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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 urothelial cancer. The objective of this study was to evaluate the use of digital droplet PCR (ddPCR)-based assays to monitor urothelial cancer 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 67 months from the start of neoadjuvant chemotherapy. A new ddPCR assay for the 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 urothelial cancer. Overall, 70% of the patients in our study harboured mutations that were assayable by ddPCR.
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Monitoring of urothelial cancer disease status after treatment by digital droplet PCR liquid biopsy assays

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Abstract

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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 67 months from the start of neoadjuvant chemotherapy. A new ddPCR assay for the *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

urothelial cancer. Overall, 70% of the patients in our study harboured mutations that were assayable by ddPCR.

Keywords;

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Word count; 2499 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 this showed that 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, were 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-µm FFPE tissue sections. DNAs from plasma, buffy coat and peripheral blood mononucleocytes (PBMCs) 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. 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, 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		
PIK3CA E542K	1	5.0
PIK3CA E545K	2	10.0
TERT -124	11	55.0
FGFR3 K650/652M	1	5.0
Mutations identified by Sanger sequencing		
TP53 Y163C	1	5.0
TP53 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)	Neoadjuvant Chemotherapy	Cystectomy	*TERT* -124, *FGFR3* K650/652M
2	M	62	pT1cG3	1	0	56 (Alive)	Nephroureterectomy		
3	M	77	pT2G3	0	0	60 (Alive)	Neoadjuvant Chemotherapy	Cystectomy	*TERT* -124, *PIK3CA* E542K
4	M	67	pT2G3	0	0	49 (Alive)	Radiotherapy	Chemotherapy	*TERT* -124
5	M	81	pT2G3	0	0	50 (Alive)	Neoadjuvant Chemotherapy	Cystectomy	*TERT* -124
6	M	67	pT2G3	0	0	57 (Alive)	Radiotherapy	Chemotherapy	
7	F	83	pT2G3	0	0	70 (Alive)	Radiotherapy		*TERT* -124
8	M	65	pT2G3	0	0	64 (Alive)	Radiotherapy		*TP53* P278T
9	M	66	pT2G3	0	0	47	Chemotherapy		*TP53* Y163C
10	M	64	pT2G3	1	0	46	Radiotherapy		*TERT* -124
11	M	68	pT2G3	3	1	57 (Alive)	Cystectomy	Radiotherapy	*TERT* -124
12	M	79	pT2G3	3	0	27	Radiotherapy		
13	M	63	pT2G3	0	1	29	Radiotherapy		*PIK3CA* E545K
14	M	69	pT2G3	0	1	18	Neoadjuvant Chemotherapy	Cystectomy	*TERT* -124
15	M	70	pT2G3	0	1	28	Cystectomy		*TERT* -124
16	M	61	pT2G3	0	1	32	Chemotherapy		
17	F	43	pT2G3	1	1	11	Neoadjuvant Chemotherapy	Cystectomy	*TERT* -124, *PIK3CA* E545K
18	M	77	pT2G3	1	1	28	Radiotherapy	Chemotherapy	*TERT* -124
19	F	82	pT2aG3	0	1	21	Cystectomy		
20	M	73	pT2aG3	4	1	58	Radiotherapy		

*as of 1/1/2018

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

PIK3CA E542K, *PI3KCA* E545K and telomerase reverse transcriptase (*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 cftDNA from a case in remission

Patient 3 was a 77-year-old male 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 confirmed in the patient's tumour DNA by ddPCR using a commercially available, validated assay (Fig. 1C, Supplementary Results). No mutant signals were observed in negative controls, 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 detected in the plasma of Patient 3 at 1 week after the start of neoadjuvant chemotherapy (Fig. 1D), however, it was not detected at 46 months post-cystectomy (Fig. 1E). A minimum of 3 independent experiments were performed and the results were replicated (Fig. 1F). 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.

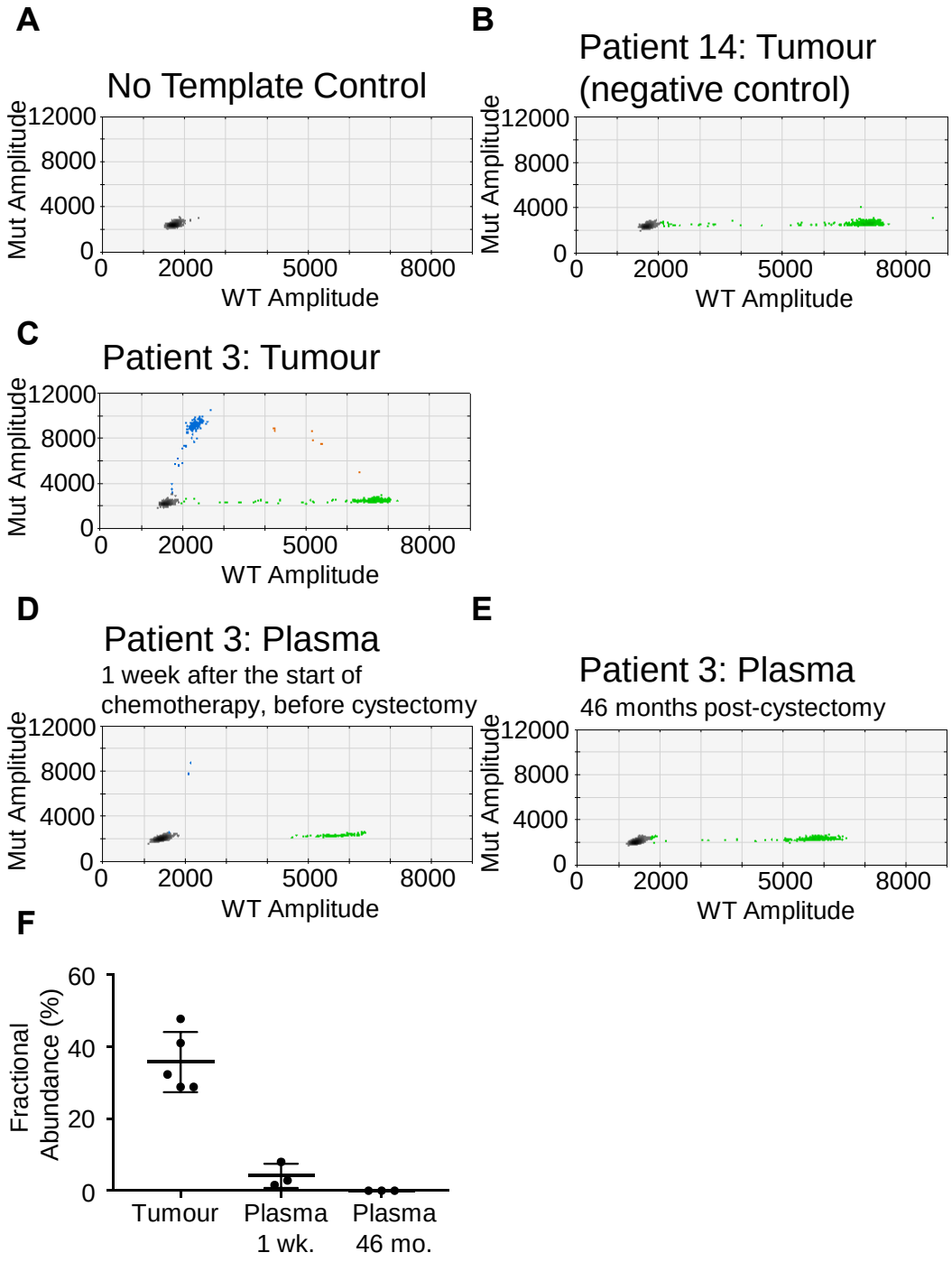

Fig. 1: ddPCR-based detection of a *PIK3CA* E542K mutation in plasma ctDNA from a case of remission. The plots (**A-E**) indicate the digital PCR droplets positive with wild type (green), mutant (blue), both mutant and wild type (red) amplicons, and those with no amplicons (black). The assays were performed 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 neoadjuvant chemotherapy, before cystectomy (**D**), and plasma collected at 46 months after cystectomy (**E**). (**F**) Summary of fractional abundance of the mutant allele within total droplets positive with amplicons is shown.

3.4. ddPCR analysis of a TP53 Y163C mutation in plasma and urine ctDNA 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 31 weeks into chemotherapy (Fig. 2B). However, mutant alleles were detected in plasma, as well as in urine, collected at 67 months after the initiation of chemotherapy (Fig. 2C-E). Clinically, the patient relapsed and has since died from UC.

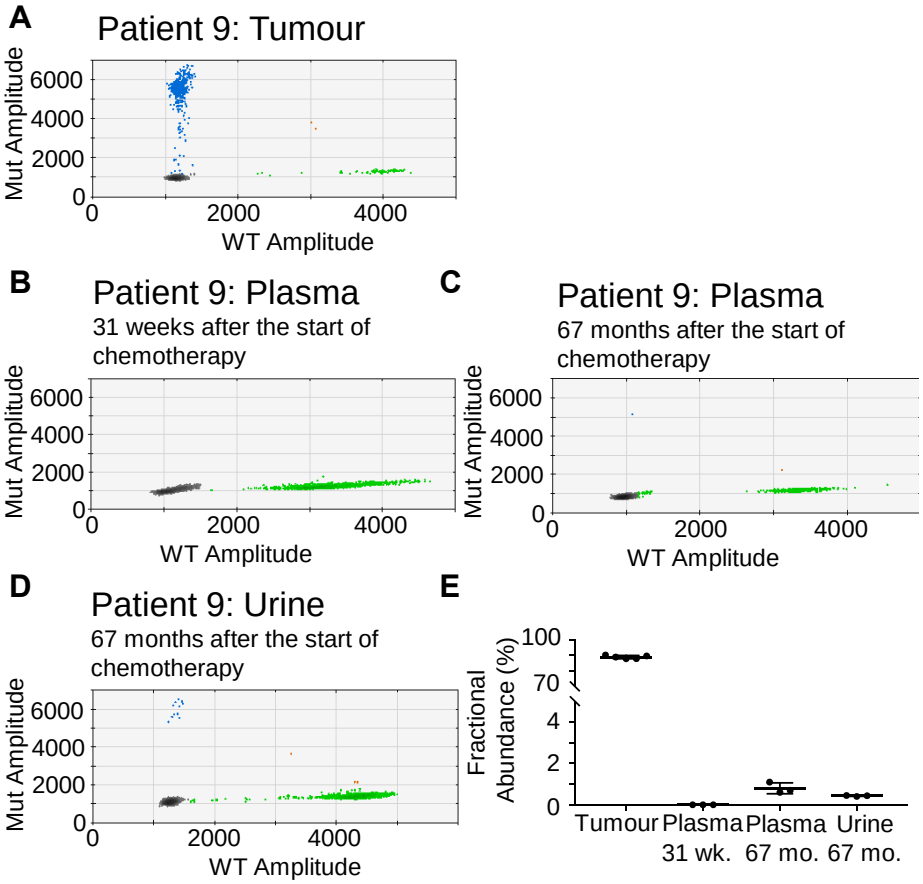

Fig. 2: ddPCR-based detection of a *TP53* Y163C mutation in plasma and urine cell-free tumour DNA from a case of relapse. The *TP53* Y163C ddPCR assays were performed to monitor the disease status of Patient 9, using DNAs extracted from tumour (**A**), plasma collected at 8 months (**B**), and plasma (**C**) and urine (**D**) collected at 67 months after the initiation of chemotherapy. (**E**) Fractional abundance of the mutant allele.

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 level of detection (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 neoadjuvant chemotherapy (Fig. 3B) with a FA of 36.0% (Fig. 3D). In contrast, the mutant droplets were not detected in the plasma at 46-months post-cystectomy (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 in Patient 11, a 68-year-old male with a pT2 G3 tumour, by SNaPshot (Table 2). Patient's tumour DNA was shown to be positive for the mutant alleles by ddPCR (Fig. 3E) with a FA of 42.1% (Fig. 3I). Although 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 (7.0% ± 7.0%) was below 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 (Fig. 3G, H), with a FA of 16.0% and 24.4%, respectively (Fig. 3I). Clinically, the patient remains in remission (Table 2).

As the patient also had a previous relapse with metastasis, his disease status is being carefully monitored by the oncologist.

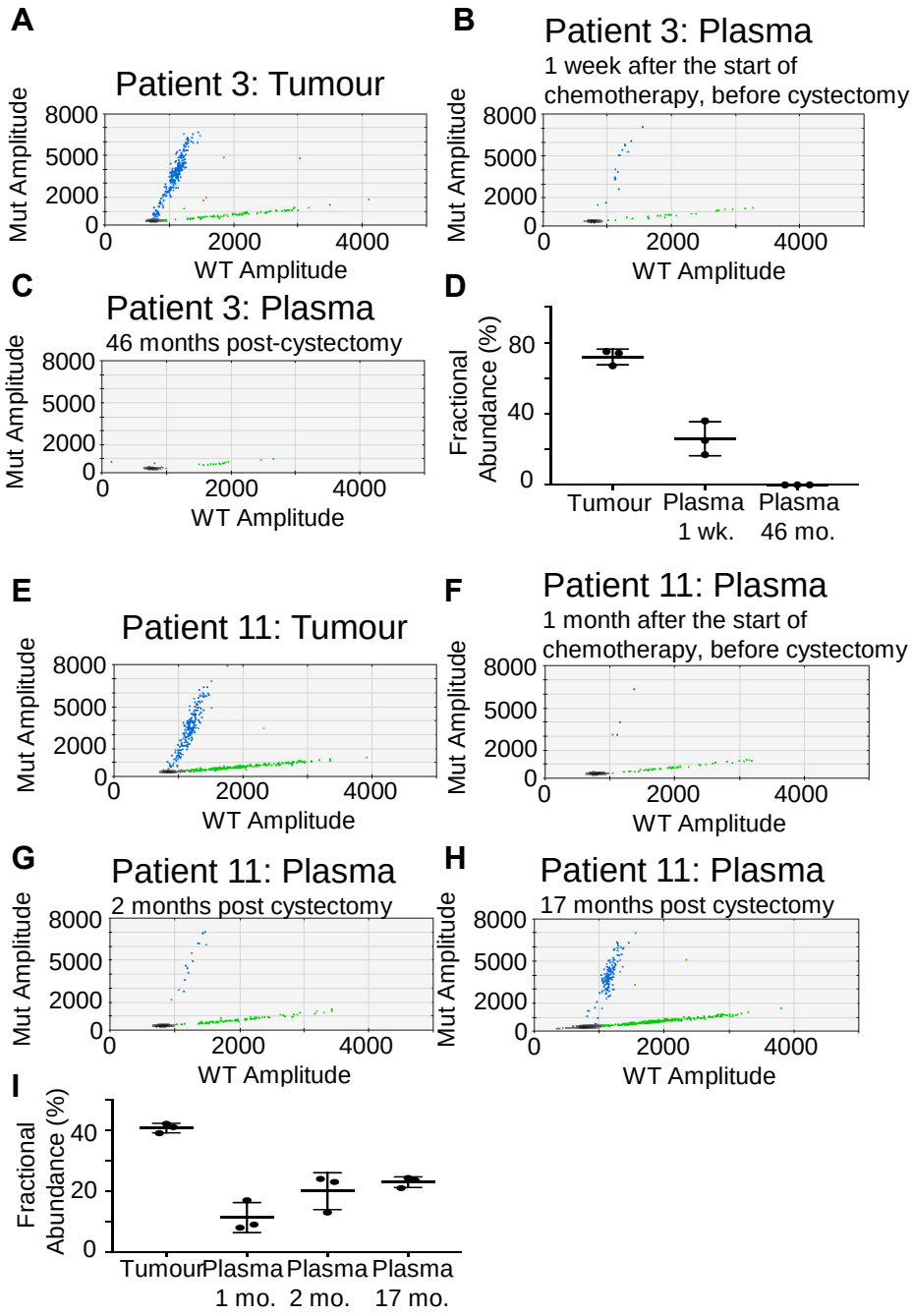

Fig 3: ddPCR-based detection of the *TERT* -124 mutation in plasma ctDNA from cases of remission and relapse. The new *TERT* -124 ddPCR assays were applied to monitor the disease status of Patient 3 (**A-D**) and Patient 11 (**E-I**). The plots show the results of the assay performed using DNAs extracted from tumour (**A, E**), plasma at 1 week after the initiation of neoadjuvant chemotherapy, before cystectomy (**B**), 46 months post-cystectomy (**C**), 1 month after the initiation of neoadjuvant chemotherapy, before cystectomy (**F**), 2 months post-cystectomy (**G**), and at 17 months post-cystectomy (**H**). Fractional abundance of the mutant allele are summarized (**D, I**).

3.6. The ddPCR assay for CNTNAP4 gene developed from WES presented a case of relapse

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, S3) in addition to the CNTNAP4 G727*. 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 neoadjuvant chemotherapy and at 38 months post-cystectomy, with the FA of 0.25% and 0.4%, respectively (Fig. 4D,E,G). However, in the urine collected at 38 months post-cystectomy, the mutant droplets were detected at FA of 4.8%, i.e. above the LOD (Fig. 4F). The result from urine suggested that this patient may be relapsing, although there was no clinical evidence of relapse at this time.

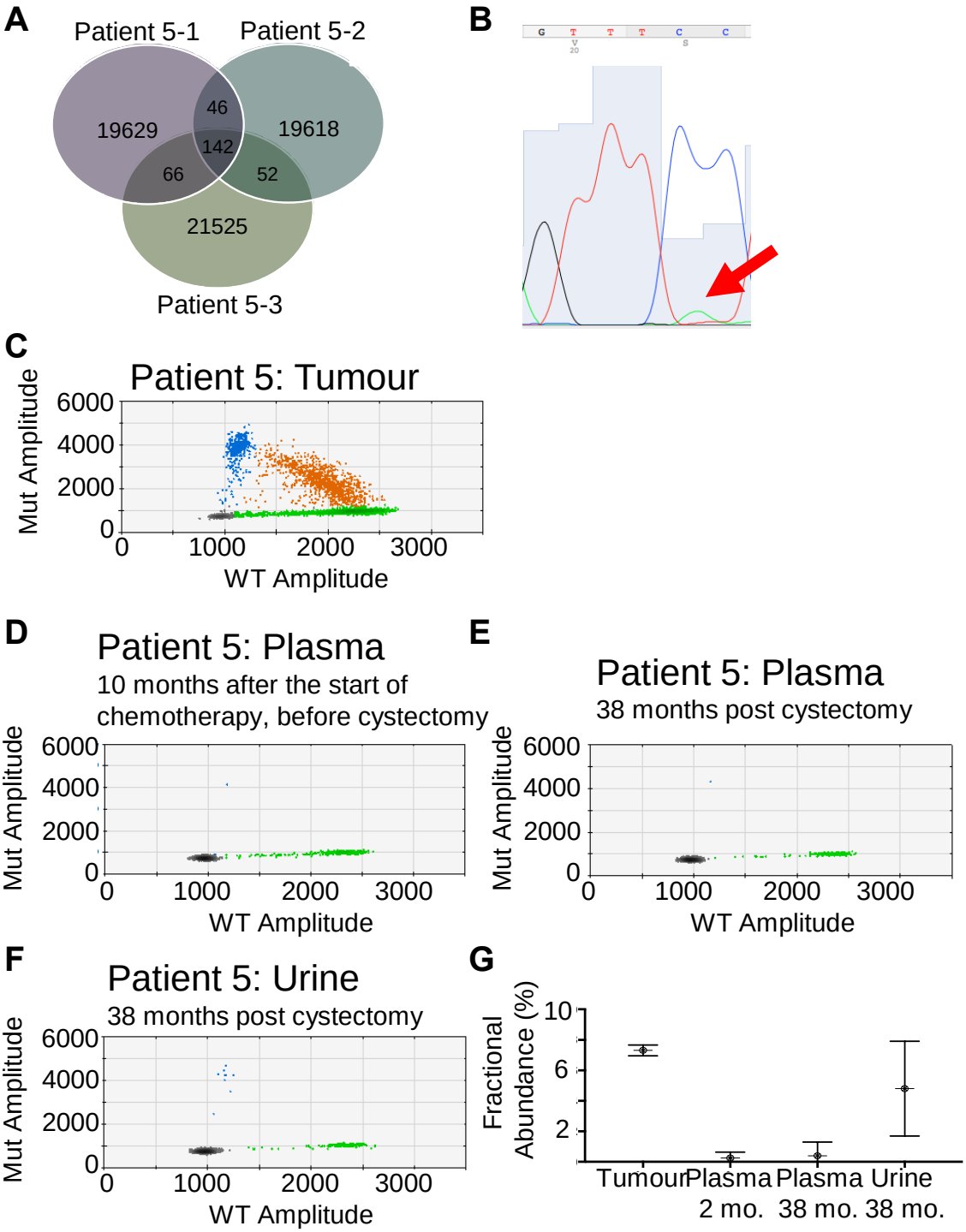

Fig. 4: ddPCR-based detection of a CNTNAP4 G727* mutation identified by WES in the plasma and urine from a case of relapse. (A) Number of tumour-specific mutations identified by WES in three adjacent areas (5-1, 5-2, 5-3) of tumours in Patient 5. 142 mutations were found in common between 5-1, 5-2 and 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 neoadjuvant chemotherapy before cystectomy (**D**), and plasma (**E**) and urine (**F**) collected at 38 months post-cystectomy. (**G**) Summary of fractional abundance of the mutant allele.

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].

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 [34]. A function as a tumour suppressor was shown in the other family member CNTNAP2 (CASPR2) [35]. According to TCGA database (portal.gdc.cancer.gov) [36], 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 [37], however, G727* mutation has not been reported so far.

Stratification strategies for neoadjuvant therapy 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 [38, 39]. 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 [40] 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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Monitoring of urothelial cancer disease status after treatment by digital droplet PCR liquid biopsy assays

John J. G. Pritchard et al

Highlights:

- Digital Droplet PCR assays of tumour-specific mutations can be used to monitor cell free tumour DNA levels in plasma and urine in bladder 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* promotor (-124) enables detection at a lower fractional abundance than SNaPshot.
- A ddPCR assay was developed to detect a novel mutation, *CNTNAP4* G727*, identified by whole exome sequencing.