Oncogenic Addiction to ERBB2 Signaling Predicts Response to Trastuzumab in Urothelial Cancer

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ABSTRACT

Urothelial carcinoma (UC) is a common and frequently lethal cancer. Despite the presence of genomic alterations creating dependency on particular signaling pathways, the use of targeted therapies in advanced and metastatic UC has been limited. We performed an integrated analysis of whole-exome and RNA sequencing of primary and metastatic tumors in a patient with platinum-resistant UC. We found a strikingly high *ERBB2* mRNA expression and enrichment of downstream oncogenic *ERBB2* signaling in this patient's tumors compared with tumors from an unselected group of patients with UC (N=17). This patient had an exceptional sustained response to trastuzumab. Our findings show that oncogenic addiction to *ERBB2* signaling potentially predicts response to *ERBB2*-directed therapy of UC.

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Exceptional responders are patients who experience deep and durable responses to treatments that are not effective for most of the population with the same malignancy.¹ Studying the molecular basis for these extreme phenotypes provides insight into the molecular determinants of response to targeted therapy.² To dissect the determinants of sensitivity to *ERBB2*-directed therapy in a patient with metastatic platinum-resistant urothelial cancer (PRUC) who achieved an exceptional response to trastuzumab, we performed whole-exome sequencing (WES) and RNA sequencing (RNA-seq) of the patient's primary and metastatic PRUC tumors. We discovered evidence of oncogenic addiction to *ERBB2* signaling in this patient's tumors.

Methods

Patient Details

Patients consented to participate in the precision medicine protocol at Weill Cornell Medicine (WCM), and the study was approved by the WCM/New York-Presbyterian Institutional Review Board (protocol 1305013903). Samples from the patient's cystectomy tumor and metastases were prospectively collected. Somatic DNA from tumor samples and germline DNA from peripheral blood mononuclear cells were submitted for WES as previously described.³ RNA was extracted from tumor samples and submitted for RNA-seq.

Whole-Exome Sequencing

DNA extractions from patients' tumors were performed using the Maxwell 16 Tissue DNA Purification Kit (Promega). Whole-exome capture libraries were constructed after sample shearing, end repair, and phosphorylation and ligation to barcoded sequencing adaptors. Ligated DNA was size-selected for lengths between 200 and 350 bp and subjected to HaloPlex Exome Target Enrichment System sequencing (Agilent Technologies). Sequencing was performed using the high-throughput HiSeq 2500 system (2 × 100 bp; Illumina). Reads were aligned

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to GRCh37/hg19 reference using the Burrows-Wheeler Aligner and processed according to the Institute for Precision Medicine IPM-Exome-pipeline, version 0.9.³

RNA-Seq Analysis of WCM Samples

We used STAR_2.4.0f1 for sequence alignment against the human genome build hg19, downloaded via the UCSC Genome Browser, and Sequence Alignment/Map SAMtools version 0.1.19 for sorting and indexing reads, Cufflinks 2.0.2 for obtaining the expression values (fragments per kilobase of exon per million fragments mapped [FPKM]), and UCSC RefGene GTF (gene transfer format) file for annotation.

Gene Set Enrichment Analysis

GSEAPreranked was used for gene set enrichment analysis (GSEA). Log₂ fold change of the WCM137-1 versus the mean of other WCM bladder cases was calculated for all genes, which became the input for GSEA. Similarly, we calculated the fold change for WCM137-2. Gene sets used were oncogenic signatures and 90 curated gene sets of druggable cancer genes. Significant pathways with $P < .05$ and false discovery rates < 0.25 are reported.

Outlier Analysis

We used STAR_2.4.0f1 for sequence alignment against the human genome build hg19, downloaded via the UCSC Genome Browser, and SAMtools version 0.1.19 for sorting and indexing reads, Cufflinks 2.0.2 for obtaining the expression values (FPKM), and GENCODE version 19 GTF files for annotation.⁴⁻⁷ The gene counts from the HTSeq count and DESeq2 Bioconductor package were used to identify differentially expressed genes.^{8,9} The hypergeometric test and GSEA¹⁰ were used to identify enriched signatures using the different pathway collections in the MSigDB database (Molecular Signatures Database).¹¹

Z-scores were calculated across the WCM bladder cohort for 74 druggable genes. For each sample, the quantiles were calculated to compute the lower and upper bounds to define an outlier. We selected genes with z-scores > 2.5 and FPKM > 100 as outlier genes.

Results

Clinical Course and Exceptional Response

A 76-year-old woman initially presented with hematuria. Cystoscopy and transurethral resection of bladder tumor (TURBT) revealed high-grade T1 urothelial carcinoma (UC) (Figure 1A). A restaging TURBT performed 6 weeks later revealed residual malignancy with superficial invasion into the lamina propria. The patient was treated with intravesical bacillus Calmette-Guérin (BCG) and

experienced remission for 25 months until she developed a local recurrence. She was retreated with a second course of intravesical BCG but 5 months later started experiencing urinary obstruction and was found to have a mass at the tip of the urethra. A biopsy of this mass revealed high-grade UC with extensive invasion of the surrounding connective tissues. FDG-PET/CT revealed bilateral inguinal lymphadenopathy. A biopsy of the inguinal lymph nodes revealed metastatic UC.

The patient was referred for systemic therapy and was enrolled in the CALGB 90601 randomized phase III trial comparing gemcitabine + cisplatin (GC) and bevacizumab with GC and placebo in patients with advanced UC. She received 6 cycles of treatment (GC $\pm$ bevacizumab). Restaging CT scans showed a partial response in the pelvic lymph nodes along with persistence of high-grade papillary muscle-invasive UCB on repeat TURBT. Because her disease remained confined to the pelvis, she underwent a robot-assisted radical cystectomy with ileal conduit urinary diversion and left inguinal lymph node excision (pathologic stage ypT2N2M0). At 5 months after surgery, she developed systemic disease progression presenting with new liver metastases (Figure 1B). To identify potentially druggable genetic alterations, we enrolled her in our Institute for Precision Medicine protocol study and performed an in-depth integrated analysis of WES and RNA-seq from her primary and metastatic PRUC tumors, which showed outlier *ERBB2* mRNA expression (measured in FPKM) (Figure 2A). In fact, *ERBB2* FPKMs from the primary and metastatic tumors were ranked 62nd and 53rd, respectively, of 21,000 genes when ranked by expression level (Figure 2A). Allele-specific copy number analysis of the *ERBB2* locus on chromosome 17 showed strikingly high levels of focal *ERBB2* amplification (Figure 2B, C) with copy number values of 14 and 20 in the primary and metastatic tumors, respectively (Figure 2C). These findings were validated by fluorescence in situ hybridization (FISH) (Figure 2D). The outlier somatic *ERBB2* copy number and mRNA expression translated into overexpression of the ERBB2 protein on immunohistochemical (IHC) analysis (Figure 2E). No activating somatic mutations in *ERBB2* were identified.

Based on these results, the patient was started on trastuzumab (6 mg/kg) in combination with docetaxel (70 mg/m²). She received 4 cycles of this regimen, which were well-tolerated, and experienced substantial clinical improvement. Surprisingly, restaging CT scans showed a complete response with total disappearance of all metastatic lesions (Figure 1B). She continued on maintenance trastuzumab every 3 weeks. Four years later, she continues to be in complete remission with no evidence of disease.

Figure 1. Patient's clinical course. (A) Clinical events in chronologic order, including the diagnosis of noninvasive, invasive, and metastatic disease; local and systemic treatments at each stage; outcomes; and last follow-up (time scale, months). (B) Axial and coronal CT images showing liver metastases (left panels, arrows) and complete response after docetaxel + trastuzumab (right panels).

Abbreviations: BCG, bacillus Calmette-Guérin; GC, gemcitabine/cisplatin; LNs, lymph nodes; NMIBC, non-muscle-invasive bladder cancer; RARC, robot-assisted radical cystectomy; UC, urothelial cancer.

We reasoned that the exceptional response phenotype observed in this patient's PRUC resulted from targeting the tumor's addiction to ERBB2 signaling. We hypothesized that oncogenic addiction to ERBB2 requires outlier overexpression of genes specifically involved in signal transduction through the ERBB2 receptor tyrosine kinase pathway. To test this hypothesis, we performed RNA-seq analysis of an unselected cohort of patients with advanced UC (n=17) from our institution and compared *ERBB2* mRNA expression in their tumors with that from our exceptional responder's tumors. *ERBB2* mRNA expression in both the primary and metastatic samples from our patient was significantly higher compared with other patients in the cohort ($P=4.229\text{e-}08$) (Figure 3A). Analysis of outlier mRNA expression ranked

the exceptional responder's *ERBB2* expression in the 95th percentile of all transcripts compared with tumors from other unselected patients in the cohort (Figure 3B).

To determine whether this *ERBB2* outlier mRNA expression level translates into actual activation of ERBB2 downstream signaling, we performed GSEA. We used the gene expression data from normal bladder tissue in the Genotype-Tissue Expression (GTEx) project database as a normal control.¹² GSEA revealed significant enrichment in the expression of *ERBB2*-target genes (*ERBB2* signature) in tumors from our exceptional responder compared with normal bladder tissue from GTEx and UBC tumors in our unselected cohort (Figure 3C). These results strongly suggest that our exceptional responder's tumors were specifically dependent on the

Figure 2. *ERBB2* overexpression in the patient's tumors. (A) *ERBB2* mRNA outlier expression in primary (left panel) and metastatic tumors (right panel). (B) Location of the *ERBB2* locus on chromosome 17. (C) Allele-specific copy number analysis of the *ERBB2* locus showing strikingly high levels of focal *ERBB2* amplification in the primary and metastatic tumors. (D) Fluorescence in situ hybridization confirms the presence of several copies of *ERBB2* (red) per nucleus compared with 2 reference copies of chromosome 17 (green) (original magnification x600). (E) Diffuse *ERBB2* staining by immunohistochemistry (original magnification x200).

Abbreviations: FPKM, fragments per kilobase of exon per million fragments mapped; LN, lymph node; UC, urothelial cancer.

outlier *ERBB2* expression and the ensuing constitutive activation of a network of *ERBB2* target genes. This resulted in a phenotype that was rapidly progressive but exceptionally sensitive to *ERBB2*-directed therapy.

Discussion

The *ERBB*/HER signaling pathway is a receptor–ligand system that consists of 4 receptors and at least 11 ligands.¹³ *ERBB2* is a member of the *ERBB* receptor family that acts as a coreceptor with *ERBB1*, *ERBB3*, and *ERBB4*. The humanized monoclonal antibody trastuzumab is directed against *ERBB2* and approved for the treatment

of several *ERBB2*-overexpressing cancers, including breast and gastric.^{14,15} Molecular alterations in *ERBB2* are frequent in invasive UC, with a reported prevalence of 9.2% to 80% for protein overexpression and up to 32% for copy number amplification.^{16,17} *ERBB2* overexpression in UC is associated with increased metastasis and recurrence.¹⁸

Several clinical trials of *ERBB2*-directed therapies in UC failed to meet their primary end points^{19–21} but demonstrated activity in a subset of patients with tumors overexpressing *ERBB2*, including some patients with durable responses.^{14,22} In a single-arm phase II study of 59 patients with locally advanced or metastatic UC,

Figure 3. *ERBB2* mRNA expression was significantly higher in tumors from the exceptional responder than in unselected patients with UC and normal bladder tissue. (A) Primary and metastatic samples from the exceptionally responding patient have significantly higher *ERBB2* mRNA levels (WCM137, top 2 green bars) than those from patients with UC from the unselected WCM cohort ($P = 4.229 \times 10^{-8}$). (B) Outlier analysis of *ERBB2* mRNA expression confirms *ERBB2* as an outlier gene in the exceptional responder's primary and metastatic tumor samples (ordered from left to right) compared with tumors from other patients in the unselected WCM UC cohort. (C) *ERBB2* target gene transcripts are enriched in gene set enrichment analysis of tumors from the exceptional responder compared with normal bladder tissue from GTEx and tumors from the unselected WCM UC cohort.

Abbreviations: FDR, false discovery rate; FPKM, fragments per kilobase of exon per million fragments mapped; GTEx, Genotype-Tissue Expression; UC, urothelial cancer; WCM, Weill Cornell Medicine.

lapatinib, a dual-receptor tyrosine kinase inhibitor of ERBB1 and ERBB2, was used as second-line treatment.¹⁹ Although the response rate (1.7%), time to progression (8.6 weeks), and overall survival (17.9 weeks) did not meet the primary end point, patients with overexpression of ERBB1 or ERBB2 had an improved median overall survival of 30.3 versus 10.6 weeks in those with low or negative expression ($P=.0001$). In another single-arm phase II trial in which treatment-naïve patients with advanced UC were treated with trastuzumab, paclitaxel, carboplatin, and gemcitabine,¹⁴ ERBB2 overexpression by IHC, gene amplification in the tumor by FISH, or presence in serum for extracellular ERBB2 domain testing by quantitative enzyme-linked immunosorbent assay was detected in 52% of patients. In this trial, 70% of patients with ERBB2-overexpressing UC tumors experienced a clinical response to the chemotherapy combination; median time to progression and median survival were 9.3 and 14.1 months, respectively. Although paclitaxel could have had a role in the exceptional response we observed in our patient, the persistence of the complete response after discontinuation of paclitaxel while receiving maintenance trastuzumab is consistent with possible oncogenic addiction to ERBB2 signaling, as suggested by the presence of ERBB2 transcriptional signature.

In another phase II trial, 61 patients with advanced UC and overexpression of ERBB2 by IHC and confirmed by FISH were randomized to receive GC or gemcitabine + carboplatin without (arm A) or with (arm B) trastuzumab.²⁰ The trial did not demonstrate significant differences between the arms. Recently, a phase II trial by Choudhury et al²¹ examined the 3-month progression-free survival (PFS3) in patients with metastatic PRUC treated with afatinib, 40 mg/d continuously until progression or intolerance. Tumor analysis for alterations in *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4* was performed using next-generation sequencing. Of 23 patients, 5 (21.7%) achieved PFS3, but because the study did not meet the prespecified end point of ≥ 7 patients reaching PFS3, the study did not advance to the second stage of enrollment. For the entire cohort, the median PFS was 1.4 months, the objective response rate was 8.6%, and the median overall survival for all patients was 5.3 months. The 6 patients with *ERBB2/3* alterations had a median time to progression of 6.6 months compared with 1.4 months in patients without alterations ($P<.001$). In this trial, 83% of patients with *ERBB2* copy number amplification and/or *ERBB3* somatic mutations achieved PFS3, whereas 0% of patients without alterations reached PFS3. The patient with the longest PFS had the highest somatic copy number of ERBB2 (copy number, >50) and was the only patient with both *ERBB2* and *ERBB3* alterations.²¹ This finding is consistent with our observation that outlier *ERBB2* mRNA and copy number levels are associated with response to ERBB2-targeted therapy.

Collectively, the results of these trials highlight the challenges in using ERBB2 status as a biomarker for trials of ERBB2-targeted therapies. It is important to recognize that *ERBB2* copy number amplification does not linearly translate into protein overexpression by IHC.^{3,21} Furthermore, the relevance of *ERBB2* as a tumor driver may vary even within *ERBB2*-amplified cases.²³ The result of the Choudhury et al²¹ study in UC showed that *ERBB2* amplification by quantitative PCR or FISH copy number was more predictive of response to *ERBB2*-directed therapy with afatinib in patients with UC compared with protein overexpression detected by IHC¹⁰; 75% of patients with amplification reached PFS3 compared with only 25% with 2+ or 3+ IHC staining.²¹ IHC testing to evaluate HER2 status is notoriously inaccurate compared with FISH.¹⁰ A meta-analysis evaluating the concordance between IHC and FISH for detecting ERBB2 in breast cancer reported rates of 36% for IHC score 2+ and 91% for IHC score 3+.²⁴ These findings highlight the challenges of using a single biomarker test to predict response to ERBB2-directed therapy.

Conclusions

Our report describes an integrated analysis of WES, RNA-seq, IHC, and FISH suggesting that oncogenic addiction to ERBB2 is associated with exceptional clinical response to trastuzumab. Because most trials testing ERBB2-directed therapies have relied on assigning ERBB2 status by IHC, it is possible that the overall negative results of these trials might reflect a failure of the biomarker methodology to predict oncogenic addiction to ERBB2 rather than a failure of the tested ERBB2-targeted therapies. A combinatorial biomarker strategy that integrates genomic and RNA-seq, FISH, and protein quantification might predict oncogenic addiction to a specific signaling pathway. Future testing of ERBB2-directed treatments in a subset of patients with UC tumors with oncogenic addiction to ERBB2 is warranted.

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