Project Title:	**ctDNA-FGFR status as a predictive biomarker for FGFR targeted therapy**
Protocol Version:	2.0
Version Date	April 18, 2024
Principal Investigator:	Bernhard Eigl MD FRCPC Medical Oncologist Provincial Director, Systemic Therapy Clinical Trials Clinical Associate Professor, UBC BC Cancer - Vancouver 600 West 10th Avenue, Vancouver, BC V5Z 4E6 P: 604-877-6000, ext. 672707 F: 604-877-0585 E: bernie.eigl@bccancer.bc.ca
Co-Investigators:	Dr. Alexander Wyatt Senior Research Scientist Assistant Professor, University of British Columbia Vancouver Prostate Centre 2660 Oak Street, Vancouver, British Columbia, V6H 3Z6 P: (604) 364-1940 E: awyatt@prostatecentre.com

Table of Contents

BACKGROUND AND RATIONALE 1

FGFR in Urothelial Cancer 1

Preliminary Data 2

HYPOTHESES AND OBJECTIVES 2

Objective 1 (Primary) 2

Objective 2 (Secondary) 2

Objective 3 (Exploratory) 3

STUDY DESIGN 3

Inclusion Criteria 4

Exclusion Criteria 4

Sample Processing and ctDNA Evaluation 4

Statistical Considerations 5

REFERENCES 6

APPENDIX A: DATA DICTIONARY 7

BACKGROUND AND RATIONALE

FGFR in Urothelial Cancer

There have been several exciting advances in the management of advanced urothelial cancer (UC) in recent years, but long-term survival is still rare for the majority of metastatic UC patients. A particularly notable advancement has been the development of small molecule tyrosine kinase inhibitors targeting the fibroblast growth factor receptor (FGFR) signaling pathway, which is an oncogenic pathway addiction in up to 20% of bladder cancers (1). Health Canada recently approved the first oral pan-FGFR inhibitor (erdafitinib) for use in the treatment of metastatic urothelial cancer with FGFR aberrations, and other inhibitors are in advanced clinical development. The efficacy (and approved indication) of these agents is restricted to cancers that have specific and predefined genomic alterations in FGFR genes and the companion diagnostic currently required to detect these alterations relies on (usually archival) tumour tissue testing (2).

While the use of FGFR inhibitors in patients with metastatic UC and FGFR alterations can result in marked responses, several key clinical and scientific questions remain:

1. **Is archival primary tumour tissue (or a tissue biopsy of an isolated metastatic site) representative of the predominant metastatic clone driving lethal disease progression?** Bladder cancers are notoriously heterogeneous and primary tissue genotype and RNA profile can vary in space and time (3, 4). Analysis of a single primary focus may therefore identify alterations not present in the wider primary tumor, let alone the metastatic lesions (and vice versa). In support of this concept, FGFR alterations appear to have a different prevalence in early versus late stage disease. We believe it is highly plausible that archival tumour tissue is "mis-calling" FGFR status in metastatic patients and therefore resulting in sub-optimal patient selection for FGFR inhibitor treatment.
2. **Are we missing an opportunity to extend the benefit of FGFR inhibitors to more patients by not knowing the full spectrum of FGFR activating alterations?** The companion diagnostic for erdafitinib evaluates tumour tissue for specific FGFR aberrations. At present, eligible tumors must have at least one of the following translocations: FGFR2-BICC1, FGFR2-CASP7, FGFR3-TACC3_V1, FGFR3-TACC3_V3, FGFR3-BAIAP2L1; or one of the following FGFR3 mutations: p.R248C, S249C, G370C, Y373C. However, since there are a number of other recurrent mutations in FGFR genes, and rarer alterations that affect relevant protein domains, we posit that the current screening strategies do not identify all FGFR activating alterations and are therefore hampering the clinical impact of FGFR inhibitors.
3. **What molecular mechanisms underlie resistance to FGFR inhibitors?** While erdafitinib is a highly effective treatment, resistance inevitably develops and primary resistance in eligible patients is also seen (2). There is currently limited preclinical data regarding the mechanism of resistance to FGFR inhibitors. Resistance to molecularly-targeted therapies in other cancers is often driven by secondary alterations to the target in

question (e.g. amplification or mutation of the Androgen Receptor in prostate cancer). We believe that a better understanding of FGFR status post-treatment, as well as concomitant genome-wide alterations is needed to guide decision making during treatment and to inform the development of future therapies.

Preliminary Data

Recent advances in next-generation sequencing have made possible the interrogation of plasma cell-free (cf) circulating tumor (ct) DNA from patients with solid tumors. We and others have shown that the ctDNA "liquid biopsy" provides a non-invasive tool to sample somatic mutations and copy number alterations. We have shown that ctDNA isolation from metastatic bladder cancer patients is feasible and reveals clinically actionable genomic information (5, 6). To date we have evaluated more than 200 blood samples from over 100 bladder cancer patients and have shown that: ctDNA can be characterized in over 85% of patients with locally advanced or metastatic disease; that bulk ctDNA actually provides a more comprehensive (and current) genomic profile than assessment of single tissue foci; and that populations predicted to respond to certain therapies can be identified through ctDNA (submitted). Although promising, this data is still limited by small numbers of any given alteration and only broad conclusions can be made. There have been no studies to date formally comparing tissue and blood-based FGFR testing other than an exploratory analysis of ctDNA testing in a clinical trial population restricted to tissue positive patients (7).

HYPOTHESES AND OBJECTIVES

Our preliminary and unpublished data strongly suggests that plasma ctDNA in metastatic UC patients is representative of predominant metastatic genotype (8-10). Therefore, we hypothesize that blood-based ctDNA testing will provide a more accurate assessment of somatic FGFR status than same patient archival primary tissue. This project has 3 specific objectives:

Objective 1 (Primary): To evaluate the diagnostic value of ctDNA testing against the "gold standard" of tissue testing.
In order to establish whether ctDNA is a valid surrogate diagnostic test, it will be formally evaluated against tissue FGFR testing. Isolation of ctDNA and testing will be undertaken to evaluate the presence of the specific FGFR aberrations that the "gold standard" tissue test calls. A ROC curve will be plotted evaluating the sensitivity and specificity of ctDNA against the gold standard.

Objective 2 (Secondary): To evaluate whether ctDNA may be a superior predictive marker by identifying actionable FGFR mutations that are currently missed on tissue assays.
It is feasible that mutations other than the ones called in the tissue tests may drive FGFR pathway addiction, or that "false positives" found on ctDNA actually reflect the limitations of archival assays. During the course of our analyses of ctDNA samples submitted to the GU Biobank of Predictive Biomarkers protocol (formerly REB#H16-00934 and now REB#H23-01402)

we have identified a cohort of patients with FGFR aberrations that are not part of the standard criteria, including several known hot-spot mutations in FGFR1/3 and a previously undocumented fusion (FGFR3-ADD1). We hypothesize that at least some of these other aberrations could cause constitutive upregulation of the FGFR signaling pathway and be amenable to erdafitinib therapy. In order to evaluate this hypothesis, Janssen Canada has agreed to provide patients with negative tissue testing but ctDNA identified FGFR aberrations with access to erdafitinib therapy, and response rate data will be collected by participating sites.

Objective 3 (Exploratory): To identify molecular mechanisms of FGFR inhibitor resistance by undertaking repeat ctDNA analysis upon disease progression in patients who initially responded to erdafitinib therapy.

Through the serial collection of ctDNA data, we have shown that patients with mUC treated with chemotherapy and/or immunotherapy do not exhibit the emergence of new driver mutations upon development of resistant disease. While this is not surprising for non-targeted therapies, it is conceivable that the development of resistance to erdafitinib might involve specific genomic events that result in upregulation of FGFR independent signaling pathways, or further mutation of FGFR to an inhibitor independent state. In order to evaluate the mechanisms of resistance to erdafitinib, ctDNA samples will be analyzed from FGFR positive patients upon progression on erdafitinib therapy and compared to baseline.

STUDY DESIGN

This project will collect clinical data and analyze plasma ctDNA for FGFR status in a subset of patients from the GU Biobank of Predictive Biomarkers protocol (formerly REB#H16-00934 and now REB#H23-01402) with metastatic UC, as well as metastatic UC patients from other contributing centres in Canada.

For patients enrolled in the GU Biobank at a BC Cancer centre, blood samples are collected at baseline and at subsequent progression and/or changes in treatment. All patients will have provided consent for analysis of serum and tumor tissue biomarkers when consent to the GU Biobank was provided. Some clinical data was obtained in accordance with the same protocols and consent, and additional clinical data pertaining to disease status, treatments received and/or planned, response to treatment(s), and results of FGFR status from tissue testing will be extracted from patient records per the data dictionary (Appendix A). Blood samples in the GU Biobank collected at the time points of interest for this analysis will be analyzed for FGFR aberrations in ctDNA by the lab of Dr. Alexander Wyatt at the Vancouver Prostate Centre according to the established laboratory procedures outlined below. Results of the ctDNA FGFR analysis will be provided to the clinicians responsible for the patient's care at BC Cancer following the established GU Biobank procedures for the return of material incidental findings. Patients identified to have a FGFR aberration through analysis of ctDNA will be eligible for access to erdafitinib through the established agreement with Janssen.

Samples from other participating centres will be collected under REB-approved projects at the respective centre, and samples will be transferred to the lab of Dr. Alexander Wyatt for analysis under established material transfer agreements. Clinical data pertaining to disease status, treatments received and/or planned, response to treatment(s), and results of FGFR status from tissue testing per the data dictionary will be provided by investigators at participating centres and entered into our central database. Results of the ctDNA FGFR analysis will be provided to the clinicians responsible for the patient's care at other participating Canadian sites who will follow site-specific procedures for the return of material incidental findings. Patients identified to have a FGFR aberration through analysis of ctDNA will be eligible for access to erdafitinib through the established agreement with Janssen. An additional ctDNA sample will be collected from FGFR positive patients upon progression on erdafitinib therapy.

Inclusion Criteria

Patients with metastatic urothelial cancer who are about to undergo tissue testing for FGFR aberrations and who have baseline blood samples drawn during the management of their disease (during progressing disease and before starting systemic therapy) are eligible to be included in this analysis. Eligible patients may either be enrolled on the GU Biobank (formerly H16-00934 and now REB#H23-01402) or enrolled on REB-approved projects at external institutions within Canada with established material transfer agreements.

Exclusion Criteria

Metastatic UC patients who will not have tissue and baseline blood available for FGFR testing will be excluded from this project.

Sample Processing and ctDNA Evaluation

Whole blood collected in Streck Cell-Free DNA BCT tubes will be kept at room temperature prior to and during processing. Samples will be centrifuged at 1600 rcf for 15 minutes; buffy coat will subsequently be aliquoted, and plasma transferred to a new tube and spun for 10 additional minutes at 3200 rcf. Plasma and buffy coat aliquots will be stored at -80°C prior to DNA extraction. Cell-free DNA (cfDNA) will be extracted from up to 6 mL of plasma with the QIAGEN Circulating Nucleic Acids kit and quantified with the the Quantus Fluorometer and QuantiFluor ONE dsDNA system. Germline (i.e. leukocyte) DNA (gDNA) will be extracted from the buffy coat fraction using the Promega Maxwell RSC Blood DNA kit and Maxwell RSC system, and quantified with a NanoDrop spectrophotometer. Libraries for next-generation sequencing will be prepared with a minimum input of 25 ng of DNA per sample, using the KAPA HyperPlus kit with IDT CS adapters (duplex adapters containing 3 bp unique molecular identifiers) and UDI primers. Library quantification will be carried out with a NanoDrop spectrophotometer, and each library run on an ethidium bromide gel to confirm success. Purified sample libraries will then be multiplexed into pools for hybridization to a custom targeted panel capturing 60 genes with known relevance to bladder cancer (Roche NimbleGen SeqCap EZ Choice), including FGFR genes (exons and introns, to enable rearrangement detection). After capture, pools will be

diluted and sequenced on an Illumina HiSeq 2500 (2 x 125 bp) instrument to a minimum of 1500x non-redundant coverage. Alignment and analysis of DNA sequencing data will be performed utilizing an established bioinformatics pipeline (6, 11). Somatic mutations will be supported by a minimum variant allele fraction of 0.1%. All somatic mutation calls will be filtered against patient-matched gDNA, as well as the background error rate, in addition to meeting thresholds for mapping quality and read-end proximity. Mutations related to clonal hematopoiesis will be excluded.

Statistical Considerations

The primary objective of this project is to evaluate ctDNA as a diagnostic test against the de facto gold standard of tissue testing. To do this the area under the ROC curve will be calculated for the new test. Assuming a 20% prevalence of FGFR mutations, we would need to collect a total of 180 samples to evaluate ctDNA testing in this fashion. This would give us 90% power to detect a difference of 0.15, under the null hypothesis of AUC <= 0.75 (good diagnostic accuracy) and the alternative hypothesis of AUC = 0.9 (excellent diagnostic accuracy) with a two-sided alpha of 0.05. Our goal will be to collect 210 baseline samples to allow for a 15% ctDNA negative rate based on our previous work (note that detection rates have significantly increased with incorporation of the latest generation of commercial sequencing adapters and digital error suppression approaches, and therefore 15% is likely to be an overestimate).
For objective 3 to provide information that would inform future studies on the evolution and targeting of mutations evolving during erdafitinib treatment, a minimal cohort of 25 participants who are receiving erdafitinib will need to be enrolled. An accrual of a further 50 participants to a total of 260 is planned after the 210 accrual target has been reached. Assuming a 20% prevalence of FGFR mutations, this would lead to around 54 participants who are FGFR positive. It is expected that around half of these will access erdafitinib and provide a progression ctDNA sample.

REFERENCES

1. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer. 2015;15(1):25-41.
2. Loriot Y, Necchi A, Park SH, Garcia-Donas J, Huddart R, Burgess E, et al. Erdafitinib in Locally Advanced or Metastatic Urothelial Carcinoma. N Engl J Med. 2019;381(4):338-48.
3. Warrick JI, Sjodahl G, Kaag M, Raman JD, Merrill S, Shuman L, et al. Intratumoral Heterogeneity of Bladder Cancer by Molecular Subtypes and Histologic Variants. Eur Urol. 2019;75(1):18-22.
4. Morrison CD, Liu P, Woloszynska-Read A, Zhang J, Luo W, Qin M, 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(6):E672-81.
5. Sundahl N, Vandekerkhove G, Decaestecker K, Meireson A, De Visschere P, Fonteyne V, et al. Randomized Phase 1 Trial of Pembrolizumab with Sequential Versus Concomitant Stereotactic Body Radiotherapy in Metastatic Urothelial Carcinoma. Eur Urol. 2019;75(5):707-11.
6. Vandekerkhove G, Todenhofer T, Annala M, Struss WJ, Wong A, Beja K, et al. Circulating Tumor DNA Reveals Clinically Actionable Somatic Genome of Metastatic Bladder Cancer. Clin Cancer Res. 2017;23(21):6487-97.
7. Santiago-Walker AE, Moy C, Cherkas Y, Loriot Y, Siefker-Radtke AO, Motley C, et al. Analysis of FGFR alterations from circulating tumor DNA (ctDNA) and Tissue in a phase II trial of erdafitinib in urothelial carcinoma (UC). Journal of Clinical Oncology. 2019;37(7_suppl):420-.
8. Lavoie JM, Vandekerkhove G, Annala M, Sundahl N, Sano T, Struss W, et al. Circulating tumor DNA in patients with metastatic urothelial cancer: concordance of genomic findings with matched tissue biopsies. ASCO Annual Meeting: JCO; 2019.
9. Lavoie JM, Vandekerkhove G, Annala M, Taavitsainen S, Sundahl N, Walz S, et al. Circulating tumour DNA (ctDNA) utility as a biomarker for metastatic urothelial carcinoma (mUC). ESMO Congress: Annals of Oncology; 2019.
10. Vandekerkhove G, Lavoie JM, Annala M, Sundahl N, Sano T, Struss W, et al. Genomic concordance between profiling of circulating tumor DNA (ctDNA) and matched tissue in metastatic urothelial carcinoma. . ASCO Genitourinary Cancers Symposium: JCO; 2019.
11. Annala M, Vandekerkhove G, Khalaf D, Taavitsainen S, Beja K, Warner EW, et al. Circulating Tumor DNA Genomics Correlate with Resistance to Abiraterone and Enzalutamide in Prostate Cancer. Cancer discovery. 2018;8(4):444-57.

APPENDIX A: DATA DICTIONARY

Time Point	*Variable Name*	*Data Type*	*Definition*
Baseline Sample	Study Code	Text	Study code (study code = center code - patient ID) e.g. 01-001
Baseline Sample	Gender	Text	Assigned sex at birth
Baseline Sample	DOB (MM/YYYY)	Date	Date of birth (month and year only)
Baseline Sample	Smoking Status	Text	Select from Current, Past, Never
Baseline Sample	Date of Diagnosis	Date	Date of 1st UC diagnosis
Baseline Sample	Location of Primary	Text	Select from Kidney/Renal Pelvis, Ureter, Bladder, Urethra
Baseline Sample	Date of Metastatic Diagnosis	Date	Date of metastatic UC diagnosis
Baseline Sample	Prior Systemic Therapy for Metastatic Disease	Text	Select from Platinum based chemotherapy, Non-platinum based chemotherapy, Immunotherapy, Clinical Trial, Other (enter info on other treatments)
Baseline Sample	Other Prior Systemic Therapy for Metastatic Disease	Text	Enter additional information on prior systemic therapy if 'Other' selected
Baseline Sample	Start Date of Prior Systemic Therapy for Metastatic Disease	Date	Date the prior systemic therapy for metastatic disease has started
Baseline Sample	End Date of Prior Systemic Therapy for Metastatic Disease	Date	Date the prior systemic therapy for metastatic disease has ended
Baseline Sample	ctDNA Sample Collection Date	Date	ctDNA Sample Collection Date
Baseline Sample	Planned Next Therapy	Text	Platinum based chemotherapy, Platinum based chemotherapy followed by Avelumab maintenance, Non-platinum based chemotherapy, Immunotherapy, Clinical Trial, None, Other (enter info on other treatments)
Baseline Sample	Other Planned Next Therapy	Text	Enter additional information on planned next therapy if 'Other' selected

Tissue Test Results	Date Tissue Test Requested	Date	Enter the date tissue FGFR testing was requested
Tissue Test Results	Date Tissue Test Result Received	Date	Enter the date tissue FGFR testing was received
Tissue Test Results	Actionable FGFR Aberration Identified	Y/N	Enter whether an actionable FGFR aberration was identified (Y/N), if no skip specific FGFR aberration section
Tissue Test Results	Specific FGFR Aberration(s) Identified	Text	Select all that apply: FGFR2-BICC translocation, FGFR2-CASP7 translocation, FGFR3-TACC#_V1 translocation, FGFR3-TACC3_V3 translocation, FGFR3-BAIAP2L1 translocation, FGFR3 mutation (p.R248C), FGFR3 mutation (S249C), FGFR3 mutation (G370C), FGFR3 mutation (Y373C), Other (please specify)
Tissue Test Results	Other FGRR Aberration(s) Identified	Text	Specify any other FGFR aberration(s) identified
Erdafitinib Treatment	Erdafitinib Treatment Planned	Y/N	Was treatment with erdafitinib planned based on an identified FGFR aberration?
Erdafitinib Treatment	Start Date Erdafitinib Therapy	Date	If patient received erdafitinib enter the date treatment was started
Erdafitinib Treatment	Best Response to Erdafitinib	Y/N	Investigator assessed response to erdafitinib therapy
Erdafitinib Treatment	Erdafitinib Response Date	Date	Date of response to erdafitinib therapy (investigator assessed)
Erdafitinib Treatment	Progression on Erdafitinib Date	Date	Date of progression on erdafitinib treatment (investigator assessed)
Erdafitinib Treatment	ctDNA Progression Sample Collected	Date	If collected, enter the date of the progression/change in treatment sample collected after progression on erdafitinib
ctDNA - Baseline Sample	Baseline ctDNA - Date Sequencing Results Received	Date	Date ctDNA sequencing results of the baseline sample were received
ctDNA - Baseline Sample	Baseline ctDNA - Collection Date of Sequenced Sample	Date	ctDNA Baseline Sample Collection Date
ctDNA - Baseline Sample	Baseline ctDNA - Actionable FGFR	Yes/No/Inconclusive	Enter whether an actionable FGFR aberration was identified (Yes/No/Inconclusive), if no or

	Aberration Identified		inconclusive skip specific FGFR aberration section
ctDNA - Baseline Sample	Baseline ctDNA - Specific FGFR Aberration(s) Identified	Text	Select all that apply: FGFR2-BICC translocation, FGFR2-CASP7 translocation, FGFR3-TACC#_V1 translocation, FGFR3-TACC3_V3 translocation, FGFR3-BAIAP2L1 translocation, FGFR3 mutation (p.R248C), FGFR3 mutation (S249C), FGFR3 mutation (G370C), FGFR3 mutation (Y373C), Other (please specify)
ctDNA - Baseline Sample	Baseline ctDNA - Other FGRR Aberration(s) Identified	Text	Specify any other FGFR aberration(s) identified
ctDNA - Erdafitinib Treatment	Progression ctDNA - Date Sequencing Results Received	Date	Date the ctDNA sequencing results of the progression sample were received
ctDNA - Erdafitinib Treatment	Progression ctDNA - Collection Date of Sequenced Sample	Date	Date the ctDNA progression sample was collected
ctDNA - Erdafitinib Treatment	Progression ctDNA - Actionable Aberration Identified	Yes/No/Inconclusive	Enter whether an actionable FGFR aberration was identified (Yes/No/Inconclusive), if no or inconclusive skip specific FGFR aberration section
ctDNA - Erdafitinib Treatment	Progression ctDNA - Specific Aberration(s) Identified	Text	Enter the specific aberration(s) identified in the ctDNA progression sample