PIK3CA Mutations Are an Early Genetic Alteration Associated with FGFR3 Mutations in Superficial Papillary Bladder Tumors

Elena López-Knowles, Silvia Hernández, Núria Malats, Manolis Kogevinas, Josep Lloreta, Alfredo Carrato, Adolina Tardón, Consol Serra, and EPICURO Study Group Investigators

Abstract
Bladder tumors constitute a very heterogeneous disease. Superficial tumors are characterized by a high prevalence of FGFR3 mutations and chromosome 9 alterations. High-grade and muscle-invasive tumors are characterized by 

Mutations in the regulatory p85 subunit have rarely been reported; class IA enzymes are the only ones thus far shown to be directly involved in carcinogenesis: the catalytic p110α subunit, encoded by the PIK3CA locus, has recently been shown to be mutated in tumors from the colon, stomach, endometrium, ovary, thyroid, breast, and glioblastomas; the p110β and p110δ subunits have been studied less extensively and have not been found to be mutated (1). Mutations in the regulatory p85 subunit have rarely been reported; their biological significance is uncertain. PIK3CA mutations occur mainly in the regions encoding for the helical and kinase domains and have been clearly shown to have oncogenic properties in a variety of assays (1, 3–5). However, little is known about the stages at which these mutations arise during carcinogenesis or about their association with tumor evolution.

Bladder cancer is an excellent paradigm for the study of tumor development and progression. The majority of tumors are urothelial cell carcinomas and are classified on the basis of bladder wall involvement (Ta, T1-T4) and nuclear grade (G1-G3). PUNLMPs have been attributed a very low risk of progression and are classified separately (6). It is currently thought that urothelial cell carcinomas progress through two different pathways: one, involving ~75% of cases, is associated with papillary growth, presentation as Ta or T1 stage tumors, and generally good prognosis. These tumors almost universally display alterations of genes in chromosome 9 and frequent mutations in FGFR3 or, alternatively, Ras genes (7). The other pathway is associated with dysplasia, muscle invasion, poor prognosis, Tp53 mutations, and genomic instability (8). Bladder cancer presents several formidable challenges: (a) many patients with low-grade papillary tumors present numerous recurrences that require continued medical control and the use of invasive procedures, and a few of them progress to develop muscle-invasive disease; (b) a subset of patients present with nonmuscle invasive high-grade Ta or T1 tumors that have a high risk of becoming muscle invasive, and the identification of risk factors predictive of disease progression is of utmost importance; (c) finally, other patients present with muscle-invasive or metastatic tumors and their disease is life-threatening (9).

In the course of a BAC array-CGH analysis of bladder cancer, we have found that the BAC harboring PIK3CA is gained or amplified.
in 54% or 9.7% of T1G3 tumors, respectively.\(^2\) We therefore hypothesized that this gene might also undergo activating mutations in bladder cancer. Here, we present evidence that PIK3CA mutations occur in ~20% of bladder tumors of low grade and stage. By contrast, mutations are less common in high-grade tumors. These findings may have important implications to develop better strategies for bladder cancer detection as well as for the design of targeted therapy.

### Materials and Methods

**Bladder cancer cell lines.** A panel of 14 bladder cancer cell lines (Table 1) was used for the analyses. Cells were obtained from the Ludwig Institute for Cancer Research New York Branch (Sloan-Kettering Institute) or from Yves Fradet (Laval University, Quebec, Canada). DNA was extracted using Qiamp DNA Mini kit (Qiagen, Hilden, Germany).

**Patients and tumors.** Cases were drawn from the EPICURO Study, which is composed of 1,356 patients with bladder cancer recruited from 1997 to 2001 in 18 hospitals in Spain (11). For this work, a subset of cases \((n = 87)\) representative of the whole study population was identified through stage/grade stratification and random selection. Based on the initial results, we subsequently analyzed all PUNLMPs from the study \((n = 43)\). Staging and grading of tumors was carried out according to the criteria of the tumor-node-metastasis classification and the WHO International Society of Urological Pathology (8). Diagnostic slides from all paraffin-embedded blocks corresponding to each case were reviewed by a panel of expert pathologists to confirm diagnosis and ensure uniformity of classification criteria. The characteristics of cases included in the study are shown in Supplementary Table S1. Written informed consent was obtained from all patients. The study was approved by the Ethics Committees of all participating institutions.

**Mutational analyses.** Microdissection, DNA extraction, and controls used for PCR have been reported elsewhere (11). Primers were designed to avoid amplification of a known PIK3CA pseudogene: 9F TGAAGTG-TATTGCTTTTCTTGT, 9R TGGATCTCTGCTTTATTTATCC; 20F TTTGTCCTCAACGCTGAGCA, 20R CGATGCTTTAATGTTGCTGTG, 22F ACTTACGAAGAGCGTTTTGGA, and 20R TTTGGACCTTAAAGGCTGATGAA. PCR reactions were done using 10 to 50 ng of DNA, 0.2 \(\mu\)mol/L of each primer, 200 \(\mu\)mol/L deoxynucleotide triphosphates, 3.5 mmol/L MgCl2, 1 \(\times\) PCR II buffer, and 1.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 94\(^\circ\)C (10 minutes) for 1 cycle, 94\(^\circ\)C (40 seconds), 60\(^\circ\)C (40 seconds), 72\(^\circ\)C (40 seconds) for 42 cycles, and a final extension step of 72\(^\circ\)C (10 minutes).

All mutations were confirmed by analyzing the products of a second independent PCR. When a previously undescribed sequence variant was found in tumor DNA, it was confirmed using independent PCR reaction products. Germ line DNA was used to determine the somatic nature of previously undescribed variants. As quality control, 6% of all PCR products were sequenced in both directions and findings were replicated. PIK3CA mutational analysis was carried out as described elsewhere (11).

**Statistical analyses.** Association between PIK3CA and FGFR3 mutational status was tested by applying \(\chi^2\) Mantel-Haenszel test. The trend of PIK3CA mutational prevalence according to stage and grade was estimated using \(\chi^2\) for linear trend test.

## Results

### Mutational analysis of PIK3CA in bladder cancer cell lines.

We analyzed exons 9 and 20 in a panel of 14 bladder cancer cell lines; 2 (14.3%) were found to have a mutation (Table 1). MGH-U4 had the H1047R hotspot mutation, which is widely reported in several tumor types (1, 9); in these cells, both FGFR3 and exons 4 to 9 ofTp53 were wild type. 253J cells harbored the E545G mutation; this codon is a hotspot in PIK3CA but the most commonly reported mutation is the E545K substitution. These cells also display wild-type FGFR3 and Tp53 genes. RT4, the only line analyzed derived from a low-grade papillary tumor, had normal PIK3CA sequences.

Table 1 summarizes the results of mutational analysis in these cell lines.

### Mutational analysis of PIK3CA, FGFR3, and Tp53 in bladder cancer cell lines

<table>
<thead>
<tr>
<th>Source</th>
<th>PIK3CA</th>
<th>FGFR3</th>
<th>H-ras*</th>
<th>Tp53</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U4</td>
<td>UCC, dysplasia</td>
<td>H1047R</td>
<td>wt</td>
<td>N.D.</td>
<td>wt</td>
</tr>
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<td>T24</td>
<td>UCC, grade 3</td>
<td>wt</td>
<td>wt</td>
<td>G12V</td>
<td>Y126X</td>
</tr>
<tr>
<td>RT4</td>
<td>UCC, grade 1</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>LGW01</td>
<td>wt</td>
<td>wt</td>
<td>N.D.</td>
<td>wt</td>
<td>++</td>
</tr>
<tr>
<td>SW 800</td>
<td>UCC, grade 1</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>++</td>
</tr>
<tr>
<td>253J</td>
<td>Metastasis</td>
<td>E545G</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>575A</td>
<td>UCC, grade 3</td>
<td>wt</td>
<td>Y375C</td>
<td>N.D.</td>
<td>IVS9+12, wt</td>
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<tr>
<td>J82</td>
<td>UCC, invasive</td>
<td>K652E</td>
<td>wt</td>
<td>E271K</td>
<td>++</td>
</tr>
<tr>
<td>VMCUB-3</td>
<td>UCC, grade 3</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>E271K</td>
</tr>
<tr>
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<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>E285K</td>
</tr>
<tr>
<td>SW 1710</td>
<td>UCC</td>
<td>wt</td>
<td>wt</td>
<td>R273C</td>
<td></td>
</tr>
<tr>
<td>JON</td>
<td>Adenocarcinoma</td>
<td>wt</td>
<td>wt</td>
<td>R280T</td>
<td></td>
</tr>
<tr>
<td>5637</td>
<td>UCC, grade 2</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: UCC, urothelial cell carcinoma.

*Data retrieved from Jebar et al. (7); N.D., not done.

1. MGH-U4 cells were derived from a grade 1 tumor and are not tumorigenic in nude mice.

2. 253J cells were derived from a grade 3 tumor; highly tumorigenic and metastatic variants from this cell line have been established.

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\* E. López-Knowles et al., in preparation.
Mutational analysis of **PIK3CA** in bladder cancer tissues. The tumors included cover the whole spectrum of bladder cancer and their distribution by T and G is representative of the disease at presentation. Overall, 11 of 87 (13%) tumors harbored **PIK3CA** mutations. The distribution according to stage was as follows: T1 (9 of 57, 16%), T2 (2 of 10, 20%), and muscle-invasive tumors (0 of 20, 0%; P = 0.098). The prevalence of mutations was higher in low-grade tumors: grade 1 (6 of 27, 22.2%), grade 2 (3 of 23, 13%), and grade 3 (2 of 37, 5.4%; P = 0.047; Fig. 1). Most mutations were found at hotspot codons: E542K (n = 1), E545K (n = 5), G1007R (n = 1), H1047L (n = 1), and H1047R (n = 3). Eleven of 87 cases harbored a G-to-A germ line polymorphism located at −55 bp from the start of exon 9, which was unrelated to the somatic mutations and of which the significance is unknown.

**Mutational analysis of PIK3CA in PUNLMP.** The results described above suggested that **PIK3CA** mutations are associated with superficial tumors. Therefore, we extended our initial study to include all PUNLMP from our study. Eleven of 43 (25.6%) tumors were mutant; when this group was included to compare the prevalence of **PIK3CA** mutations according to tumor progression (PUNLMP, T2, Ta1, and muscle invasive), the P value of the trend test was highly significant (P = 0.019). The mutations identified were E542K (n = 3), E545K (n = 4), E545G (n = 1), H1047L (n = 1), and H1047R (n = 2). These findings support the notion that **PIK3CA** mutations can occur early in the course of urothelial carcinogenesis (i.e., before muscle invasion occurs).

**Association of PIK3CA mutations with alterations in FGFR3.** FGFR3 mutations are associated with low-stage and low-grade urothelial tumors and thought to occur early in tumor development (7, 12). Therefore, the pattern of **PIK3CA** mutations suggested that this genetic change might constitute an alternative pathway to urothelial cell carcinoma development. FGFR3 exons 7 and 10 were amplified and sequenced and the results of the analysis of mutations in both genes are shown in Table 2 and Supplementary Table S2. All but one of the **PIK3CA** mutations found in PUNLMP occurred among **FGFR3**-mutant samples. Among tumors of higher stage or grade, 8 of 11 **PIK3CA**-mutant tumors also harbored **FGFR3** mutant alleles, supporting the notion that mutations in these two genes do not represent alternative pathways of tumor progression. The results were analyzed also according to different tumor strata. Tumors were categorized into three groups on the basis of the T stage, grade, prevalence of **FGFR3** mutations, and prognosis (13, 14): T1,G1 and T2,G2 tumors; high-grade nonmuscle invasive tumors (T3,G3 and T4,G3 tumors); and muscle-invasive tumors. Eighteen of 69 (26%) **FGFR3mut** tumors were **PIK3CA**mut, versus 4 of 58 (6.9%) **FGFR3wt** tumors (P = 0.005). The findings were similar in the three tumor groups described above (Table 2). These results indicate that **PIK3CA** mutations are strongly associated with **FGFR3** mutations.

**Discussion**

**PI3K** and **Akt** kinases link crucial signaling pathways involved in cancer development and progression: they are activated by receptor tyrosine kinases (in part through ras proteins), modulate p53 activity through murine double minute-2 phosphorylation, modulate the pRb pathway through induction of cyclin D and inhibition of its degradation, and regulate the Wnt pathway through glycogen synthase kinase 3β (2). Furthermore, the **PI3K** pathway affects fundamental processes such as protein synthesis and cellular growth, mediated by mammalian target of rapamycin and S6 kinase.

A recently reported mechanism for **PI3K** pathway activation in cancer cells is the presence of activating mutations (1, 3–5, 15). Here, we have searched for activating mutations in **PIK3CA** in bladder tumors. We find that mutations occur in ~20% of superficial tumors and have a very low prevalence among muscle-invasive tumors. Within the former group, **PIK3CA** mutations tend to occur in a subset of cases harboring **FGFR3** mutations, supporting the notion that they do not represent an alternative pathway of tumor progression. The lower prevalence of **PIK3CA** mutations in muscle-invasive tumors further strengthens the notion that papillary and muscle-invasive tumors are two different molecular entities.

Somatic **FGFR3** mutations present in bladder tumors also occur as germ line alterations in patients with skeletal dysplasias (16). It has been shown that in chondrocytes, **FGFR3** activation leads to growth arrest, differentiation, and apoptosis, whereas **PI3K** pathway activation can bypass these effects and enhance cell survival (17). **FGFR3** mutations are associated with bladder tumors of good prognosis (12, 13, 18). By contrast, **FGFR3** mutations fail to predict the risk of recurrence, progression, or death among patients with higher stage or grade in superficial tumors (11), suggesting that their effect is overridden by additional genetic alterations.

![Figure 1. Prevalence of **PIK3CA** and **FGFR3** mutations in bladder tumors according to T stage and grade.](cancerres.aacrjournals.org)

**Table 2.** Association of **PIK3CA** and **FGFR3** mutations in bladder tumors

<table>
<thead>
<tr>
<th></th>
<th><strong>FGFR3</strong>&lt;sub&gt;mut&lt;/sub&gt;/<strong>PIK3CA</strong>&lt;sub&gt;wt&lt;/sub&gt;</th>
<th><strong>FGFR3</strong>&lt;sub&gt;mut&lt;/sub&gt;/<strong>PIK3CA</strong>&lt;sub&gt;mut&lt;/sub&gt;</th>
<th><strong>FGFR3</strong>&lt;sub&gt;wt&lt;/sub&gt;/<strong>PIK3CA</strong>&lt;sub&gt;mut&lt;/sub&gt;</th>
<th><strong>FGFR3</strong>&lt;sub&gt;wt&lt;/sub&gt;/<strong>PIK3CA</strong>&lt;sub&gt;wt&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUNLMP</td>
<td>21</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>T1,G1/G2</td>
<td>24</td>
<td>8</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>T2/T3,G3</td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>≥T4</td>
<td>2</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>18</td>
<td>54</td>
<td>4</td>
</tr>
</tbody>
</table>
Based on these observations and the findings in chondrocytes (17), it is conceivable that activation of the PI3K pathway in bladder cancer may contribute to the malignant behavior of FGFR3-mutant tumors.

A striking observation is that the prevalence of PIK3CA mutations decreases with increasing stage and grade. It is tempting to speculate that the PI3K pathway may contribute to bladder cancer progression through different molecular mechanisms: in low-grade, low-stage tumors, PIK3CA mutations would play a major role; in higher-stage, higher-grade tumors, genomic changes, such as PIK3CA gain/amplification and PTEN loss, may play a more crucial role. This notion would be supported by the finding that PTEN loss of expression has been reported to be associated with muscle-invasive tumors (19). Furthermore, we have found that 10q23 losses, where the tumor suppressor PTEN maps, are associated with high tumor stage and grade, with a prevalence of 58% in T1G3 and muscle-invasive tumors. Other genes may also play a role: TSC1 is mutated in a small proportion of bladder tumors (20), leading to activation of mammalian target of rapamycin, a downstream component of the PI3K pathway. However, mutations in TSC1 have not been reported to be associated with stage or grade. Further work will be necessary to precisely establish the role of the PI3K pathway in bladder cancer progression.

The cell lines of which the PIK3CA mutations status is reported herein may be useful for these studies. However, it is known that bladder cancer lines do not adequately represent the spectrum of bladder cancer from patients: few of them are derived from papillary low-grade superficial tumors and only 2 of 14 lines tested had mutations in FGFR3, a proportion that is much lower than that found in bladder tumors.

The association of PIK3CA mutations with superficial tumors suggests that they may also be of diagnostic and prognostic value.

The mutations found in bladder cancer occur in the same codons in which they have been described in other tumors (1). Because only a few nucleotides are mutated, diagnostic strategies for early detection of tumor recurrences may be warranted. Furthermore, PIK3CA mutations may identify a subset of FGFR3-mutant tumors with discrete clinical behavior and thus be of prognostic use. Whyte et al. (21) have used microarrays to describe a gene expression profile characteristic of cultured cells harboring mutations in PIK3CA. These cells also showed significantly increased sensitivity to various compounds, including topoisomerase I inhibitors, when compared with cells with wild-type PIK3CA (21). It is thus conceivable that a PIK3CA mutation-associated expression profile can also be identified in bladder tumors.

Based on the findings reported herein, large, preferably prospective, studies should be conducted to determine the prognostic value of PIK3CA mutations in combination with FGFR3 mutations in bladder cancer. Our findings also support the notion of combining drugs targeting FGFR3 and PIK3CA for the treatment of superficial urothelial cell carcinoma.

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**References**

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