Development of a Urine-Based Liquid Biopsy for Early Detection and Monitoring of Bladder Cancer and Metastatic Urothelial Carcinoma (mUC)

Vincent Funari, Vy Tran, Nathan Lam, Hesam Mahmodi, Mariam Otmishi, Kenda Scruggs, Kimberly Prado, Robert Embree

Introduction
Bladder cancer(BC), particularly NMIBC, reoccurs ~70% of the time. This means regular patient follow-up with invasive methods e.g.cystoscopy. Alternatively, non-invasive urine tests e.g. cytology, Bladder17® (IHC), BladderFISH® can help stratify patients early.

New targeted therapies, like enfortumab vedotin (EV) and FGFR3 TKI inhibitors revolutionized BC therapy. FGFR3 mutations found in 50-60% of all BC and 60-80% of low-grade NMIBC tumors have better prognoses.

Early FGFR3 mutation status can aid in patient management while standard of care like cystoscopy and biopsies can be delayed as long as 4-6 weeks from the time of scheduling. Blood based liquid biopsies using Next-Gen sequencing (NGS) can be expensive and have long TAT. While effective, EV and Chemotherapy are highly toxic. In patients with compromised health, immunotherapy or FGFR3 inhibitors are preferable. Here we investigate the potential of a non-invasive, inexpensive at home collection with quick TAT.

Methods
190 urine samples from BC(2-50 mL) patients stored for ~10 days in a methanol fixative (G.Fix by SSN) were spun and washed 2X with PBS. For larger feasibility studies, healthy urine samples collected in ZYMO Research, nRICH, Paxgene collection kit or 2–3 day old BC samples in the G.Fix were separately pooled and divided into aliquots to undergo different extraction conditions and PCRs to determine the best methodology. 16 patient had paired urine and BC FFPE samples and were analyzed with Qiagen therascreen FGFR3 FDA kit. Nucleic Acid was analyzed using UV spec, Qubit, PCR, and Fragment Analyzer/Bioanalyzer.

Results
13/54 BC patient urines contained 1-2 FGFR3 mutations. 6/16 urine/FFPE paired samples were discordant, while 4/7 had matching mutations. Average RNA concentration was 63.88 ng/µL, 260/280 ratio was 1.75, and 260/230 ratio was 0.761.

Zymo yielded highest RNA concentrations and 260/230 ratios, but lower overall yield compared to Thermo; in addition Zymo is not high throughput or user friendly as the automated Nrich or Thermo extractions. Overall, greater differences are seen in sample collections and patients. In general, there was an inverse correlation between 260/230 ratio and Ct values suggesting the presence of qRT-PCR inhibitors. The ~10 day old or 1-2 day old G.fix BC urine samples had the highest Ct values vs healthy 12 hr old urines collected in Zymo cups. Average RNA was 120bp w/little evidence of 18S/28S. nRICH urine preservative and extraction protocol produced the largest RNA fragment profile and was fully automated. Pelleted Urine samples produced the highest yield of RNA.

DNA based-PCR resulted in an average of ~10x more sensitive result than RNA based qRT-PCR.

Conclusions
Obtaining TNA from BC and normal urines to identify FGFR3 mutations is possible though the quality of RNA maybe compromised. qRT-PCR testing in urine with an FDA approved FFPE kit, is not as sensitive as DNA based PCR testing, due to lower quality of RNA and possibility of RT inhibitors. More studies are needed to understand why the quality of RNA is poor even in fresh samples, and if that resulted in lower concordance with paired samples. Liquid biopsy can reveal more mutations and secondary malignancies in heterogenous samples.

Figure 3A

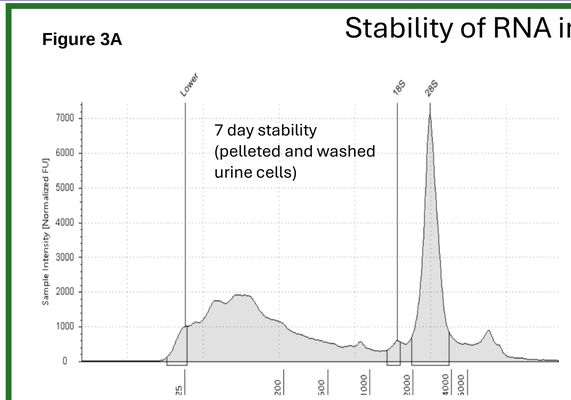


Figure 3B

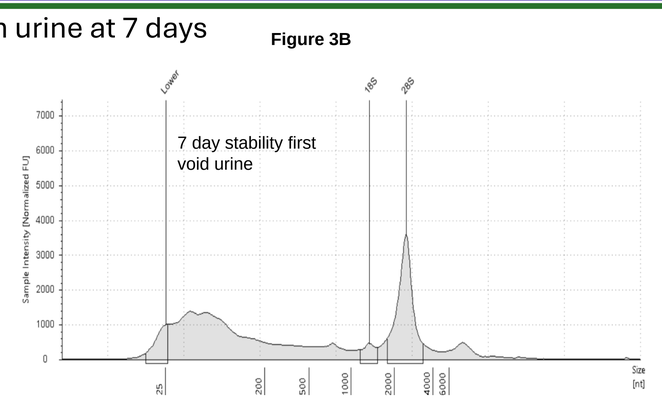


Figure 3A. RNA profile of 50 mls pelleted and PBS washed cells after 7 days with nRICH. **Figure 3B.** RNA profile of Urine extracted with NRICH urine kit after 7 days

Fig 3. Conclusion: Comparing RNA extraction profiles between Figures 4A-B versus Figure 1, there is a higher degradation ratio of <200bp versus >200bps when sample is stored up to 7 days compared to 24 hours, however even at 7 days there is a significant percent of fragments that are larger than 200bps. Additionally, nRICH extractions are limited to 30mls currently however pelleting larger volume of cells (e.g. 50ml) and washing them with PBS and leaving them in nRICH preservative until extraction yielded more RNA as expected.

Number of patients	Mutations identified
41	Negative
3	S249C
2	Y373C
2	FGFR3-BAIAP2L1, FGFR2-CASP7
2	FGFR2-CASP7
1	R248C
1	G370C
1	FGFR3-TACC3v1
1	FGFR3-TACC3v3

Table 1. FGFR2/3 mutations identified in urine cell pellets from bladder cancer patients identified using a proprietary BladerFISH test (loss of p16, chromosomes 3, 7, and 17). Urine was preserved in G.FIX a methanol based preservative optimized for FISH.

Urine and subsequent biopsy FGFR3 mutation results

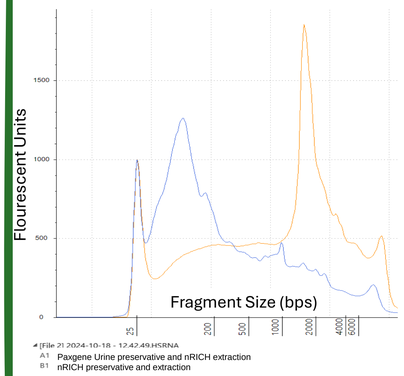
Urine	Detected Mutation	Subsequent Biopsy from patient	Detected Mutation	Original Tumor if patient relapsed	Detected mutation
Urine	Neg	FFPE	FGFR2-BICC1	N/A	N/A
Urine	Neg	FFPE	FGFR3-BAIAP2L1	FFPE	Neg
Urine	Neg	N/A	N/A	FFPE	Neg
Urine	Neg	FFPE	Neg	N/A	N/A
Urine	S249C	FFPE	S249C	N/A	N/A
Urine	R248C	FFPE	R248C	N/A	N/A
Urine	Neg	FFPE	G370C	N/A	N/A
Urine	Neg	FFPE	Neg	N/A	N/A
Urine	Neg	FFPE	S249C	N/A	N/A
Urine	G370C	FFPE	S249C	N/A	N/A

Table 2. FGFR2/3 mutations identified in urine cell pellets and subsequent biopsy from bladder cancer patients.Two cases were relapse and had a different mutation signature than the original biopsy. Urine was preserved in G.FIX a methanol based preservative optimized for FISH.

Table1-2 Conclusions: The therascreen® bladder cancer FDA screen is clinically validated for two gene fusions (FGFR3-TACC3v1 and FGFR3-TACC3v3) both of which we identified in bladder cancer patient's urine. The most common mutation observed was FGFR3 S249C which is consistent with literature for bladder cancer biopsies. However, in RUO mode FGFR2 fusions and FGFR3-BAIAPL2 can be identified, and collectively were more common than the clinically validated FGFR3 mutations. This suggests that an LDT could have wider clinical utility than the therascreen® FDA kit. While some of the urine biopsies had a similar genetic background to the subsequent bladder biopsy, in one case there was a completely different FGFR3 genetic profile in urine compared to the FFPE biopsy of the same patient. In 4 cases, their failed to identify a matching mutation in urine as the solid tumor biopsy. More investigation is needed in these cases to establish if the urine was of sufficient quality and quantity to adequetly detect the mutations or if there were differences in collection that may have affected the result.

Interestingly, two cases had the same two unique fusions, neither of which would be reported by the therascreen® FDA kit (FGFR3-BAIAP2L1, FGFR2-CASP7). So those patients who normally would be eligible for EV treatment, would not get the opportunity for the EV targeted therapy if they were tested only with the FDA kit. Another observation is that those two cases have multiple translocations in two different chromosomes (e.g. 10 and 4). Having a FISH or IHC assay to investigate if these are in the same cancer cell could help characterize if the tumor is heterogenous or there are multiple bladder cancer sites that need to be biopsied. This could be clinically useful for assisting a urologist during a cystoscopy o look for more than one tumor site/biopsy. In future studies, comparing paired FFPE staging and FISH signal strength to see if they correlate with presence or absence of mutations in Urine could help determine which patients should be tested for FGFR3 mutation status to help begin to stratify the patients with potential therapeutic or prognostic options.

Quality of RNA extracted from from Exosomes and Cells in First Void Urine



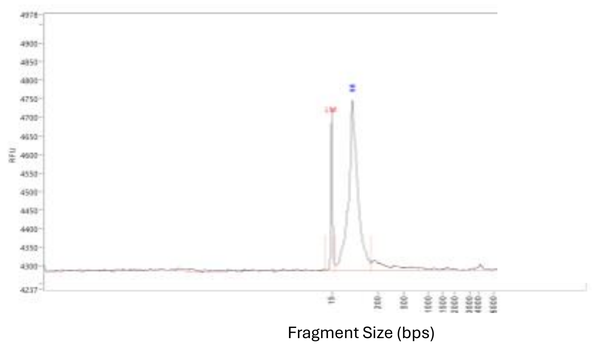


Figure 1. RNA profiles of PAXgene Urine liquid biopsy preservative and nRICH Urine preservative extracted with nRICH revolution sample prep

Figure 2. Example of most RNA profiles from Zymo Urine extraction, Thermo Fisher MagMax RNA, Qiagen RNeasy kit independent of nRICH or SSN solutions G.FIX methanol based preservative.

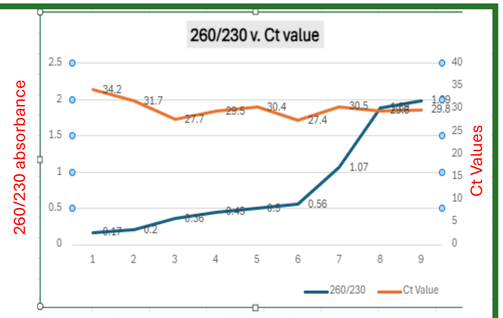


Figure 4. Comparison of qRT- PCR Ct values from housekeeping gene(GUSB) to 260/230 absorbance ratios from Nanodrop Spectrophotometer

Fig. 4 Conclusions: Impurities in all urine extractions results in inhibitors that decrease sensitivity of qRT-PCR(RNA), we do not see the same inhibition in qPCR (DNA). Second bead washes do not remove inhibitors. The likely contaminant is probably Urea which can inhibit reverse transcription.

Fig.1-2 Conclusions: RNA pro
preservative produced distinct 18/28S ribosomal peaks which are indicative of high quality RNA, regardless of preservative or extraction methods. nRICH preservative and extraction of 30 mls of first void urine demonstrated the largest RNA fragments with most fragments being larger than 200 bps. Most qPCR or NGS assays rely on fragments being at least 100bp, therefore RNA profiles that contain 50% of material smaller than 100bp makes assay development difficult. Assays that take advantage of small RNA fragment sizes or possibly DNA could be useful in developing a non-invasive urine liquid biopsy assay

Emeritus MEDICAL TECHNOLOGY
EmeritusDX Together, We Save Lives
Freedom PATHOLOGY PARTNERS