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**Monitoring of urothelial cancer disease status after treatment by digital droplet PCR
liquid biopsy assays**

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Abstract

Objectives: Real-time monitoring of disease status would be beneficial for timely decision making in the treatment of urothelial cancer (UC), and may accelerate the evaluation of clinical trials. Use of cell free tumour DNA (cftDNA) as a biomarker in liquid biopsy is minimally invasive and its successful use has been reported in various cancer types, including UC. The objective of this study was to evaluate the use of digital droplet PCR (ddPCR)-based assays to monitor UC after treatment.

Method and Materials: Blood, urine and matching formalin fixed, paraffin embedded (FFPE) diagnostic specimens were collected from 20 patients diagnosed with stage T1 (n=2) and T2/T3 (n=18) disease. SNaPshot assays, Sanger sequencing and whole exome sequencing (WES) were used to identify tumour-specific mutations, and somatic mutation status was confirmed using patient-matched DNAs extracted from buffy coats and peripheral blood mononucleocytes. The ddPCR assays of the tumour-specific mutations were used to detect the fractional abundance of cftDNA in plasma and urine.

Results: SNaPshot and Sanger sequencing identified point mutations in 70% of the patients that were assayable by ddPCR. Cases of remission and relapse monitored by assays for *PIK3CA* E542K and *TP53* Y163C mutations in plasma and urine concurred with clinical observations up to 48 months from the start of chemotherapy. A new ddPCR assay for the telomerase reverse transcriptase (*TERT*) promoter (-124) mutation was developed. The *TERT* assay was able to detect mutations in cases below the limit of detection by SNaPshot. WES identified a novel mutation, *CNTNAP4* G727*. A ddPCR assay designed to detect this mutation was able to distinguish mutant from wild-type alleles.

Conclusions: The study demonstrated that ddPCR assays could be used to detect cftDNA in liquid biopsy monitoring of the post-therapy disease status in patients with UC. Overall, 70% of the patients in our study harboured mutations that were assayable by ddPCR.

Keywords;

Urothelial carcinoma, cell free DNA, circulating tumour DNA, molecular diagnosis, digital droplet PCR, whole exome sequencing

Word count; 2851 words

1. Introduction

Urothelial carcinoma (UC) is the 10th most common cancer, with 550,000 new cancer cases worldwide in 2018 [1]. Risk of progression from non-muscle-invasive bladder cancer (NMIBC) to MIBC is high in T1 disease, up to 17% at one year, increasing to 45% at 5 years [2]. High-risk NMIBC and MIBC are treated aggressively, often by cystectomy with peri-operative chemotherapy in the case of MIBC. Effectiveness of treatments of metastatic disease is limited [3, 4]. The recent technological advance in identifying cell free tumour DNA (cftDNA) offers the opportunity to enable real-time diagnosis in combination with new and existing diagnostic modalities, which may facilitate the selection of therapy options and more accurate prediction of disease prognosis [5].

DNA is released into the bloodstream or urine as cell-free DNA (cfDNA), and DNA released from tumours, cftDNA, can be differentiated from normal cfDNA using tumour-specific mutations as markers [6]. Use of cftDNA-based markers in liquid biopsy samples may enable a minimally invasive approach to disease monitoring, and facilitates longitudinal, repeated sampling. cftDNA is detectable in many cancer types, including UC [7], albeit the levels may vary [8]. Studies showed that real-time monitoring of tumour burden is possible, when measured as variant allele frequency or fractional abundance (FA) of cftDNA, and it can represent DNA from both primary and metastatic tumours [6, 9, 10].

Detection of the UC hotspot mutations *FGFR3* S249C and Y373C, and *PIK3CA* E545K in plasma and urine cfDNA was used in disease surveillance, and the level of cftDNA was associated with later disease progression in NMIBC and recurrence in patients that were undergoing cystectomy [11]. A custom-designed panel of one to six patient-specific ddPCR assays, developed based on the pre-screening of the tumour-specific mutations by next-generation sequencing, was successfully utilised in longitudinal monitoring of NMIBC [12]. An extension of this strategy that used 84 personalized ddPCR assays targeting 61 genes in

cftDNA detected a relapse in MIBC patients with a lead-time of 101 days after cystectomy over radiographic imaging [13]. The level of cftDNA in liquid biopsy could be detected by a panel of general or UC-specific mutations and structural alterations, such as copy number alterations (CNAs) [13-18].

The underlying principle of ddPCR is a single PCR reaction split into 10,000 to 20,000 discreet measurements in "droplets", which enables quantification of a single mutant DNA sequence amongst thousands of wild-type sequences [6]. While relatively limited in the flexibility of mutational coverage in monitoring patients, it does have an advantage in simplicity and sensitivity. In this study, we have tested the feasibility and practicality in performing commercially available ddPCR-based assays and in designing new ddPCR assays where pre-existing assays were not available.

2. Materials and Methods

2.1. Patients and DNA samples

Samples were collected under the approvals by The West of Scotland Research Ethics Service (REC 10/S0704/18) and MI84 ECMC Blood Biomarkers Study. The survival status of patients is as of 1/1/2018. The matched formalin fixed, paraffin embedded (FFPE) samples were acquired from the NHS Greater Glasgow and Clyde Biorepository (16/WS/0207 app 122). Blood samples were processed at the Queen Elizabeth University Hospital or The Analytical Services Unit (ASU), University of Glasgow. Macrodissection of tumour areas, identified by the pathologist, was performed from five to ten 8- μ m FFPE tissue sections. DNAs from plasma, buffy coat, peripheral blood mononucleocytes (PBMCs), and urine were extracted as described in Supplementary Methods.

2.2. SNaPshot assay and Sanger sequencing

Hotspot mutations in the *TERT* promotor, *PIK3CA*, *FGFR3* and *RAS* genes were assayed by SNaPshot analysis [19] in tumour DNAs. Sanger sequencing was used to identify TP53 mutations. The patient-matched buffy coat DNAs were used as control for somatic mutations. Further details of SNaPshot assay and Sanger sequencing are provided in Supplementary Methods.

2.3. Whole Exome Sequencing (WES)

WES and the subsequent bioinformatic analysis was performed by Glasgow Polyomics Facility, University of Glasgow. Whole exome capture was carried out using the SeqCap EZ Exome+UTR kit (Roche, Pleasanton, CA, USA), sequenced using the NextSeq 500 platform (Illumina, San Diego, CA, USA) and 2x 75 bp reads were generated. The sequence data was aligned to the GRCh37 (hg19) genome using BWA (Version 0.7.10-r789) and variants were called using the Genome Analysis Toolkit (GATK, Broad Institute, Cambridge, USA). Tumour-specific mutations were identified by removing germ-line variants identified in PBMCs. Variant annotation and effect prediction was completed using SnpEff [20].

2.4. The ddPCR

A typical ddPCR experiment consisted of a control amplification without the addition of template DNA, negative (tumour DNA that does not carry the mutation) and positive (tumour DNA that carries the mutation) controls, along with the assays performed with plasma and urine cfDNA. DNA input of liquid biopsy was 0.5-8 ng per reaction. BioRad C1000 Touch thermos cycler was used for PCR in droplets generated, which were analysed by the QX200 droplet digital PCR system and QuantaSoft (BioRad, Watford, UK). FA of the mutant allele was calculated as the number of droplets positive with mutant amplicon, divided by total

droplets positive with amplicons. The assays were repeated in at least three independent experiments. The CNTNAP4 assay was carried out in duplicate or triplicate in a single assay. Further details of ddPCR assays are described in Supplementary Methods.

3. Results

3.1. Patient characteristics

A cohort of 20 patients (2 NMIBC and 18 MIBC) was available (Table 1). Eight patients had recurrent disease, 5 of whom had a single recurrence, while others had 3 or more. Of 11 deceased patients, 8 (73%) had metastasis. Metastasis was identified in 11% (1 out of 9) of patients who are alive. Cystectomy with neoadjuvant chemotherapy (NAC), radiotherapy with or without chemotherapy were the most common treatment types (70% combined) (Table 2).

Table 1: Clinicopathological characteristics of patients in this study

	All patients (n = 20)	
	Number	%
Sex		
Male	17	85.0
Female	3	15.0
Age (Years)		
Range	43-83	
Median	67	
Mean	68	
T Stage		
pT1	2	10.0
pT2	18	90.0
Grade		
2	1	5.0
3	19	95.0
Recurrence/Outcome		
Clinical Recurrence	8	40.0
No Recurrence	12	60.0
Current Status		
Alive	9	45.0
Deceased	11	55.0
Presence of Metastasis		
No metastasis	11	55.0
Metastasis present	9	45.0
Survival Time, mean (months)		
No metastasis	53.8	
Metastasis present	31.5	
Treatments		
Cystectomy only	2	10.0
Cystectomy and Neo. Adj. Chemotherapy	5	25.0
Cystectomy and Radiotherapy	1	5.0
Radiotherapy only	6	30.0
Radiotherapy and Chemotherapy	3	15.0
Chemotherapy only	2	10.0
Nephroureterectomy	1	5.0
Presence of mutations identified in each patient		
Two mutations	3	15.0
Single mutation	11	55.0
No mutation	6	30.0
Mutations identified by SNaPshot		
<i>PIK3CA</i> E542K	1	5.0
<i>PIK3CA</i> E545K	2	10.0
<i>TERT</i> -124	11	55.0
<i>FGFR3</i> K650/652M	1	5.0
Mutations identified by Sanger sequencing		
<i>TP53</i> Y163C	1	5.0
<i>TP53</i> P278T	1	5.0

Table 2. Details of patients and tumour samples

Patient ID	Sex	Age	Tumour Stage/Grade	Recurrence	Metastasis	Survival* (Months)	Treatment 1	Treatment 2	Mutations identified in tumour by SNaPshot/Sanger sequencing
1	M	57	pT1bG2	1	0	62 (Alive)	NAC	Cystectomy	<i>TERT</i> -124, <i>FGFR3</i> K650/652M
2	M	62	pT1cG3	1	0	56 (Alive)	Nephroureterectomy		
3	M	77	pT2G3	0	0	60 (Alive)	NAC	Cystectomy	<i>TERT</i> -124, <i>PIK3CA</i> E542K
4	M	67	pT2G3	0	0	49 (Alive)	Radiotherapy	Chemotherapy	<i>TERT</i> -124
5	M	81	pT2G3	0	0	50 (Alive)	NAC	Cystectomy	<i>TERT</i> -124
6	M	67	pT2G3	0	0	57 (Alive)	Radiotherapy	Chemotherapy	
7	F	83	pT2G3	0	0	70 (Alive)	Radiotherapy		<i>TERT</i> -124
8	M	65	pT2G3	0	0	64 (Alive)	Radiotherapy		<i>TP53</i> P278T
9	M	66	pT2G3	0	0	47	Chemotherapy		<i>TP53</i> Y163C
10	M	64	pT2G3	1	0	46	Radiotherapy		<i>TERT</i> -124
11	M	68	pT2G3	3	1	57 (Alive)	Chemotherapy and Radiotherapy	Cystectomy	<i>TERT</i> -124
12	M	79	pT2G3	3	0	27	Radiotherapy		
13	M	63	pT2G3	0	1	29	Radiotherapy		<i>PIK3CA</i> E545K
14	M	69	pT2G3	0	1	18	NAC	Cystectomy	<i>TERT</i> -124
15	M	70	pT2G3	0	1	28	Cystectomy		<i>TERT</i> -124
16	M	61	pT2G3	0	1	32	Chemotherapy		
17	F	43	pT2G3	1	1	11	NAC	Cystectomy	<i>TERT</i> -124, <i>PIK3CA</i> E545K
18	M	77	pT2G3	1	1	28	Radiotherapy	Chemotherapy	<i>TERT</i> -124
19	F	82	pT2aG3	0	1	21	Cystectomy		
20	M	73	pT2aG3	4	1	58	Radiotherapy		

*as of 1/1/2018

NAC, neoadjuvant chemotherapy

3.2. SNaPshot and Sanger sequencing identified assayable mutations in 70% of samples

PIK3CA E542K, *PI3KCA* E545K and *TERT* promoter (-124) mutations were identified by SNaPshot assays in 5%, 10% and 55% of patients, respectively (Table 1, Table 2). *TP53* mutations (P278T and Y163C) were identified by Sanger sequencing in 10% of the patients. No *FGFR3* S249C or RAS gene mutations were detected. Overall, no mutations were identified in 30% of the cases.

3.3. ddPCR analysis of a *PIK3CA* E542K mutation in plasma cfDNA from a case in remission

Patient 3 was a 77-year-old male, diagnosed with non-metastatic muscle invasive disease, with a pT2 G3 tumour that carried a *PIK3CA* E542K mutation as identified by SNaPshot (Table 2). The presence of the *PIK3CA* E542K mutation was evaluated by ddPCR using a commercially available, validated assay, with a limit of detection (LOD) of 0.1%. A LOD of the mutant allele defines the lowest fraction of mutant DNA that an assay can reliably detect. The *PIK3CA* E542K mutation was confirmed in the patient's tumour DNA with an FA of 35.7 % (Fig. 1C, Supplementary Results). No mutant signals were observed in the absence of template DNA, or in the tumour DNA from Patient 14, that was wildtype for the *PIK3CA* E542K mutation by SNaPshot (Fig. 1A, B). The mutant allele was still detected in the plasma of Patient 3 (FA of 4.2%) above the LOD of 0.1% at 1 week after the start of NAC (Fig. 1D). The patient subsequently had cystectomy at 3.5 months after NAC. The mutant allele was not detected at 45 months after cystectomy (48 months after the start of NAC) in plasma or urine (Fig. 1E, F). A minimum of 3 independent experiments were performed and the results were replicated (Fig. 1G). The patient is currently in remission after 5 years and believed to be disease free. Therefore, ddPCR-based monitoring of the *PIK3CA* E542K mutation concur with clinical observations.

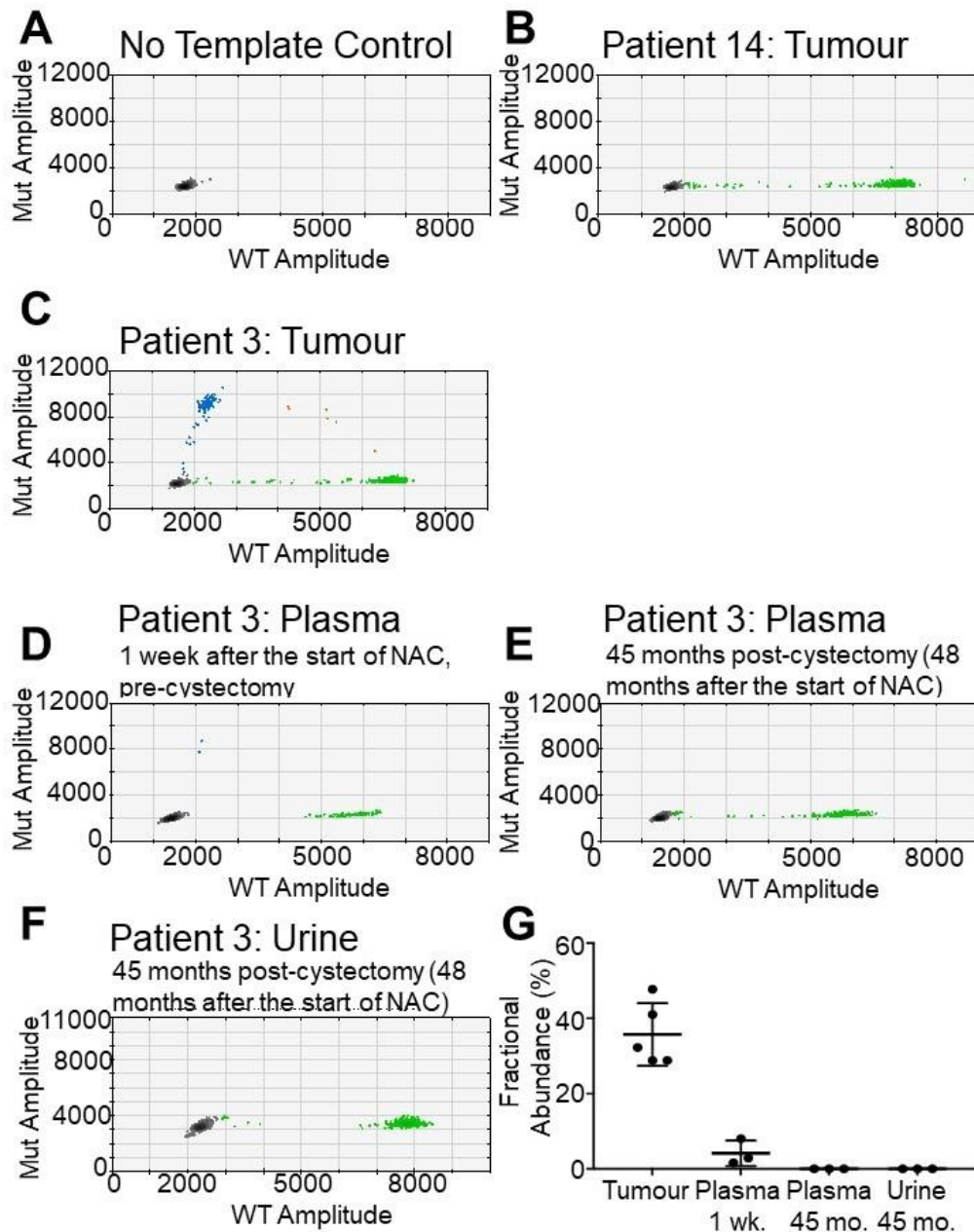


Fig. 1: ddPCR-based detection of a *PIK3CA* E542K mutation in plasma and urine ctDNA from a case of remission. The plots (A-F) indicate the digital PCR droplets positive with wild type (green), mutant (blue), both mutant and wild type (red) amplicons, and those with no amplicons (black). The number of positive droplets in the plots depends on the total DNA input, therefore fractional abundance (FA) is to be used for comparisons among samples. The assays were performed along with negative controls in which no template DNA was added (A) and tumour DNA known to be negative for this mutation (Patient 14) (B). The *PIK3CA* E542K assays were performed to monitor the disease status of Patient 3, using DNAs extracted from tumour (C), plasma collected at 1 week after the start of NAC, before cystectomy (D), plasma (E) and urine (F) collected at 45 months after cystectomy (48 months from the start of NAC). (G) Summary of FA of the mutant allele within total droplets positive with amplicons is shown. Error bars are mean with standard deviation based on 5 independent experiments for tumour DNA and 3 independent experiments for plasma and urine.

3.4. ddPCR analysis of a TP53 Y163C mutation in plasma and urine cftDNA from a relapsed case

Patient 9 was a 66-year-old male with a pT2 G3 tumour that was identified with a *TP53* Y163C mutation by Sanger sequencing (Table 2). The *TP53* Y163C mutation was confirmed in the patient's tumour DNA by a commercially available, validated ddPCR assay (Fig. 2A). No mutant alleles were detected by ddPCR in plasma collected at 3 weeks into cisplatin gemcitabine chemotherapy (Fig. 2B). However, mutant alleles were detected in plasma and urine collected at 37 months after the initiation of chemotherapy (FA of 0.80 and 0.43%, respectively) above the LOD of 0.1% (Fig. 2C-E). Clinically, the patient had a very good response to chemotherapy but relapsed and has died from UC at 45 months from the initiation of chemotherapy. Therefore, the results of the ddPCR assay matches the clinical observations.

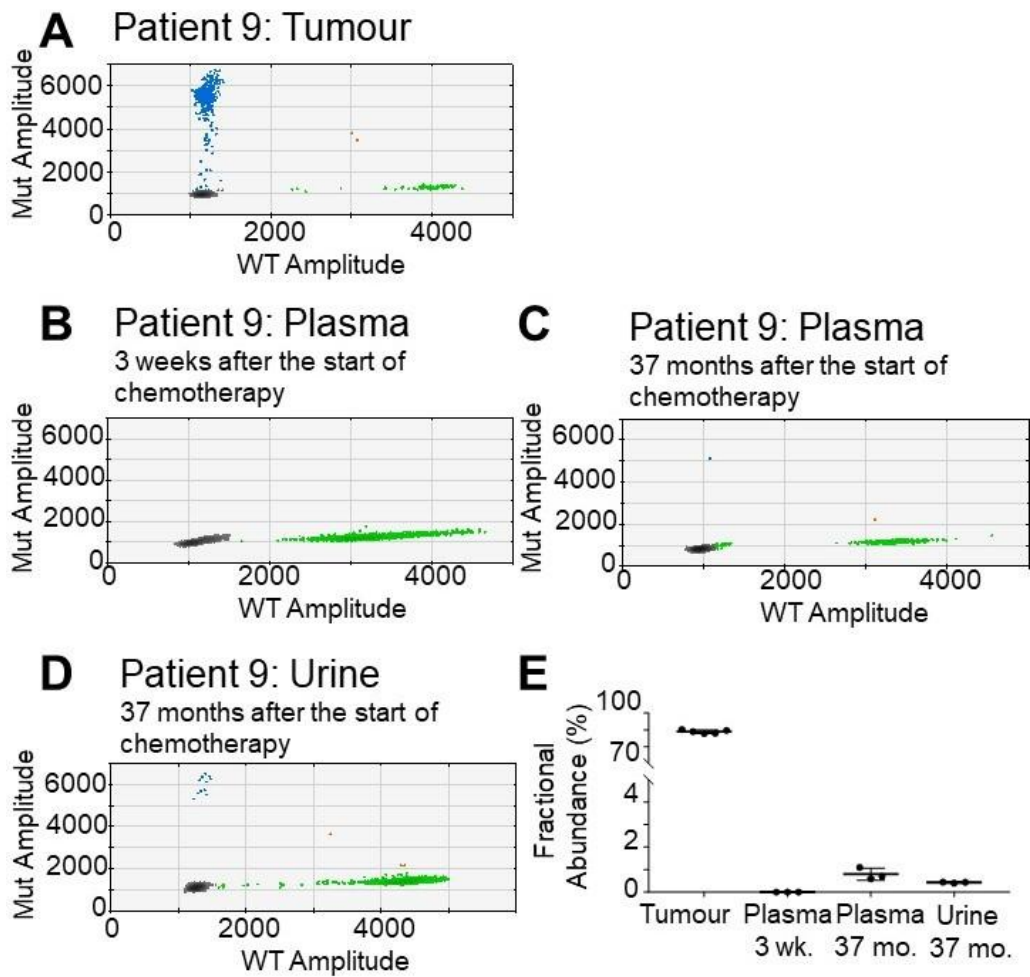


Fig. 2: ddPCR-based detection of a *TP53* Y163C mutation in plasma and urine cell-free tumour DNA from a case of relapse. The *TP53* Y163C ddPCR assays were performed to monitor the disease status of Patient 9, using DNAs extracted from tumour (A), plasma collected at 3 weeks (B), and plasma (C) and urine (D) collected at 37 months after the initiation of chemotherapy. (E) Fractional abundance of the mutant allele. Error bars are the mean and standard deviation based on 3 independent experiments.

3.5. Application of the new *TERT* -124 ddPCR assay to the monitoring of plasma from cases of patients in remission

The *TERT* -124 mutation was identified in 55% of our patients (n=11/20) by SNaPshot assay (Table 1). In order to take advantage of this common mutation, a ddPCR assay was designed with a new set of probes and previously reported PCR amplification primers [21, 22] (Supplementary Results, Fig S1, Fig S2). The new assay was able to detect the presence of the *TERT* -124 mutation correctly with the LOD of 0.5% (Fig S1). The LOD of SNaPshot is reported to be between 5-10% [19]. Our results showed that the new ddPCR assay could detect the mutant allele in cases below the detection limit of SNaPshot (Fig S2).

A *TERT* -124 mutation had been identified in the tumour of Patient 3 by SNaPshot (Table 2), as well as the *PIK3CA* E542K mutation (Fig. 1). The new ddPCR assay showed mutant droplets in the patient's tumour DNA (Fig. 3A) with the FA of 74.1% (Fig. 3D). *TERT* -124 mutant alleles were detected in the plasma collected at 1 week from the start of NAC (Fig. 3B) with a FA of 36.0% (Fig. 3D). In contrast, the mutant droplets were not detected in the plasma at 45 months post-cystectomy (48 months after the start of NAC) (Fig. 3C, D). These results are in accordance with our observations for the *PIK3CA* E542K mutation in Patient 3 (Fig. 1).

Furthermore, a *TERT* -124 mutation was identified by SNaPshot in the initial pT2 G3 tumour of Patient 11 (Table 2). The patient was a 68-year-old male who had metastatic disease (para-aortic lymph nodes). The patient was treated with palliative chemotherapy, which led to a complete response to treatment and went on to a consolidative radiotherapy to his metastatic lymph nodes and his bladder tumour. The patient developed non-muscle invasive disease in his bladder and had undergone cystectomy at 38 months from the start of chemotherapy. It is unclear whether the tumour is a second primary "recurrent" urothelial tumour or regrowth of a residual tumour. Patient's initial tumour DNA was shown to be positive for the mutant alleles

by ddPCR (Fig. 3E) with a FA of 42.1% (Fig. 3I). Some mutant droplets were observed in the plasma collected at 1 month after the start of chemotherapy prior to cystectomy (Fig. 3F). The FA value (11.3%) was above the LOD for the assay (Fig. 3I). The number of mutant signals detected by ddPCR increased in the plasma collected at 2 months and 17 months post-cystectomy (40 months and 57 months from the start of chemotherapy) (Fig. 3G, H), with a FA of 20.0% and 23.0%, respectively (Fig. 3I). Clinically, the patient remained in remission at 16 months from cystectomy (56 months from the chemotherapy). As it is probable that the patient has residual micrometastatic disease, his status is being carefully monitored by the oncologist.

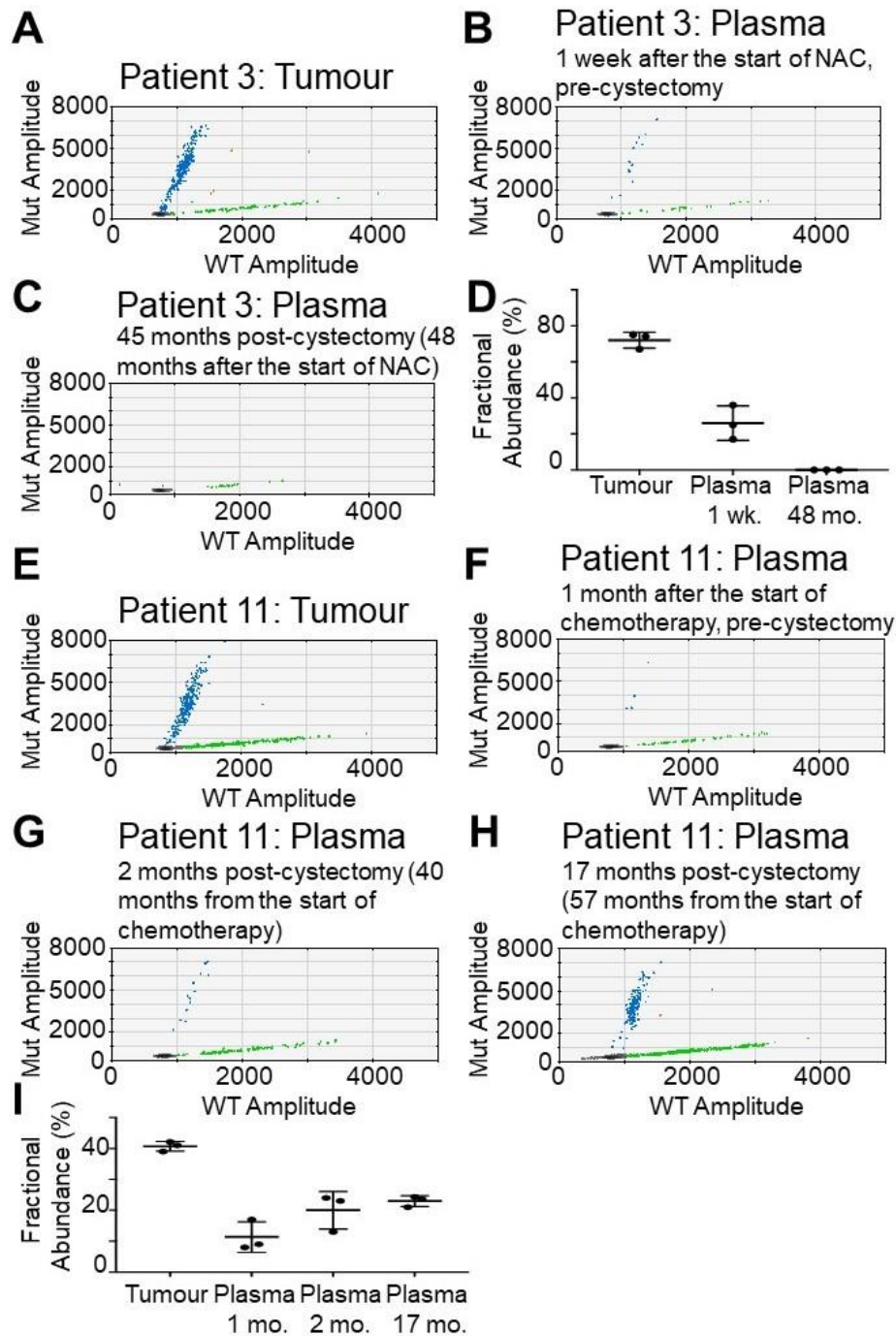


Fig 3: ddPCR-based detection of the *TERT* -124 mutation in plasma ctDNA from cases of remission and relapse. The new *TERT* -124 ddPCR assays were applied to monitor the disease status of Patient 3 (A-D) and Patient 11 (E-I). In Patient 3, the plots show the results of the assay performed using DNAs extracted from tumour (A), plasma at 1 week after the initiation of NAC, before cystectomy, (B) plasma collected at 48 months from the start of NAC (45 months post-cystectomy) (C), and FA of the mutant allele summarized (D). In Patient 11, the plots show the assay results of tumour DNA (E), plasma at 1 month after the initiation of chemotherapy, before cystectomy (F), 2 months post-cystectomy (40 months from the start of chemotherapy) (G), at 17 months post-cystectomy (57 months from the start of chemotherapy) (H), and FA of the mutant allele summarized (I). Error bars are the mean and standard deviation based on 3 independent experiments.

3.6. The ddPCR assay for *CNTNAP4* gene was developed from WES

To identify patient-specific tumour mutations in an unbiased fashion, WES was performed in DNAs extracted from FFPE tumours (n=3, Patient 4, 5, and 6) (Fig. 4, Supplementary Results, Fig S3A). Three adjacent areas within one FFPE block from Patient 5 were also sampled to assess the variability of mutations. We found that adjacent areas 5-1, 5-2 and 5-3, contained 19883, 19858 and 21785 tumour-specific mutations, respectively. This was close to the reported mutation rate [23]. Among these, 142 mutations were identified as common (Fig. 4A). Following a systematic filtering, we selected a *Contactin-Associated Protein-Like 4 (CNTNAP4) G727** mutation for assay development. We confirmed the presence of this mutation in the tumour DNA by Sanger sequencing, as a secondary peak showing a C>A mutation (Fig. 4B). A new ddPCR assay designed was able to distinguish mutant and normal genomic DNAs with the LOD of 1% FA of mutant allele (Supplementary Results, Fig S4).

Patient 5 was an 81-year-old male with a pT2 G3 tumour that carried a *TERT* -124 mutation (Table 2, Fig S2A) in addition to the *CNTNAP4 G727**. Cystectomy followed 3.5 months after NAC. The presence of the *CNTNAP4 G727** mutation was confirmed in the patient's tumour DNA by ddPCR (Fig. 4C) with a FA of 7.31% (Fig. 4G). Mutant droplets were observed in plasma collected 2 months into NAC and at 38 months post-cystectomy, with the FA of 0.25% and 0.4%, respectively, below the LOD (Fig. 4D, E, G). However in the urine collected at 38 months post-cystectomy, the mutant droplets were detected at FA of 1.2% (Fig 4F). *TERT* -124 ddPCR assay was positive in tumour (FA 41.6%) but negative (FA 0%) in plasma at 2 months and urine at 38 months (data not shown). There was no clinical evidence of relapse at this time, and the disease status is being carefully monitored by the oncologist.

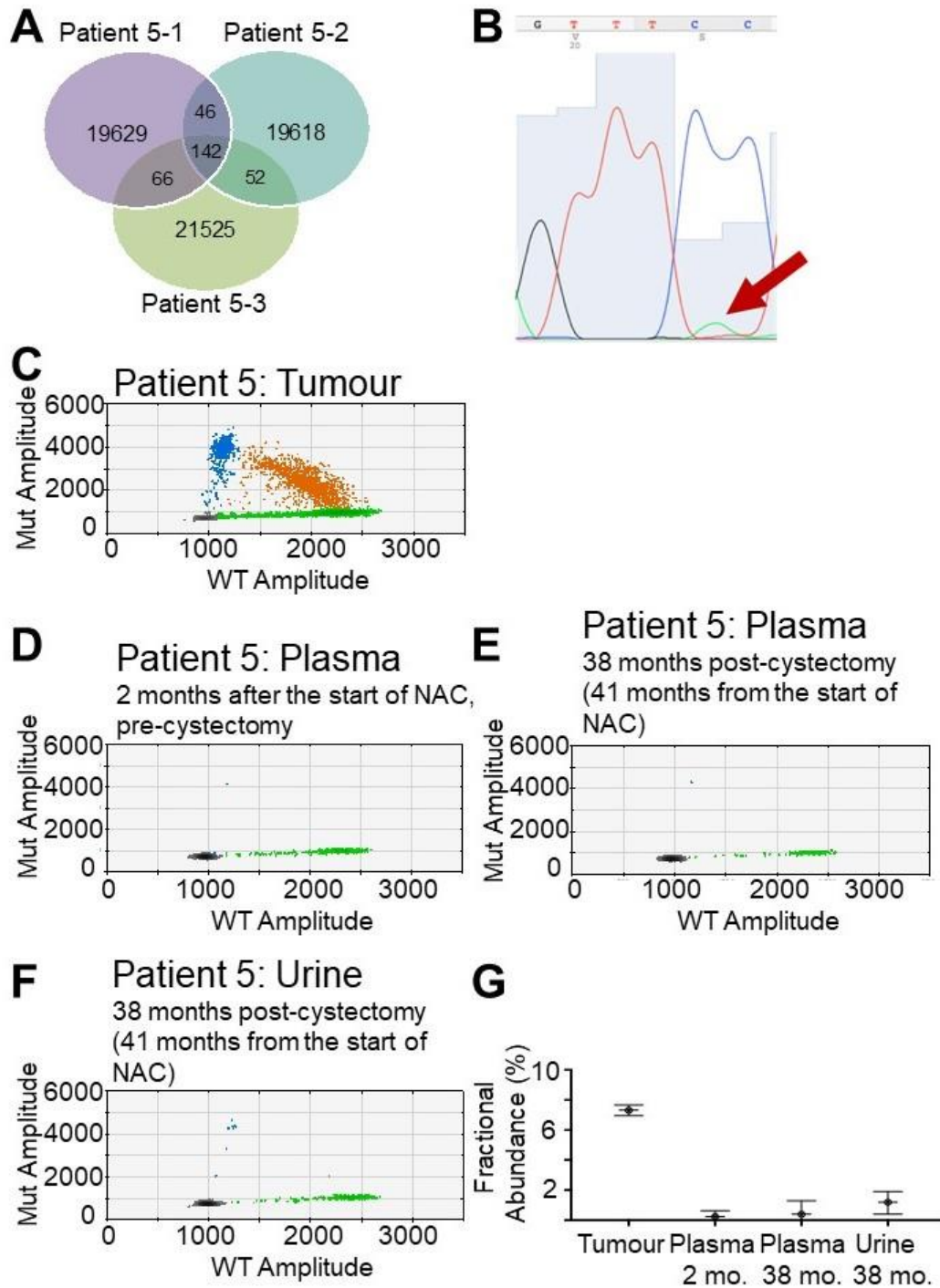


Fig. 4: ddPCR-based detection of a *CNTNAP4* G727* mutation identified by WES in the plasma and urine. (A) Number of tumour-specific mutations identified by WES in three adjacent areas (5-1, 5-2, 5-3) of tumours in Patient 5. 142 mutations were found in common between 5-1, 5-2 and 5-3. (B) Sanger sequencing confirmed the presence of the *CNTNAP4* G727* mutation in tumour area 5-1 (arrow). The plots show the results of the new *CNTNAP4* G727* ddPCR assay performed on DNAs extracted from tumour (C), plasma collected at 2 months after the start of NAC before cystectomy (D), and plasma (E) and urine (F) collected at 38 months from cystectomy (41 months from the start of NAC). (G) Summary of FA of the mutant allele. Error bars are 95% confidence intervals calculated by the Poisson distribution based on the number of positive droplets from duplicates in a single experiment.

4. Discussion

While a good range of ddPCR assays are commercially available for the detection of hotspot mutations in *PIK3CA* and *FGFR3*, a limited range was available for *TP53* mutations, likely due to these mutations being distributed across the gene [24, 25]. Some commercial ddPCR assays are validated *in silico*, however, this did not guarantee that the assays would work. We were not able to design a functional *FGFR3* K650/652M ddPCR assay due to persistent false-positive signals.

Inhibition of PI3K was shown to suppress bladder tumours particularly effectively in the presence of *PIK3CA* hotspot mutations, as evaluated in human urothelial cell lines and in xenograft mouse models [26]. Various inhibitors for PI3K/AKT/mTOR signalling pathways are currently in clinical trials for advanced solid tumours, including those of the bladder [27, 28], and evaluation of *PIK3CA* mutational status as a biomarker is a rational approach in patient selection, at least in the trial set up.

TERT is a part of telomerase protein complex that plays a role in extending the telomere length [29]. *TERT* promotor at -124 and -146 are mutated frequently in UC across all stages, but not in normal bladder tissues [29-31]. These mutations are predicted to increase the level of *TERT* transcripts, leading to pro-tumour telomerase activity [29]. Several studies have investigated the usefulness of *TERT* promotor mutations as a urinary cell free biomarker, using SNaPshot [21, 22], ddPCR [30] and next generation sequencing with urine sediment DNA [32]. Telomerase inhibitors are in clinical trials based on the likely involvement of telomerase activity in tumour progression [29]. The ddPCR assay in this study produced meaningful results with as little as 1 ng of DNA as starting material, in contrast to SNaPshot that required 5 ng [33]. The LOD provides important guidance to the decision of positivity of the mutant allele in samples. The ddPCR-based assays can have a range of LODs depending on the ddPCR assay platforms and the nature of samples, such as different amount of DNA input. For

example, the LOD for *PIK3CA* E542K ddPCR assay, the same BioRad assay we used in this study, was reported as 0.1% in the study of metastatic biliary cancers using serum as a liquid biopsy [34], while for EGFR T790M and L858R mutations, the LOD was 0.5% using fragmented DNA of 100-200bp representing plasma cfDNA size in the study of non-small cell lung carcinoma [35].

CNTNAP4 (CASPR4) is a member of Neurexin-IV/Caspr/Paranodin (NCP) family of cell adhesion and recognition molecules and is expressed in neuronal subpopulations in specific brain regions [36]. A function as a tumour suppressor was shown in the other family member CNTNAP2 (CASPR2) [37]. According to TCGA database (portal.gdc.cancer.gov) [38], 28 different *CNTNAP4* mutations were identified in 6.80% (28/412) of UC. *CNTNAP4* mutations were also identified at a high frequency in lung, colorectal cancer, and melanoma (68-82%), and at a lower frequency in breast cancer (15%). Mutation at the G727 site, G727V has been identified in colorectal cancer [39], however, G727* mutation has not been reported so far.

Stratification strategies for NAC and surveillance in conjunction with cftDNA analysis have been proposed [14, 16]. Use of cftDNA monitoring in the course of checkpoint immunotherapy is also possible [40, 41]. A novel concept of an integrated monitoring system, such as continuous individualised risk index (CIRI) is based on the advance of cftDNA-based technologies [5]. The cost effectiveness of ddPCR should be carefully evaluated, as repeat cystoscopies are expensive for health services [42] and tests for several mutations in genes such as *FGFR3*, *PI3KCA*, and *TP53* are already available as molecular diagnostic services offered by the National Health Service in the UK. Given that the cfDNA technology has still not been implemented widely in the health service, clinical applicability of ddPCR-based approaches may lie in the feasibility of use of such assays in a clinical setting.

Conflict of Interest

No conflicts of interest were declared.

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Author contributions

JP, CH and CO planned and performed experiments, GH performed WES bioinformatics, SF advised on histopathology. RJ supervised clinical data. JP, CH and TI analysed the results and wrote the manuscript, TI, MAK, HL, and RJ supervised the overall project. All authors reviewed and/or edited the manuscript.

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Supplementary Methods

Preparation of plasma, buffy coat, peripheral blood mononucleocytes (PBMCs), and urine

Nine ml of blood was added to a S-Monovette EDTA tube (Sarstedt, Numbrecht, Germany) and mixed by inverting several times. The samples were centrifuged at 1600g for 10 minutes at 4°C in a swing out head centrifuge. The plasma was placed into cryotubes (Alpha Labs, Eastleigh, UK) in 1-ml aliquots and centrifuged for a further 10 minutes at 13200 rpm at room temperature. The resulting supernatant was stored in cryotubes at -80°C. The buffy coat was collected by aspiration after plasma was removed, and stored in 1-ml cryotubes at -80°C. PBMCs were processed from 6 ml of blood in an EDTA vacutainer (BD, Workingham, UK). Five ml of HISTOPAQUE-1077 (Sigma-Aldrich, Gillingham, UK) was added to a 15-ml tube and layered with 5 ml of blood. Four layers were visible after centrifugation at 600g for 20 minutes. The plasma (top layer) was discarded. PBMC (the second layer above a clear bottom layer) was transferred into a clean 15-ml tube. Ten ml of ice-cold PBS was added, and the sample was centrifuged at 500g for 10 minutes at 4°C. The pellet was resuspended in 1 ml of ice-cold PBS and transferred to a 1.5-ml Eppendorf tube and centrifuged for 5 minutes at 500g at 4°C. The supernatant was discarded and the PBMCs (pellet) were stored at -80°C. Voided urine collected from patients (10 ml) was added with 200 µl of 0.5 M EDTA, pH 8 (Thermo Fisher Scientific, UK #15575020) to achieve a final concentration of 10 mM EDTA. Urine was stored at -80°C until DNA extraction.

DNA Extraction

DNA was extracted using the QIAamp FFPE Tissue Kit (Qiagen, Manchester, UK) and eluted in 70-100 μ l of buffer ATE or molecular-grade water. DNA was extracted from PBMCs using the QIAamp Blood Mini Kit (Qiagen) and eluted in 100 μ l buffer AE. Plasma and urine DNAs were extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and eluted in 40 μ l buffer AVE. DNA was stored at -20°C. DNA was quantified using Qubit High Sensitivity DNA or Broad Range DNA Kits (ThermoFisher Scientific, Loughborough, UK). For WES, genomic DNA samples from patient tumour and matched PBMCs were assessed by Agilent TapeStation (Agilent, Santa Clara, CA USA) and Qubit 3.0 (ThermoFisher, Loughborough, UK).

SNaPshot assay

Ten ng of DNA was mixed with 1X PCR buffer (Promega, Southampton, UK), 1.5 mM MgCl₂, 0.17 mM dNTPs, 0.7 μ M of each primer, 5% glycerol, 1 unit GoTaq DNA polymerase (Promega), in a total volume of 15 μ l. The thermal cycler conditions were an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 45 seconds, 60°C for 45 seconds and 72°C for 45 seconds and a final extension for 10 minutes at 72°C. PCR products were treated with 3 units of shrimp alkaline phosphatase and 2 units of exonuclease I to remove excess dNTPs and primers. SNaPshot assay was carried out using an Applied Biosystems SNaPshot Multiplex Kit (ThermoFisher Scientific). The reaction consisted of 2.5 μ l of SNaPshot Ready Multiplex Mix, 1X BigDye sequencing buffer, 1 μ l of probe mix and 1 μ l of PCR product in a total volume 9 μ l. The extension reactions were performed in a thermal

cycler and consisted of 35 cycles of denaturation at 95°C for 10 seconds and annealing at 58.5°C for 40 seconds. Products were treated with 1 unit of shrimp alkaline phosphatase and diluted 1 in 10. The diluted product (1 µl) was mixed with 9.8 µl HiDi formamide (ThermoFisher Scientific) and 0.2 µl LIZ-standard (ThermoFisher Scientific). Analysis was carried out on ABI3100 (ThermoFisher Scientific).

Sanger sequencing

PCR was used to amplify regions covering exons 5 and exon 8 [1] and exon 7 [2] of *TP53* with following cycle: 95°C for 5 minutes; 30 cycles of 93°C for 45 seconds, 57°C (exon 5), 59°C (exon 7) or 55°C (exon 8) for 45 seconds and 72°C for 1 minute; and 72°C for 5 minutes. PCR products were purified using QIAquick PCR purification kit (Qiagen, Manchester, UK).

PIK3CA E542K and TP53 Y163C ddPCR assays

The assays used were; *PIK3CA* E542K, #dHsaCP2000073 FAM Mutant, #dHsaCP2000074 HEX Wild Type, and *TP53* Y163C, #dHsaCP2000099 FAM Mut, #dHsaCP2000100 HEX WT (BioRad, Watford, UK). Assays were performed in reactions containing 2 µl of DNA, 12.5 µl SuperMix no dUTP (BioRad), 1.25 µl FAM Primers and 1.25 µl of HEX Primers and 1µl of Alu1 (NEB, Hitchin, UK) or Tru1 (ThermoFisher Scientific). PCR was performed as follows; 95°C for 10 minutes then 40 cycles of 94°C for 30 seconds and 55°C for 1min followed by 98°C for 10 minutes, and a final hold at 4°C. The LOD of *PIK3CA E542K and TP53 Y163C ddPCR assays* is 0.1% (PrimePCR ddPCR Mutation Assay

Validation Data, BioRad). The criteria for positive calling was >3 droplets, as suggested by BioRad, or >1 droplet if the result was replicable in more than 3 independent experiments.

Design of new ddPCR assays

The ddPCR assays (PCR primers and probes) for *TERT* -124 and *CNTNAP4* G727* were designed using Primer 3Plus [3] with the Human GRCh37 as a reference sequence. The specificity of the sequences was evaluated by PrimerBlast and an *in-silico* PCR run (genome.ucsc.edu/cgi-bin/hgPcr).

TERT -124 ddPCR assay

The *TERT* -124 assay was optimised using a TERT vector DNA. The TERT vector DNA construct (a gift from Dr Alan Bilsland and Professor W. Nicol Keith, University of Glasgow, UK) was generated from pGL3-hTERT [4] using a QuikChange Lightning Site Directed Mutagenesis kit (Agilent) and the primers TERT-124 Forward (5'-ccccgcccagcccctccgggccctccag-3') and TERT-124 Reverse (5'-ctgggagggcccgaaggggctgggcccggg-3'). The complete 574bp promoter region was sequenced. The promoter was excised from a clone found to be positive for the mutation using XhoI/HindIII digest and re-inserted in the pGL3 (Promega) plasmid backbone. The *TERT* -124 ddPCR assay was carried out using a forward primer GTCCTGCCCCTTCACCTT, reverse primer CAGCGCTGCCTGAAACTC [5, 6], mutant probe CAGCCCCTTCCGGGCCCT and wild type probe CAGCCCCCTCCGGGCCCT. The PCR was as follows; 95°C for 10 minutes then 50 cycles of 94°C for 30 seconds and 60°C for 1min followed by 98°C for 10 minutes, and a final hold of 4°C.

CNTNAP4 G727 ddPCR assay*

The assay was carried out using a forward primer CCTCTGAGTTGGTGGGTAGG, reverse primer CACCATTCATTCCGGTCAG, mutant probe CTTGTGGATTAGAGTGAAACTGCATTG and wild type probe TGTGGATTAGAGGGAAACTGCATT. The assay was optimised using oligo DNA synthesised by IDT, Leuven, Belgium, and Human-Random-Control-DNA-5 (HRC-5) (Public Health England, Salisbury, UK). The PCR was as follows; 95°C for 10 minutes then 50 cycles of 94°C for 30 seconds and 61°C for 1min followed by 98°C for 10 minutes and a final hold of 4°C.

Supplementary Results

ddPCR analysis of a PIK3CA E542K, TP53 P278T, and FGFR3 K650/652M, in additional patients

The *PIK3CA* E545K ddPCR assay also successfully detected mutations in tumour DNA samples from Patients 13 and 17, and correctly called Patient 19 negative for the mutation (data not shown). No plasma samples were available for these patients for monitoring.

A *TP53* mutation in exon 8 (P278T) was identified in Patient 8, however a ddPCR assay was not commercially available. Patient 1 was identified with a *FGFR3* K650/652M mutation (Table 2). While an *in silico* designed ddPCR assay was commercially available, we were not able to eliminate the false positive signal. Several in-house assays were designed, however the issue of false positive signal persisted.

A new TERT -124 ddPCR assay can detect TERT -124 mutations in cases below the SNaPshot assay detection limit

Using a new set of probes and previously reported PCR amplification primers [5, 6], a new ddPCR assay for *TERT* promotor -124 mutation was designed and tested. An optimal annealing temperature was identified as 60°C with 50 cycles for the PCR (Fig. S1A). An optimal ratio of the wildtype to mutant probes of 50:50 was established (Fig. S1B). The primer to probe ratio was optimal at 1.8:1 (Fig. S1C). We determined the sensitivity of the assay by spiking mutant vector DNA into wild type vector DNA. Plotting the observed fractional abundance (FA) of the mutant allele against the expected FA, presented as a solid line (Fig. S1D), we have examined the linear relationship between observed and expected mutant FA. We observed that the lower points of observed FA were away from, and not in alignment with, the linear decline of the expected FA. Based on this, we determined the limit of detection (LOD) of this assay as 0.5%.

Next, we performed the ddPCR assay using 9 samples in which the SNaPshot assay had previously indicated the presence of a *TERT* -124 mutation. Using the tumour DNA that ranged between 1 ng to 20 ng as a template, the production of mutant droplets was clearly observed (Fig. S2B and data not shown). The FA of the mutant allele of the 9 tumours ranged from 27% to 67% (Fig. S2A).

A further 9 patients were identified as negative for the *TERT* -124 mutation by SNaPshot analysis of their tumour DNA (Table 1, Table 2). Using the ddPCR assay, no mutant droplets were observed in 5 out of 9 patients (Patients 6, 9, 19, 20 and 21) in accordance with SNaPshot (Fig. S2C, D and data not shown). In contrast, 4 out of 9 (Patients 2, 8, 12 and 13) produced mutant droplets (Fig. S2C and data not shown). The FA of the

mutant allele in the tumour of Patient 2 was 7%, and therefore above the LOD of the ddPCR assay (Fig. S2E). The FA was below the LOD in the other 3 cases (Patient 8, 12, 13).

Taken together, the above results showed that the new *TERT* -124 ddPCR assay was able to detect the presence of the *TERT* -124 mutation correctly.

The ddPCR assay for CNTNAP4 G727 mutation*

WES performed in DNAs extracted from FFPE tumours (n=3) were compared with the results from the corresponding patient's PBMCs for the presence of germ-line mutations (Fig S3A). From a total of 19674 (Patient 6) and 39776 (Patient 4) tumour-specific mutations identified, systematic filtering of small nucleotide polymorphisms (SNPs) resulted in 54 and 249 mutations, respectively, that could be used in the development of ddPCR assays to track the presence of cftDNA (Fig. S3B, C). In consideration of regional variations in mutations within the same tumour samples (Fig. 4A), we further filtered the mutations to include those that were in at least 10% of the total number of reads and with a depth of >50.

A ddPCR assay was designed for *CNTNAP4* G727* mutation (Fig. S4). The optimal annealing temperature was established as 61°C with 50 cycles (Fig. S4A). The optimal wild type and mutant probe ratio was identified as 50:50 (Fig. S4B). The primer to probe ratio was optimal at 2:1 (Fig. S4C). The new assay was tested using tumour DNA from Patient 5 and HRC-5 DNA as a negative control. The LOD of the assay was determined as 1% FA of the mutant allele, as the observed values were not aligning with the expected at mutant FA lower than 1% (Fig. S4D).

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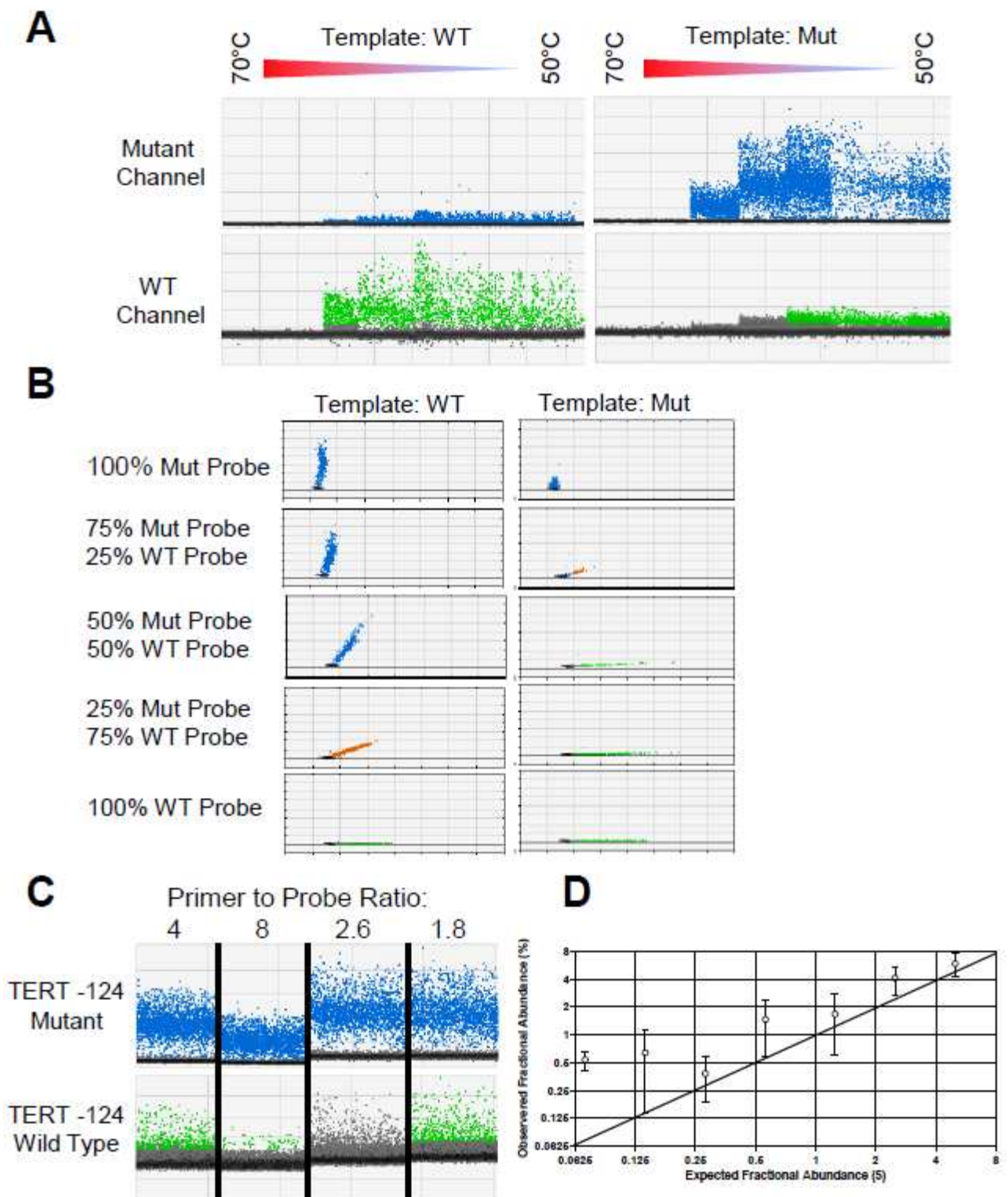


Fig. S1: Optimisation of the new *TERT* -124 ddPCR assay. (A) Identification of optimal annealing temperature across a temperature gradient from 50°C to 70°C. **(B)** Establishment of wildtype (WT) probe to mutant (Mut) probe ratio to achieve the best specificity for each WT and Mut template DNA. **(C)** Identification of the optimal primer to probe ratio. **(D)** Observed and expected fractional abundance of the mutant allele was used to determine the level of detection (LOD).

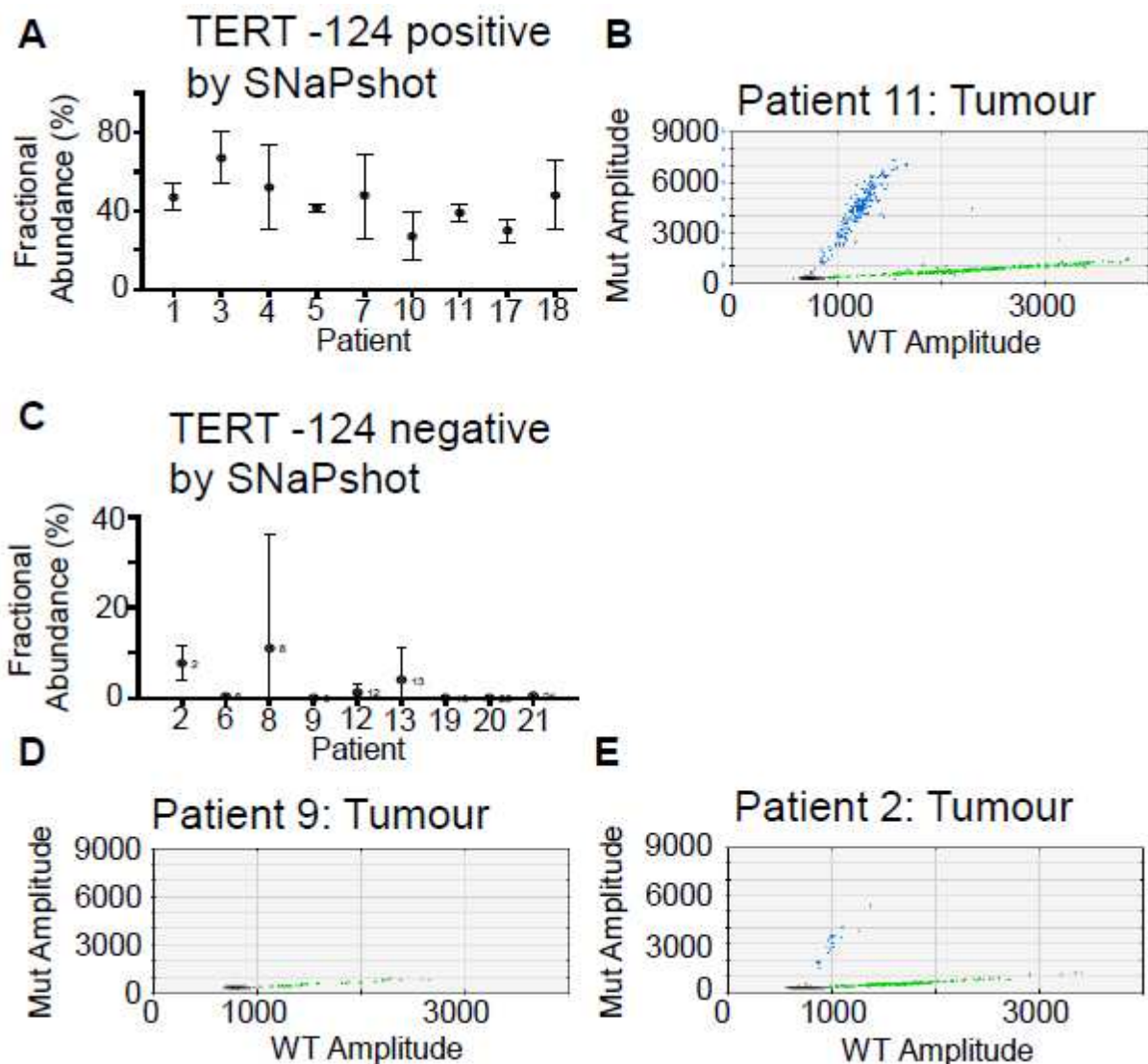


Fig. S2: Detection of the *TERT* promoter -124 mutation by a new ddPCR assay. We have used a new ddPCR assay to investigate the *TERT* -124 mutation status in tumour DNAs that had been previously identified by SNaPshot as positive (**A, B**) or negative (**C-D**). Fractional abundance of the mutant allele (**A, C**). The representative plots of *TERT* -124 -positive (Patient 11), and -negative (Patient 9) cases are shown (**B, D**). Tumour DNA from Patient 2 was identified as positive by the ddPCR assay, while previously identified as negative by SNaPshot (**E**).

A

Patient	Sex	Age	Tumour Stage/Grade	Tumour DNA tissue	Germ Line control
4	Male	67	pT2 G3	FFPE	PBMC
5	Male	81	pT2 G3	FFPE	PBMC
6	Male	67	pT2 G3	FFPE	PBMC

B

Step	Filter
1	Remove Intergenic and IG mutations
2	Remove RNA Mutations
3	Remove synonymous mutations
4	Remove splice variants
5	Remove downstream mutations
6	Remove intron mutations
7	Remove 5' and 3' UTR mutations
8	Remove upstream mutations
9	Remove transcription factor binding site mutations
10	Remove low effect mutations
11	Remove mutations lacking complete data
12	Remove mutations not identified in the COSMIC database
13	Remove all mutations with a read depth <50

C

Filter Stage	Patient-Sample				
	4	5-1	5-2	5-3	6
Raw Tumour	193252	113215	97472	114171	103848
Tumour-Germ line	39776	19883	19858	21785	19674
SNP's	31382	18254	18958	20840	18593
Insertions	7007	1134	518	509	695
Deletions	1386	495	382	436	386
Variant rate (/Mb)	12.9	6.4	6.4	7.0	6.4
Final Filter	249	73	81	54	54

Fig. S3: Whole exome sequencing (WES) of tumours from three patients. (A) Summary details of three patient's tumour samples that have been subjected to WES. (B) The approach used to filter the sequencing results to identify candidate mutations for ddPCR assays. (C) Numbers of mutations identified in tumours from three patients. Three proximate areas of the tumour from Patient 5 (5-1, 5-2, 5-3) were also compared.

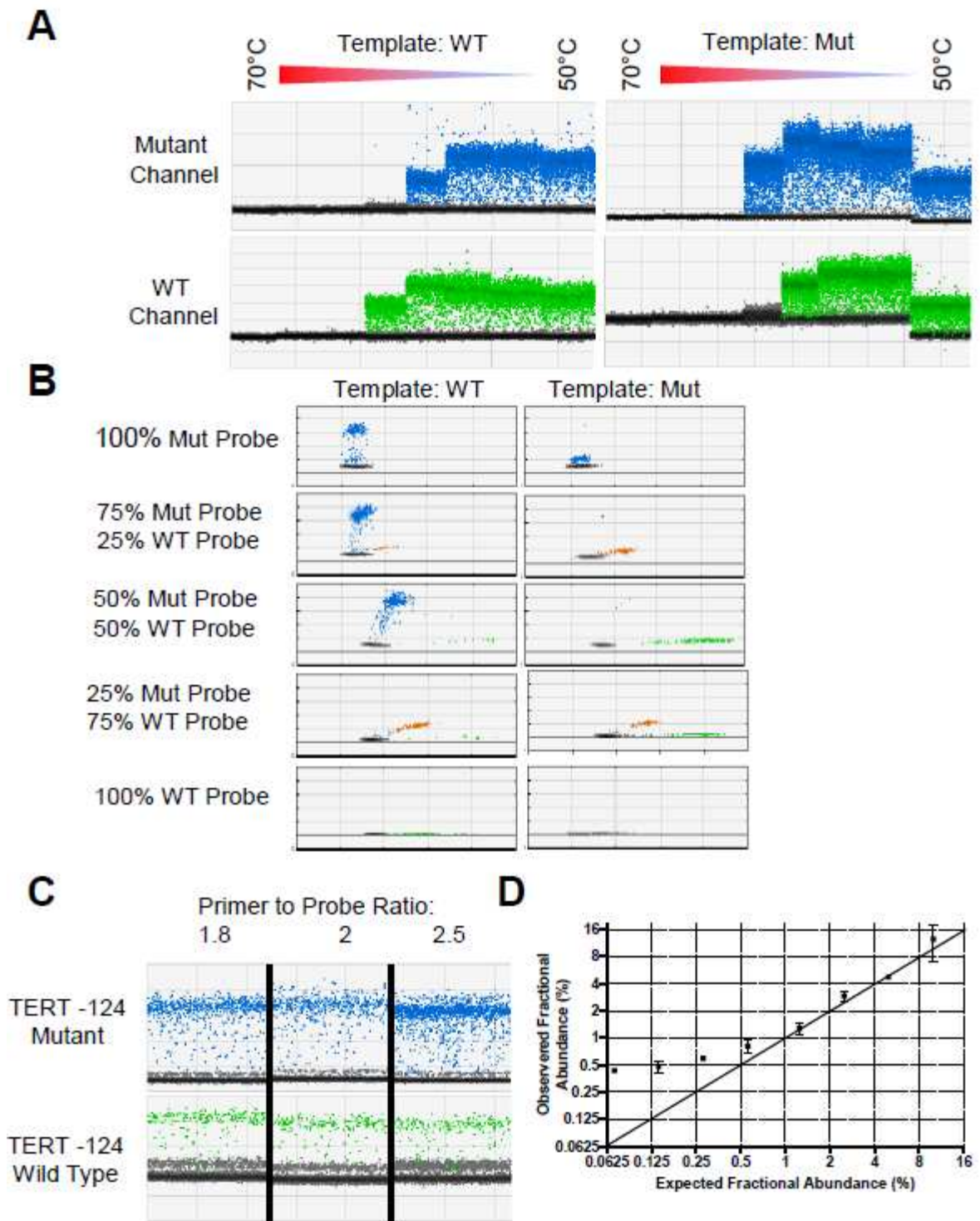


Fig. S4: Characteristics of the developed *CNTNAP4* G727* ddPCR assay. (A) Identification of optimal annealing temperature using a temperature gradient. (B) Establishment of WT probe to Mut probe ratio to achieve the best specificity for each WT and Mut template DNA. (C) Identification of the optimal primer to probe ratio. (D) Observed and expected fractional abundance of the mutant allele was used to determine the LOD.

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Highlights:

- Digital Droplet PCR assays of tumour-specific mutations can be used to monitor cell free tumour DNA levels in plasma and urine in urothelial cancer patients.
- Seventy percent of the patients in our study harboured mutations that were assayable by ddPCR.
- Commercially available ddPCR assays for *PIK3CA* E542K and *TP53* Y163C can be used to assess disease status.
- A new ddPCR assay for *TERT* promoter (-124) enables detection at a lower fractional abundance than SNaPshot.
- A ddPCR assay was developed to detect a novel mutation, *CNTNAP4* G727*.