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

Gordon, Naheema S; Baxter, Laura A; Goel, Anshita; Arnold, Roland; Kaur, Baljit; Liu, Wenyu; Pirrie, Sarah J; Hussain, Syed; Viney, Richard; Ford, Daniel; Zarkar, Anjali; Wood, Mary A; Mitin, Timur; Thompson, Reid F; James, Nicholas D; Ward, Douglas G; Bryan, Richard T

*DOI:*
10.1111/bju.15589



*Document Version*
Publisher's PDF, also known as Version of record

*Citation for published version (Harvard):*
Gordon, NS, Baxter, LA, Goel, A, Arnold, R, Kaur, B, Liu, W, Pirrie, SJ, Hussain, S, Viney, R, Ford, D, Zarkar, A, Wood, MA, Mitin, T, Thompson, RF, James, ND, Ward, DG & Bryan, RT 2022, 'Urine DNA for monitoring chemoradiotherapy response in muscle-invasive bladder cancer: a pilot study', *BJU International* , vol. 129, no. 1, pp. 32-34. https://doi.org/10.1111/bju.15589

# I'M A TESTICULAR CANCER SURVIVOR

# SEE ME

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

**The European Society for Mediacal Oncology recommends** measurement of testosterone levels during follow-up.[8]

**PRESCRIBING INFORMATION TESTOGEL® (testosterone) 16.2 MG/G, GEL**

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

**Presentation:** Transdermal gel in a multi-dose container, one pump actuation delivers 1.25g of gel containing 20.25mg of testosterone. **Indication:** Testosterone replacement therapy for male hypogonadism when testosterone deficiency has been confirmed by clinical features and biochemical tests. **Dosage and administration:** Cutaneous use. The recommended dose is two pump actuations of gel (i.e. 40.5mg of testosterone) applied once daily. The daily dose should not exceed four pump actuations (81 mg testosterone) per day. Adjustment of dosage should be achieved by increments of one pump actuation, usually based on measurements of blood testosterone levels and/or clinical response. The gel should be administered by the patient himself, onto clean, dry, healthy skin on the right and left upper arms and shoulders. **Contraindications:** Cases of known or suspected cancer of the prostate or breast, known hypersensitivity to testosterone or to any other constituent of the gel. **Warnings and precautions for use:** Testosterone deficiency should be clearly demonstrated by clinical features and confirmed by 2 separate blood testosterone measurements. Testosterone levels should be monitored at baseline and at regular intervals during treatment. In addition, in patients receiving long-term androgen treatment the following laboratory parameters should be checked regularly: haemoglobin, haematocrit (to detect polycythaemia), liver function tests and lipid profile. Testogel may affect results of laboratory tests of thyroid function. Risk of pre-existing prostatic cancer should be excluded and the prostate gland and breast monitored during Testogel treatment. Androgens may accelerate the progression of sub-clinical prostate cancer and benign prostate hyperplasia. Testogel should be used with caution in cancer patients at risk of hypercalcaemia and associated hypercalciuria due to bone metastases; regular monitoring of blood calcium levels is recommended in these patients. Testogel may cause oedema with or without congestive cardiac failure in patients suffering from severe cardiac, hepatic or renal insufficiency or ischaemic heart disease. If this occurs, treatment must be stopped immediately. Testogel should be used with caution in patients with ischaemic heart disease. Testosterone may cause a rise in blood pressure and should be used with caution in men with hypertension. Testogel should be used with caution in patients with thrombophilia or risk factors for venous thromboembolism (VTE), as there have been post-marketing reports of thrombotic events in these patients during testosterone therapy. In thrombophilic patients, VTE cases have been reported even under anticoagulation treatment, therefore continuing testosterone therapy after first thrombotic event should be carefully evaluated. In case of treatment continuation, further measures should be taken to minimise the individual VTE risk. Spermatogenesis may be suppressed leading to adverse effects on semen parameters. Gynaecomastia occasionally develops and occasionally persists. Irritability, nervousness, weight gain, prolonged or frequent erections may indicate excessive androgen exposure requiring dosage adjustment. Testogel should be used with caution in patients with epilepsy and migraine. Do not apply to the genital areas as the high alcohol content may cause local irritation. Testogel is flammable until dry. Testogel can be transferred to other persons by close skin to skin contact. There is limited experience regarding safety and efficacy of Testogel in patients over 65 years of age. Testogel is not indicated for use in women or in children under 18 years of age. Testogel is not a treatment for male impotence or sterility. FOR THE FULL LIST OF WARNINGS AND PRECAUTIONS PLEASE CONSULT SECTION 4.4 OF THE FULL SPC. **Interactions:** May increase the activity of oral anticoagulants. Concomitant administration of testosterone and ACTH or corticosteroids may increase the risk of developing oedema. Testogel may cause changes in insulin sensitivity, glucose intolerance, glycaemic control, blood glucose and glycosylated haemoglobin levels. **Pregnancy and lactation:** Pregnant women must avoid any contact with Testogel application sites. This product may have adverse virilising effects on the foetus. **Undesirable effects:** Local skin reactions include: acne, alopecia, dry skin, skin lesions, contact dermatitis, hair colour changes, rash, sweating, hypertrichosis, application site hypersensitivity, application site pruritus. The following commonly (≥1/100; <1/10) occur with Testogel: emotional symptoms, prostate specific antigen (PSA) increased, increased haematocrit, increased haemoglobin and increased red blood cell count. The following uncommonly (≥1/1000 to <1/100) occur with Testogel: malignant hypertension, flushing, phlebitis, diarrhoea, abdominal distention, oral pain, gynaecomastia, nipple disorder, testicular pain, increased erection and pitting oedema. Other adverse reaction identified during post-approval use of Testogel : testis disorder, headache, dizziness, paraesthesia, vasodilation (hot flushes), deep vein thrombosis, dyspnoea, polycythaemia, anaemia, musculoskeletal pain, gynaecomastia, testis disorder, prostate enlargement, oligospermia, benign prostate hyperplasia, impaired urination, anxiety, depression, aggression, insomnia, nausea, asthenia, oedema, malaise and weight increase. In case of severe application site reactions, treatment should be reviewed and discontinued if necessary.

**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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*BJU Int* 2021 doi:10.1111/bju.15589

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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 ± 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/crctu/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 (Norgenbiotek.com). Urine samples were centrifuged for 10 min at 2000***g*** 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 pellett 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* promotor 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 × 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× and 3000×, respectively.

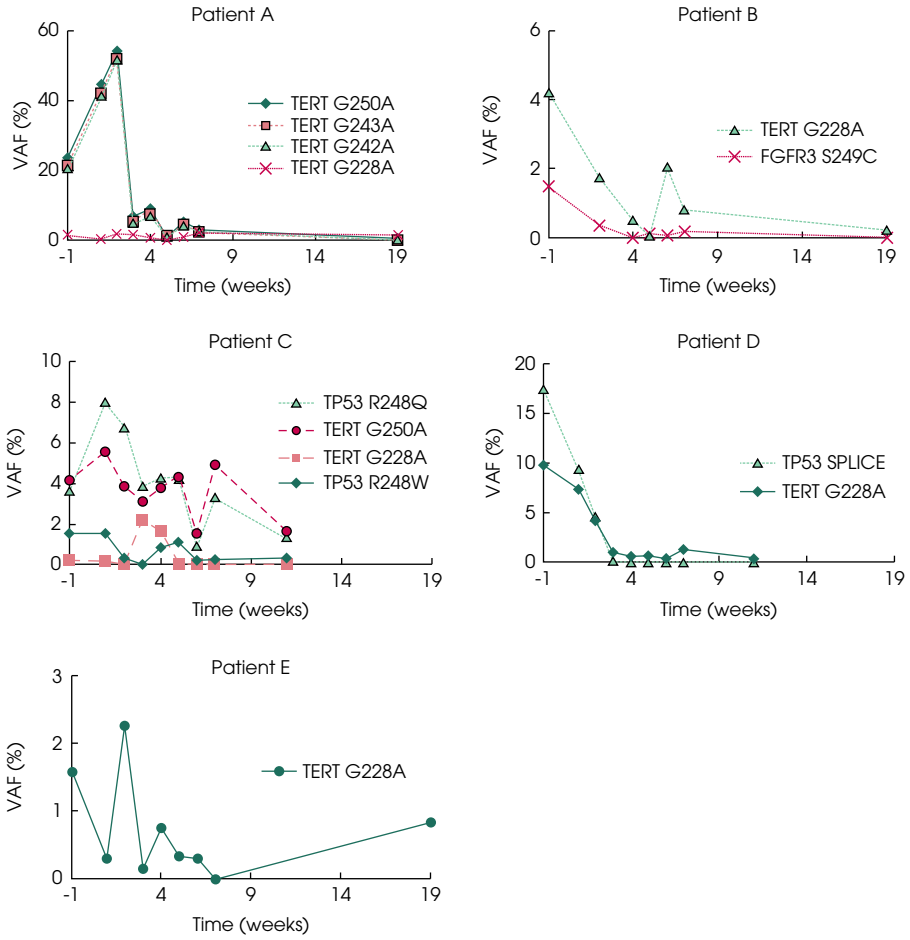
Multiplex-PCR-based libraries were prepared using AmpliseqHD reagents (ThermoFisher) and workflow. Two panels of target-specific primers and 2 × 20 ng urine cpDNA were used to amplify the same target regions as for the capture-based approach. The libraries were sequenced using 540 chips on an S5 Ion Torrent system (2 × 100 bp; ThermoFisher). Alignments, consensus-building and variant calling were performed using Ion Reporter software. Average raw and consensus read depths were 79 300× and 5500×, 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 ≥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.

## Acknowledgements

The authors thank Katie Marquis, Florence Pethick and Giorgio Pea from Thermo Fisher UK, Nonacus Limited, and West Midlands Regional Genetics Laboratory. The liquid biopsy work was funded by a Cancer Research UK Early Detection Committee – CRUK-OHSU Spark Award (C16909/A27035). The TUXEDO trial was funded by Cancer Research UK (CRUK/09/021) and cetuximab was supplied by Merck Serono Ltd.

## 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).

## Funding

The liquid biopsy work was funded by a Cancer Research UK Early Detection Committee – CRUK-OHSU Spark Award (C16909/A27035). The TUXEDO trial was funded by Cancer Research UK (CRUK/09/021) and cetuximab was supplied by Merck Serono Ltd.

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