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PI3K signaling pathway is activated by *PIK3CA* mRNA overexpression and copy gain in prostate tumors, but *PIK3CA*, *BRAF*, *KRAS* and *AKT1* mutations are infrequent events

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The phosphatidylinositol 3-kinase (PI3K)–AKT and RAS–MAPK pathways are deregulated in a wide range of human cancers by gain or loss of function in several of their components. Our purpose has been to identify genetic alterations in members of these pathways in prostate cancer. A total of 102 prostate tumors, 79 from prostate cancer alone (group G1) and 23 from bladder and prostate cancer patients (G2), are the subject of this study. In 20 of these 23, the bladder tumors were also analyzed. *PIK3CA, KRAS, BRAF* and *AKT1* mutations were analyzed by direct sequencing, and *BRAF* also by pyrosequencing. *PIK3CA* quantitative mRNA expression and fluorescence *in situ* hybridization (FISH) gains were tested in 25 and 32 prostate tumors from both groups (G1 and G2), respectively. Immunohistochemistry for pAKT was performed in 55 prostate tumors. Of 25 prostate tumors, 10 (40%) had *PIK3CA* mRNA overexpression that was statistically associated with Gleason score ≥ 7 ($P = 0.018$). *PIK3CA* copy gain was detected in 9 of 32 (28%) prostate tumors. Of 20 bladder tumors, 3 (15%) displayed mutations in *PIK3CA*, *KRAS* and *AKT1*, the corresponding prostate tumors being *wt.* We also detected a previously not reported *PIK3CA* polymorphism (IVS9 + 91) in two prostate tumors. In all, 56% of prostate tumors overexpressed pAKT. There is a statistical association ($P < 0.0001$) of strong pAKT immunostaining with high Gleason score, and with *PIK3CA* alterations (mRNA overexpression and/or FISH gains). *PIK3CA* gene is deregulated by mRNA overexpression and DNA gain in ~40 and 28% of prostate tumors, respectively. High-grade prostate tumors are associated with *PIK3CA* mRNA overexpression, but not with FISH status. *PIK3CA*, *BRAF*, *KRAS* and *AKT1* mutations are very infrequent events in prostate tumors. However, PI3K signaling pathway is activated by *PIK3CA* FISH gain and/or mRNA overexpression, leading to an increased pAKT protein expression.
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The phosphatidylinositol 3-kinase (PI3K) signaling pathway has been described as one of the most frequently deregulated pathways in cancer.[1] PI3K can be activated through interaction with tyrosine kinase receptors[2,3] or through the binding of active RAS to its catalytic subunit.[4] PI3K activity can be counterbalanced by the action of PTEN.[5]

Only the catalytic subunit p110α, also called *PIK3CA*, has been reported to be mutated and

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amplified in different human tumor types.[2,6] The RAS–MAPK and PI3K–AKT pathways are strongly interconnected and have a central role in tumorigenesis. RAS is activated by growth factors and hormone signaling, binding to RAF proteins (also named MAPKKKs). The activation of RAF proteins causes the subsequent phosphorylation and activation of MEK1 and 2 (MAPKKs)/ERK1 and 2 (MAPKs).[7] The major downstream target of RAS is MAPKs, but it is also known to activate other targets like PI3K.[8] Furthermore, it has also been published[9] that AKT can inhibit the Raf–MEK–ERK pathway, through the inhibition of RAF phosphorylation.[9]

The PI3K signaling pathway can be activated by two main different mechanisms: activating mutation and amplification. More than 80% of mutations are clustered in exons 9 and 20 (helical and kinase domain) with three hot spot mutations, E542K, E545K and H1047R.[6] To date, only three studies have reported *PIK3CA* alterations in prostate cancer.[10–12] Two of them described *PIK3CA* amplification, and only the most recent study by Sun *et al*[12] found for the first time *PIK3CA* mutations in $\sim$3% of prostate tumors. Data from the 'Catalogue of Somatic Mutations in Cancer' (www.sanger.ac.uk) reported a mutation frequency of 2%.

KRAS mutations have been described in up to 13% of prostate cancers, with differences in the prevalence rates between Japanese and American men.[13–16] Two reports have been published about *BRAF* mutations in prostate tumors, but only Cho *et al*[16] described 10% of mutations in their Asian population. Most of *BRAF* mutations in human cancer consist in the missense mutation V600E[17] (see http://www.sanger.ac.uk/genetics/CGP/cosmic/). It has been reported that *BRAF* mutations occur in many tumor types in which *KRAS* is frequently mutated.[16,18]

To date, only one article has reported *AKT1* mutations in prostate cancer. Boormans *et al*[19] described the *E17K* change in 1 of 92 prostate tumors. The mutated sample presented a pAKT overexpression, suggesting activation of the *AKT* pathway.[19]

In summary, the constitutive activation of the PIK3–AKT and RAS–RAF pathways can result from different and/or complementary aberrant events, including constitutive active mutation, gene amplification, overexpression or loss of function. Although other authors have studied different components of the PI3K–AKT and RAS–RAF pathways, this is the first article in which various members of both pathways have been analyzed together in the same set of prostate samples in order to test their involvement in prostate cancer.

Materials and methods

Tumor Samples and Patients

A total of 102 cases of prostate cancer from the files of the Department of Pathology at the Hospital del Mar in Barcelona, Spain, are the subject of this report. All cases were collected between 1997 and 2008. None of the patients received previous chemotherapy or hormone therapy. In all, 72 cases were formalin-fixed, paraffin-embedded (FFPE) samples, 30 were frozen tissues, and none of them were matched. From these 72 cases, 65 were biopsy or prostatectomy specimens from patients with only prostate cancer (group G1; 7 were needle biopsies), and 23 were from patients who, in addition to prostate cancer, had another independent tumor in the bladder (group G2; 18 from cystoprostatectomy specimens and 5 from patients with radical prostatectomy and transurethral bladder biopsy). Stage and grade of the 23 bladder tumors from group 2 (G2) are shown in Table 1. In 6 patients from G2 with high grade and stage bladder cancer, we selected a previous bladder tumor biopsy with lower grade and stage for comparing molecular changes in *PIK3CA* in both bladder tumor samples. According to the Gleason grade, the 102 prostate samples were: 36 tumors with Gleason $=6$, 52 with Gleason $=7$ and 14 with Gleason ≥ 8.

Mutational Analysis (PCR Reaction and Direct Sequencing)

In adequate tumor areas, manual microdissection and DNA extraction from FFPE and frozen tissues was performed as previously described.[20]

In this study, we focused on analyzing the hot spot mutation codons and surrounding exonic regions of *PIK3CA, KRAS, BRAF* and *AKT1* in 81 prostate tumor samples from group G1 and 23 from G2. In group G2, we also performed mutational analysis of 20 corresponding bladder tumors. In addition, we also analyzed the 5′ upstream region of exon 20 of *PIK3CA* in 56 samples (50 from group G1 and 6 from G2).

As previous studies have reported the presence of a pseudogene extending from exons 9 to 13 for *PIK3CA*, we designed specific primers for exon 9 in order to amplify only the standard gene sequence of *PIK3CA*.[21–23]

The primer sequences, annealing temperature, PCR product size, exons and hot spot codons analyzed are shown in Table 2.

PCR reactions, product purification (Qiagen, Crawley, UK) and mutational analysis were performed as previously described with the Big Dye Terminator Kit v.3.1 (ABIPRISM 377, PerkinElmer Applied Biosystems).[20] Each PCR product was sequenced in both forward and reverse directions. In all cases where a change was identified in the first PCR reaction, an independent PCR amplification and sequencing experiment was performed.

BRAF Pyrosequencing Assay

A total of 54 samples were analyzed by pyrosequencing and direct sequencing. PCR amplification primers and annealing temperatures are shown in

Table 1 Mutational status and pathological features in specimens of the double tumor group (G2)

Double tumor group (G2)						
Case no.	*Gleason score*	*Bladder tumor stage and grade*	*PIK3CA, KRAS, BRAF and AKT mutational analysis in matched prostate and bladder tumors*		*PIK3CA mutational analysis in previous bladder tumor*	*Previous bladder tumor stage and grade*
			Prostate tumor	*Bladder tumor*		
59	3+4	TaG1	WT	WT	—	—
60	3+3	T3G3	WT	WT	—	—
61	3+3	TaG2	WT	Not done	—	—
62	3+3	T4G3	WT	WT	WT	T1G3
63	4+5	T3G3	WT	WT	—	—
64	3+3	T2G3	WT	WT	—	—
65	3+4	T2G2	WT	WT	WT	TaG2
66	3+3	T4G3	WT	G12V (*KRAS*)	—	—
67	3+3	TaG3	WT	WT	—	—
68	3+3	T2G3	WT	WT	WT	T1G3
69	3+3	TaG3	WT	WT	—	—
70	3+4	T4G3	WT	WT	—	—
71	3+3	T1G3	WT	WT	—	—
72	3+3	T3G3	WT	WT	—	—
73	4+5	T4G3	WT	WT	WT	TaG3
74	3+3	T2G3	WT	E17K (*AKT*)	—	—
75	3+3	T3G3	WT	Not done	—	—
76	3+3	T2G3	WT	WT	WT	TaG2
77	4+3	T4G3	WT	WT	WT	T1G2
78	5+4	T2G3	WT	Not done	—	—
79	3+3	T3aG3	WT	E545K (*PIK3CA*)	—	—
80	3+4	T1G3	WT	WT	—	—
81	3+4	T2G3	WT	WT	—	—

The shaded words represent the specific mutation and the gene abbreviation.

Table 2 Primer sequences, amplified fragment size and annealing temperatures for *PIK3CA*, *KRAS*, *BRAF* and *AKT1,* and *BRAF* pyrosequencing

Exon		*Primer sequence*	*Fragment (bp)*	*Hot spot codon*	*Temperature*
PIK3CA					
9	F	5′-AAAGCAATTTCTACACGAGATCCT-3′	191	542, 545	56 °C
	R	5′-GGAATAAATAAAGCAGAATTTACA-3′			
20A	F	5′-GCTCCAAACTGACCAAAC-3′	209	—	56 °C
	R	5′-CTGAGCAAGAGGCTTTGG-3′			
20B	F	5′-TTCGAAAGACCCTAGCCT-3′	182	1047	58 °C
	R	5′-GCTCACTCTGGATTCCACAC-3′			
KRAS					
1	F	5′-CTGAAAATGACTGAATATAAACTTGT-3′	152	12, 13	58 °C
	R	5′-GGTAAATCTTGTTTTAATATGCATAT-3′			
BRAF					60 °C
15	F	5′-CCTTTACTTACTACACCTCAG-3′	153	600	
	R	5′-GTGGATGGTAAGAATTGAGG-3′			
BRAF pyro	F	5′-AAATAGGTGATTTTGGRCTAGCTACA-3′	140	600	63 °C
	R	5′-GCATCTCAGGGCCAAAAATTTA-3′			
	S	5′-CCACTCCATCGAGATT-3′			
AKT1					
1	F	5′-TCTGACGGGTAGAGTGTGCGT-3′	109	17	63 °C
	R	5′-CTACTTCCTCCTCAAGAATGA-3′			

Table 2. PCR reactions were performed in a 25 μl volume using 10–50 ng of DNA. The products underwent electrophoresis in an agarose gel to confirm successful amplification of the 140 bp PCR product. The PCR products were sequenced by the Pyrosequencing PSQ96 HS System (Biotage AB) following the manufacturer's instructions.

Total RNA Extraction and Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from 25 frozen prostate tumor samples (Table 3) with Ultraspec and RNeasy Mini kit (Qiagen, Chatsworth, CA, USA) from 10 to 15 sections of 10 μm. All samples contained a

Table 3 Number of prostate samples according to Gleason score, as analyzed by mutational analysis, FISH and qRT-PCR of *PIK3CA* and pAKT immunohistochemistry

	Total of samples	*Mutational analysis*	*FISH analysis*	*qRT-PCR*	*Immunohistochemistry of pAKT*
Gleason score					
6	36	32	13	10	14
7	52	42	15	12	30
≥8	14	11	4	3	11
Total					
0	102	81	32	25	55

minimum of 70% of tumor cells. We also selected four normal samples as controls. Total RNA purity and quality were assessed with the NanoDrop® ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

PIK3CA mRNA expression was analyzed by quantitative real-time PCR in the 25 samples, on the ABI PRISM 7000 Sequence Detection System using the TaqMan® Gene Expression Assays probe and primer mix (Applied Biosystems) according to the manufacturer's specifications. The Assay Identification number was HS00180679_m1. Human B2M was used as endogenous control. The samples were run in triplicate and the mean value was calculated for each case. Non-carcinomatous prostate tissues (4 cases/run) were used to normalize the data using the ΔΔCt method. Values >1.9 reflect an overexpression of *PIK3CA* mRNA.

PIK3CA Fluorescence *In Situ* Hybridization (FISH) Analysis

FISH analysis was carried out on FFPE tissues in 32 cases (Table 3). *PIK3CA* probe was prepared from bacterial artificial chromosome (BAC) clones (pooled RP11-680J18 and RP11-386L21) selected from the CHORI BAC/PAC resource (http://bacpac.chori.org) and positioned to G-banded metaphase spreads via FISH. BAC DNA was labeled directly in SpectrumGreen-dUTP® (Abbott Molecular, Des Plaines IL, USA). Then, we made a mixture of *PIK3CA* probe with a commercial centromeric α-satellite probe specific for chromosome 3 (Abbott Molecular, Spectrum red), in which *PIK3CA* is located. Pretreated tissue sections and mixture of probes were co-denatured at 78 °C and hybridized overnight at 37 °C (Hybrite chamber; Abbot Molecular). After posthybridization washes, slides were counterstained with 4,6-diamino-2-phenylindole (DAPI) (Abbott Molecular).

Results were analyzed in a fluorescence microscope (Olympus, BX51) using the Cytovision software (Applied Imaging, Santa Clara, CA, USA). A minimum of 100 nuclei were scored by two different observers. We used adjacent normal tissues from five cases as controls, to establish the cutoff value (mean +3 s.d.) for polysomy (3 to 4 copies; 3.3%) and for monosomy (45%).

Immunohistochemistry of pAKT in Prostate Tumors

Immunohistochemical staining for pAKT was performed using the phospho-AKT (Ser473) antibody (Cell Signaling Technology, Beverly, MA, USA). A total of 55 samples were tested for pAKT expression (14 tumors with Gleason =6, 30 with Gleason =7 and 11 with Gleason ≥8; Table 3). pAKT was detected in both cytoplasm and nucleus. The results were graded, considering separately cytoplasmic and nuclear immunostaining, as 0 (negative), 1 (weak), 2 (moderate; Figure 5a), and 3 (strong; Figure 5b). The score (histoscore) for each of them was the sum of the product of the staining intensity and corresponding tumor percentage (histoscore $=[1\times(\%1+\text{ cells})]+[2\times(\%2+\text{ cells})]+[3\times(\%3+\text{ cells})]$).[24] For this study, the tumor score was obtained from the addition of the nuclear and cytoplasmic histoscores. A final tumor score 0–50 was considered negative or weak, between 50 and 100 moderate and >100 was considered strong positivity.

Results

PIK3CA, KRAS and *AKT1* Mutational Analysis

No mutations were detected in *PIK3CA, KRAS* or *AKT1* in the prostate tumors from patients with only prostate cancer (G1) or in the tumors from patients with bladder and prostate cancer (G2). On the other hand, we identified a previously not reported polymorphism in intron 9 of *PIK3CA* (IVS9+91) in two prostate tumors (one in each group, G1 and G2), generated by a transition C→T. The corresponding normal adjacent prostate tissue and normal seminal vesicle tissue (in the case from G1 group) and the matched muscle-invasive (T2G3) bladder tumor and normal bladder tissue (in the case from G2), were also analyzed. All of them presented the IVS9+91, confirming the polymorphic nature of this change. Both prostate tumors harbored, in addition, the previously reported polymorphic change T1025T,

which was also present in the normal tissue from the two cases and in the bladder tumor from one of them. There seems to be an association between the presence of polymorphisms IVS9 + 91 and T1025T, because both were present at the same time and never occurred separately.

As stated above, in the study of the matched bladder and prostate tumors from G2, none of the prostate tumors harbored any mutations. In the respective bladder tumors from this group, we detected three mutated samples (3 of 20, 15%), with mutations in *PIK3CA*, *KRAS* and *AKT1* genes, respectively (Figure 1). All mutations were found in hot spot codons: E545K in *PIK3CA*, G12V in *KRAS* and E17K in *AKT1*. None of the bladder tumors had mutations in *BRAF*. Previous papers on *PIK3CA* alterations in bladder cancer have reported from 13 to 25% of mutations in this gene, but with a higher prevalence in superficial than in deeply infiltrating bladder tumors.[25,26] In our study, most of the bladder samples were high-grade and high-stage tumors (Table 1). For this reason, we also investigated *PIK3CA* mutations in previous superficial bladder tumors in six patients from G2, also with negative results.

BRAF Mutational Analysis by Direct Sequencing and Pyrosequencing Technique

In order to confirm that no *BRAF* mutations were present, we not only performed direct sequencing but also pyrosequencing in prostate and bladder tumor samples. Different studies have found that pyrosequencing is a more sensitive and specific method than direct sequencing for detecting mutations. The limit for mutant allele detection ranges between 2.5 and 5% with the pyrosequencing technique, compared with the 10–20% obtained with direct sequencing.[27–29] As a positive control, we used a colon tumor harboring the V600E mutation, which was detected by both direct sequencing and pyrosequencing (Figure 2). Direct sequencing was performed in all prostate tumors and in 17 of the bladder tumors from G2, and we did not detect any mutation. Pyrosequencing was performed in 39

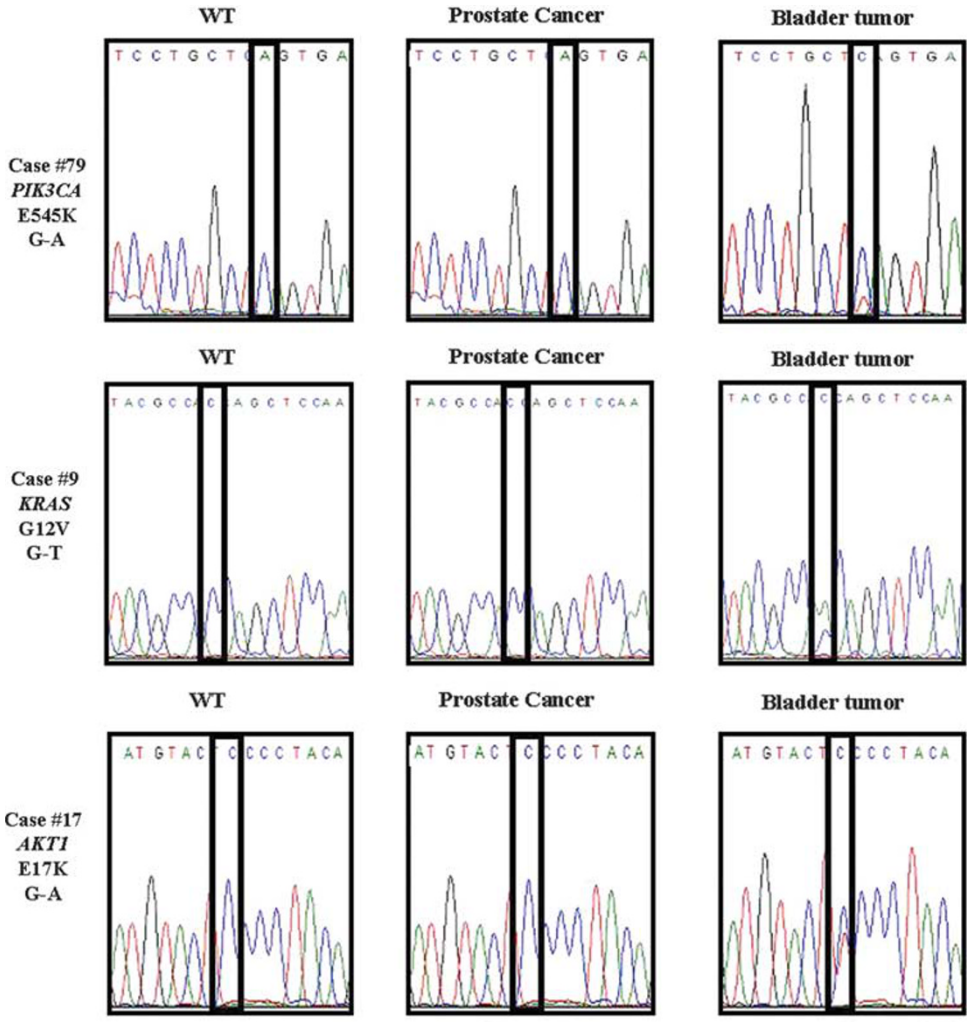

Figure 1 *PIK3CA, KRAS* and *AKT1* mutations in bladder tumors from patients with prostate and bladder cancer.

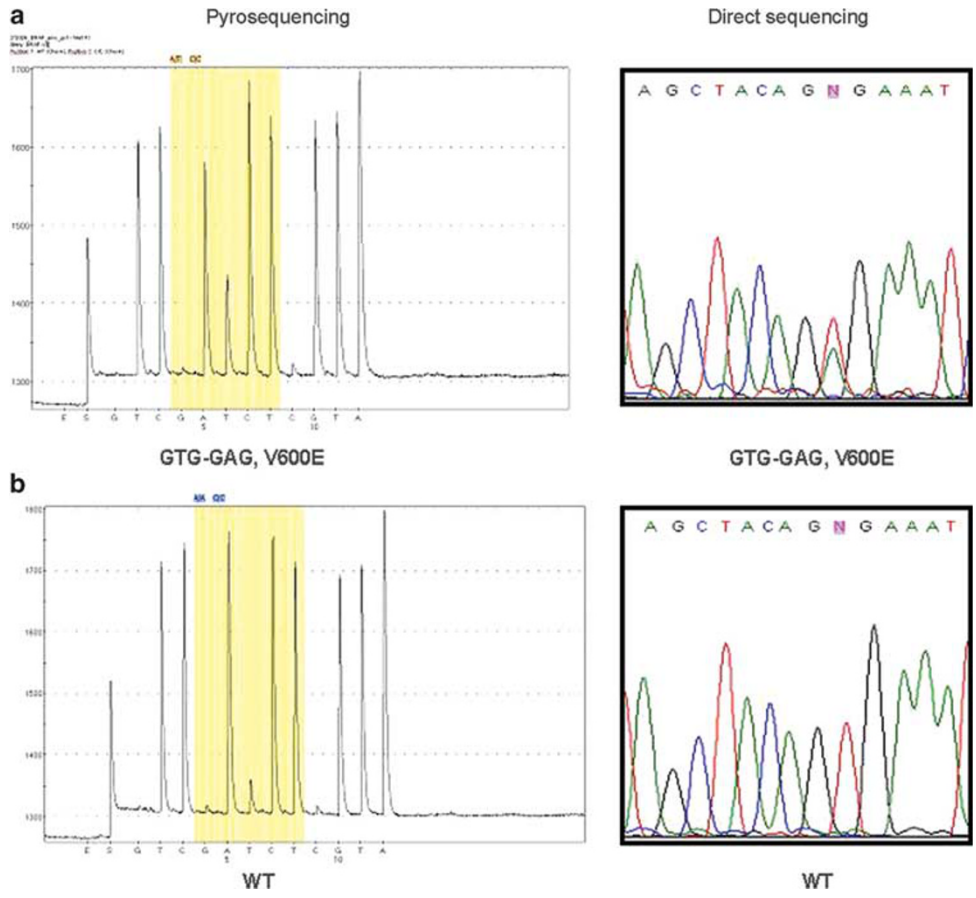

Figure 2 Pyrograms and direct sequences of two samples. (**a**) Positive control: the change is detected by both pyrosequencing and direct sequencing. (**b**) Sample no. 4: the change is not detected by pyrosequencing or by direct sequencing.

prostate and 9 bladder tumors, all of them also analyzed by direct sequencing, and none of these 48 samples presented the codon 600 mutation.

PIK3CA Quantitative mRNA Expression

The mRNA *PIK3CA* expression was investigated in 25 prostate tumors. From these 25 samples, 10 tumors were Gleason $=6$, 11 were Gleason $=7$ and 3 were Gleason ≥ 8. The mRNA expression index ranged between 0.56 and 3.9. Based on the statistical analysis, the cutoff assumed for mRNA overexpression was >1.9. Of 25 prostate tumors, 10 (40%) showed *PIK3CA* overexpression, and all but one of them were Gleason ≥ 7 (1 case was Gleason $=6$, 7 were Gleason $=7$ and 2 were Gleason $=9$; Figure 3). Thus, *PIK3CA* mRNA overexpression was statistically associated with high-grade Gleason score in prostate tumors (Fisher's exact test, P-value $=0.018$). In 19 of these 25 samples, the mutation analysis was also performed with negative results.

PIK3CA FISH Analysis

From the 81 prostate tumors in which mutational study was done, we were able to perform FISH analysis in 32 samples. Of these 32 cases, 9 (28%) showed gain of *PIK3CA* gene because of chromosome 3 polysomy, being *PIK3CA*/centromere 3 ratio <1.5 (Figure 4). All of these cases had 3 to 4 copies of chromosome 3 in 16–50% of cells. From these nine samples, three had Gleason $=6$ and six samples had Gleason $=7$. No statistical correlation was found between polysomy and Gleason score. From the ten cases in which we have information on *PIK3CA* by both FISH and qRT-PCR analysis, six cases showed concordant results (Table 4). Two cases with *wt* FISH showed mRNA overexpression. In the two other tumors we found copy number gain but not mRNA overexpression, and thus other molecular mechanisms might be regulating *PIK3CA* mRNA expression in these cases.

Immunohistochemistry of pAKT and Association with *PIK3CA* Alterations

We could perform immunohistochemistry of pAKT in 55 prostate tumors (Figure 5a and b). In 40 of them, the mutational analysis was available and showed negative results. The total (nucleus + cytoplasm) histoscore of pAKT in the tumor samples ranged from 0 to 300. Considering strong expression,

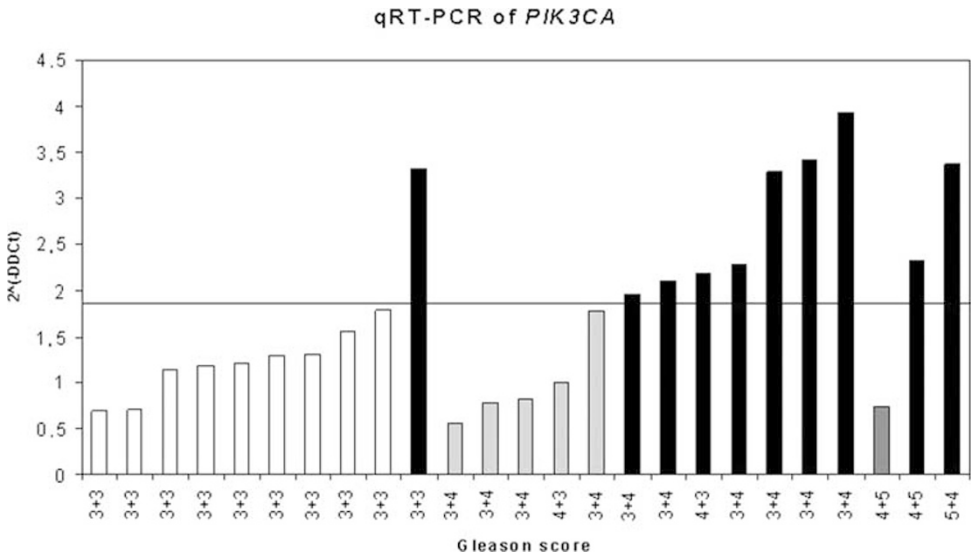

Figure 3 Histogram illustrating qRT-PCR results. *PIK3CA* mRNA overexpression was considered for a cutoff of >1.9.

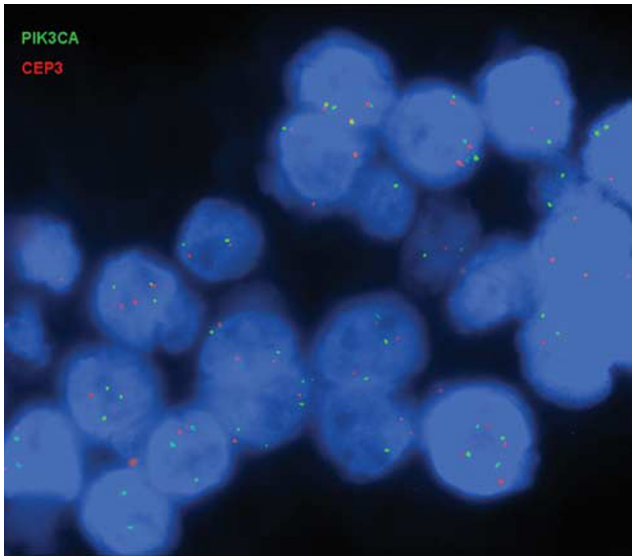

Figure 4 Representative image of one sample with *PIK3CA* copy number gain. Green signals represent bacterial artificial chromosome (BAC) for *PIK3CA* and red signals represent chromosome 3 centromere probe.

a histoscore >100, there was a statistical correlation between high pAKT protein expression and the Gleason score ($P<0.0001$), as 1 of 14 Gleason =6 (7.1%), 12 of 32 Gleason =7 (37.5%) and 9 of 9 Gleason ≥8 (100%) showed high pAKT levels. In addition, there was a correlation between high intensity of pAKT expression and *PIK3CA* overexpression (qRT-PCR) and/or amplification (FISH analysis) ($P<0.0001$). For this statistical analysis, we classified a sample as having a *PIK3CA* change when one or both techniques (FISH gains or mRNA overexpression) were positive (Table 4).

Table 4 Summary of prostate tumors in which *PIK3CA* FISH and/or qRT-PCR were available and in which pAKT immunohistochemistry was performed

Case no.	*Gleason score*	*FISH analysis*	*qRT-PCR analysis*	*Histoscore pAKT*
12	3+4	wt	Not done	Low
15	4+3	wt	wt	Low
25	3+4	wt	Not done	Low
34	3+4	Gain	Not done	High
35	4+3	Gain	Not done	High
36	3+3	Not done	wt	Low
37	3+4	wt	Overexpressed	High
38	3+3	Not done	wt	Low
39	3+4	wt	Overexpressed	High
40	3+3	wt	wt	Low
41	3+4	Gain	Overexpressed	High
42	3+4	wt	Not done	Low
43	3+4	Not done	wt	Low
44	3+3	Gain	wt	Low
45	3+3	wt	wt	Low
48	3+3	Gain	Not done	High
50	3+3	wt	wt	Low
53	3+3	Gain	wt	Low
55	4+5	Gain	Overexpressed	High
67	3+3	wt	Not done	Low
78	5+4	Not done	Overexpressed	High

The shaded gain refers to gain in the number of gene copies for a specific gene in FISH analysis, while overexpressed refers to the mRNA expression levels as defined by quantitative RT-PCR or pAKT immunohistochemical histoscore values.

Discussion

This paper is the first to analyze the combined impact of the activation of PI3K–AKT and RAS–RAF pathways, rather than their individual members, on prostate cancer. It is also the first to report *PIK3CA* alteration by mRNA overexpression in ~40% of prostate cancer cases, in addition to *PIK3CA* copy number gain also in around 28% of tumors. *PIK3CA*

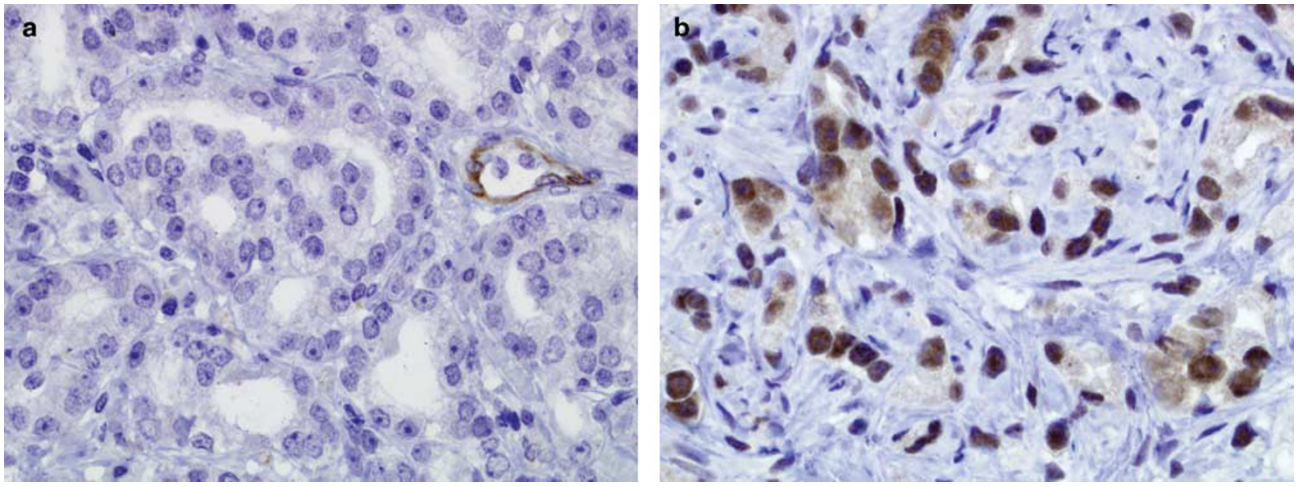

Figure 5 (**a**) Prostate adenocarcinoma, Gleason score 4 + 3 = 7, showing lack of nuclear and cytoplasmic expression of pAKT in tumor cells. Note moderate (2 +) cytoplasmic positivity in endothelial cells (× 400). (**b**) Prostate adenocarcinoma, Gleason score 4 + 5 = 9 (tertiary pattern 3), showing strong (3 +) pAKT nuclear expression in 20%, moderate (2 +) nuclear expression in 70%, weak (1 +) nuclear expression in 10%, moderate (2 +) cytoplasmic expression in 40% and weak (1 +) cytoplasmic expression in 60% of tumor cells (× 400).

mRNA overexpression and/or FISH gain are associated with high levels of pAKT protein, and *PIK3CA* mRNA overexpression is associated with high-grade Gleason score in prostate tumors (Fisher's exact test, $P = 0.040$).

The lack of mutations in *PIK3CA*, *AKT1*, *KRAS* and *BRAF* in our series of prostate cancer indicates that mutations in these genes are not key elements in prostate cancer pathogenesis. These results are in concordance with previous published studies, most of them based on a smaller number of cases or not precisely defining the clinicopathological features of the samples. In our study we have selected a larger, well-defined group of patients. Moreover, to the best of our knowledge, this study is the first to investigate different molecules of the PI3K signaling pathway in the same subset of patients. Another new approach of our study is to compare patients harboring only a prostate tumor (G1) and those with both prostate and bladder cancer (G2). This is based on a previous study from our group[20] in which a higher frequency of mutations of *FGFR3* was found in patients in whom prostate cancer occurred in association with tumors in bladder, skin and other tissues.

In several tumor types, alterations in different elements of PI3K–AKT and RAS–MAPK pathways are common events.[30,31] The constitutive activation of the PI3K pathway can result from different and/or complementary aberrant events. At present, there are only three reports on *PIK3CA* alterations in prostate cancer. Edwards *et al*[10] found amplification of *PIK3CA* in 39% of hormone-sensitive tumors and 50% of hormone-independent tumors by CGH arrays; Muller *et al*[11] also reported the lack of *PIK3CA* mutations in 12 cases of prostate cancer. A recent study[12] has reported *PIK3CA* mutations in prostate tumors for the first time, although only in 1 of 40 cases, along with *PIK3CA* amplification in 13% of the cases. Our results are in agreement with these reports on the null or very low occurrence of mutations in *PIK3CA*. In addition, we have also found gene copy number gain in ~28% of tumors, and for the first time we also report *PIK3CA* mRNA overexpression in a high proportion of prostate tumors.

We have not found *KRAS* or *BRAF* mutations. Discrepancies in the frequency of mutation of *KRAS* and *BRAF* have been reported for prostate cancer. *KRAS* mutations have been found in up to 8% of prostate tumors (see http://www.sanger.ac.uk/genetics/CGP/cosmic/).[13–16] The first study on *BRAF* alterations in prostate cancer[18] revealed no mutations in the prostate adenocarcinomas of 17 Caucasian patients (0/17). The second study, in Asian patients, found a frequency of *BRAF* and *KRAS* mutation of 10 and 7%, respectively.[16] Our series was made up of Caucasian patients and this could account for the lack of mutations in these genes. We confirmed this negative result for *BRAF* in a subset of cases by means of pyrosequencing.

The most recent reports on *AKT* suggest that only *AKT1* isoform shows mutations in certain types of human cancer, in a low proportion of cases.[32] We did not detect *AKT1* mutations in our samples, and in fact only a single case of prostate cancer with mutated *AKT1* has been reported in the literature.[19] Thus, our results confirm that *AKT1* mutations are very uncommon in prostate cancer, and that *AKT* may be activated through other mechanisms. Phosphorylation– activation of AKT could be the result of diverse alterations in different key genes of the PI3K–AKT pathway. In that sense, the only *AKT1* mutated prostate tumor reported to date[19] showed pAKT overexpression. Some authors have shown that AKT is upregulated in prostate cancer compared with benign prostatic tissue (BPT), prostatic intraepithelial neoplasia (PIN) and primary tumors

graded 2–5 according to Gleason.[33,34] Interestingly, no change in the degree of AKT expression was detected in studies in which tumors had Gleason scores ranging from 6 to 10.[35,36] On the other hand, the staining intensity for pAKT has been reported to be significantly greater in prostate tumors with high Gleason grade (Gleason 8–10).[35–37] These reports are in concordance with the results of the present study, in which there was statistical correlation between pAKT expression and Gleason score $P<0.0001$. Finally, we have also found a statistical correlation between pAKT and *PIK3CA* mRNA overexpression and/or *PIK3CA* copy gain $P<0.0001$. Most of the cases analyzed with both techniques showed concordant results. Therefore, we can speculate that FISH gain could lead to an increase of *PIK3CA* mRNA expression and subsequent pAKT activation. On the other hand, it is also interesting to note that two cases with *wt* FISH showed mRNA overexpression and high levels of pAKT and two cases with FISH gain but not mRNA overexpression did not show high levels of pAKT intensity. Thus, other molecular mechanisms might be regulating *PIK3CA* mRNA expression in these cases.

Some of the patients in our study presented, in addition to prostate cancer, a simultaneous or previous urothelial neoplasm. Therefore, we have also performed the mutational study in the corresponding bladder tumors and we have found mutations in *PIK3CA, KRAS* and *AKT1* in some cases, but not in the respective prostate tumors. Data from the literature reveal that independent bladder tumors harbor ~13–25% of *PIK3CA* mutations.[25,26] We found a lower percentage of mutations of this gene (5%) in the bladder tumors from patients with prostate cancer. This difference could be related to the fact that bladder tumors in patients with and without prostate cancer could progress through different pathways, but it could also be explained by differences in the bladder tumors included in our study. Most of the bladder tumors from G2 were muscle invasive, whereas *PIK3CA* mutations are more frequent in superficial tumors.[25,26] To investigate whether the differences in the prevalence of *PIK3CA* mutations were because of the invasive nature of the G2 bladder tumors, we selected six of these cases in which a previous, superficial, low-grade bladder tumor was available, and we did not find *PIK3CA* mutations in any of them. Interestingly, the only bladder tumor with mutated *PIK3CA* in our study was deeply invasive and high grade (T3G3).

The main goal of this study has been to comprehensively assess the PI3K–AKT and RAS–RAF signaling pathway status in prostate tumors with and without associated bladder cancer. This is the first work in which several different genes rather than individual genes in the pathways have been investigated in a large group of prostate tumors. According to our results, mRNA overexpression emerges as a new type of PIK3CA alteration in prostate tumors. A high proportion of prostate tumors are altered through *PIK3CA* mRNA overexpression and/or DNA gain, and mRNA overexpression is associated with high Gleason score (≥ 7), whereas copy number gain is not. Although PI3K and AKT have been suggested to be promising molecular targets in the management of prostate cancer,[36,38] *PIK3CA*, *BRAF*, *KRAS* and *AKT1* mutations represent very infrequent events in this tumor. We have shown, in addition, that *PIK3CA* amplification and/or mRNA overexpression are associated with higher levels of pAKT protein expression. However, more studies will be needed to further characterize if these changes could lead to a PI3K–AKT pathway activation that could justify the clinical use of *PIK3CA* and AKT inhibitors.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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