Spectrum of Phosphatidylinositol 3-Kinase Pathway Gene Alterations in Bladder Cancer

Fiona M. Platt,1 Carolyn D. Hurst,1 Claire F. Taylor,2 Walter M. Gregory,3 Patricia Harnden,1 and Margaret A. Knowles1

Abstract Purpose: The phosphatidylinositol 3-kinase (PI3K) pathway can be activated by alterations affecting several pathway components. For rational application of targeted therapies, detailed understanding of tumor biology and approaches to predict efficacy in individual tumors are required. Our aim was to assess the frequency and distribution of pathway alterations in bladder cancer.

Experimental Design: We examined the pathway components (PIK3CA, PTEN, TSC1, RHEB, and LKB1) and putative upstream regulators (FGFR3 and RAS genes) for mutation, allelic loss, copy number alteration, and expression in bladder tumors and cell lines.

Results: No mutations were found in RHEB and only a single mutation in LKB1. PIK3CA mutations were detected in 25% of tumors and 26% of cell lines with a significant excess of helical domain mutations (E542K and E545K). There was over-representation but not amplification of the gene. Loss of heterozygosity of the PTEN region and homozygous deletion were found in 12% and 1.4% of tumors, and reduced expression in 49%. Forty-six percent of cell lines showed alterations that implicated PTEN. Sixteen percent of tumors and 11% of cell lines showed TSC1 mutation, and 9q loss of heterozygosity was common (57%). Pathway alterations were independently distributed, suggesting that the mutation of two pathway members may have additive or synergistic effects through noncanonical functions.

Conclusions: PI3K pathway alterations are common in bladder cancer. The lack of redundancy of alterations suggests that single-agent PI3K-targeted therapy may not be successful in these cancers. This study provides a well-characterized series of cell lines for use in preclinical studies of targeted agents. (Clin Cancer Res 2009;15(19):6008–17)

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a crucial role in cell growth, proliferation, and survival (1). Signaling through this pathway is commonly upregulated during tumorigenesis (2, 3). Genomic alterations have been detected in multiple genes in the pathway, including the tumor suppressor genes PTEN and LKB1, which are inactivated by mutation and/or deletion in sporadic cancers (4, 5), and the proto-oncogenes AKT1, AKT2, PDK1, PIK3CA, and PIK3R1, which are activated by mutation or amplification (6–9). In addition, oncogenic activation of the RAS genes by point mutation activates the pathway through the interaction of RAS with p110α (10). Increased signaling through the ERBB receptors also activates the pathway. Activation of the mTOR pathway can also be achieved downstream of RAS through extracellular signal-regulated kinase–mediated negative regulation of the TSC1/TSC2 complex (11). Several of the activated proto-oncogenes represent potential targets for therapy, and there is currently much interest in developing agents to target these proteins.

Activation of the PI3K pathway and the types of genetic change found are somewhat tissue-specific. For example, some tumor types show predominantly point mutations in PTEN, whereas others show homozygous deletions (4). Similarly, some tumor types have been reported to show mutual exclusivity of different events in the pathway, whereas others do not (12). To use PI3K pathway–targeted agents rationally in the future, comprehensive information on the pathway activation status and the mechanisms of activation in different tumor types is needed. Furthermore, well-characterized tumor cell lines with defined pathway alterations are required for preclinical studies.

In bladder cancer, several components of the pathway are implicated. Loss of heterozygosity (LOH) at the PTEN locus is
common in advanced bladder cancer (13), and homozygous dele-
tion has been identified in some cases (14–16). However, bial-
elic inactivation by small mutations in the retained allele in
tumors with LOH is infrequent, and it is possible that PTEN is
haploinsufficient in this tissue. Recently, activating mutations of
PIK3CA have been identified in bladder tumors, particularly in
tumors of low grade and stage (17). We have identified inac-
vitating mutations in the tuberous sclerosis complex (TSC) gene
TSC1 in a subset of bladder tumors of all grades and stages (18),
and shown that >50% of bladder tumors have lost one allele of
TSC1 (19). Inactivation of this gene has not been found in other
human cancers. Finally, several receptor tyrosine kinases that
signal through the PI3K pathway, including ERBB2 and epider-
mal growth factor receptor, are altered in bladder cancer (20),
and shown that >50% of bladder tumors have lost one allele of
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**Translational Relevance**

Phosphatidylinositol 3-kinase (PI3K) pathway acti-
vation can be achieved by alterations affecting sever-
al pathway components. Therefore, it is critical to
understand how pathway activation occurs in differ-
et tumor types so that targeted therapeutic ap-
proaches can be applied rationally. We have exam-
ined multiple alterations affecting the pathway
components (PIK3CA, PTEN, TSC1, RHEB, and LKB1)
and putative upstream regulators (FGFR3 and RAS
genes) for mutation, allelic loss, copy number alter-
ation, and expression in a large panel of bladder tu-
mors and cell lines. Our findings indicate that this
pathway is altered at high frequency and through
several different mechanisms. There was a lack of re-
dundancy of alterations, implying that different path-
way members may have additive or synergistic
effects through noncanonical functions. This indi-
cates that single-agent PI3K-targeted therapy may
not be successful in these cancers. Our study also
provides a well-characterized series of cell lines for
use in preclinical studies of targeted agents.

**Materials and Methods**

**Patient samples and DNA isolation.** The study was approved by the
Local Research Ethics Committee, and informed consent was obtained
from all patients. Cold cup biopsies of urothelial carcinoma (69 prima-
ry tumors, 23 recurrent tumors) were collected, snap frozen, and stored
in liquid nitrogen. The remainder of the tumor was embedded in par-
affin for diagnostic assessment. Samples were graded and staged by a
single pathologist (22, 23). The tumor panel consisted of 3 pTaG1, 34
pT1G3, 7 pT2G3, 6 TpT3G3, 24 TpT4G3, 15 pT2G2, and 3 G2 tumors with no
underlying stroma (pT0). All were transitional cell carcinomas.

Frozen sections were inspected for at least 70% tumor purity. DNA
was extracted with the use of the QIAamp DNA mini kit (Qiaegen,
Crawley, West Sussex, United Kingdom). Venous blood samples were
collected in EDTA tubes, and DNA was extracted with the use of a Nu-
ucleon DNA extraction kit (Nucleon Biosciences, Lanarkshire, United
Kingdom) or by a salt precipitation method.

**Cell lines.** Thirty-four urothelial carcinoma–derived cell lines
(253J, 5637, 639V, 92-1, 96-1, 97-1, 97-2, 97-7, BC-
3C, BFTC905, BFTC909, CAL29, DSH1, HT1197, HT1376, J82, JMSU1,
JON, KUI-19, MGH-H3, RT12, RT4, SCAber, SD, SW780, SW1710,
T24, TCCSUP, UMIC3, VMCC1B1, VMCC1B1-II, and VMCC1BIII) and
other human nonurothelial tumors (HuC29) derived from the nonmalignant urothelial epithelium of a patient with bladder cancer were investigated. DNA was extracted
either by standard phenol/chloroform extraction or with the use of a QIAamp DNA mini kit (Qiagen).

**Mutation analyses.** TSC1, RHEB, and LKB1 mutation screens used
fluorescent single-strand conformational polymorphism analysis as de-
scribed (24). TSC1 was screened in 27, RHEB in 8, and LKB1 in 9 frag-
ments. PIK3CA, FGFR3, HRAS, KRAS, and NRAS were analyzed with
the use of high-resolution melting curve analysis as described (25). PIK3CA
was analyzed in 20 fragments covering all coding sequences. FGFR3
hotspot exons 7, 10, and 15, and RAS gene hotspot exons 2 and 3 were
analyzed. Samples with aberrant melting profiles were sequenced with
the use of the BigDye Terminator v1.1 Cycle Sequencing kit (Applied
Biosystems, Warrington, United Kingdom). DNA from matched blood
samples was analyzed to confirm somatic mutation status. Primer
sequences are given in Supplementary Table S1.

**LOH analysis.** LOH was investigated with the use of microsatellite
markers in the TSC1 region on 9q34 (D9S1830, D9S1199, D9S149, and
D9S566), the TSC2 region on 16q13.3 (D16S3024), and the PTEN region
on 10q23.3 (D10S1765, D10S215, D10S541). Forward primers were
fluorescently labeled, and PCR products were run on an ABI 3130 se-
quencer (Applied Biosystems) and analyzed with the use of the ABI
GeneMapper v3.7 software. LOH was scored as a reduction in signal of
at least 60% from one allele. In cell lines in which matched constitu-
tional DNA was not available, LOH in the PTEN region was predicted by
the presence of only a single allele at all loci examined. Homozygous
deletion analysis was carried out as described (15).

**Array comparative genomic hybridization.** Two types of 1-Mb reso-
lution comparative genomic hybridization (CGH) array (Centre for
Microarray Resources, University of Cambridge, Cambridge, United
Kingdom; ref. 26) were used for genome-wide analysis of DNA copy
number. The clone coverage on the two arrays was essentially the same,
with a few exceptions (details available on request). Hybridization was
as described (27). Reference samples were normal lymphoblastoid cell
DNA or paired normal DNA from patients’ blood. Breakpoints,
and regions of gain and loss were detected by BlueFuse software
(Cambridge BlueGnome 2006, Cambridge, United Kingdom), an array
CGH–Smooth algorithm with calling thresholds set to 0.15 or 0.2 log2
ratio (for Centre for Microarray Resources and Sanger archives, respec-
tively). Alterations were confirmed visually by two observers.

**Immunohistochemistry.** Sections were deparaffinized and rehy-
drogenated, and endogenous peroxidase activity was blocked in 3% hydro-
peroxide. Sections were boiled in 10 mmol/L citric acid buffer
(pH6) for 2 min; nonspecific binding was blocked with an avidin/bio-
tin solution (Vector Laboratories, Peterborough, United Kingdom), fol-
lowed by 10% normal serum (DakoCytomation, Glostrup, Denmark).
Slides were incubated with antibodies to PTEN (1:100; Cell Signaling
Technology, Danvers, CA) or hamartin (1:100; Epitomics, Burlingame,
CA) for 1 h at room temperature, followed by species-specific secondary
antibody for 30 min. Incubation with StreptABComplex (DakoCytoma-
tion) for 30 min was followed by visualization with 3,3′-diaminoben-
zidine (Vector Laboratories) and counterstaining with hematoxylin. All
runs included no primary antibody control.

**Immunoblotting.** Cell lines were analyzed for hamartin protein ex-
pression by western blotting as described (18). PTEN was analyzed with
the use of the same antibodies used for immunohistochemistry at
1:1,000 dilution. Protein loading was assessed with the use of mouse
anti-actin, (1:1,000; Sigma-Aldrich, Gillingham, Dorset, United Kingdom).

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Mutations were identified in 9 cell lines (26%) and 25 tumors (27%). Eight different mutations were found in exons 2, 9, 12, and 20 (Table 1). The polymorphisms identified are listed in Supplementary Table S2. The majority of mutations were in hotspot codons 542 and 545 (E542K and E545K) in the helical domain. One cell line contained E545Q, and one tumor contained both E542K and E545K. Four tumors and one cell line contained H1047R, a kinase domain hotspot mutation. Mutations in one cell line and two tumors, resulting in amino acid changes P124L, Q643R, and M1043I, were confirmed as somatic by comparison with matched normal DNA. The mutation A1066V found in the cell line 639V could not be confirmed as somatic because paired normal DNA was not available.

This spectrum of mutations differs from that found in other cancers (4) (Fig. 1A). Therefore, exons 9 and 20 were screened in an additional 92 tumors. This panel contained a larger proportion of T1 and T2 tumors (20 T2, 41 T1, 31 ≥ T2, 19 G2, 73 G3). The mutations found were 14 E545K, 4 E542K, 3 H1047R, 1 M1043I, 1 E545Q, and 1 D1017H, with 1 tumor containing E545K and E542K, confirming a significant excess of helical domain mutations in urothelial carcinoma. In this extended panel, there was a significant relationship with tumor grade but not with stage [for grade, P = 0.037; for stage, χ² (trend) = 3.46, P = 0.063].

Some tumor types show genomic amplification of PIK3CA (28). As part of a larger study, detailed results of which will be reported elsewhere, array CGH data were available for 72 of the tumors. This showed over-representation of the gene in 6 of 72 tumors and 16 of 35 cell lines, including 1 tumor and 4 cell lines that also had a mutation. There were no high-level amplifications.

**RAS and FGFR3.** PIK3CA interacts directly with RAS (10, 29) and, in the presence of RAS mutation, both the mitogen-activated protein kinase and PI3K pathways are activated. FGFR3, which is commonly activated by mutation in bladder tumors (30), also activates the RAS–mitogen-activated protein kinase pathway in urothelial cells. Therefore, we assessed the mutation status of the RAS genes and FGFR3 (Table 2). Eleven tumor samples had a mutation in a RAS gene, seven of which also had PIK3CA mutation. Fifty tumors had FGFR3 mutation, 17 of which also had PIK3CA mutation. FGFR3 mutation showed significant association with low tumor grade (P < 0.001) and stage (P < 0.001). FGFR3 and RAS mutations were previously found to be mutually exclusive (21), but here two samples with FGFR3 mutation also contained low-copy number RAS mutation, possibly indicating that these samples contained >1 tumor clone.

Six of the cell lines contain a RAS mutation (21), and four have FGFR3 mutation (ref. 21 and this study). RAS mutation was found with PIK3CA mutation in one line (HT1197), and PIK3CA mutation with FGFR3 mutation in two lines (639V and J82).

**PTEN.** Previous studies of urothelial carcinoma have found infrequent mutation in the PTEN gene (14–16). Thus, we did not screen for small sequence alterations. However, LOH at the PTEN locus is common in muscle-invasive bladder tumors (13, 15), and occasional homozygous deletion has been reported. We screened tumors for LOH and assessed heterozygosity at three highly polymorphic loci in the region of PTEN in cell lines to provide a prediction of LOH. Eleven tumors (12%) showed LOH. The relationship with tumor grade and
Fig. 1.  A, Pie charts showing the distribution of PIK3CA mutations found in this study (top left); those listed in COSMIC for positions M1043, H1047, E542, and E545, excluding bladder tumor mutations (top right); the range of PTEN expression profiles recorded (bottom left; for scoring criteria see Materials and Methods); and the overall distribution and combinations of alterations identified in PI3K pathway genes (bottom right).  B, patterns of staining for PTEN protein. Control is normal ureter. Other images are bladder tumors illustrating the scoring criteria applied. Scores are nuclear followed by cytoplasmic intensity. In tumor samples with scores of 1,2; 1,1; 0,1; and 0,0, stromal cells with intense staining act as positive controls. All images were captured at the same magnification.
Table 2. Mutations of FGFR3 and RAS genes in bladder tumors and cell lines

<table>
<thead>
<tr>
<th>Protein effect</th>
<th>No. of tumors</th>
<th>Cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R248C</td>
<td>3</td>
<td>639V</td>
</tr>
<tr>
<td>S249C</td>
<td>35</td>
<td>97-7</td>
</tr>
<tr>
<td>G372C</td>
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</tr>
<tr>
<td>S373C</td>
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<td>0</td>
</tr>
<tr>
<td>Y375C</td>
<td>6</td>
<td>MGH-U3</td>
</tr>
<tr>
<td>K652E</td>
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<td>382</td>
</tr>
<tr>
<td>K652N</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>RAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRAS G12V</td>
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<td>T24</td>
</tr>
<tr>
<td>HRAS G12S</td>
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<td>0</td>
</tr>
<tr>
<td>HRAS G13V</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HRAS Q61L</td>
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<td>0</td>
</tr>
<tr>
<td>HRAS Q61R</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>KRAS G12A</td>
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<td>0</td>
</tr>
<tr>
<td>KRAS G12C</td>
<td>0</td>
<td>UMUC3</td>
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<td>0</td>
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<td>J82</td>
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<tr>
<td>KRAS G12S</td>
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<td>0</td>
</tr>
<tr>
<td>KRAS G12V</td>
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<td>0</td>
</tr>
<tr>
<td>KRAS Q61H</td>
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<tr>
<td>NRAS Q61L</td>
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<td>BFTC905 HT1197</td>
</tr>
<tr>
<td>NRAS Q61R</td>
<td>0</td>
<td>KU19-19</td>
</tr>
</tbody>
</table>

Stage was not significant [for grade, \( P = 0.270 \); for stage, \( \chi^2 \) (trend) = 5.6, \( P = 0.102 \)]. Array CGH showed under-representation of the PTEN region in 11 samples (15%), including homozygous deletion in 1. Data were available for eight of the tumors with LOH, and five showed under-representation. Five samples showed under-representation but retained both alleles.

Immunohistochemistry was used to assess PTEN protein expression in all tumors for which paraffin-embedded blocks were available (\( n = 89 \)). As location-specific functions for PTEN have been described (reviewed in ref. 31), nuclear and cytoplasmic staining were assessed separately. Normal ureteric and bladder urothelium were used as positive controls, and the lowest level of staining observed in control urothelium was scored as 2. Staining in tumor samples was scored as 2 if it was at least as strong as the weakest staining in controls, 1 if detectable but reduced, and 0 if staining was absent (Fig. 1B). Endothelial cells stained strongly and acted as internal controls. Nuclear staining was reduced more frequently than cytoplasmic staining (Supplementary Fig. S1). Fifty-one percent of tumors showed normal or increased levels of staining, and the remainder (49%) showed reduced staining in the nucleus, cytoplasm, or both (Fig. 1A). Seven tumors (8%) showed complete absence of expression. Reduced expression was associated with higher grade (\( P = 0.01 \)) but not stage (\( \chi^2 \) (trend) = 2.1, \( P = 0.14 \); Fig. 2; Supplementary Table S3). Of the tumors with LOH, eight showed reduced or absent PTEN expression and three had normal expression.

Of the seven tumors with no expression, array CGH data were available for five, one of which showed homozygous deletion. One showed reduction in copy number compatible with the detected LOH in the region. The other three retained heterozygosity, which may indicate a small region of homozygous deletion not involving the microsatellite markers used or homozygous deletion of the marker, and the detection of two alleles contributed by contaminating normal DNA in the sample.

Allele counts at three microsatellite loci within 1 Mb of PTEN (D10S1765, D10S215, D10S541) were used to predict LOH in
cell lines. Array-based CGH analysis provided DNA copy number information, and immunoblotting was used to assess protein expression (Table 3). Ten lines were homozygous at all loci, indicating probable LOH, and one (UMUC3) showed homozygous deletion of all three microsatellite markers. This line is known to contain homozygous deletion encompassing the entire gene (15). Of the 10 lines with single alleles, information on PTEN has been reported for T24, which contains a mutation (16), and for J82, which has intragenic homozygous deletion (15). Paired normal DNA was available for DSH1 and J82, allowing confirmation of LOH. Immunoblotting revealed no protein expression in DSH1, and homozygous deletion of exon 3 was found by PCR (data not shown). Seven of the homozygous nonmutant cell lines (5637, 97-24, 647V, 96-1, VMbI, SW780, and JMSU) retained some PTEN expression. Array CGH revealed a region of copy number loss encompassing PTEN in 5637, SW780, JMSU, and 647V. In total, 10 of 35 cell lines showed under-representation at the PTEN locus by array CGH. Therefore, 16 of the 35 cell lines had demonstrable alterations that implicated PTEN.

TSC1. Mutations were identified in 3 of 29 cell lines analyzed previously (18). In the additional six cell lines screened here, two mutations were found in one (639V; overall frequency: 4 of 35; 11.4%). Mutations were identified in 15 of 92 (16%) tumors. These comprised eight nonsense, four missense, and three frameshift mutations. Q55X, which was found twice in this tumor panel, was identified previously in a cell line (18). No mutations were found in the constitutional DNA from the same individuals. Mutations are shown in Table 4 together with 11 mutations from our previous study to show the overall mutation spectrum in urothelial carcinoma. We found 7 different polymorphisms in 57 of 92 tumors (Supplementary Table S4). TSC1 mutation showed no significant relationship to tumor stage or grade (for grade, $P = 0.77$; for stage, $P = 0.42$).

It has been suggested that TSC1 is haploinsufficient in some lesions in patients with germline mutation (32), and we previously found apparent heterozygosity for mutations in tumor samples (18). As the samples used in this previous study were not microdissected, it was not clear whether apparent heterozygosity resulted from normal tissue contamination. Here all samples contained at least 70% tumor cells. To assess the biallelic loss of function of the gene, we carried out LOH analysis with the use of four microsatellite markers in the TSC1 region (D9S1830, D9S1199, D9S149, and D9S66). Fifty-seven percent

### Table 3. Status of PTEN in bladder tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Allelic status of PTEN region</th>
<th>Copy number reduction*</th>
<th>Mutation status</th>
<th>Protein expression</th>
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<td>RT4</td>
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<td>+</td>
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<td>N48I (50)</td>
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<td>-</td>
<td>WT*</td>
<td>+</td>
</tr>
<tr>
<td>J82</td>
<td>LOH</td>
<td>HD (15)</td>
<td>HD (635-1212Δ578)*</td>
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<td>SW1710</td>
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<td>WT</td>
<td>+</td>
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<tr>
<td>UMUC3</td>
<td>HD of markers assessed</td>
<td>HD (15)</td>
<td>HD (1-1212Δ1212)*</td>
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<tr>
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<td>HD (165-209Δ45)*</td>
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<td>ND</td>
<td>+</td>
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<tr>
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<td>WT*</td>
<td>+</td>
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<td>+</td>
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<td>ND</td>
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<td>+</td>
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<tr>
<td>MGH-U3</td>
<td>Heterozygous</td>
<td>-</td>
<td>ND</td>
<td>+</td>
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</table>

Abbreviations: HD, homozygous deletion; WT, wild type; ND, not done.
*+ denotes reduced copy number; - denotes no copy number change.
†COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/).
showed LOH, including all but one of the mutant tumors. This latter tumor (containing F216A) was analyzed again after microdissection to obtain pure tumor cell DNA, and retention of heterozygosity was confirmed by sequencing (data not shown). The distribution of TSC1 LOH according to tumor grade and stage was 18 of 42 G1 or G2 (42.8%), 30 of 44 G3 (68%; \( P = 0.03 \)), 18 of 41 pTa (44%), 19 of 28 pT1 (68%), 10 of 14 \( \geq T2 \) [71%; \( \chi^2 \) (trend) = 4.7, \( P = 0.03 \)], and 1 of 3 pTx tumors.

Array CGH \((n = 72)\) revealed copy number loss in the TSC1 region in 36 tumors, 29 of which had LOH (2 tumors not informative). Tumors with under-representation were evenly distributed between stages and grades: 17 of 35 G1 or G2 (49%), 19 of 37 G3 (51%; \( P = 1.0 \)), 16 of 35 pT1 (46%), 14 of 24 pT1 (58%), and 5 of 11 \( \geq T2 \) (45%; \( P = 0.6 \)).

Immunohistochemistry was carried out on paraffin-embedded tissues. Examples are shown in Supplementary Fig. S2. Normal ureter showed strong uniform cytoplasmic staining for hamartin. Fourteen tumors showed complete absence of staining. Of these, 11 had mutation. In one case (tumor with Y185X) the block contained both positive and negative fragments (Supplementary Fig. S2), suggesting that the fresh frozen sample used for mutation analysis was probably derived from the negative component. Insufficient tissue was available from one mutant tumor, and three tumors with mutation were found to express hamartin protein. These had Q951X, F216A, and 1531insA, which is predicted to result in termination at amino acid 510. Three tumors with no detected mutation showed absence of staining. These all had 9q LOH and copy number loss. All other tumors showed cytoplasmic staining.

The protein products of TSC1 and TSC2 (hamartin and tuberin, respectively) act in a complex downstream of AKT to inhibit signaling through mTOR through the GTPase activating protein activity of TSC2 toward RHEB (33, 34). Because tuberin represents the active partner in this complex, somatic inactivation of TSC2 could have more profound effects than inactivation of TSC1. Consistent with this, germline TSC2 mutations cause a more severe phenotype than TSC1 mutations (35). TSC2 is a large gene and has not been examined in sporadic cancers. To assess the possible involvement of TSC2 in bladder cancer, we carried out LOH analysis for a single microsatellite marker \((D16S3024; \text{maximum heterozygosity, 86%})\) within 0.5 Mb of TSC2. Eleven informative cases (15%) showed LOH. Array-based CGH analysis showed under-representation of the TSC2 region in 7 of 72 cases (9.7%), 4 of which also had LOH.

### Relationships of PI3K pathway alterations.
Complete data for the 92 tumors are given in Supplementary Table S5, and the distribution of alterations is shown in Fig. 1A. We examined the relationship of individual events to tumor grade and stage, and the relationships among events; this was done with data for 89 tumors. It was of particular interest to assess the relationships of the known PI3K pathway members PIK3CA, TSC1, and PTEN. All possible combinations of these events were found in both tumors and cell lines (Supplementary Tables S5 and S6), and they were not significantly associated in any combination (Supplementary Tables S7 and S8). Previously, we found that RAS and FGFR3 mutations are mutually exclusive events in urothelial carcinoma (21), and this was confirmed here. The relationship of RAS mutation to PIK3CA mutation and

### Table 4. Mutations in TSC1 in bladder tumors and cell lines

<table>
<thead>
<tr>
<th>Exon</th>
<th>DNA variation</th>
<th>Protein effect</th>
<th>No. of tumors</th>
<th>Cell lines</th>
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<tr>
<td>3</td>
<td>73-77ΔS</td>
<td>Frameshift</td>
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<tr>
<td>4</td>
<td>C104G</td>
<td>S35C</td>
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<td></td>
<td>A203G</td>
<td>H68R</td>
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<tr>
<td>5</td>
<td>A314G</td>
<td>H105R</td>
<td>1</td>
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</tr>
<tr>
<td>6</td>
<td>T473G</td>
<td>F158C</td>
<td>1*</td>
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</tr>
<tr>
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<td>C555G</td>
<td>Y185X</td>
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<tr>
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<td>Y195X</td>
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<td>H206D</td>
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<td></td>
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<tr>
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<td>F216A</td>
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<td></td>
</tr>
<tr>
<td>9</td>
<td>IV57-1G&gt;A</td>
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</tr>
<tr>
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<td>F285V</td>
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<td></td>
<td>C866A</td>
<td>S289X</td>
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<td>C1250T</td>
<td>T4171*</td>
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<td>C2851T</td>
<td>Q951X</td>
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</table>

*Previously published (18, 38).
†This variant has been described in TSC patients and was also found in the bladder cancer patient’s germline.
other events in the PI3K pathway has not been examined previously. A significant association of RAS mutation to PIK3CA mutation was found \( (P = 0.003) \). However, data from an additional 92 bladder tumors (not shown) did not confirm this \( (P = 0.238) \). Although FGFR3 is not known to activate the PI3K pathway in urothelial cells, an association of FGFR3 mutation with PIK3CA mutation has been reported previously \( (17) \). Here, this did not reach significance \( (P = 0.07) \). There was no association of FGFR3 mutation with TSC1 mutation, but there was a significant inverse relationship between FGFR3 mutation and loss of PTEN expression.

Stepwise linear regression analysis revealed that FGFR3 mutation and LOH on 9q together showed increased ability to predict tumor grade and stage, with FGFR3 mutation and retention of heterozygosity on 9q associated with lower grade and stage \( (r^2 \text{ for grade} = 29\%; r^2 \text{ for stage} = 36\%) \) compared with either variable alone. For mutant FGFR3 alone, \( r^2 \) for grade and stage were 19\% and 24\%, respectively. For 9q LOH alone, \( r^2 \) for grade and stage were 6\% and 7\%, respectively. Within the mutant FGFR3 tumors, 16 of 31 (52\%) stage Ta cases had LOH compared with 10 of 11 (91\%) T1 and 3 of 3 (100\%) T2 \( \chi^2 \text{ (trend) } = 6.7, P = 0.01 \). Within FGFR3 wild-type tumors, 2 of 10 (20\%) Ta had LOH compared with 9 of 17 (53\%) T1 and 7 of 11 (64\%) T2 \( \chi^2 \text{ (trend) } = 3.92, P = 0.05 \). For grade within the mutant tumors, 17 of 34 (50\%) G2 had LOH compared with 13 of 14 (93\%) G3 \( \chi^2 \text{ (trend) } = 3.92, P = 0.05 \). For grade within the mutant tumors, 17 of 34 (50\%) G2 had LOH compared with 13 of 14 (93\%) G3 \( \chi^2 \text{ (trend) } = 3.92, P = 0.05 \).

Within wild-type tumors, 1 of 8 (13\%) G2 had LOH compared with 17 of 30 (57\%) G3 \( \chi^2 \text{ (trend) } = 3.92, P = 0.05 \).

**Discussion**

The appropriate selection of patients for targeted therapies requires knowledge of the molecular characteristics of tumors and their predicted dependence on individual proteins. Inappropriate patient selection may not only lead to lack of response, but may be detrimental due to feedback regulation and cross-talk between pathways. Here we sought to define the PI3K pathway status in bladder tumors and cell lines as a first step in understanding the mechanisms by which constitutive pathway activation occurs in urothelial carcinoma, and to define molecular analyses that could aid in the selection of patients for targeted therapies. Inhibition of the PI3K pathway through the development of p110 \( \alpha \) and mTOR inhibitors is a current major focus in drug development.

We have shown that multiple components of the PI3K pathway are altered in bladder cancer and that some changes are tissue-specific. Genomic alterations in three of the key genes in the pathway (PIK3CA, TSC1, PTEN) are not mutually exclusive, implying that combined mutations have additive or synergistic effects, and that the noncanonical effects of these components are drivers of bladder cancer.

LKB1 is a serine threonine kinase that activates AMP-activated protein kinase and negatively regulates mTOR signaling through TSC1/TSC2 \( (36) \). Germline mutation causes Peutz-Jeghers syndrome, a multiple hamartoma syndrome in which patients develop a range of benign tumors, predominantly in the jejunum but also in other tissues, including the ureter and bladder \( (37) \). We found only a single somatic mutation, a missense change \( (Q100E) \), indicating that LKB1 is not a critical mutation target in urothelial carcinoma, unlike other sporadic cancers. The functional significance of this missense change is unknown.

This residue is identical in rat, mouse, cow, chicken, xenopus and fugu orthologues, but not in Drosophila. In other tumor types, however, the majority of mutations reported are protein-truncating (stop or frameshift). We did not examine LOH in the region of the gene in this tumor panel, and it will be of interest to examine 19p LOH in urothelial carcinoma in the future.

Bladder cancer is the only sporadic cancer in which mutations have been identified in either of the TSC genes. In this and our previous study \( (18) \), a wide range of inactivating mutations of TSC1 was identified, including nonsense, missense, frameshift, and splicing mutations, as well as in-frame deletions. As all tumors with mutation except one showed loss of the wild-type allele, we conclude that in most cases, biallelic inactivation is required. One case, which contained the mutation F216A, retained both alleles of TSC1 and continued to express hamartin. We have shown altered localization of this protein when re-expressed in TSC1-null urothelial cells, and unlike other somatic missense mutants, there is no splicing defect or reduced protein stability \( (38) \). This points to altered function rather than haploinsufficiency in this case.

Although we did not screen TSC2 for mutations, our finding of infrequent LOH indicates that biallelic inactivation of TSC2 is unlikely to be common. TSC2 mutation analysis in tumors with LOH at 16p and expression analysis should now be undertaken. The widely studied functions of both TSC1 and TSC2 are attributed to the TSC1/TSC2 complex that regulates mTOR activity through RHEB. Although some independent functions have been ascribed to TSC2, independent function of TSC1 is not clear. The finding of mutations in bladder but not other cancers, and the lack of mutual exclusivity of TSC1 mutations with either PIK3CA or PTEN alterations may indicate that TSC1 does have an independent function in the urothelium.

We carried out a comprehensive screen of all coding sequences of PIK3CA and found an overall mutation frequency of 25.3\% in tumors and cell lines, all but two of which were in exons 9 and 20. In a previous study of 87 bladder tumors (65.5\% Ta, 11.5\% T1, 23.0\% T2), mutations were identified in exons 9 and 20 in 13\% of cases \( (17) \). In the extended panel of tumors analyzed here for exons 9 and 20 mutations, that contained a different distribution of tumor stages (35.4\% Ta, 39.2\% T1, 25.4\% T2), we found mutations in 25\%. There was a high frequency of E545K and E542K relative to H1047R with E543K, by far the most common mutation \( (53\% \text{ of mutations found}) \). This mutation spectrum is different from that found in several other types of cancer in which H1047R is most common \( (Fig. 1A) \).

M1043I has been identified previously in colorectal \( (39, 40) \) and endometrial cancer \( (41) \). Other rare mutations found were P124L and A1066V in cell lines, and E545Q, Q643R, and D1017H in tumors. The P124L and A1066V mutations found in J82 and 639V confirm the results for these cell lines reported by the Cancer Genome Project (COSMIC), but these mutations have not been reported in other samples. However, a missense mutation in codon 124 \( (P124T) \) has been reported in colorectal cancer \( (40) \). D1017H has been reported in endometrial cancer \( (42) \). E545G, found in a single cell line, has been reported previously in urothelial carcinoma \( (17) \). Both E545Q and E545Q have been reported in other cancers \( (4) \) and in epidermal nevi \( (43) \). Q643R represents a novel mutation.

PI3Ks are heterodimeric lipid kinases composed of a catalytic \( (p110) \) and a regulatory subunit \( (p85) \). In unstimulated cells,
p110α is regulated through an inhibitory effect of p85. This is lost upon the stimulation of upstream receptors, when p85 is recruited either by direct binding to an activated receptor or indirectly through adapter molecules, bringing PI3K to the membrane. Structural studies of p110α provide evidence for an interaction of the N-terminal SH2 domain of p85 with the helical domain of p110α (44, 45), and it is thought that this is responsible for p85-mediated inhibition of p110α. A recent study has shown that helical domain (E545K, E542K) and kinase domain (H1047R) mutations induce gain of function through distinct mechanisms with differential dependence on p85 binding and interaction with RAS (46). Helical domain mutations are independent of p85 binding but require RAS interaction. In contrast, the kinase domain mutation is transforming even in the absence of RAS binding but remains dependent on interaction with p85. Therefore, we hypothesize that the predominance of helical domain mutations in bladder tumors may be determined by selective pressures relating to the cellular context. This could be related to the activation of the RAS–mitogen-activated protein kinase pathway during tumor development either through RAS mutation or signaling through epidermal growth factor receptor, which is commonly upregulated in these cancers, or by other mechanisms. In the panel of 92 tumors presented in detail here, 7 cases showed both RAS and PIK3CA mutations. However, this apparent association was not confirmed in the additional 92 tumors analyzed, although the different distributions of grades and stages in this series make direct comparison difficult. Therefore, it will be important to evaluate these potential interactions in a larger series in the future.

Although mouse models (47) and human tumor studies (15, 16) provide clear evidence for the involvement of PTEN deficiency in bladder cancer development, assessment of the involvement of PTEN in tumor samples is difficult because biallelic inactivation is not common. Thus, we used several approaches to infer reduced PTEN function. Reduced PTEN expression was found (49%), confirming previous findings (47), and was significantly associated with high tumor grade. The lack of significant association with tumor stage may reflect the relatively small number of muscle-invasive tumors analyzed. The frequency of LOH and/or deletion at the PTEN region were not high (12% and 15%, respectively), suggesting that other mechanisms are responsible for this reduced protein expression.

Unlike PTEN loss, the expression of constitutively activated AKT in the presence of wild-type PTEN is not sufficient to drive tumorigenesis (48), indicating that noncanonical functions of PTEN are critical for tumor development. PTEN plays an essential role not only through its cytoplasmic effects on the PI3K pathway signaling but through nuclear functions in which it stabilizes centromeres by interaction with CENP-C, and down-regulates mitogen-activated protein kinase and cyclin D1 to induce G0-G1 arrest (reviewed in ref. 31). PTEN interacts with p53 and plays a key role in the maintenance of genomic stability by interaction with CHK1 (49). The lack of mutual exclusivity of the PI3K pathway events examined here indicates that not only PTEN but each component is likely to have nonlinear functions.

In conclusion, several components of the PI3K pathway are altered in bladder tumors, with no evidence for redundancy of function. Thus, although a large proportion of urothelial carcinoma may be “addicted” to signaling through this pathway, single-agent therapy (e.g., with PI3K inhibitors) may not be successful. Combinations of inhibitors of pathway members, upstream regulators, or interacting pathways may be required. In this context, it will be particularly interesting to examine the relationship of the ERBB receptors to the changes examined here. This study not only provides insight into the mechanisms by which the PI3K pathway function is altered in bladder tumors but also provides a well-characterized series of cell lines for preclinical studies of agents that target components of the pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Clinical Cancer Research

Spectrum of Phosphatidylinositol 3-Kinase Pathway Gene Alterations in Bladder Cancer

Fiona M. Platt, Carolyn D. Hurst, Claire F. Taylor, et al.


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