Next-Generation Sequencing and In Silico Analysis Facilitate Prolonged Response to Pazopanib in a Patient With Metastatic Urothelial Carcinoma of the Renal Pelvis

Andrew W. Hahn, BA^a; Smith Giri, MD^a; Dilan Patel, MD^b; Heather Sluder, BSN, RN^c; Ari Vanderwalde, MD, MPH^{a,c}; and Mike G. Martin, MD^{a,c}

Abstract

With the advent of widespread tumor genetic profiling, an increased number of mutations with unknown significance are being identified. Often, a glut of uninterpretable findings may confuse the clinician and provide little or inappropriate guidance in therapeutic decision-making. This report describes a method of protein modeling by in silico analysis (ie, using computer simulation) that is easily accessible to the practicing clinician without need for further laboratory analysis, which can potentially serve as a guide in therapeutic decisions based on poorly characterized tumor mutations. An example of this model is given wherein poorly characterized *KIT*, *PDGFRB*, and *ERBB2* mutations were discovered in a patient with treatment-refractory metastatic transitional cell carcinoma of the renal pelvis. The *KIT* and *PDGFRB* mutations were predicted to be pathogenic using in silico analysis, whereas the *ERBB2* mutation was predicted to be benign. Based on these findings, the patient was treated with pazopanib and achieved a partial response that lasted for 7.5 months. We propose that in silico analysis be explored as a potential means to further characterize genetic abnormalities found by tumor profiling assays, such as next-generation sequencing. (J Natl Compr Canc Netw 2015;13:1181–1185)

Urothelial carcinoma of the renal pelvis (UCRP) and the ureter is a rare disease, representing 5% of all urothelial malignancies. The incidence of UCRP in the general US population is estimated to be 1.15 per 100,000 person-years.¹ Although radical nephroureterectomy is the therapy of choice for nonmetastatic UCRP, the rate of tumor recurrence is still high. For metastatic UCRP (mUCRP), cisplatin-based chemotherapies are often used as first-line agents with limited response, largely based

on experience from urothelial tumors of the bladder.² However, second-line chemotherapies for urothelial malignancies have significantly lower response rates and no significant survival benefit over supportive care.²

Whole-genome studies of metastatic urothelial cancer have shown profound genomic heterogeneity at the nucleotide and chromosomal levels.^{3,4} Furthermore, the mutation profiles of histologically similar tumors may vary widely, explaining the variable response to chemotherapy observed in small clinical trials.⁵ The availability of next-generation sequencing (NGS) has made it feasible for clinicians to analyze genetic profiles of an individual's cancer. When these results are coupled with the continual development of targeted therapies, personalized medicine is possible. However, interpretation of the numerous genetic variants that NGS provides and selection of an appropriate targeted therapy presents a novel challenge to clinicians. This report documents the successful use of NGS coupled with in silico analysis to select a targeted therapy, pazopanib, from nu-

From the ^aDepartment of Internal Medicine, The University of Tennessee Health Science Center, Memphis, Tennessee; ^bDepartment of Internal Medicine, Washington University in St. Louis, St. Louis, Missouri; and ^cDepartment of Hematology/Oncology, The West Cancer Center, Memphis, Tennessee. Submitted November 18, 2014; accepted for publication May 21, 2015.

The authors have disclosed that they have no financial interests, arrangements, affiliations, or commercial interests with the manufacturers of any products discussed in this article or their competitors.

Correspondence: Andrew W. Hahn, BA, The University of Tennessee Health Science Center/West Cancer Center, 1588 Union Avenue, c/o Dr. Mike G. Martin, Memphis, TN 38104. E-mail: ahahn1@uthsc.edu

merous potential mutations in a patient with heavily pretreated mUCRP.

Case Report

A 61-year-old woman presented to our clinic in November 2010 with intermittent hematuria for 2 months. Further evaluation revealed a left high-grade UCRP, and the patient underwent a radical nephroureterectomy (pT2NXM0). The patient received 3 cycles of adjuvant gemcitabine plus cisplatin (GC) but relapsed with biopsy-proven urothelial pulmonary metastases. Subsequently, the patient received sequential chemotherapies, including 6 months of palliative carboplatin and gemcitabine with a partial response (PR), 4 months of pemetrexed with a PR, 5 months of paclitaxel with stable disease (SD), and 2 months of methotrexate with progressive disease (PD). Each chemotherapy change was mandated by PD on CT. Her performance status remained excellent despite her PD and extensive chemotherapy exposure.

Because of a lack of validated therapeutic options, a Precipio NGS-421 whole-exome sequencing (WES) analysis (Yale School of Medicine, New Haven, CT) was conducted on the biopsied metastatic tissue that revealed potentially targetable mutations in *ERBB2* (UniProtKB #P04626), *KIT* (#P10721), and *PDGFRB* (#P09619), and other genes (Table 1). Precipio uses DNA extracted from tumor cells with custom HaloPlex reagents and is sequenced on the Ion Torrent Personal Genome Machine (Illumina platform).^{6,7} The significance of the mutations was

then predicted in silico using Harvard’s PolyPhen-2 (1,2,3) algorithm on a personal computer. PolyPhen-2 is a “tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations.”⁸ PolyPhen-2 uses “protein sequences from UniProtKB/UniRef100 Release 2011_12..., structures from PDB/DSSP Snapshot 03-Jan-2012...and UCSC MultiZ multiple alignments of 45 vertebrate genomes with hg19/GRCh37 human genome...” to make predictions.⁸ The *KIT* mutation had not been previously described or targeted in UCRP but is sensitive to pazopanib in other malignancies.⁹ PolyPhen-2 predicted the mutations in *KIT* and *PDGFRB* to be pathogenic and the *ERBB2* mutation to be benign. Our patient was started on off-label pazopanib, which has activity against *KIT* and *PDGFRB* mutations, and remained on therapy for more than 7.5 months with minimal side effects and stabilization of her previous weight loss and her CT scan findings (SD in the bones and liver and a 50% reduction in lung metastases). Her response to pazopanib was her longest response to any agent. After progression, her performance status deteriorated and she elected hospice care.

Discussion

mUCRP has a grim prognosis, with a 3-year survival rate of less than 10%.¹ First-line chemotherapy for mUCRP constitutes GC or methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC).^{2,10} Clinical trials among patients with all urothelial malignancies have

Table 1 Selected Mutations Found in Patients With mUCRP Using Next-Generation Sequencing					
Gene	Variant	Effect	Clinical Significance ^a	PolyPhen-2 Score	Prediction
<i>KIT</i>	c.2662C>T p.R888W	Point mutation	Previously described in other malignancies and associated with clinical responses	1.000 (sensitivity, 0.00; specificity, 1.00)	Probably damaging
<i>PDGFRB</i>	c.595G>A p.V199M	Point mutation	Not previously described, clinical trials are open	0.997 (sensitivity, 0.41; specificity, 0.98)	Probably damaging
<i>PDGFRB</i>	c.596T>G p.V199G	Point mutation	Not previously described, clinical trials are open	0.988 (sensitivity, 0.73; specificity, 0.96)	Probably damaging
<i>ERBB2</i>	c.3557C>A p.A1186D	Point mutation	Unknown functional significance, clinical trials are open	0.049 (sensitivity, 0.94; specificity, 0.83)	Benign
<i>CHEK1</i>	c.749C>A p.P250Q	Point mutation	Unknown functional significance, clinical trials are open	1.000 (sensitivity, 0.00; specificity, 1.00)	Probably damaging
<i>PIK3CG</i>	c.2191G>A p.D731N	Point mutation	Unknown functional significance, clinical trials are open	0.831 (sensitivity, 0.84; specificity, 0.93)	Possibly damaging

^aAs reported by Precipio.
Abbreviation: mUCRP, metastatic urothelial carcinoma of the renal pelvis.

shown response rates ranging from 40% to 72% for GC and MVAC, but no trials have specifically examined the response rates among patients with UCRP.^{11,12} Dose-related and cumulative renal toxicities are the most common dose-limiting adverse effects in the treatment of UCRP. Among the first-line agents, cisplatin and methotrexate are nephrotoxic agents that are renally excreted. Because most patients with UCRP are elderly and many have had prior radical nephroureterectomy, the concern for renal toxicity frequently limits the use of these chemotherapeutic regimens.¹¹

The poor outcomes and significant toxicities associated with first-line treatment of mUCRP create a need for novel second-line therapies, yet the rarity of the disease has limited guidance from clinical trials. The paucity of meaningful trials has created an opportunity for NGS to provide clinicians with meaningful data for the personalized treatment of heavily pretreated metastatic disease. However, the wealth of information provided by NGS presents clinicians with a new challenge in deciphering which genomic alterations should be acted upon. For example, Ross et al¹³ recently published the results of genomic profiling in 125 patients with cancer of unknown primary, and found a mean of 4 genetic alternations per patient. This case presents an example of not only how NGS can produce a wealth of potentially useful data but also how in silico analysis of the results of NGS can help guide physicians in selecting appropriate, off-label targeted therapy.

NGS technologies were first commercially introduced in 2007.¹⁴ NGS platforms, such as Illumina or Ion Torrent, have increased the sensitivity and scalability for DNA sequencing of cancer cells to provide practical genetic information.¹⁵ An array of NGS applications exist, including DNA sequencing, which can be used for whole-genome sequencing, WES, or sequencing of specific targeted “hot spots” of the genome.¹⁴ WES is most useful in clinical diagnostics because it analyzes exons, which only account for approximately 2% of all genomic DNA but still encompass the DNA relevant to targeted therapies. We used Precipio (one of the many companies that offer NGS) WES of 421 genes to yield our initial NGS results.¹⁶ Precipio uses the Illumina and Ion Torrent NGS platforms to produce its WES results. WES results provide data on point mutations present within exons of the tissue

specimen provided. Precipio, like most NGS services available, provides only the mutations present and advises whether these mutations are the focus of any targeted therapies or current clinical trials. The challenge that oncologists face is deciphering which mutations of the many listed by NGS are driving a particular malignancy and should be the focus of targeted therapy. For example, our patient’s NGS revealed mutations in *ERBB2*, which is seen in 9% of urothelial carcinomas (UCs), and in *KIT* and *PDGFRB*, which are seen in other cancer types.¹⁷

Some nucleotide sequence variants identified by NGS can be readily labeled as pathogenic if they truncate the reading frame or disrupt gene structure; however, many of the variants identified are missense mutations whose significance is more difficult to interpret. In silico analysis algorithms for missense mutations, such as PolyPhen-2, can help guide oncologists in determining which mutations are likely pathogenic or benign.¹⁸ In silico algorithms use at least 1 of the following 4 classes of abnormalities to predict pathogenicity: comparison of physiochemical characteristics of wild-type and variant amino acids, evolutionary conservation at the location of mutation, the evolutionarily tolerated amino acid range at the mutation, or the protein’s structural considerations.¹⁹ For many algorithms, evolutionary fitness serves as a surrogate for pathogenicity.

The in silico analysis algorithm used in this case was PolyPhen-2, which is one of the many algorithms available but has a free, user-friendly interface available online that can be used on personal computers.⁸ PolyPhen-2 is a decision tree algorithm that combines protein structural attributes with a prebuilt sequence alignment that only includes mammalian species. The protein structural attributes are elucidated from crystal structures and include residues in active sites, binding sites, and alpha helices, and solvent accessibility.^{18,19} PolyPhen-2 then calculates a Position-Specific Independent Counts (PSIC) score that is the difference between the wild-type and mutant amino acid and converts the score into 1 of 3 categories: benign, possibly damaging, or probably damaging, as seen in Table 1. PolyPhen has been validated multiple times.^{18,20,21} In a recent study, Chan et al²⁰ used curated locus-specific databases that covered 254 substitutions in 5 genes to reveal an overall predictive score of 75.0% when using PolyPhen.

For our patient, *in silico* analysis predicted a benign change of *ERBB2* compared with probably damaging mutations in *KIT* and *PDGFR*. When combined with preclinical studies indicating that UC is an angiogenic tumor overexpressing vascular endothelial growth factor (VEGF), we selected the targeted therapy pazopanib to treat our patient's heavily pretreated mUCRP.¹¹ Pazopanib is an oral angiogenesis inhibitor that targets VEGF receptor, platelet-derived growth factor receptor, and *KIT* and is indicated for the treatment of metastatic renal cell carcinoma and soft tissue sarcoma.²² The results of 3 small clinical trials evaluating pazopanib in the treatment of metastatic UC are summarized in Table 2.^{23–25} The contrasting results of the trials evaluating pazopanib alone do not provide a definitive answer on the utility of pazopanib in the treatment of metastatic UC.^{23,24} Interestingly, 4 out of the 7 PRs in the study by Necchi et al²⁴ occurred in patients with UC of the upper urinary tract. *PDGFRB* is a receptor targeted by pazopanib, yet it was not a biomarker evaluated in the clinical trials performed.^{23,24} Genetic heterogeneity of heavily pretreated metastatic UC may explain the contrasting results seen in the clinical trials reported to date.

Pazopanib is not the only selective tyrosine kinase inhibitor (TKI) targeting the VEGF axis that has been used to date for metastatic UC. Gallagher et al²⁶ used sunitinib, a TKI that inhibits VEGFR, *PDGFR*, *KIT*, *FLT3*, and *RET*, in a recent phase II study of 77 patients with metastatic UC, including 16 with mUCRP. A PR was seen in only 4 of the 77 patients. However, SD was seen in another 18 patients, indicating that sunitinib has clinical activity against metastatic

UC and that the VEGF axis is a viable target for novel therapies.

Another promising therapeutic class for the treatment of metastatic UC is anti-programmed death-ligand 1 (PD-L1) antibodies.²⁷ Expression of PD-L1 enables cancer cells to elude immune surveillance and is thought to play a significant role in UC. A recent study has shown anti-PD-L1 immunotherapy to have promise in the treatment of metastatic UC, although a detailed discussion of this is outside the scope of this article.²⁷

Conclusions

mUCRP is a rare disease with a poor prognosis due to a paucity of effective second-line therapies. Genomic platforms may yield clinically meaningful, individualized results that benefit patients facing such a challenging disease. Even though NGS can highlight potential targets, clinicians still face a challenge in deciphering which genomic alterations should be acted upon. Our case presents a unique use of *in silico* analysis with PolyPhen-2 to help oncologists determine which mutations should be targeted from WES results. We propose that *in silico* testing may be a useful, free, and easily accessible way to prioritize abnormalities found by NGS when clinical annotation is not definitive.

Acknowledgments

Author Contributions: MGM treated the patient and performed *in silico* analysis; MGM, AWH, and SG wrote the initial manuscript; MGM, AWH, DP, AV, HS, and SG completed all revisions.

Table 2 Outcomes of Completed Clinical Trials (Phase I or II) of Pazopanib in Advanced or Metastatic Urothelial Carcinoma

	Pili et al ²³	Necchi et al ²⁴	Gerullis et al ²⁵
Number of patients	19	41	5
Chemotherapy used	Pazopanib, 800 mg/d	Pazopanib, 800 mg/d	Pazopanib, 200 mg + vinflunine, 280 mg
Response rate (PR or CR)	PR: 0 CR: 0	PR: 7 CR: 0	PR: 0 CR: 0
Adverse effects: number and types	9 grade 3: anemia, thrombocytopenia, leukopenia, fatigue	Grade 3: HTN (3), fatigue (2), GI and vaginal fistulas (4)	Trial discontinued at phase I due to 2 of 5 patients with dose-limiting toxicities

Abbreviations: CR, complete response; GI, gastrointestinal; HTN, hypertension; PR, partial response.

In Silico Analysis in Therapeutic Decisions

References

1. Raman JD, Messer J, Sietatycki JA, Hollenbeak CS. Incidence and survival of patients with carcinoma of the ureter and renal pelvis in the USA, 1973-2005. *BJU Int* 2011;107:1059-1064.
2. Gallagher DJ, Milowsky MI, Bajorin DE. Advanced bladder cancer: status of first-line chemotherapy and the search for active agents in the second-line setting. *Cancer* 2008;113:1284-1293.
3. Morrison CD, Liu P, Woloszynska-Read A, et al. Whole-genome sequencing identifies genomic heterogeneity at a nucleotide and chromosomal level in bladder cancer. *Proc Natl Acad Sci U S A* 2014;111:E672-681.
4. Ross JS, Wang K, Al-Rohil RN, et al. Advanced urothelial carcinoma: next-generation sequencing reveals diverse genomic alterations and targets of therapy. *Mod Pathol* 2014;27:271-280.
5. Chang F, Li MM. Clinical application of amplicon-based next-generation sequencing in cancer. *Cancer Genet* 2013;206:413-419.
6. Tse C, Shoemaker AR, Adickes J, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* 2008;68:3421-3428.
7. Wallace MD, Pfeifferle AD, Shen L, et al. Comparative oncogenomics implicates the neurofibromin 1 gene (NF1) as a breast cancer driver. *Genetics* 2012;192:385-396.
8. PolyPhen-2: Prediction of Functional Effects of Human nsSNPs. Available at: <http://genetics.bwh.harvard.edu/pph2/>. Accessed September 9, 2015.
9. Ganjoo KN, Villalobos VM, Kamaya A, et al. A multicenter phase II study of pazopanib in patients with advanced gastrointestinal stromal tumors (GIST) following failure of at least imatinib and sunitinib. *Ann Oncol* 2014;25:236-240.
10. Kwak C, Lee SE, Jeong IG, Ku JH. Adjuvant systemic chemotherapy in the treatment of patients with invasive transitional cell carcinoma of the upper urinary tract. *Urology* 2006;68:53-57.
11. Dasanu CA, Ong-Bacay A, Codreanu I. Newer developments in the therapeutics of the transitional cell carcinoma of renal pelvis. *J Oncol Pharm Pract* 2012;18:97-103.
12. Sternberg CN, Yagoda A, Scher HI, et al. Methotrexate, vinblastine, doxorubicin, and cisplatin for advanced transitional cell carcinoma of the urothelium. Efficacy and patterns of response and relapse. *Cancer* 1989;64:2448-2458.
13. Ross JS, Wang K, Gay L, et al. Comprehensive genomic profiling of carcinoma of unknown primary site: new routes to targeted therapies. *JAMA Oncol* 2015;1:40-49.
14. Bao R, Huang L, Andrade J, et al. Review of current methods, applications, and data management for the bioinformatics analysis of whole exome sequencing. *Cancer Inform* 2014;13(Suppl 2):67-82.
15. Yadav SS, Li J, Lavery HJ, et al. Next-generation sequencing technology in prostate cancer diagnosis, prognosis, and personalized treatment. *Urol Oncol* 2015;33:267.e1-13.
16. Precipio Diagnostics. SmartGen. Available at: <http://www.precipiodx.com/Smartgen.html#sthash.u6OFvI6n.dpbs>. Accessed September 9, 2015.
17. Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* 2014;507:315-322.
18. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010;7:248-249.
19. Tavtigian SV, Greenblatt MS, Lesueur F, Byrnes GB. In silico analysis of missense substitutions using sequence-alignment based methods. *Hum Mutat* 2008;29:1327-1336.
20. Chan PA, Duraisamy S, Miller PJ, et al. Interpreting missense variants: comparing computational methods in human disease genes CDKN2A, MLH1, MSH2, MECP2, and tyrosinase (TYR). *Human Mutat* 2007;28:683-693.
21. Chao EC, Velasquez JL, Witherspoon MS, et al. Accurate classification of MLH1/MSH2 missense variants with multivariate analysis of protein polymorphisms-mismatch repair (MAPP-MMR). *Human Mutat* 2008;29:852-860.
22. Sternberg CN, Davis ID, Mardiak J, et al. Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *J Clin Oncol* 2010;28:1061-1068.
23. Pili R, Qin R, Flynn PJ, et al. A phase II safety and efficacy study of the vascular endothelial growth factor receptor tyrosine kinase inhibitor pazopanib in patients with metastatic urothelial cancer. *Clin Genitourin Cancer* 2013;11:477-483.
24. Necchi A, Mariani L, Zaffaroni N, et al. Pazopanib in advanced and platinum-resistant urothelial cancer: an open-label, single group, phase 2 trial. *Lancet Oncol* 2012;13:810-816.
25. Gerullis H, Eimer C, Ecker TH, et al. Combined treatment with pazopanib and vinflunine in patients with advanced urothelial carcinoma refractory after first-line therapy. *Anticancer Drugs* 2013;24:422-425.
26. Gallagher DJ, Milowsky MI, Gerst SR, et al. Phase II study of sunitinib in patients with metastatic urothelial cancer. *J Clin Oncol* 2010;28:1373-1379.
27. Powles T, Eder JP, Fine GD, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 2014;515:558-562.