bladder cancer. The unravelling of the exact role that *RAF* and *RKIP* genes play in urinary bladder cancer, can provide specific points at which novel therapies could be targeted against it.

Conflict of interest statement All authors state that they do not have any financial interests or connections, direct or indirect that might raise the question of bias in the present work.

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