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Advanced urothelial carcinoma: next-generation sequencing reveals diverse genomic alterations and targets of therapy

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Although urothelial carcinoma (UC) of the urinary bladder generally portends a favorable prognosis, metastatic tumors often follow an aggressive clinical course. DNA was extracted from 40 μm of formalin-fixed, paraffin-embedded (FFPE) sections from 35 stage IV UCs that had relapsed and progressed after primary surgery and conventional chemotherapy. Next-generation sequencing (NGS) was performed on hybridization-captured, adaptor ligation-based libraries for 3320 exons of 182 cancer-related genes plus 37 introns from 14 genes frequently rearranged in cancer to at an average sequencing depth of 1164 $\times$ and evaluated for all classes of genomic alterations (GAs). Actionable GAs were defined as those impacting the selection of targeted anticancer therapies on the market or in registered clinical trials. A total of 139 GAs were identified, with an average of 4.0 GAs per tumor (range 0–10), of which 78 (56%) were considered actionable, with an average of 2.2 per tumor (range 0–7). Twenty-nine (83%) cases harbored at least one actionable GA including: *PIK3CA* (9 cases; 26%); *CDKN2A/B* (8 cases; 23%); *CCND1* (5 cases; 14%); *FGFR1* (5 cases; 14%); *CCND3* (4 cases; 11%); *FGFR3* (4 cases; 11%); *MCL1* (4 cases; 11%); *MDM2* (4 cases; 11%); *EGFR* (2 cases, 6%); *ERBB2* (*HER2/neu*) (2 cases, 6%); *NF1* (2 cases, 6%) and *TSC1* (2 cases, 6%). Notable additional alterations included *TP53* (19 cases, 54%) and *RB1* (6 cases; 17%). Genes involved in chromatin modification were altered by nonsense mutation, splice site mutation or frameshift indel in a mutually exclusive manner in nearly half of all cases including *KDM6A* (10 cases; 29%) and *ARID1A* (7 cases; 20%). Comprehensive NGS of 35 UCs of the bladder revealed a diverse spectrum of actionable GAs in 83% of cases, which has the potential to inform treatment decisions for patients with relapsed and metastatic disease.
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Urothelial carcinoma (UC) is the most common form of urinary bladder malignancy with 73 510 new cases and 14 880 UC-related deaths reported in the United States in 2011, and is more prevalent in men than women.[1] Known risk factors include cigarette smoking and exposure to other environmental toxins, poisons and workplace-related chemicals.[2–4]

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The majority of bladder UC presents as low-grade exophytic papillary tumors that extend into the bladder lumen and do not invade the bladder's smooth muscle wall.[5] The non-invasive papillary tumors and non-invasive *in situ* UCs are typically treated with the installation of intraluminal (intravesical) chemotherapy and immunotherapy.[6–9] Although this approach controls the disease in most patients for long periods of time, many patients ultimately experience disease progression heralded by both transformation of the tumor from low grade to high grade and the development of muscle-invasive disease.[6] Both patients who experience disease progression and the patients who initially present with muscle-invasive UC are most often treated surgically by either partial or total

cystectomies.[10] Although many patients are cured by this approach, a significant proportion of post-cystectomy patients subsequently relapse and develop metastatic disease.[6,11] Although many patients with metastatic UC respond to the cytotoxic chemotherapy regimens used for this disease, a significant number of relapsed and metastatic UC are either resistant to the non-targeted systemic chemotherapy at the time systemic therapy begins or develop resistance to chemotherapy over time.[12–15]

Characterization of the genomic drivers of UC development and progression has long been of interest to cancer biologists, urologists, genitourinary oncologists and pathologists.[16–20] Protein expression studies mostly performed on formalin-fixed, paraffin-embedded (FFPE) specimens using immunohistochemistry (IHC) have been widely used to predict prognosis.[16–20] Increased DNA copy number determined by fluorescence *in situ* hybridization (FISH) has been used for both early UC detection[21,22] and prediction of prognosis.[23,24] Studies on mRNA and miRNA expression in UC have also been carried out using RT-PCR and genomic microarrays, and have uncovered expression profiles linked to disease outcome.[25–28] There has been considerable interest in the mutational status of the tumor suppressor gene *TP53* as a prognostic factor and guide to surgical management of the disease.[29,30] Although *TP53* single gene sequence assays have been applied to UC, most laboratories have used IHC to detect the expression of presumed mutant TP53 protein to help guide treatment planning in some cases.[31] In contrast, 'one-off' single gene assays widely applied for the current management of non-small-cell lung and colorectal cancers and metastatic melanoma have not, to date, been utilized for the clinical management of metastatic UC.[32] Thus, given the limited accuracy of TP53 IHC for predicting actual *TP53* mutations, the lack of current systemic therapy impact obtained from determining the *TP53* mutation status and the current lack of single gene assays available to drive treatment selection for metastatic UC, a number of investigators have queried whether comprehensive genomic profiling of hundreds of cancer-related genes could potentially assist medical oncologists in the selection of targeted therapies for patients with metastatic UC.

Materials and methods

The pathologic diagnosis of each case of relapsed and metastatic UC was confirmed on routine hematoxylin- and eosin-stained slides. All samples sent for DNA extraction contained a minimum of 20% DNA derived from tumor cells. DNA was extracted from 40 μm of FFPE tissue using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega) and quantified using a standardized PicoGreen fluorescence assay (Invitrogen). Library Construction was performed as described previously, using 50–200 ng of DNA sheared by sonication to ~100–400 bp before end-repair, dA addition and ligation of indexed, Illumina sequencing adaptors.[33] Enrichment of target sequences (3320 exons of 182 cancer-related genes and 37 introns from 14 genes recurrently rearranged in cancer representing approximately 1.1 Mb of the human genome) was achieved by solution-based hybrid capture with a custom Agilent SureSelect biotinylated RNA baitset.[33] The selected libraries were sequenced on an Illumina HiSeq 2000 platform using 49×49 paired-end reads. Sequence data from genomic DNA was mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner and were processed using the publicly available SAMtools, Picard and Genome Analysis Toolkit.[34,35] Point mutations were identified by a Bayesian algorithm; short insertions and deletions determined by local assembly; gene copy number alterations (amplifications) by comparison to process matched normal controls; and gene fusions/rearrangements were detected by clustering chimeric reads mapped to targeted introns as described previously.[36] Actionable GAs were defined as impacting anticancer drugs on the market or in registered clinical trials. Local site permissions to use clinical samples were used for this study.

Results

The 35 UC patients had a mean age of 64.0 years (range 27–82 years) with 6 female (17%) and 29 male (83%) patients (Table 1). Thirty-four (97%) tumors were high grade, 1 tumor was low grade (3%) and 35 (100%) patients were stage IV at the time of genomic analysis. NGS was performed on the primary UC in 23 (66%) cases including 15 transurethral bladder tumor resections (TURBT), 1 urethral biopsy and 7 radical cystoprostatectomies. Twelve (34%) patients had NGS performed on metastatic site biopsies including four lymph node biopsies, four liver biopsies and biopsies of metastases to the brain, lung, psoas muscle and abdominal wall, respectively.

A total of 139 GAs were identified in the UC series, with an average of 4.0 GAs per tumor (range 0–10) (Table 1, Figure 1 and Supplementary Table 1), of which 78 (56%) were considered to be actionable, with an average of 2.2 actionable alterations per tumor (range 0–7). Twenty-nine (83%) UC cases harbored at least one actionable GA potentially impacting selection of targeted therapies including: *PIK3CA* (9 cases; 26%); *CDKN2A/B* (8 cases; 23%); *CCND1* (5 cases; 14%); *FGFR1* (5 cases; 14%); *CCND3* (4 cases; 11%); *FGFR3* (4 cases; 11%); *MDM2* (4 cases; 11%); *EGFR* (2 cases, 6%); *ERBB2* (*HER2/neu*) (2 cases, 6%); *NF1* (2 cases, 6%) and *TSC1* (2 cases, 6%).

Table 1 Clinicopathologic features and genomic alterations in 35 cases of urothelial carcinoma of the urinary bladder

Study no.	*Gender*	*Age (years)*	*Specimen used for NGS*	*Tumor type*	*Tumor grade*	*Tumor stage at the time of NGS*	*Genomic alterations*	*Actionable alterations*
1	M	40	Cystectomy	UC	HG	IV	5	2
2	M	51	TURBT	UC	HG	IV	2	1
3	F	56	TURBT	UC	HG	IV	5	2
4	M	64	TURBT	UC	HG	IV	6	3
5	F	42	Lymph node biopsy	UC	HG	IV	3	1
6	M	64	Liver biopsy	UC	HG	IV	5	1
7	M	68	TURBT	UC	HG	IV	0	0
8	M	80	Psoas muscle biopsy	UC	HG	IV	1	0
9	F	65	Abdominal wall biopsy	UC	HG	IV	0	0
10	F	71	Lymph node biopsy	UC	HG	IV	3	2
11	M	68	TURBT	UC	HG	IV	0	0
12	M	80	Cystectomy	UC	HG	IV	3	1
13	M	83	Brain biopsy	UC	HG	IV	9	8
14	M	61	TURBT	UC	HG	IV	5	3
15	M	60	Lung biopsy	UC	HG	IV	2	1
16	M	73	Urethra biopsy	UC	HG	IV	6	2
17	M	57	TURBT	UC	HG	IV	6	5
18	M	71	TURBT	UC	HG	IV	4	1
19	M	76	TURBT	UC	HG	IV	6	3
20	M	53	Cystectomy	UC	HG	IV	6	3
21	M	27	TURBT	UC	HG	IV	6	3
22	M	71	Cystectomy	UC	HG	IV	3	1
23	M	72	Liver biopsy	UC	HG	IV	4	3
24	M	58	TURBT	UC	HG	IV	6	5
25	M	77	Cystectomy	UC	HG	IV	5	1
26	M	77	Lymph node biopsy	UC	HG	IV	3	1
27	M	82	Cystectomy	UC	HG	IV	1	1
28	F	71	TURBT	UC	HG	IV	0	0
29	M	74	Mediastinal lymp node	UC	HG	IV	7	1
30	M	75	Cystectomy	UC	HG	IV	1	0
31	F	43	Liver biopsy	UC	HG	IV	4	2
32	M	58	TURBT	UC	LG	IV	3	2
33	M	72	TURBT	UC	HG	IV	6	4
34	M	48	TURBT	UC	HG	IV	1	1
35	M	52	Liver biopsy	UC	HG	IV	4	1

The amplifications, mutations and fusions in the *FGFR1* and *FGFR3* genes (9 patients; 26% of all patients) were the most common class of actionable GA discovered in this study. The *FGFR1* fusion (case 16) was an *FGFR1-NTM* fusion in a 73-year-old male patient with metastatic UC in which both the histopathologic diagnosis and NGS assay were performed on the same urethral biopsy (Figure 2a). This tumor also featured potentially actionable amplifications of *CCND3*, *CDK4*, *MCL1* and *MDM2*. The *T141R* mutation (case 17) in *FGFR1* was detected in a 57-year-old man using a transurethral bladder tumor resection specimen. This tumor also had potentially actionable amplifications of *ERBB2*, *RAF1* and *CCND1* plus a homozygous deletion in the tumor suppressor gene *CDKN2A/B* (Figure 2b). The *FGFR1* gene amplifications were uniformly six or greater copies per cell and were identified in three cases: case 6 from a liver biopsy specimen in a 54-year-old man whose tumor also had *MYCN* amplification and non-actionable mutations in *ARID1A*, *RB1* and *TP53*; case 12, an 80-year-old man who underwent a cystoprostatectomy and whose tumor also featured non-actionable mutations in *KD6MA* and *TP53*; and case 13, an 80-year-old man with brain metastasis whose tumor also featured seven potentially actionable additional GA including loss of *CDKN2A/B*, mutation in *PIK3CA* and amplification of *RAF1*. The two cases with *FGFR3* fusions included a *FGFR3-TACC3* fusion that has been previously described in glioblastoma[37] and urothelial bladder cancer.[38] This *FGFR3-TACC3* fusion was found in case 23, a 72-year-old male patient with metastatic disease to the liver whose tumor also featured potentially actionable amplification of *CCND1* and mutation of *PIK3CA*. The second *FGFR3* fusion was the *FGFR3-JAKMIP1* fusion seen in case 34, a 48-year-old man whose original bladder biopsy specimen was used for genomic profiling. This tumor had no additional GA. Two patients had UC, which featured known activating mutations in *FGFR3*: case 24, a 58-year-old male patient whose tumor also had amplifications in the *MDM2* and *PIK3CA*, loss of *CDKN2A/B* and a mutation in *TSC1* (Figure 2c); and case 25, a 77-year-old man whose tumor also

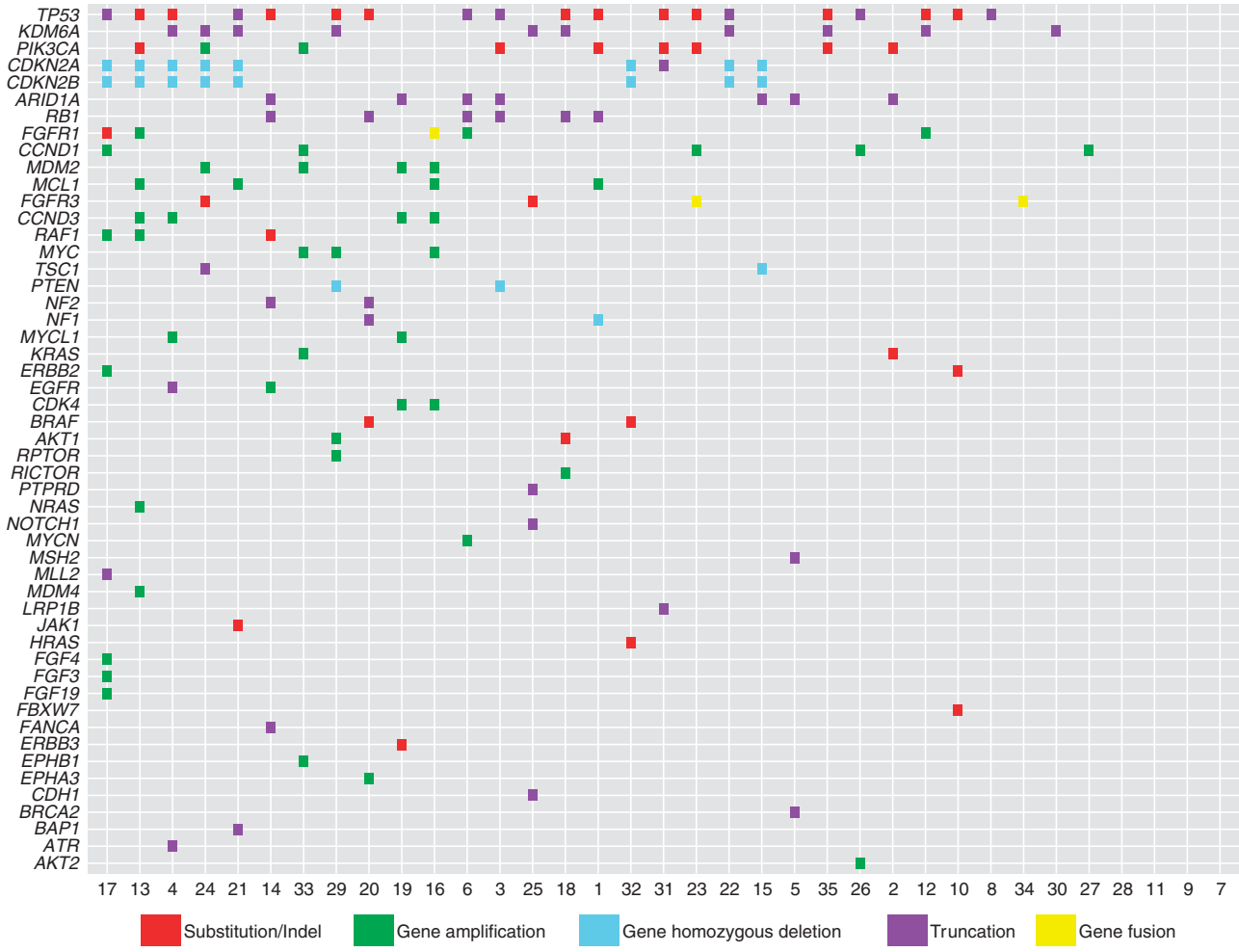

Figure 1 Tile plot of genomic alterations in 35 cases of urothelial carcinoma.

harbored multiple non-actionable gene mutations including a mutation in *CDH1*.

Additional notable actionable alterations included two cases (6%) with alterations in *ERBB2* (*HER2/neu*), which included one *ERBB2* gene amplification (Figure 2b) and one *ERBB2* gene mutation (S310F) (Figure 2d), two cases (6%) with alterations in *EGFR*, which included one *EGFR* amplification and one *EGFR* mutation (Q486*), two cases (6%) with alterations in *TSC1* (one frameshift indel and one homozygous deletion), two cases with amplification in *RAF1* and one case (4%) with a mutation in *JAK1*. The tumor with an *ERBB2* amplification (case 17 described above) also had amplifications in five other genes, including *RAF1* and *CCND1*, a homozygous deletion in *CDKN2A/B* and the described *FGFR1* mutation (Figure 2b). The tumor with an S310F *ERBB2* mutation was obtained from a metastatic UC to a lymph node in a 71-year-old woman (Figure 2d). This tumor also had mutations in the *FBXW7* and *TP53* genes.

Other notable additional alterations included *TP53* (19 cases; 54%), *RB1* (6 cases; 17%) and *MCL1* (4 cases; 11%). Six (17%) tumors had two distinct *TP53* GA. In the one case of UC, a GA in *TP53* was the sole GA in this tumor. Of the 19 UC with *TP53* GA, 6 (32%) also featured a mutation in the *RB1* gene, and 6 (32%) had mutations in the *PIK3CA* gene (Table 1). Genes involved in chromatin modification were altered by nonsense mutation, splice site mutation or frameshift indel in a mutually exclusive manner in nearly half of all cases including *KDM6A* (10 cases; 29%) and *ARID1A* (7 cases; 20%).

Discussion

For patients receiving standard of care chemotherapy, the five-year progression-free survival rates for patients with systemic UC of the urinary bladder are in the 10–11% range.[12–15] The overall survival rates are 7% for patients with visceral metastases and 21% for patients without visceral metastases.[15] Although multiple first- and second-line chemotherapy regimens are available, most often using a platin-containing regimen, the efficacy is limited

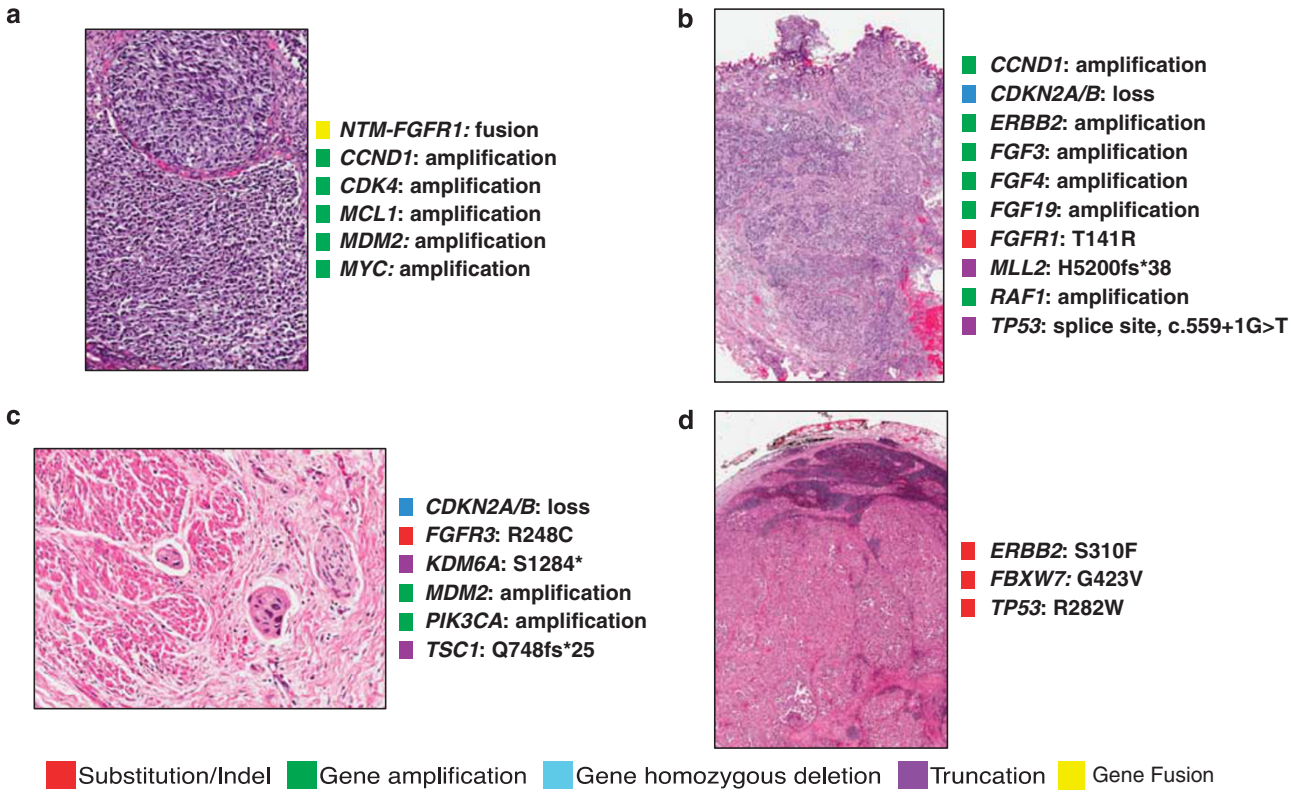

Figure 2 Case examples of UC with significant genomic alterations. (**a**) *FGFR1-NTM* fusion in UC. This UC (case 16) presented as a urethral mass in a 73-year-old man who developed metastatic high-grade disease. This tumor was sequenced to a depth of 1037 ×, which revealed six alterations including an *FGFR1-NTM* gene fusion. Additional alterations included amplifications of the *CCND3*, *CDK4*, *MCL1*, *MDM2* and *MYC* genes. (**b**) *ERBB2* (*HER2*) amplification in UC. This UC (case 17) is a high-grade, advanced-stage tumor from a 57-year-old man. The histology is taken from the transurethral resection specimen used for the NGS assessment. This tumor also featured other potential opportunities for targeted therapies including *CDKN2A/B* loss, *FGFR1*, *MLL2* and *TP53* mutations, and amplifications in *CCND1*, *FGF3*, *FGF4*, *FGF19* and *RAF1* mutation. (**c**) *PIK3CA* amplification and *FGFR3* mutation in UC. This case (case 24) is a 58-year-old male patient with a high-grade stage IV UC. NGS was performed on the primary tumor using a transurethral resection FFPE specimen sequenced to a depth of 1059 ×. This tumor featured six GAs, one of which was an amplification of the *PIK3CA* gene. This is the first *PIK3CA* amplification reported in a case of UC. The tumor also had a *R248C* mutation in the *FGFR3* gene. This tumor's histology does resemble the large bulky intraluminal growth pattern previously described for *FGFR3*-mutated UC, but does not clearly show the koilocytotic nuclear changes also attributed to those tumors. This tumor featured additional potential actionable GA in the *CDKN2A/B*, *MDM2* and *TSC1* genes as well as GA in *KDM6A*. (**d**) *ERBB2* (*HER2*) mutation in UC. This UC (case 10) is a high-grade, advanced-stage tumor from a 71-year-old woman. The histology is taken from the lymph node metastasis specimen used for the NGS assessment. Note that this tumor has a micropapillary architecture. This UC features the S310F base substitution in the *ERBB2* (*HER2*) gene. This tumor also featured mutations in the *FBXW7* and *TP53* genes.

and the toxicity is significant.[12–15] The emergence of the significantly less toxic therapies that target the GA driving an individual patient's disease have produced major responses and prolongation of survival for non-small-cell lung cancer and other solid tumor patients, clearly positively impacting the clinical outcome of disease. The purpose of this study was to search for GA that permit the application of targeted therapy in a series of 35 relapsed UC cases with a single comprehensive NGS-based diagnostic assay encompassing the deep sequencing of hundreds of cancer-related genes at a high, uniform depth of coverage.

In this study, GAs were identified in a wide range of both actionable and non-actionable oncogenes and tumor suppressors. Encouragingly, 29 (83%) of the UC cases had at least one actionable GA. Of these cases, the gene most frequently altered was *PIK3CA*, which was altered by a base substitution in seven cases and gene amplification in two cases. *PIK3CA* encodes the catalytically active subunit of phosphatidylinositol 3-kinase (PI3K) and is the central member of the PI3K pathway that regulates a number of critical cellular functions, including cell growth, proliferation, differentiation, motility and survival.[37,38] Activating *PIK3CA* mutations have been observed in 20–25% of urothelial carcinomas, and such mutations occur mostly in the helical domain.[37] These activating mutations in *PIK3CA* may predict sensitivity to inhibitors of both PI3K and its downstream signaling pathway (the mTOR/Akt pathway).[38] The mTOR inhibitors temsirolimus and everolimus have been tested in several clinical trials in urothelial carcinoma, and are approved by the FDA for use in other solid tumor types. Inhibitors of PI3K and Akt as well as second-generation

mTOR inhibitors are currently in clinical trials in solid tumors, alone or in combination with other therapies.

Amplifications, mutation and gene fusions of the fibroblast growth factor receptor (FGFR) genes *FGFR1* and *FGFR3* (9 patients; 26% of all patients) encompass the most common actionable GA in this series within a gene family. *FGFR1* is an upstream regulator of the RAS, MAPK and Akt signaling pathways and have important roles in the regulation of the cell cycle and angiogenesis.[39] *FGFR1* mutations are rare in UC, with none currently listed in the COSMIC database (October 2012). The *T141R* mutation seen in case 17 of this series represents the first example of this genomic alteration. The *FGFR1-NTM* fusion identified in case 16 also appears to be a novel finding with no previous published report of this fusion for any tumor type including UC. *FGFR1* oncogenic fusions have been frequently reported in myeloproliferative neoplasms,[40] glioblastoma[41] and rhabdomyosarcoma.[42] As a protein assayed by IHC, *FGFR1* is frequently overexpressed in urothelial carcinoma, and has been associated with MAPK pathway activation and the epithelial–mesenchymal transition.[43–45] Three UCs in this series featured *FGFR1* amplifications, consistent with *FGFR1* being commonly amplified in human cancers, and has been previously reported in approximately 3% of UCs.[39,45] FGFR mRNA overexpression has also been reported in UC.[43] An FDA-approved therapy for another indication, ponatinib, does inhibit *FGFR1* and may be useful in the setting of *FGFR1* amplification. Clinical trials of this and other FGFR inhibitors are also currently underway.

In contrast to low alteration rates of *FGFR1*, *FGFR3* has been reported to be the most commonly mutated gene in UC.[45] The COSMIC database lists a 47% *FGFR3* mutation rate in UC (COSMIC, November 2012). The current UC study, however, found that only 11% of cases featured *FGFR3* mutations or fusions in this heavily treated, advanced-stage cohort of patients. This lower rate of *FGFR3* mutation/fusion in the current series is supported by the concept that UC develops through at least two molecular pathways, one related to *FGFR3*, typically in less invasive tumors, and one related to *TP53*, characterized by higher grade invasive tumors,[46] both of which may eventually progress to high-grade disease. In a recent study of a subset of high-grade UC, which harbored *FGFR3* mutations, the tumors were noted to feature a histologic appearance of bulky exophytic disease and koilocytotic nuclear changes.[47] Study of the histology of the four UC cases with *FGFR3* mutation/gene fusion in the current series revealed that one case (case 24) did display the large papillary intraluminal tumor features but without the prominent koilocytotic nuclear changes (Figure 2c). Similar to the UC cases with *FGFR1* GA, the tumors with activating mutations of *FGFR3* may also prove to be sensitive to Fgfr family inhibitors, and clinical trials of these agents, including pazopanib (FDA approved for use in renal cell carcinoma and soft tissue sarcoma), are currently underway.

TP53 and RB are well-known tumor suppressors, but GAs in both are not directly targetable at present. *TP53* alterations were identified in 54% of the cases of metastatic UC. The frequency of *TP53* mutation is consistent with the 44% currently reported *TP53* mutation frequency in the COSMIC database (October 2012) and the 50% TP53 nuclear staining rate by IHC,[48] the latter of which is thought to correlate with the inappropriate increased stability of TP53 via mutation of *TP53*. Although *TP53* mutation has been a significant adverse prognostic factor in most studies, problems in assessment of *TP53* status by IHC have prevented both the universal acceptance of this prognostic significance and the clinical utility of IHC-based *TP53* assessment. GA of TP53 could potentially be better correlated with outcomes, but such a finding is beyond the scope of this study.

Mutations in the *RB1* gene were identified in 17% of UC cases in this study, including four nonsense mutations, one splice site mutation and one frameshift alteration. Loss-of-function GAs in the *RB1* tumor suppressor gene have been reported in 26% of 43 cases of UC listed on the COSMIC database as of October 2012. Loss of *RB1* expression, detected by IHC, has been linked to disease progression and adverse prognosis in urinary bladder UC.[49–51]

Alterations in chromatin regulators that likely serve as tumor suppressors in urothelial carcinoma were frequently observed in this case series. Both mutations in *ARID1A* and *KDM6A* were observed in 49% of our cases, and occurred only in a mutually exclusive manner as seen in a previous study.[52] *ARID1A* mutations occurred in 20% of the relapsed UC surveyed here. *ARID1a* is a member of the SWI/SNF family and is believed to regulate gene transcription via the control of chromatin structure. Although loss or inactivation of *ARID1A* by mutation has been reported in a variety of tumors, there are no reports of *ARID1A* mutation in UC in the COSMIC database (October 2012), although 5% (18/97) of UC did harbor *ARID1A* mutations in a recent study.[52] Twenty-nine percent of the UC in this study harbored mutations in the *KDM6A*, which encodes a histone H3 lysine 27 demethylase (also known as UTX).[37] This frequency may be correlated with selection of advanced, relapsed UC in our study, as, in contrast, *KDM6A* mutations (at 11%) are relatively rare in UC in the COSMIC database as of February 2013. However, another study identified a 21% frequency of *KDM6A* mutations in their series.[52] Interestingly, these investigators suggest that *KDM6A* mutations may be more associated with low-grade and early-stage tumors, although their study population was primarily Asian, and could potentially reflect increased incidence of a preceding Schistosomal infection. In this sequencing study of 97 cases of UC, 24% of cases featured

inactivating mutations of the *KDM6A* gene, which was the most frequently mutated gene identified and was particularly associated with early-stage and low-grade tumors.[52] The eight chromatin remodeling genes evaluated in this study were altered in 59% of the 97 UC patients, and the presumed alterations in chromatin remodeling before cell division was linked to potential UC development and progression.[52]

Amplifications of the *CCND1* in 14% and *MDM2* in 11% of UC were also identified, both of which may be targetable by agents currently in clinical development. *CCND1* encodes cyclin D1, which interacts with the cyclin-dependent kinases Cdk4 and Cdk6, resulting in the phosphorylation and inactivation of Rb and the progression of the cell cycle. Although the expression of cyclin D1, detected by IHC, has been reported in approximately 80% of UC,[53,54] *CCND1* amplification has been reported in approximately 10% of UC of the bladder primarily in high-grade tumors.[55,56] There are no approved therapies that directly target cyclin D1, which is the protein product of *CCND1*; however, *CCND1* amplification might predict sensitivity to inhibitors of Cdk4 and Cdk6, which are currently being tested in clinical trials. *MDM2*, a regulator of TP53, has been reported as being amplified in up to 10% of UCs.[57–59] *MDM2* copy number gain has been associated with adverse outcome in some studies of UCs, but the assessment of a correlation of Mdm2 expression with disease stage has yielded conflicting results, and Mdm2 expression alone does not appear to be a significant biomarker of prognosis in UC patients.[59] *MDM2* antagonists are being studied preclinically and in clinical trials for multiple tumor types. Amplification of *MDM2* may increase sensitivity to these agents, but more data are required to confirm this initial observation.

Several other potentially actionable GAs associated with FDA-approved drugs were identified in lower frequencies in this case series. Two cases (6%) with alterations in *ERBB2* (*HER2/neu*), which included one *ERBB2* gene amplification (Figure 2b) and one *ERBB2* mutation S310F (Figure 2d), were identified. *ERBB2* encodes the receptor tyrosine kinase Her2 and amplification of this gene has been associated with adverse prognosis and benefits from targeted therapy in breast cancer.[60] *ERBB2* amplification, detected by FISH, has been reported in 8–9% of primary UC increasing in incidence with advanced disease stage.[61] Her2 protein overexpression, detected by IHC, has been identified in nearly 20% of UCs of the bladder with a similar significant enrichment in higher grade and muscle-invasive tumors.[62] *ERBB2* amplification is widely accepted as a predictor of sensitivity to Her2-targeted drug therapies, including trastuzumab, lapatinib and pertuzumab, which are approved for use in breast cancer and gastroesophageal cancer (trastuzumab only). Her2-targeted therapy with trastuzumab, lapatanib and other therapies are under investigation for the treatment of *ERBB2*-amplified UCs, but phase 3 clinical trial data have yet to emerge.[63] In case 10 of this study, the UC in a 71-year-old female patient with stage IV high-grade UC, an S310F external domain mutation in the *ERBB2* gene was identified and is the first known mutation of *ERBB2* in UC. Recent *in vitro* data suggest that *ERBB2* S310F is an activating mutation, which is sensitive to irreversible dual Egfr/Erbb2 inhibitors.[64] *ERBB2* mutations have not been previously reported in urothelial carcinoma (COSMIC, PubMed, August 2012), yet may predict sensitivity to Her2-targeted drug therapies analogous to ongoing clinical studies in both non-small-cell lung and breast cancers.

Additional actionable GAs found in this series of UC included one frameshift indel and one homozygous deletion of *TSC1*, which may be associated with sensitivity to mTOR inhibitors.[65–68] A recent study demonstrated the utility of cancer genomic profiling by linking such mutations in *TSC2* to improved survival for UC patients under everolimus treatment.[69] Amplification of *RAF1*, which has been linked to high-grade tumor, advanced-stage tumor and poor survival in UC,[70–72] was also observed in the series, and can be targeted by kinase inhibitors such as Sorafenib, a multikinase inhibitor whose targets include the Raf1 protein (CRAF). Sorafenib has been approved for use in renal cell carcinoma and hepatocellular carcinoma and is under investigation in clinical trials in multiple solid tumor types.

In summary, there has been keen interest in both developing and identifying targeted therapies to benefit patients with metastatic UC.[73,74] Deep genomic profiling with a comprehensive NGS-based diagnostic assay of metastatic UC identified an unexpectedly high frequency of potentially actionable GAs that can both influence therapy selection and direct patients to enter clinical trials using targeted therapies. Moreover, these opportunities for UC patients to receive targeted therapies include both commercially available agents approved for other indications and drugs in both early and late stages of clinical development. The diversity and spectrum of the actionable UC GAs identified in this study open pathways for new approaches towards treating this highly malignant and often relentless disease notable for being refractory to conventional, non-targeted treatments.

Disclosure/conflict of interest

The authors JS Ross, K Wang, GA Otto, J He, G Palmer, R Yelensky, D Lipson, S Ali, S Balasubramanian, JA Curran, L Garcia, K Mahoney, SR Downing, M Hawryluk, VA Miller and PJ Stephens are employees and have stock ownership in Foundation Medicine.

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