Research Report

Molecular Correlates of *In Vitro* Responses to Dacomitinib and Afatinib in Bladder Cancer

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Abstract.

Background: The HER family of proteins (EGFR, HER2, HER3 and HER4) have long been thought to be therapeutic targets for bladder cancer, but previous clinical trials targeting these proteins have been disappointing. Second generation agents may be more effective.

Objective: The aim of this study was to evaluate responses to two second-generation irreversible tyrosine kinase inhibitors, dacomitinib and afatinib, in bladder cancer cell lines.

Methods: Cell lines were characterized by targeted next generation DNA sequencing, RNA sequencing, western blotting and flow cytometry. Cell survival responses to dacomitinib or afatinib were determined using (3-[4,5-dimethylthioazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) or [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosylfate (PMS) cell survival assays.

Results: Only two cell lines of 12 tested were sensitive to afatinib. Sensitivity to afatinib was significantly associated with mutation in either HER2 or HER3 ($p < 0.05$). The two cell lines sensitive to afatinib were also responsive to dacomitinib, along with an additional 4 other cell lines out of 16 tested. No characteristic was associated with dacomitinib sensitivity. Molecular profiling demonstrated that only two genes were high in both afatinib and dacomitinib sensitive cells. Further, higher expression of RAS pathway genes was noted for dacomitinib responsive cells.

Conclusions: This study confirms that cell line screening can be useful in pre-clinical evaluation of targeted small molecule inhibitors and suggests that compounds with similar structure(s) and target(s) may have distinct sensitivity profiles. Further, combinational targeting of additional molecularly relevant pathways may be important in enhancing responses to HER targeted agents in bladder cancer.

Keywords: Sequence analysis, RNA, CHD5 protein, human, TCHH protein, human, ErbB Receptors, cell line, tumor, genes, ras

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INTRODUCTION

Bladder cancer is predicted to have over 79,000 new cases and cause over 16,000 deaths in 2017. It is the second most common genitourinary cancer, fourth most common cancer in men, and the eighth highest cause of cancer death in men [1]. Mortality for invasive and metastatic bladder cancer remains high [2]. The recent observations of impressive and durable responses to new immune therapies were the first improvement in metastatic bladder cancer treatments in the past 20 years. However, responses to these promising new agents are limited to 20–30 percent of patients [3]. New and more effective treatments as well as predictive and prognostic markers must be found.

Precision medicine, which matches targeted therapies to the individual molecular profile of a patients' tumor, has shown promise in many tumor types [4]. For bladder cancer, members of the HER/erbB family have been recognized as potential targets. The HER family includes four proteins: HER1/epidermal growth factor receptor (EGFR); HER2/erbB2; HER 3; and HER 4. Of these four proteins, three (EGFR, HER2 and HER4) have intracellular portions capable of cell signaling, while HER3 lacks active intracellular kinase domains. In response to ligand binding, members of this family form dimers which activate a tyrosine kinase signaling cascade [5–7].

EGFR overexpression has long been observed in bladder cancer, and appears to contribute to progression and invasion in bladder cancer [8]. HER2 expression has been reported in bladder cancer, and some studies suggest expression may be associated with more aggressive metastatic disease and shorter survival [9–11]. The Cancer Genome Atlas Project identified amplification in EGFR, amplification and mutation in HER2, and/or HER3 in multiple muscle-invasive bladder cancer tumors. Additionally, these alterations were mostly found in different tumors, which results in nearly 20% of patients demonstrating alteration in one of these genes [12]. These results have been confirmed in an independent study [13]. In spite of these data, early clinical trials targeting either EGFR or HER2 were mixed. Initial studies were performed using antibody-based agents that target the external domains of EGFR (cetuximab) or HER2 (trastuzumab). Cetuximab, when added to a combination of gemcitabine and cisplatin, produced greater toxicity with no improvement in overall survival or progression-free survival compared to the chemotherapies alone in patients with advanced urothelial cancers [14]. In a single-armed phase II clinical trial, trastuzumab added to paclitaxel, carboplatin and gemcitabine showed a high 70% initial response rate, but the time to progression and survival rates were no better than historical results for standard chemotherapy in metastatic urothelial cancer [15]. Since these agents target only one HER family member, dimerization with an untargeted HER partner might explain clinical resistance [7]. Experimental data have shown that EGFR may also partner with other non-HER family receptors [16, 17], providing additional means of resistance to therapies targeting EGFR activation.

A second generation of small molecule HER family drugs targeting the tyrosine kinase activity of HER family members have been generated [18]. This approach should be effective against any HER family dimers regardless of the participating receptors. Lapatinib is a reversible HER family tyrosine kinase inhibitor (TKI) that has shown promise in pre-clinical studies in bladder cancer [19]. However, clinical trials with this drug in unselected patients with advanced bladder cancer have been disappointing [20, 21].

New irreversible TKI HER family drugs are now available for study [18]. These drugs were developed specifically to target EGFR mutations that allow cancer cells to evade other EGFR targeting therapies [18, 22–24]. Even though such EGFR mutations are not common in bladder cancer [12] the high level of HER family expression in bladder cancer, along with previous lackluster results with traditional HER targeting agents, compels studies of other HER targeting drugs. Additionally, our expanding knowledge of the genetics of advanced bladder cancer may reveal subtypes more likely to respond to HER targeted agents. For example, up to 40% of cases of the aggressive micropapillary subtype have HER2 mutations when fewer than 10% of non-micropapillary bladder cancers have such mutations [13, 25].

Two of the irreversible HER targeting agents, dacomitinib and afatinib, have very similar chemical structures and even bind to identical sites on the mutant EGFR molecule [26, 27]. Dacomitinib has been shown to have promise in bladder cancer pre-clinical studies [28]. Afatinib is another irreversible pan-EGFR inhibitor which has activity in tumors. A phase II trial of single agent afatinib in metastatic platinum-refractory metastatic bladder cancer showed that patients with HER2 or HER3 molecular alterations derived clinical benefit from afatinib [29]. The identification of genomic, transcriptomic and protein markers to indicate which

patients with metastatic bladder cancer might benefit from EGFR-targeted therapies is essential for further drug development in this patient population. In this study, we explored the endogenous genetic and protein expression profiles of a panel of bladder cancer cell lines and compared them to the *in vitro* cytotoxicity responses to dacomitinib and afatinib to identify potential biomarker predictors of sensitivity to these kinase inhibitors.

MATERIALS AND METHODS

Chemicals

Dacomitinib was provided by Pfizer (New York, NY) and afatinib purchased from Selleckchem (Houston, TX). Staurosporine, MTT (3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyl tetrazolium bromide) and phenazine methosulfate (PMS) were purchased from Sigma Chemicals (St. Louis, MO). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was purchased from Promega (Madison, WI).

Cell lines

All cells were cultured in Dulbecco's Minimal Essential Medium (DMEM; Corning, Manassas VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/Fungizone (Corning, Manassas, VA). Cell lines 253J, 5637, J82, RT4, SW780 and T24 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). UM-UC-1,3,5,6,7,9,10,12,13,14,15,17 and 18 were obtained from the originator, Dr. H. Barton Grossman, The University of Texas-MD Anderson Cancer Center, Houston, TX. All cell lines were appropriately characterized or found to be unique by DNA short tandem repeat analysis (IDEXX Bioresearch, Columbia, MO) within 6 months of use. All cultures were monitored and found to be free of bacterial and mycoplasma infection (last tested 2017/8/24).

Cell survival assays

Cell survival assays that were performed used standard techniques [30, 31]. Briefly, 0.1 mL of cells were plated in a 96-well plate at 1000–2000 cells/well in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin but without phenol red, and allowed to incubate overnight. The next day, 0.1 mL of various concentrations of drugs (dacomitinib or afatinib), diluted in DMEM without phenol red, were added to wells at least in triplicate. Cells without drug treatment (receiving 0.1 mL of DMEM without phenol red) served as a negative control, and cells treated with $2.5\mu M$ staurosporine diluted in DMEM without phenol red to determine maximal cytotoxicity as a positive control. After four days, the number of surviving cells was quantitated using an indicator; for dacomitinib, MTT was used as previously described [30] and for afatinib, MTS + PMS [31]. The results were analyzed by graphing the log of inhibition versus response with a nonlinear fit calculation to determine the IC_{50} (GraphPad Prism Software v5, La Jolla, CA). For each drug, mean IC_{50} values were used to classify bladder cancer cell lines as having a sensitive, moderate, or resistant response. 3-bin k-means clustering as implemented in the R programming language [32] was used to classify cell lines as sensitive, moderate, or resistant to dacomitinib.

Flow cytometry

Levels of cell surface expression of HER proteins were evaluated at the University of Michigan Cancer Center core facility on a Fortesse Cell analyzer (Becton Dickinson Biosciences, San Jose, CA) using FAC Diva 8.0.1 software (Becton Dickinson Biosciences, San Jose, CA). The following antibodies were used: Phycoerythrin or fluorescein isothiocyanate labeled rat monoclonal antibody to human EGFR (ICR 10, Abcam, Eugene, OR); allophycocyanin (APC) labeled anti-HER2 (Becton Dickinson Biosciences, San Jose, CA); APC labeled anti-human HER3 (R&D Systems, Minneapolis, MN); APC labeled anti-human HER4 (R&D Systems, Minneapolis, MN), and isotype controls mouse IgG2A (R&D Systems, Minneapolis, MN) and mouse IgG1 (Miltenyi Biotech, San Diego, CA). Cells were incubated with antibodies for 30 minutes-1 hour in the dark at room temperature on a rotator. After incubation, the cells were washed with phosphate buffered saline containing 1% fetal bovine serum (1% FBS/PBS), centrifuged, and resuspended in 1% FBS/PBS. The samples were kept on ice in the dark prior to analysis on the flow cytometer.

Western blotting

Western blotting of whole cell extracts was performed as previously described [33]. Briefly, cells were extracted by scraping into ice-cold lysis buffer and centrifuged x 14,000 g for 15 minutes. Equal

amounts of protein (25μg) for each cell line were separated by SDS-PAGE and transferred to FL-PVDF membranes (Bio-Rad, Hercules, California). The membrane was incubated with primary antibodies overnight at 4ºC, washed, and incubated with secondary antibodies for one hour at room temperature. Antibodies used as probes were mouse anti-EGFR (Ab-15 Clone H9B4, ThermoFisher Scientific, Fremont, CA); mouse anti-erbB2 (Ab-17 Clone e2-4001+3B5, ThermoFisher Scientific, Fremont, CA) and mouse anti-β-actin (Sigma-Aldrich, St. Louis, MO). Blots were imaged using LI-COR IRDye 800CW and 680LT fluorescent secondary antibodies and a fluorescence imaging system (LI-COR, Lincoln, NE).

Targeted next generation sequencing (NGS)-DNA

Genomic DNA was isolated from bladder cancer cell lines using the Qiagen DNeasy kit (Qiagen, Valencia, CA, USA) with RNA-Seq treatment. Targeted multiplexed PCR based sequencing (Ampliseq) was performed using the DNA component of the Oncomine Comprehensive Panel (OCP), which assesses mutations, short insertions/deletions (indels) and copy number alterations in ∼135 oncogenes and tumor suppressors as described [34]. High confidence somatic mutations/indels and copy number alterations were prioritized as described (e.g. hotspot mutations or amplifications in oncogenes; hotspot mutations, deleterious mutations or deep deletions in tumor suppressors) [34].

Genomic DNA was used for library generation using the Ion Ampliseq library kit 2.0 (Life Technologies, Foster City, CA) according to manufacturer's instructions with barcode incorporation as described. Templates were prepared using the Ion PGM Template OT2 200 Kit or Ion PI Template OT2 200 kit (Life Technologies, Foster City, CA) on the Ion One Touch 2 according to the manufacturer's instructions. Sequencing of multiplexed templates was performed using the Ion Torrent Personal Genome Machine (PGM) on Ion 318 chips with the Ion PGM Sequencing 200 Kit v2 or on the Ion Torrent Proton machine using Ion PI chips using the Ion PI Sequencing 200 Kit v2 (Life Technologies, Foster City, CA) according to the manufacturer's instructions.

Variant calling

Sequencing data was analyzed using Torrent Suite 5.0.2 with alignment by TMAP using default parameters, and variants called via the Torrent Variant Caller plugin (version v5.0.2.1) using default low-stringency somatic variant settings. Called variants were filtered to remove low-quality or panel-specific errors as previously described [34], including flow-corrected sequencing depth of <40 reads and variants at variant fractions of <10% in tumor suppressors or <5% in oncogenic hotspots. Synonymous and non-coding variants passing these initial filters were also removed, as were all variants present in the ExAC database at >0.1% (except those with pathogenic Clinvar annotation). The variant classification, gene/protein consequences and effect in protein function were identified using SNPnexus (http://www.snp-nexus.org) [35], Ensembl (http://useast.ensembl.org/index.html) [36], USCS Genome Browser (https://genome.ucsc.edu) [37] and RefSeq-NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq) [38] annotation tools. The findings were corroborated by bibliographic search.

Copy number analysis

Normalized, GC-content corrected read counts per amplicon for each sample were divided by those from a pool of normal male genomic DNA samples (FFPE and frozen tissue, individual and pooled samples), yielding a copy number ratio for each amplicon. Gene-level copy number estimates were determined as described previously [39–41] by taking the coverage-weighted mean of the per-probe ratios, with expected error determined by the probe-to-probe variance. Genes with a log_2 copy number ratio estimate of <–1 or >0.8 were considered to have high level loss and gain, respectively.

RNA-Seq profiling, differential expression, and gene set enrichment

Total RNA was isolated from bladder cancer cell lines using QiZol (Qiagen, Valencia, CA, USA) and the RNeasy Kit (Qiagen) with DNAseq digestion. Paired end RNA sequencing libraries were prepared and sequenced using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) as described [34].

Fragment reads were trimmed using Trimmomatic v0.36 with the following parameters: ILLUMINACLIP:TruSeq3-PE.fa:2 : 30 : 10 LEA-DING:3 TRAILING:3 SLIDINGWINDOW:4 : 15 MINLEN:36 [42]. Fragments were aligned to Gencode Release 19 GRCh37.p13 using STAR v2.5.2a with the following parameters: –outSAMtype BAM Unsorted –outFilterType BySJout –outFilterMultimapNmax 20

–outFilterMismatchNmax 999 –outFilterMismatch-NoverLmax 0.04 –alignIntronMin 20 –alignIntronMax 1000000 –alignMatesGapMax 1000000 –alignSJoverhangMin 8 –alignSJDBoverhangMin 1 –outSAMstrandField intronMotif [43]. FPKM values were quantified using Cufflinks v2.2.0 with the following parameters: –max-bundle-frags 10000000 –library-type fr-unstranded [44]. FPKM values were normalized using the upper quartile normalization method [45].

Gene expression analysis was performed on each sample using the Cufflinks pipeline, as previously described [46]. Briefly, cDNA fragment size distribution means and standard deviations were estimated from unspliced alignment to the genome. Next, reads were prepared with Tophat 2.0.4's "prep_reads" software, using the previously estimated fragment size distributions, prior knowledge of Ensembl gene annotations (hg19, GRCh37.69), and the following parameters: –min-anchor 8; –splice-mismatches 0; –min-report-intron 50; –max-report-intron 500000; –min-isoform-fraction 0.15; –max-multihits 20; –max-seg-multihits 40; –segment-length 25; –segment-mismatches 2; –min-closure-exon 100; –min-closure-intron 50; –max-closure-intron 5000; –min-coverage-intron 50; –max-coverage-intron 20000; –min-segment-intron 50; –max-segment-intron 500000; –max-mismatches 2; –max-insertion-length 3; –max-deletion-length 3; –no-closure-search; –no-coverage-search; –no-microexon-search; –library-type fr-firststrand. Alignment was then performed with Tophat 2.0.4, using Bowtie2, and again using the previously estimated fragment size distributions and Ensembl gene annotations. Finally, the expression of each Ensembl gene was estimated using Cufflinks version 2.0.2, using the following parameters: –library-type fr-firststrand; and –multi-read-correct. Gene-level FPKM values were then rounded to the nearest tenths place and summarized in expression matrices.

Fragment counts were generated per Ensembl gene using the SubRead featureCounts tool [47]. DESeq2 was used to quantify differential expression between sensitive and resistant cell lines [48]. Briefly, DESeq2 models sample read counts using a negative binomial distribution and a generalized linear model to estimate expression strengths, fold changes, and pValues. pValues were adjusted for multiple testing using the BH method to calculate qValues. For each drug, genes with a fold change ≥ 2 and a qValue ≤ 0.05 were considered to be differentially expressed. Additionally, for afatinib differential expression we required that all candidate genes have ≥ 0.5 FPKM value in both sensitive cell lines to prevent a high expressing sensitive line from masking a cell line with no expression for a given gene.

To interpret the meaning of genes with high relative expression in drug sensitive cell lines, we calculated statistical enrichment with KEGG and gene ontology (GO) annotated gene sets using a Fisher's exact test. GO gene sets were downloaded 9/30/2016 using the GO.db Bioconductor package [49–51]. KEGG gene sets were downloaded using the KEGG web API on 9/30/2016 [52]. All gene set gene IDs were mapped to Entrez gene IDs and only Entrez gene IDs that mapped to both Gencode Ensembl IDs and a given gene set source were considered in the Fisher's exact test calculation. P-values were adjusted for multiple testing using FDR for each gene set source and only gene sets with a qValue ≤ 0.05 were considered to be significant.

RESULTS

Characterization of cell lines

A panel of human bladder cancer cell lines were characterized by their gene copy number, gene mutations and expression of HER family genes in order to examine sensitivity to afatinib and dacomitinib. Only one cell line, UC5, had a high level amplification of EGFR, and six other cell lines showed a low gain in copy number for EGFR (Table 1). One cell line, UC18, had a low gain in copy number for HER2. Genomic amplification or deletions were not observed for HER3 and HER4. The bladder cancer cell line 5637 harbors an ERBB2 S310F mutation, and both UC15 and UC3 had missense mutations in ERBB3 (Table 2). All mutations are in the coding regions of the genes, resulting in alterations in the extracellular protein domains [12] and are predicted to be activating [13, 25, 53, 54].

These results largely corresponded to RNA levels observed from RNA-Seq analyses of these cell lines with UC5 displaying the highest levels of EGFR and UC18 displaying the highest level of ERBB2 and UC3 very low levels (Fig. 1A). RNA sequencing demonstrated variable mRNA levels for HER3 with highest expression seen in UC9. HER4 mRNA expression was exceedingly low across all cell lines examined (Fig. 1A). These results were confirmed in a selected panel of cells by cell-surface flow cytometry (data not shown).

Protein expression for HER family members was evaluated by western blotting. As expected based on genomic and gene expression analysis, UC5 cells dis-

played the highest level of EGFR protein by western blotting, consistent with its amplified status (Fig. 1B). UC18, which harbored a low gain of HER2 genomic DNA also displayed the most HER2 protein (Fig. 1B). Two cell lines, UC9 and UC10, have relatively higher levels of HER3 (data not shown).

These results suggest that UC5 (amplification and overexpression of EGFR), 5637 (HER2 mutation), UC18 (low gain copy number of HER2 with high protein expression), UC15 and/or UC3 (HER3 mutation) might be sensitive to inhibition of the EGFR/HER pathway. Additionally, UC7, had relatively high expression of EGFR, HER2 and HER3 by western blotting and RNA sequencing, suggesting possible sensitivity to EGFR/HER signaling inhibition.

Table 1
ErbB Family genomic alterations in the bladder cancer cell line panel

Cell Line	
253J	Low gain EGFR
UC1	Low gain EGFR
UC5	Amplified EGFR
UC7	Low gain EGFR
UC9	Low gain EGFR
UC10	Low gain EGFR
UC12	Low gain EGFR
UC18	Low gain ERBB2

Table 2
Somatic mutations in ERBB genes in bladder cancer cell line panel

| Cell Line	Gene (HUGO nomenclature)	SNP/Genome change	Protein change/ Protein sequence	Mutation type	Variant classification	Mutation function
5637	ERBB2	g.chr17 : 37868208C>T	p.S310F (extracellular domain)	Substitution	Missense mutation	Exonic
UC15	ERBB3	g.chr12 : 56478854G>A	p.V104M (extracellular domain)	Substitution	Missense mutation	Exonic
UC3	ERBB3	g.chr12 : 56478817G>A	p.M91I (extracellular domain)	Substitution	Missense mutation	Exonic
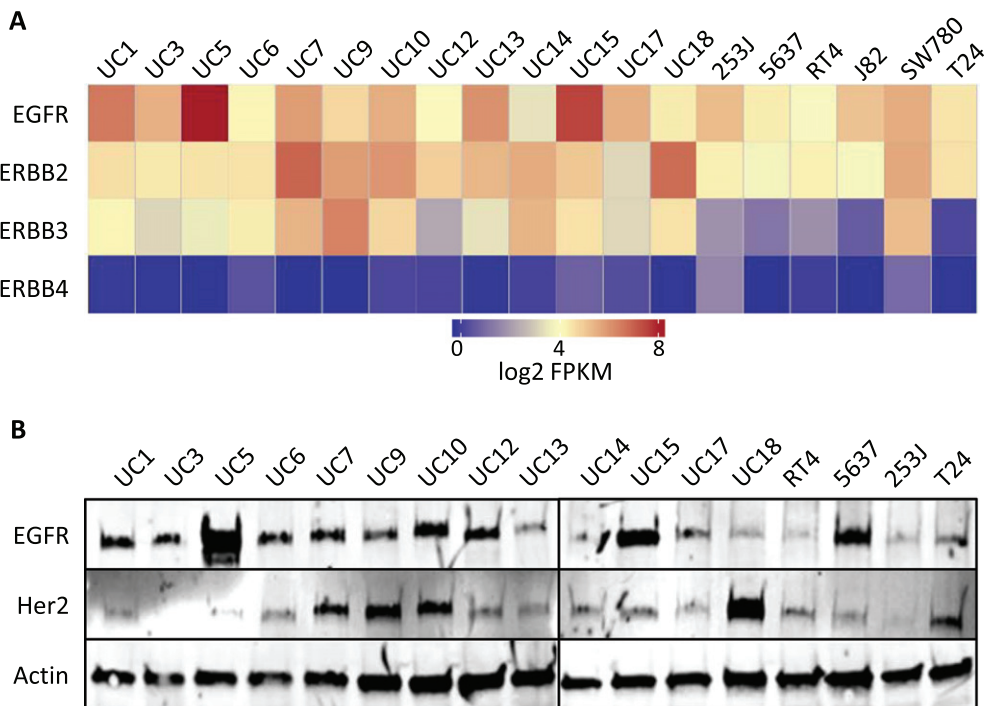
Fig. 1. Endogenous RNA and protein expression of ErbB family members. (A) Heat map of the expression of ERBB genes [in $\log_2$(FPKM)] in bladder cancer cell lines from RNA-Seq analysis. ErbB family members are listed in the rows (EGFR, ERBB2, ERBB3, ERBB4) and cell lines are listed along the columns. (B) Western blot of whole cell lysates from human bladder cancer cell lines, whose names are listed above each lane, which were probed with EGFR and HER2 antibodies. Actin was used as a loading control.

Fig. 2. Cellular responses to afatinib and dacomitinib. MTS and MTT results for both (A) afatinib and (B) dacomitinib on a number of cell lines. The IC$_{50}$ values were generated in triplicate and error bars represent the standard error of the mean. The three categories of dacomitinib response were obtained by 3-bin k-means clustering.

Response to HER targeted therapy

Having determined that our cell line panels included an appropriate sampling of HER family mutations and expression, we used these cells to test responses to afatinib and dacomitinib. Mean IC$_{50}$ values for afatinib varied from 0.39 to 6.14 μM (Fig. 2A). Only two cell lines, UC15 and 5637, were sensitive to afatinib (mean IC$_{50}$ = 0.86 μM). These cells have mutations in HER2 (S310F, 5637) or HER3 (V104M, UC15) affecting the extracellular domains (Table 2; [12]). This result is consistent with the clinical observation that the majority of patients whose tumors were sensitive to afatinib had mutations in HER3 [29]. One other cell line, UC3, has a mutation in HER3 but is not sensitive to afatinib. UC3 also harbors a mutation in *KRAS* which is associated with resistance to EGFR inhibitors in advanced lung cancers and metastatic colon cancers [55]. Interestingly, despite amplification and overexpression of EGFR, UC5 was not sensitive to afatinib, suggesting that amplification of EGFR alone may be insufficient to promote sensitivity to afatinib. Analysis of the cell line responses demonstrated a significant difference between cells with HER2 or HER3 mutations compared to those cells that do not have mutations ($p = 0.045$, Fisher's exact test; Table 3).

Dacomitinib is another irreversible pan-HER inhibitor which was identified by similar screening methods and shows strong chemical structural similarity to afatinib (Fig. 2B). That similarity extends to binding to the same site on mutant EGFR molecules in structural modeling analysis [26, 27]. Despite these similarities, the responses of the bladder cancer cell line panel to dacomitinib were distinctly different from afatinib. The mean IC$_{50}$ values for dacomitinib ranged from 0.95–13.8 μM, and showed a wider spectrum of sensitivities that were grouped into sensitive, intermediate and resistant groups by k-means clustering (Fig. 2B). As expected, the UC15 and 5637 cell lines that were sensitive to afatinib were also sensitive to dacomitinib. In contrast to afatinib, four additional cell lines, including UC5 (amplified EGFR and high EGFR protein expression but no mutation) also displayed sensitivity to dacomitinib. Two cell lines (UC6 and UC13) demonstrated sensitivity to dacomitinib although they had moderate to low expression of HER gene transcription and protein levels, and no mutations were detected. These data confirmed classification from our previous study,

which identified UC6 as sensitive, UC9 as intermediate, and UC3 as resistant to dacomitinib [28].

Preliminary evaluation of predictors for response to dacomitinib revealed a trend toward higher EGFR protein expression. Of the six cell lines sensitive to dacomitinib, four showed higher EGFR expression, while only two out of eight of the moderate and resistant cells had higher EGFR expression. However, these results were not statistically significant, possibly due to the low number of cell lines in each group.

After the cell lines were categorized as sensitive or resistant (Fig. 3A, B), they were profiled using RNA-Seq, and differential analysis was used to identify differences in endogenous gene expression between cell lines that were sensitive to each drug versus resistant cell lines. We focused on genes with increased expression in cell lines sensitive to each drug. Gene expression changes with greater or equal to 2 fold and an FDR adjusted *p*-value less than or equal to 0.05 were considered significant. Using these criteria, we identified 38 genes expressed at higher levels in afatinib sensitive cells (Table S1) and 163 genes expressed at higher levels in dacomitinib sensitive cells (Table S2). We made profiles of the top 38 RNA-Seq differentially expressed genes in afatinib and dacomitinib sensitive cell lines (Fig. 3C, D). The expression profile for the two afatinib sensitive cells were similar to each other but different from the expression profiles of the resistant cells. The expression profiles of the dacomitinib sensitive cells showed that 5637 was different from the other sensitive cells, and appeared to be more similar to the resistant cells.

Of these expression profiles, only two genes were in common between dacomitinib and afatinib responses (out of 163 up-regulated in dacomitinib responsive cells and 38 in afatinib responsive cells), as shown in Fig. 3E. The lack of overlap in the gene signatures of cells sensitive to the two agents is consistent with hypothetical differences in the pathways involved in responses to afatinib and dacomitinib, resulting in in different spectra of activities of the two drugs. The two genes in common are CHD5, encoding a helicase DNA binding protein, and TCHH, encoding trichohyalin, a structural keratin cross-linking protein. TCHH is a member of the "epidermal differentiation complex" located on chromosome 1. Other members of this complex include S100 proteins and other protein components of the cornified envelope [56]. Trichohyalin is one of the "fused" proteins, showing characteristics of calcium binding and signaling, similar to S100 proteins, and keratin cross-linking proteins [57]. Increased differential expression of trichohyalin in afatinib and dacomitinib sensitive cells may reflect greater squamous differentiation and a more "basal" phenotype [58].

The RAS Signaling Pathway was found to be statistically enriched with eight genes expressed at significantly higher levels in dacomitinib sensitive cell lines (Fig. 4). Increased expression of fibroblast growth factor receptor 3 (FGFR3) is common in bladder cancer, and FGFR3 fusions have also been reported [12]. Interestingly, of the cell lines with FGFR3 fusions, SW780 is moderately sensitive to dacomitinib and RT4 is resistant [59]. The fusions are different in the two cell lines: RT4 harbors an FGFR3-TACC fusion, while SW780 has an FGFR3-BAIAP2L1 fusion. The different responses to dacomitinib may reflect functional differences in the two fusions but also suggests that FGFR3 fusion does not play a major role in sensitivity to dacomitinib. FGF19 is one of several FGFR3 ligands [60]. Activation of FGFR3 results in signaling through RAS, which is regulated by RAS A4 [61]. Increased expression of RAS A4 may be an attempt by cells to compensate for RAS and/or FGFR3 hyperactivity. The downstream result that is observed in the dacomitinib sensitive cells is expression of several members of the calcium independent phospholipase A2 family (iPLA2). These enzymes contribute to cell proliferation and metastasis through generation of bioactive lipids such as HETE and prostaglandins [62–64].

DISCUSSION

In this study, we explored responses to two second-generation irreversible HER-family targeting TKIs, afatinib and dacomitinib using a subset of the bladder cancer cell line panels. In spite of the structural similarities of the two drugs, and their emergence from similar drug screening, the responses to the two drugs were distinctly different. Further, the expression profiles of responsive cells to the two drugs showed nearly no overlap, with only two genes in common.

Sensitivity to afatinib was observed in two cell lines, both of which harbor mutations affecting the extracellular domain of either HER2 or HER3. These results are consistent with a recent bladder cancer clinical trial with afatinib [29]. However, one cell line, UC3, with a HER3 mutation was highly resistant to afatinib, presenting an important caveat to the selection of bladder cancer patients solely with tumoral
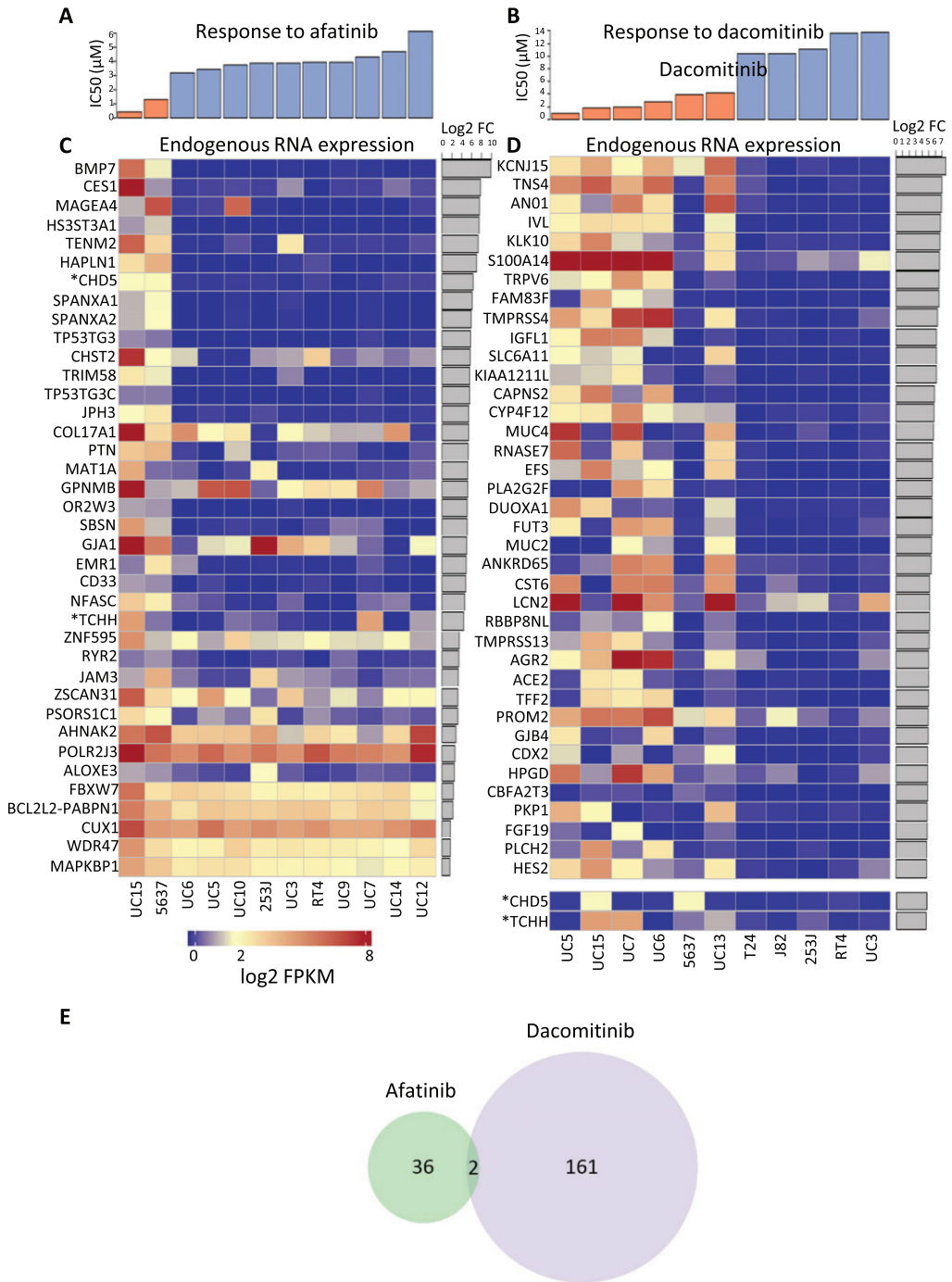
Fig. 3. Cell lines were categorized as either sensitive or resistant to each TKI and then endogenous RNA expression was compared between sensitive and resistant cell lines. Cytotoxic response to (A) afatinib or (B) dacomitinib treated cells which are separated into groups of sensitive (orange) or resistant (blue). DESeq was used to quantify differences between the endogenous RNA expression of sensitive and resistant cell lines for each drug. Afatinib and dacomitinib had 38 and 163 genes identified, respectively, using fold change ≥2 and an FDR adjusted *p*-value ≤0.05 threshold. Median expression for all cell lines was 2. All $\log_2$(FPKM) values represented have been upper quartile normalized. Heat map depiction of the top 38 up-regulated differentially expressed genes for (C) afatinib and (D) dacomitinib sensitive cell lines versus resistant bladder cell lines. Two additional upregulated genes marked with "*", CHD5 and TCHH, were included for dacomitinib despite not being in the top 38 up-regulated genes. These genes were included because they are (E) the only 2 genes overlapped between the up-regulated, differentially expressed genes for each drug. *P*-value for overlap was not significant (≥0.01).
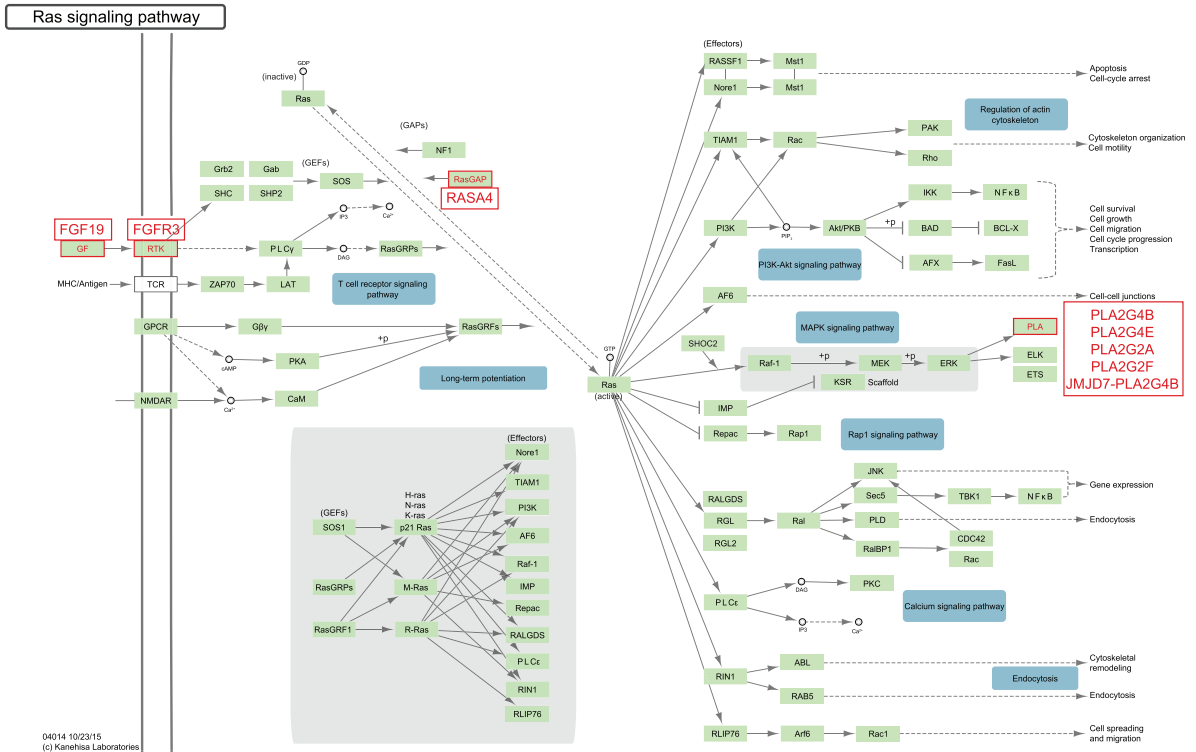
Fig. 4. DESeq analysis revealed RAS pathway involvement. The diagram is courtesy of the Kyoto Encyclopedia of Genes and Genomes (KEGG) [52, 73, 74]. Cell lines sensitive to dacomitinib have a higher expression of several RAS signaling pathway components. FDR adjusted p-value = 0.005 using Fisher's exact test. All genes highlighted in red were differentially expressed with fold change ≥ 2 and FDR qValue ≤ 0.05.

HER2 or HER3 mutations for afatinib treatment. The UC3 cell line has several unusual properties. UC3 harbors a K-ras mutation [59] which is not common in muscle-invasive bladder cancers [12]. UC3 has been shown to have a mesenchymal phenotype [65], and has previously demonstrated resistance to both IMC-CC225 antibody treatment (cetuximab) and dacomitinib [28, 66]. Finally, the HER3 mutation in the resistant UC3 line is different from the HER3 mutation in the sensitive UC15 cell line, which may reflect functional differences in the mutations. Whether these factors or others contribute to afatinib resistances will be the subject of future studies. Additionally, the afatinib clinical trial also identified two responsive patients who lacked HER3 mutations but had amplification of HER2 or EGFR in their tumors [29]. Our study did not replicate that finding, as UC5 bladder cancer cells, with amplified EGFR, and other cell lines with low gain of EGFR, failed to respond to afatinib.

Sensitivity to dacomitinib was not clearly linked to any one cellular factor. A non-statistically significant trend towards higher EGFR protein expression was

Table 3
Evaluation of afatinib sensitivity with Her2 or Her3 mutation

	Sensitive to AFAT	Resistant to AFAT
Her2 or 3 mutant	2	1
Not mutant	0	9

Fisher's exact test one-tailed $p = 0.0455$.

detected, but several non-responding cells also had higher EGFR levels. UC3 was one of these resistant cell lines with higher EGFR levels, but this resistance may be due to the *KRAS* mutation it harbors [55]. The UC1 cell line, despite high EGFR, showed only a moderate response to dacomitinib. Additionally, two of the sensitive cell lines, 5637 and UC13, had very modest EGFR protein levels. While EGFR protein may contribute to dacomitinib responses, other processes are clearly influential. The emergence of the RAS family signaling pathway in the RNA-Seq evaluation may have revealed additional contributors. RAS family proteins directly regulate key cellular processes that include growth, migration, senescence and cell death [67]. RAS family

signaling has previously been implicated in bladder cancer tumors [12, 68]. One study reported that 39% of their muscle-invasive bladder cancer samples had alterations in the RAS pathway, with mutation or amplification in FGFR3, HER2, EGFR and HER3 being most common [12]. However, one of the dacomitinib resistant cell lines, RT4, is known to have the FGFR3-TACC3 gene fusion that results in constitutively active FGFR3 [59]. A second cell line, SW780, which showed moderate resistance to dacomitinib, has a different FGFR3 fusion [59]. Those responses suggest that the activity in the RAS pathway is probably not solely at the FGFR3 receptor. Additional studies are needed to detail the role of this pathway in response to dacomitinib treatment.

The observed difference in response to afatinib and dacomitinib suggests that combination treatment might be useful. However, a more fruitful approach may be to target HER heterodimerization which is a hypothetical mechanism of resistance to HER therapies. HER family members will form heterodimers not only with other members of the family, but also with other receptors, including cMET, PDGFR, and IGF1R [17, 69, 70]. This receptor promiscuity adds another level of complexity to therapeutic targeting. Targeting several of these "rogue" dimerization partners in the therapeutic mix may be a promising approach.

New reports suggest that EGFR activation may also induce immune suppression through PDL-1 or PD-1 [71, 72]. Since recent clinical results show an excellent response in a subset of bladder cancer patients to PDL-1 or PD-1 inhibitors [3], sequential treatment with HER family targeting might be another therapeutic strategy.

In summary, our data show that two irreversible HER family TKI agents with similar structures have distinctly different efficacies in a panel of bladder cancer cell lines. While our afatinib results are consistent with the results of a clinical trial [29], our data indicate that patient selection for afatinib treatment based only on over-expression or mutation of HER family members may not guarantee therapeutic responsiveness. Our results indicate that several groups of patients may benefit from treatment with dacomitinib, including those with tumoral HER2 or HER3 mutation or amplification of EGFR, along with other factors, possibly included in the RAS pathway. Additional studies with combination treatments will assist in determining the optimal pathways for future treatments.

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/BLC-170144.

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