

## Urine DNA for monitoring chemoradiotherapy response in muscle-invasive bladder cancer

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## SEE ME

Around 20% of **testicular cancer** survivors experience **testosterone deficiency**<sup>1</sup>, which can result in metabolic syndrome and poor cardiac health<sup>2-7</sup>

**The European Society for Medical Oncology recommends** measurement of testosterone levels during follow-up.<sup>8</sup>

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For full prescribing information, including side effects, precautions and contraindications, please consult the Summary of Product Characteristics (SPC).

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**NHS Price:** £31.11 per 88g pump pack. **Legal category:** POM. **Marketing Authorisation Number:** PL 28387/0007. **Marketing Authorisation Holder:** Besins Healthcare, Avenue Louise, 287, Brussels, Belgium. **Date of preparation of Prescribing Information:** February 2021 TES/2021/016

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## Research Communication

# Urine DNA for monitoring chemoradiotherapy response in muscle-invasive bladder cancer: a pilot study

Accumulating evidence implies the utility of DNA-based urine biomarkers for initial detection of bladder cancer (BC) and surveillance of non-muscle-invasive BC [1,2]. We have previously described gene panels with utility for these indications, identifying UBC-associated mutations in 96% of all BCs, such that the associated urine test is not reliant on the initial identification of mutations in primary tumour tissue [1]. By contrast, the utility of urine as a liquid biopsy for the surveillance of patients with muscle-invasive BC (MIBC) treated by bladder preservation (radiotherapy  $\pm$  chemotherapy) remains understudied; one previous publication describes microsatellite analysis of urinary DNA to detect bladder recurrences in five out of six radiotherapy patients [3].

We undertook a pilot study to evaluate whether measuring common BC-associated mutations in urinary DNA can contribute to the monitoring of treatment responses in patients with organ-confined MIBC treated with curative intent. Our objectives were to: (i) investigate the potential of urine DNA analysis before, during and after treatment as indicators of treatment response; (ii) investigate the prognostic value of an absence of detectable genomic alterations post treatment; and (iii) compare two orthogonal methodologies for detecting variants in urinary DNA (capture and Illumina sequencing vs PCR and Ion Torrent sequencing).

As part of the TUXEDO trial (a phase I/II feasibility study of cetuximab plus 5FU and mitomycin C or cisplatin with concomitant radiotherapy in patients with organ-confined MIBC; ethics approval 11/LO/1313, protocol at <https://www.birmingham.ac.uk/research/crcu/trials/tuxedo/index.aspx>), urine samples were collected from patients with MIBC before, during and after treatment with chemoradiotherapy. Briefly, urine samples (50 mL) were collected prior to treatment on the first day of weeks  $-1$  to  $+7$  (treatment completion) and at one post-treatment visit using urine preservation tubes (Norgenbiotech.com). Urine samples were centrifuged for 10 min at 2000g and DNA extracted from the pellet using the QuickDNA kit (Zymoresearch.com) and quantitated by Qubit. Capture-based libraries were prepared using 25 ng urine cell pellet DNA (cpDNA) and Nonacus Cell3Target (Nonacus.com). Target enrichment was via a custom panel covering c.10 kb and including hotspots/regions of 29 genes: coding regions of 23 genes plus the *TERT* promoter and an additional five non-coding mutation hotspots, as previously defined by large-scale tumour sequencing [1,4]. Libraries were

sequenced on an Illumina Nextseq system ( $2 \times 150$  bp); reads were aligned to hg19 genome version using BWA. Variant allele frequencies (VAFs) were extracted using the bam-readcount tool for the disease-associated single-nucleotide variants (SNVs) identified previously [1,4]. We considered a 0.5% VAF to be the limit of detection for SNVs [5], and a 1% VAF to be the limit of quantitation. We report quantitative changes in the VAFs of urinary SNVs during treatment for all SNVs that exceeded 1% VAF at any time in each patient. Average raw and consensus read depths were  $27\ 100\times$  and  $3000\times$ , respectively.

Multiplex-PCR-based libraries were prepared using AmpliseqHD reagents (ThermoFisher) and workflow. Two panels of target-specific primers and  $2 \times 20$  ng urine cpDNA were used to amplify the same target regions as for the capture-based approach. The libraries were sequenced using 540 chips on an S5 Ion Torrent system ( $2 \times 100$  bp; ThermoFisher). Alignments, consensus-building and variant calling were performed using ION REPORTER software. Average raw and consensus read depths were  $79\ 300\times$  and  $5500\times$ , respectively. We excluded AmpliseqHD data for the *TERT* promoter as this GC-rich amplicon did not give sufficient consensus reads for reliable variant calling.

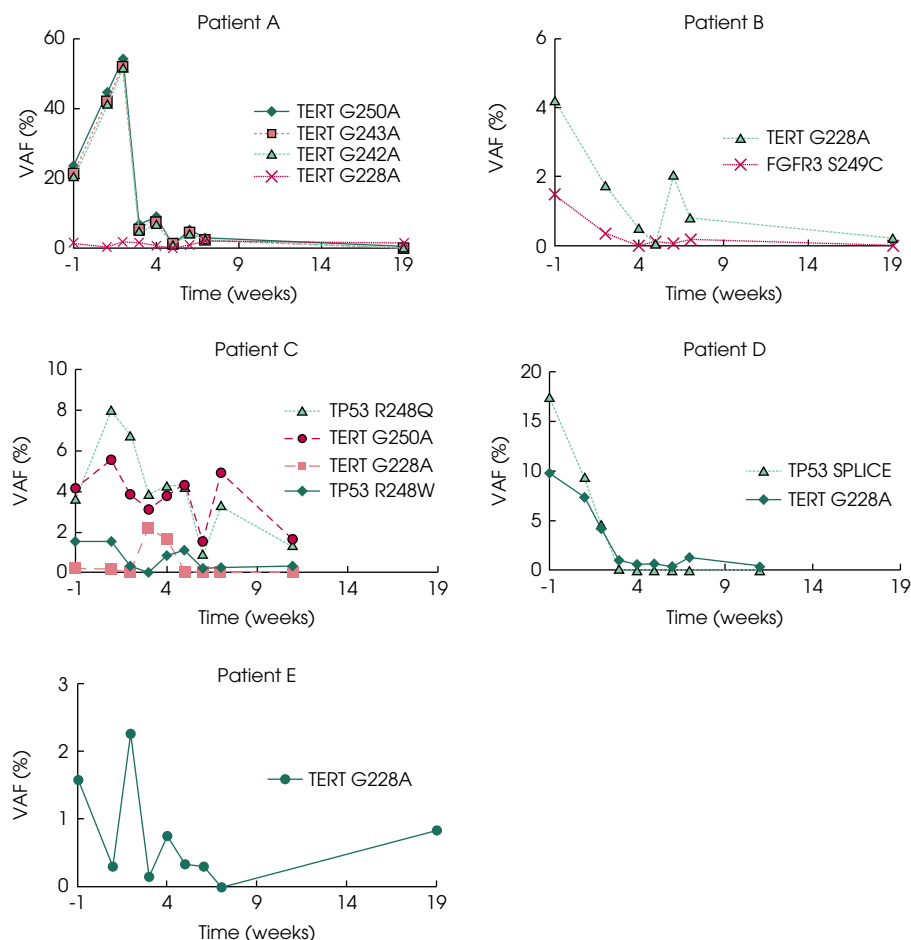
In this way, common UBC-associated mutations were determined longitudinally in six patients. For one patient, no mutations were detected at any time point. For coding and *TERT* promoter mutations, cpDNA of four out of five patients contained high levels of tumour DNA (VAFs 4–20%) at baseline (post-transurethral resection, pre-chemoradiotherapy). VAFs in cpDNA decreased to lower levels in all patients by the end of treatment (Fig. 1).

In patient A, multiple mutations in the *TERT* promoter were detected at  $>20\%$  VAF at baseline. The unusual co-occurrence of the 242/243 and 250 *TERT* mutations was confirmed by Sanger sequencing (data not shown). These mutations were detected at higher VAFs after 1 week of cetuximab-loading and after 1 week of radiotherapy, then decreased rapidly to approximately 5% VAF, remained clearly detectable through the later stages of treatment, and were present at 1.4% VAF after treatment completion. Patient A relapsed with local recurrence (grade/stage unknown) 7 months later.

In patient B, *TERT* 228A and *FGFR3* S249C mutations were present at baseline; both were undetectable post-treatment. Patient B remained disease-free 16 months post-treatment.



**Fig. 1** Trends in coding or *TERT* promoter mutations by percentage variant allele frequency (VAF) at each time point by capture-based Illumina sequencing using a previously described 23-gene panel (1). Panels (A–E) represent patients A–E, respectively.



In patient C, *TERT* and *TP53* mutations were present at baseline and, despite showing a downward trend, remained detectable at 1.7% VAF after treatment. Patient C was diagnosed with malignant ascites 3 months post-treatment.

In patient D, *TERT* and *TP53* mutations were present at baseline, dropped rapidly during treatment, and were undetectable after completion of treatment. Patient D was diagnosed with local recurrence (G3pT1) 5 months post-treatment.

In patient E, a *TERT* promoter mutation was present at low VAF at baseline; this mutation did not show a clear trend over time and remained detectable at most time points. Patient E was diagnosed with local recurrence (G3pT1) 9 months post-treatment.

PCR-based library preparation (AmpliseqHD) combined with Ion Torrent sequencing verified Illumina-based mutation detection and quantitation in cpDNA, except for the *TERT* promoter which amplified poorly (data not shown). VAFs measured by the two methods correlated well ( $r^2 = 0.96$ ), and

AmpliseqHD confirmed 84%, 94% and 98% of SNVs detected by capture-based sequencing at  $\geq 0.5\%$ , 1% and 2% VAF, respectively. Copy number profiles (a by-product of off-target reads from capture-based target enrichment) for all cpDNA were inspected manually; copy number variant levels mirrored SNV levels (data not shown).

In summary, two out of the four patients who relapsed (three local, one distant, 3–9 months after completing treatment) had undetectable urinary VAFs on treatment completion. This finding is particularly surprising for the two out of three bladder recurrences with undetectable urinary VAFs on treatment completion given that, in other cancer settings, the ‘clearance of mutations’ in liquid biopsy samples is associated with significantly improved outcomes [6]. The corollary is that two out of the three patients with detectable mutations on treatment completion experienced relapse within 7 months. Notwithstanding, we suggest that urine-based liquid biopsy monitoring of post-radiotherapy MIBC patients remains challenging, and should be combined with plasma ctDNA monitoring [7], or primary tumour tissue sequencing

(to permit personalized urinary liquid biopsies with much lower detection thresholds [8]), or both. Methodologically, both targeted capture-based and PCR-based library preparation and next-generation sequencing can be used to identify common BC-associated mutations in urinary cpDNA. These pilot data suggest the need for further liquid biopsy research in this specific MIBC setting.

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## Conflict of Interest

Timur Mitin: UpToDate, Inc (royalties for chapter authorship), Novocure (study grant and advisory board), AstraZeneca (advisory board). Richard T. Bryan: Olympus Medical Systems (advisory board), Janssen (advisory board), UroGen Pharma (research grant), QED Therapeutics (research grant), Nonacus Limited (advisory board). Douglas G. Ward: Nonacus Limited (advisory board).

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Abbreviations: BC, bladder cancer; cpDNA, chloroplast DNA; MIBC, muscle-invasive bladder cancer; SNV, single-nucleotide variant; VAF, variant allele frequency.