ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

A new immunomagnetic microfluidic device for characterizing *EGFR* mutations in circulating tumor cells from patients with non-small cell lung cancer

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Abstract

Incorporating precision oncology into cancer management has begun to improve clinical outcomes. Accurate sampling techniques that detect molecular aberrations are crucial for effective implementation. Circulating tumor cells (CTCs), derived from primary or metastatic sites and present in the blood, are proposed as useful diagnostic tools, though their use has been limited due to their rarity, especially in early-stage cancers. This study presents a novel immunomagnetic microfluidic device that efficiently isolates CTCs for analyzing epidermal growth factor receptor (*EGFR*) mutations in patients with non-small cell lung cancer (NSCLC). The device was designed and laser-cut from polymethylmethacrylate. Validation experiments involved spiking PC-9 cells (an established lung cancer cell line containing GLU 746-ALA 750 deletion mutations in exon 19 of the *EGFR* gene) into media and isolating these cells. Exons 18 – 21 of *EGFR* were amplified using a polymerase chain reaction to demonstrate the device's rapid mutation detection capability. Next-generation sequencing was used to characterize these exons in a cohort of 38 NSCLC patients, successfully isolating CTCs from all. Among these patients, 30 (79%) had *EGFR* mutations, with exon 19 showing the highest mutation rate (87%) and exon 21 the highest point mutation rate (23%). Our device captured CTCs effectively in <1 h, enabling mutation detection. Further studies are needed to assess the prognostic significance of these mutations, but this technology has potential applications in various solid tumors.

Keywords: Precision oncology; CTC; NSCLC; EGFR; Microfluidics; Immunomagnetic

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1. Introduction

Presently, the management of patients with non-small cell lung cancer (NSCLC) uses knowledge of a patient's mutational profile to guide the selection of therapy that is most likely to be effective.[1] Over the past two decades, several clinical trials have reported

improved clinical outcomes, particularly progression-free survival (PFS) and overall survival (OS), in patients with exon 18 – 21 mutations in the epidermal growth factor receptor (*EGFR*) gene who were treated with tyrosine kinase inhibitors (TKIs).[2,3] These TKIs are agents that specifically bind in, or close to, the ATP cleft of EGFR.[4] Such mutations stabilize the binding of TKIs to ATP, thereby inhibiting constitutive autophosphorylation and blocking the amplified catalytic activation of the tyrosine kinase domain after ligand binding.[5]

The response of NSCLC patients with mutations in exon 18 – 21 of the *EGFR* gene to TKIs is highly varied.[6] Common mutations, such as deletions in exon 19 (LREA regions), which removes codons 746 – 750, and the codon 858 mutation on exon 21 where leucine replaces arginine, account for around 85% of *EGFR* mutations in NSCLC and have been reported to be associated with better tumor responses (longer time to progression/survival) to TKIs when compared to other *EGFR* mutations.[7,8] Similarly, several clinical studies have reported that tumors with mutations in exon 18 are responsive to TKIs, whereas deletions and insertions in exon 18 have been associated with short-lived responses to TKIs.[9-11] Exon 19 deletions around the non-LREA regions are less responsive to TKIs when compared to deletions in these regions.[12] Mutations in exon 19 are rare and account for approximately 0.5% of all *EGFR* mutations.[13] Tumors with exon 19 mutations have heterogeneous responses to TKIs, ranging from moderately responsive (L747F, E746G, and P733L) to fully resistant (D761Y and L747S).[14,15]

Around 15% of NSCLC patients with an *EGFR* mutation have either an exon 20 mutation and/or an insertion. Aberrations in exon 20 have been associated with tumors that are mostly non-responsive to TKIs.[16] The T790M mutation on codon 790 of exon 20 is the most clinically relevant mutation on this exon,[17] with around 10% of patients with advanced lung cancer having this mutation.[18] Interestingly, more than 50% of patients with an exon 19 deletion or L858R point mutation undergoing treatment with TKIs acquire the T790M mutation and thus develop resistance to first- and second-generation TKIs.[19] However, patients with the T790M mutation are responsive to third-generation TKIs (osimertinib).[20] Tumors with the C797S mutation and exon 20 insertions (D770_N771insNPG at residues 762 – 775) are also generally non-responsive to TKIs.[21] Exon 21 mutations are rare and have been associated with lower sensitivity to TKIs (L861Q, L862V, A859X, and V851X) when compared to L858R mutations.[22] Because of their scarcity, the sensitivity of tumors with many exon 21 mutations (e.g., E866K, H870Y, H825L, H870R, G863S, and P848L) to TKIs is yet to be determined.[22]

As a result of the benefits experienced by NSCLC patients with EGFR mutations, such as longer time to progression and/or survival, through tailored therapies that suit their molecular pathology along with cost reduction associated with prudent use of TKIs, means that molecular testing has been recommended by several regulatory bodies as the standard of care.[23-25] The effectiveness of such patient stratification is highly dependent on detailed and efficient capture of the genomic environment of the malignancy in real time.[26]

In the clinic, the tumor biopsy used for detecting the mutational profile of patients with malignancies, currently the gold standard may not be adequate for effective molecular testing.[27] First, around 40% of patients with NSCLC may not be eligible for surgery, because their malignancies are at an advanced stage, and they are too weak to undergo surgery.[28] Furthermore, a single lesion or segment of a tissue biopsy may not provide sufficient information on tumor heterogeneity.[29] Re-sampling to monitor mutational changes that have some influence on progression and resistance is not practicable for the patient;[30] thus, alternatives are being actively explored. Cell-free DNA (cfDNA) is one alternative that has been utilized successfully for mutational analysis. However, its widespread use has been limited because of issues with low sensitivity particularly with regard to identifying resistant clones with specific mutations.[31,32]

Circulating tumor cells (CTCs) have been explored as a prognostic indicator for PFS and OS[33,34] and as a diagnostic tool for the detection of mutations with varying degrees of success. Some researchers indicate that the CTC sample matrix may be superior to tumor biopsy as the number of mutations detected increase, with the hypothesis being that CTCs reflect cells derived from the primary malignancy and metastatic sites.[35,36] However, other studies have reported equal or fewer numbers of mutations obtained from CTCs when compared to tissue biopsy and/or cfDNA.[37,38] The implementation of CTC sampling as a diagnostic tool has been limited by their relatively low numbers in the blood, especially in the early stages of tumor development, relatively cumbersome work flows, and the high costs of the techniques. In addition, some of the approaches for isolating CTCs from the blood have reported moderate purity values of ≤60% and yield of CTCs at ≤70%.[39,40] Most approaches for isolating CTCs from the blood of patients with NSCLC for subsequent downstream detection of *EGFR* mutations have employed the principles of immune isolation and/or size disparity between CTCs and other blood cells for isolation and polymerase chain reaction (PCR) techniques to detect mutations in exons 18 – 21.[41,42] The translation of these devices to routine use

in the clinics has been hampered by challenges related to reproducibility, low throughput, and intricate work mechanisms.[43] This study aimed to describe the design and functionality of an immunomagnetic microfluidic device that isolates epithelial cell adhesion molecule (EpCAM)-positive CTCs from the blood of patients with NSCLC and to evaluate exon 18 – 21 mutations of the *EGFR* gene with the aim of providing a rapid and robust way of obtaining clinically relevant information that can aid in treatment decisions.

2. Materials and methods

2.1. Materials

HPC Laser LS3060 60W CO_2 laser cutter (HPC Laser, Halifax, UK); polymethylmethacrylate (PMMA) sheet (Vink Plastics, Manchester, UK); PC-9 cell lines (obtained from European Collection of Authenticated Cell Cultures); neodymium iron boron (NdFeB) magnets (Integrated Magnetics, California, USA); RPMI 1640 media (Lonza, Slough, UK); Dynal anti-EpCAM magnetic beads (Thermo Fischer Scientific, Loughborough, UK); 4,6-diamidino-2-phenylindole (Vector Laboratories, Newark, USA); fluorescein-conjugated pan-cytokeratin monoclonal antibody (San Diego, USA); rhodamine-conjugated mouse anti-human CD45 antibodies (BD Biosciences, Fremont, California USA); and PCR mix and primers (Stab Vida Genetics, Laboratory, Lisbon, Portugal).

2.2. Manufacture of the PMMA chip

The dimensions of the PMMA chip were first drawn using an AutoCAD DXF file and then transferred to the laser cutter software to cut the design from a 3-mm PMMA sheet. Two pieces of PMMA measuring 98 × 98 mm were cut using an HPC Laser LS3060 60W CO_2 laser cutter. Afterward, the pieces were bonded together using ethanolic treatment and then underwent ultraviolet irradiation for 25 s as described previously.[44] A single PMMA sheet measuring 57 × 66 mm was also fabricated to form the lid.

2.3. Fabrication of the electromagnetic arm of the device

The magnetic arm is made of a C-shaped polycarbonate piece (measuring 10 cm in height and 4 mm in thickness) to which the NdFeB magnets (Integrated Magnetics, USA), measuring 20 × 5 × 4 mm, are attached at the top and bottom arms (Figure 1). The movement of the magnetic arm is controlled by a high-voltage power supply that moves the arm in the x, y, and z axes. A magnetic actuator program ensures precise movement of the arm. The magnetic device and controller unit were manufactured by Micro-Lab Devices Leeds, UK.

2.4. Cell culture

A lung adenocarcinoma cell line (PC-9)[45] obtained from the European Collection of Authenticated Cell Cultures (ECACC, www.phe-culturecollections.org.uk) was cultured in RPMI-1640 media (Lonza, UK) containing 10% (v/v) fetal bovine serum (Labtech.com, USA), with a final concentration of 50 µg/mL penicillin and 250 µg/mL streptomycin (Lonza, UK). Cells were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO_2 (Galaxy 170 S, New Brunswick Scientific, Stevenage, UK). Cells were used for experiments when they were 80% – 90% confluent.

2.5. Spiking experiments

PC-9 cell lines were harvested from culture media, and a cell viability count was undertaken. Cells were always at least 85% viable when used. PC-9 cells were added to 12 mL of RPMI-1640 media (Lonza, UK) at the following concentrations: 1×10^6, 2×10^5, 4×10^4, and 8×10^3 cells/mL. Thereafter, the cells were isolated using the microfluidic device. Cells isolated were thereafter subjected to PCR to validate the device's ability to process CTCs for downstream analysis and detection of mutations. A range of cell concentrations were used for spiking experiments to validate the utility of the device in isolating EpCAM-positive cell lines. Some of these concentrations reflected concentrations of EpCAM-positive CTCs reported in the blood of NSCLC patients (1 – 80,000 cells/mL).[46,47]

2.6. Patient recruitment/sample processing for CTC analysis

Fifty-nine patients aged between 47 and 81 years diagnosed with NSCLC and admitted to the Castle Hill Hospital were recruited for the study after ethical approval had been received from the North East-Newcastle and North Tyneside Local Research Ethics Committee (REC13/NE/0242). Written informed consent was obtained from all participants (Appendix A1). Patients' demographic/clinicopathological data were obtained from their medical records by Professor Michael Lind and presented in a pseudo-anonymized format.

A total of 13.5 mL of whole blood was collected into three 3.2% trisodium vacutainer sample bottles (BD, USA). Each sample bottle contained 4.5 mL of blood. Within 15 min of sample collection, the blood was transported on ice to the laboratory where processing was done in a class II biological safety cabinet (ESCO Scientific). Blood was pooled in a sterile 50-mL Falcon tube, and 2 mL of phosphate-buffered saline was added to reduce blood viscosity. The tube was mixed thoroughly by inverting the tube three times and then immediately loaded into the chip (Section 2.7).

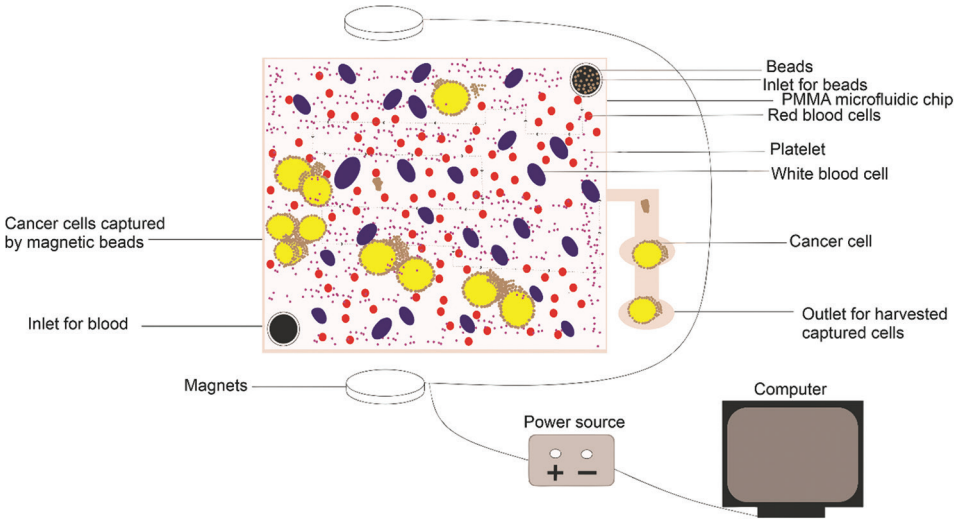


Figure 1. Schematic of the isolation of epithelial cell adhesion molecule (EpCAM)-positive cells from a heterogeneous mixture using a novel immunomagnetic microfluidic chip. The magnetic field, from the immobilized neodymium iron boron magnets positioned at the top and bottom of the polymethylmethacrylate chip, was moved across the device so that the anti-EpCAM magnetic beads (brown beads) bound to the circulating tumor cells in the blood would be collected (EpCAM-positive cancer cells in yellow). The mobility of the magnetic arm and the precision by which the arm skims across the chip are under the control of a high-voltage source and magnetic actuator app installed on the computer, respectively.

2.7. Isolation of EpCAM-positive cell line/CTCs from media/blood using the microfluidic device

Dynal magnetic beads (6 µL) coupled with an anti-EpCAM antibody (Dynabeads™ Epithelial Enrich, ThermoFisher Scientific) were placed in the bead inlet on the PMMA chip, and 13 mL of media spiked with PC-9 cells or patient blood samples were placed in the inlet for blood shown in Figure 1. EpCAM-positive cells were isolated using the immunomagnetic unit.

2.8. Immunostaining of EpCAM-positive cells

EpCAM-positive cells isolated from the device were concentrated onto microscope slides using a cytospin machine (ThermoFisher Scientific, USA). After preparation, the slides were allowed to air dry for 2 h, fixed in 100% methanol for 10 min, and finally allowed to dry for 3 h. After drying, the slides were rinsed with tap water to remove all traces of methanol. The slides were then flooded with Horse serum (blocking reagent) for 10 min (ThermoFisher Scientific, UK). Following blocking, 100 µL of fluorescein-conjugated pan-cytokeratin monoclonal antibody (Biolegend, USA) was added to the slides for 30 min to stain for cytokeratin-positive tumor cells. After incubation at room temperature, the slides were rinsed thrice in tris-buffered saline (TBS) and stained again for 30 min with 100 µL of rhodamine-conjugated mouse anti-human CD45 antibody to evaluate the presence of peripheral blood mononuclear cells. CD45, also known as leucocyte common antigen, was used to ascertain the level of any contaminating white blood cells isolated along with the CTCs. After staining, the slides were washed thrice in TBS and then rinsed in tap water before the addition of 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, USA) to stain the nuclei. The images were identified and evaluated using the Zeiss fluorescence microscope with Zeiss software for identifying CTCs and leucocyte contamination.

2.9. *EGFR* mutation detection in tumor biopsy samples

DNA was extracted from formalin-fixed and paraffin-embedded tissue biopsy samples using the cobas® EGFR DNA extraction kit in accordance with the manufacturer's protocol. Thereafter, extracted DNA was analyzed for selected mutations in exons 18 – 21 of the *EGFR* gene using the cobas® *EGFR* Mutation Test.

2.10. PCR experiments to identify exon 18 – 21 mutations in the *EGFR* gene of EpCAM-positive cells

PC-9 cell lines, bearing mutations in exons 18 – 21 of the *EGFR* gene, and CTCs from patients with NSCLC were used to demonstrate the device's ability to isolate CTCs for downstream analysis. Genomic DNA (gDNA) extracted from EpCAM-positive cells were isolated from a heterogeneous mixture of cells using the device as described earlier, and exons 18 – 21 of the *EGFR* gene were amplified using 0.75 µL of the following primers at a concentration of 10 pmol/µL: **exon** 18: forward (fwd) primers-GCTGAGGTGACCCTTGTCTC, reverse (rev)-TGGAGTTTCCCAAACACTCAG (300bp); **exon 19**:

fwd-GCTGGTAACATCCACCCAGA rev-TTATCTC CCCTCCCCGTATC (261 bp); exon 20: fwd-CACA CT GACGTGCCTCTCC rev-TTATCTCCCCTCCCCGTA TC (251 bp); exon 21: fwd-AGCCATAAGTCCTCG ACGTG rev-CCTGGTGTCAGGAAAATGCT (320 bp) (primer sequences were obtained from Stab Vida Genetics Laboratory, Portugal). Genomic DNA (3 µL) was added to 47 µL of standard PCR mix (provided by Stab Vida Genetics Laboratory, Portugal) Thermocycling temperatures were as follows: initial denaturing at 98 °C for 15 min, then 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, and amplification at 75 °C for 1 min, with a final elongation step at 70 °C for 5 min. PCR products were evaluated using gel electrophoresis on 2% (w/v) agarose gel.

2.11. Next-generation sequencing (NGS) to determine exon 18 – 21 mutations in patient samples

Amplicon and library generation were performed according to the procedure of Nextera XT (15031942) (Illumina, USA). The amplicon generated was sequenced using an Illumina sequencer, with the data being processed using Trim galore (version 0.4.3.1) and Prinseq (version 0.20.4). After sorting, data were aligned to the reference with BWA (MEM) version 0.7.17.1. Variants were detected using the VAR direct version from 07.03.2018 (sequencing was done at Stab Vida Genetics Laboratory, Portugal).

2.12. Statistical analysis

Data were analyzed using Prism version 9.0 (Graph pad software, San Diego California USA).

3. Results

3.1. Device description and validation

The device was designed to isolate EpCAM-positive cells in blood contained within a PMMA device using a 4.5-µm diameter magnetic bead covalently bound to an anti-EpCAM antibody sweeping through the blood on a chip (Figure 1). As the magnetic bead sweeps through the blood, it binds with any EpCAM-positive cells. The movement of the magnetic beads can be attributed to the magnetic field generated by NdFeBr magnets attached to the electromagnetic arms of the unit. The arm moves across the chip in step delays of 6 s to allow sufficient time for all magnetized cells to be dragged through the fluid and kept together until they arrive at the outlet where the collected cells were easily isolated for further analysis (Figure 1). The total time taken for isolation of EpCAM-positive cells from 13 mL of fluid using the device was 50 min. Spiking experiments using the PC-9 cell line spiked into media showed that the device was able to capture EpCAM-positive cells (Figure 2). Having established the optimal parameters for collection, the CTCs from PC-9 were analyzed for mutations in 4 exons of the *EGFR* gene. Figure 3A shows the gene amplification of each exon independently using gDNA obtained from the PC-9 cell line, showing that all mutations were detectable. Figure 3B shows a multiplex PCR using the same primers on cells isolated from experiments where the PC-9 cells were spiked into media at various concentrations (1×10^6, 2×10^5, 4×10^4, and 8×10^3 cells/mL).

3.2. Isolation of EpCAM-positive cells from the blood of patients with NSCLC

CTCs isolated from the blood of patients were immunostained to identify markers of epithelial cells and ensure that the cells isolated were tumor-derived. Figure 4A shows a brightfield image of a clump of cells and beads isolated from the patient's blood. Figure 4B shows that the isolated cells were tumor-derived as the clump stained well with a pan-cytokeratin antibody (which stains for epithelial-derived cells). Figure 4C shows that only a few cells stained positive for CD45 antigen using rhodamine-conjugated mouse anti-human CD45 antibodies (a marker for hematological cells), most possibly showing a few leukocytes co-isolated with the tumor cells. Figure 4D shows that most of the cells stained positive for DNA content using DAPI. Figure 4E presents a merged image of the three fluorescence channels, clearly showing that the epithelial tumor cells are the predominant cell type isolated.

3.3. Patient characteristics

Fifty-nine patients recruited for the study were diagnosed following a tissue biopsy. Their clinicopathological characteristics are shown in Table 1, and the process of recruitment is described in Figure 5.

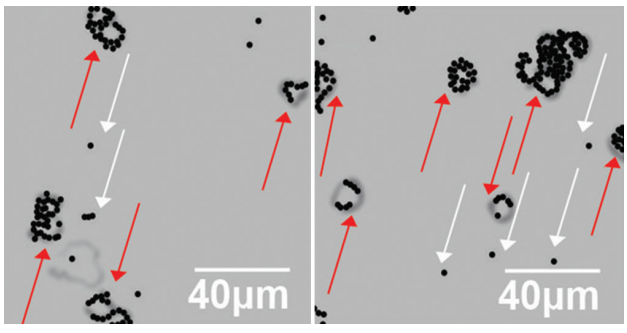


Figure 2. PC-9 cells bound to epithelial cell adhesion molecule (EpCAM)-coated beads. PC-9 cell lines (expressing relatively high levels of EpCAM) were spiked in media and isolated from media using the device. The results Panel A- show the capture of PC-9 cell lines by the device (red arrow depicts cell lines positive for EpCAM captured by beads. White arrow depicts free beads unattached to cells (scale bar: 40 µm). The same was also observed in Panel B (scale bar: 40 µm) (representative data from four repeats)

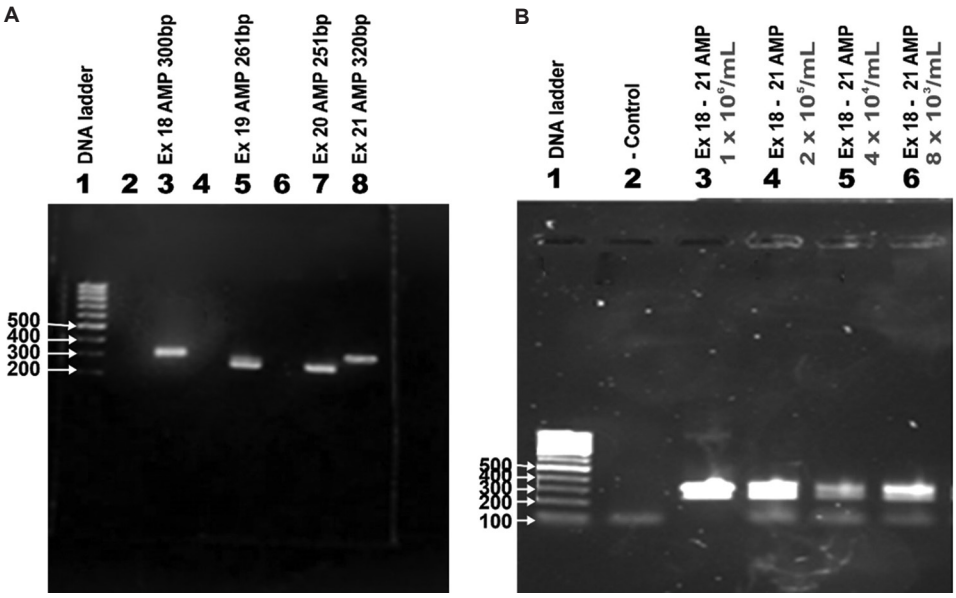


Figure 3. Validation experiments to show that isolated epithelial cell adhesion molecule-positive cells can be used for downstream polymerase chain reaction (PCR) analysis. DNA from isolated PC-9 cell lines had exons 18 – 21 of the *EGFR* gene amplified individually and then as a multiplex. (A) Lane 1, DNA ladder; lane 2, negative control; lanes 3, 5, 7, and 8 show amplification of exons 18, 19, 20, and 21 regions individually from a single sample. (B) Lane 1, DNA ladder; lane 2, negative control; lanes 3, 4, 5, and 6 show the multiplex PCR of amplification of exons 18, 19, 20, and 21 of PC-9 cell lines spiked at the following concentrations (1×10^6, 2×10^5, 4×10^4, 8×10^3 cells/mL) in media and thereafter isolated from the media using the device (representative data from 3 repeats).

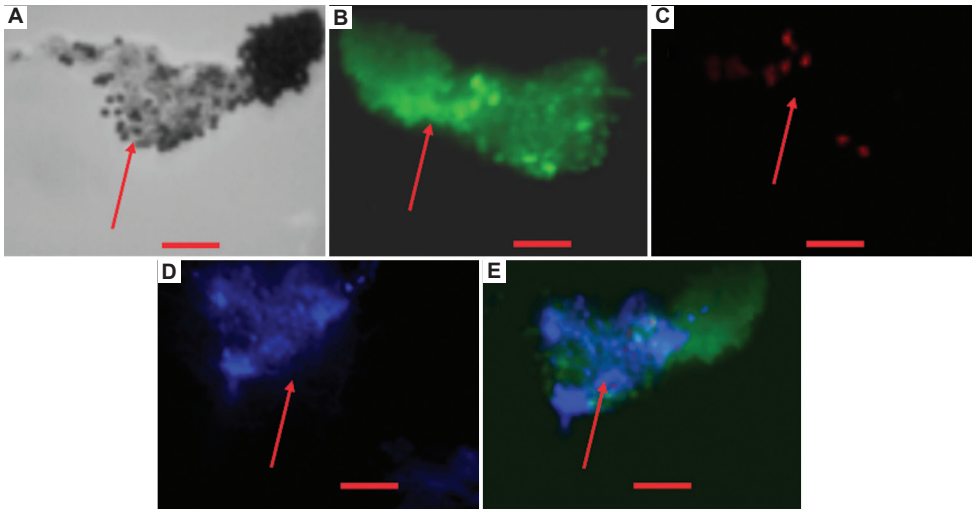


Figure 4. Immunostaining of cells isolated from the blood of patients with non-small cell lung cancer (scale bar: 40 μm for panels A-E). (A) brightfield imaging of epithelial cell adhesion molecule-positive cells surrounded by magnetic beads, isolated from blood of patients. (B) cells staining positive for a fluorescein-conjugated pan-cytokeratin monoclonal antibody (BioLegend, USA). (C) few cells staining positive with a rhodamine-conjugated anti-CD45 antibody. (D) 4,6-Diamidino-2-phenylindole-stained-nuclei of cells. (E) Merged image of the three fluorescence channels (representative data from three independent repeats).

3.4. Detection of mutations in CTCs

Among the 38 patients who had their CTC-enriched samples analyzed for *EGFR* mutations, 30 (79%) presented with a mutation. Mutated events (expressed in %) among all mutations detected ranged from 1% to 55% (Table 2). Exon 19 had the highest number of genetic variants seen in 26 patients (87%), with E746_A750 delELREA deletions being the most common variation. Exon 21 had the highest number of point mutations with 7 (23%), whereas exon 20 had the highest number of single nucleotide variants/single

Table 1. Clinicopathological characteristics of NSCLC patients who underwent CTC analysis for mutations

	N (%)
Age	
Mean age	65.4 years
Range	47 – 76 years
Sex	
Male	20 (52.6)
Female	18 (47.3)
Smoking status	
Unknown	21 (55.3)
Yes	13 (34.2)
No	4 (10.5)
Type of NSCLC	
Adenocarcinoma	36 (94.7)
Large cell	1 (2.6)
Other	1 (2.6)
Stage of cancer	
IIb	2 (5.2)
IIIA	5 (13.1)
IIIb	7 (18.4)
IV	24 (59.3)
Metastatic regions*	
Lung	10 (26.3)
Lymph nodes	2 (5.3)
Bone	9 (23.7)
Liver	1 (2.6)
Kidney	1 (2.6)
Pleura	4 (10.5)
Brain	2 (5.3)
Neck	1 (2.6)
Mediastinal	9 (23.7)
PDL1 expression ≥1%	30 (78.9)
Other mutations	
ROS1	1 (2.6)

*One tumor had metastasized to two different regions.
Abbreviations: NSCLC: Non-small cell lung cancer; CTC: Circulating tumor cell; PDL1: Programmed death ligand 1; ROS1: ROS proto-oncogene 1 receptor tyrosine kinase.

nucleotide polymorphisms at 4 (13%) (Figure 6A and B). The most frequent point mutations on exon 21 were L858R and P848L (3 occurrences, 8%) (**Figure 6B**). Among the CTC samples analyzed, 9 (31%) had mixed *EGFR* mutations. All patients with a mixed mutation had an exon 19 deletion (Table 3). This study also detected uncommon mutations of varied clinical significance (Table 4).

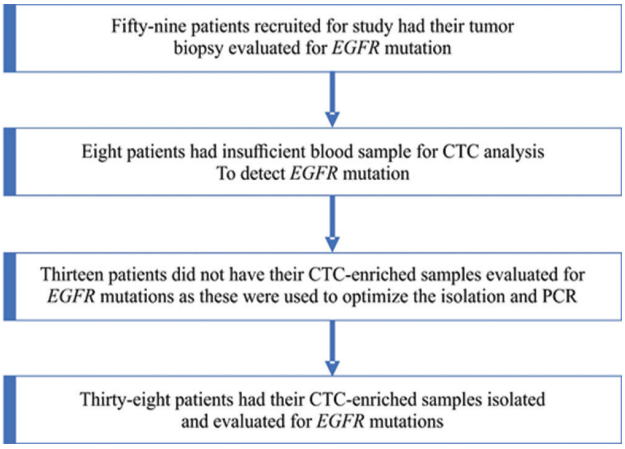


Figure 5. Flow chart for patient recruitment
Abbreviations: *EGFR*: Epidermal growth factor receptor; CTC: Circulating tumor cell.

3.5. Comparison of mutations detected using NGS in CTCs with cobas® EGFR mutation testing of matched tumor biopsies

Similarities and differences in *EGFR* mutations detected in CTCs and matched tumor biopsies were evaluated. Our results (Table 5 and Figure 7) showed that significantly more mutations were detected in the CTCs than in the matched biopsies (Fisher's exact test, $P = 0.0173$; Figure 7). *EGFR* mutations were diagnosed in 30 CTC samples, whereas only 4 matched biopsies (Patients: 17, 27, 40, and 59; Table 5) showed a mutation. The current study showed that NGS analysis of isolated CTCs had a higher likelihood ratio of detecting mutations than did the cobas® EGFR analysis of the tumor biopsy (Fisher's exact test, likelihood ratio 1.855; Figure 7). In addition, only one of the four tissue biopsy samples positive for an *EGFR* mutation had a similar mutation result obtained from its matched CTC sample (Patient 27; Table 5). The mutations obtained from the other three biopsy samples were discordant from their matched CTC samples; however, for the eight CTC samples with no *EGFR* mutation detected, the same lack of mutations was observed in the matched tumor biopsies (Table 5 and Figure 7).

4. Discussion

The present study was designed to describe the ability of a new immunomagnetic microfluidic device to isolate CTCs for downstream analysis. In addition, the clinical potential for the use of CTCs as a sample matrix for the diagnosis of *EGFR* mutations in NSCLC was also demonstrated.

The results in Table 2 show that among the 38 patients whose CTC samples were evaluated for *EGFR* mutations, 30 (79%) contained at least one mutation. This differs

Table 2. *EGFR* mutations detected in CTCs

Patient ID	Exon	Mutations	Percentage of mutations detected using NGS	No. of CTCs counted
1		WT		
3		**WT**		
8		**WT**		
9	**21**	**L858R**	**ND**	**NR**
10		**WT**		
11		**WT**		
13	**19**	**Deletion** (E746_A750delELREA)	**ND**	**NR**
14		**WT**		
15	**19**	**Deletion** (E746_A750delELREA)	**ND**	**NR**
16	**19**	**Deletion** (E746_A750delELREA)	**ND**	**NR**
17	**19**	**Deletion** (E746_A750delELREA)	**ND**	**NR**
	21	**L858R**	**ND**	**NR**
19	**19**	**Deletion** (E746_A750delELREA)	**ND**	**NR**
20	**21**	**L858R**	**ND**	**NR**
21		**WT**		
22		**WT**		
24	**19** **20**	**Deletion** (E746_A750delELREA) C.2389T > A	**2.01** **1.3**	**7**
25	**20**	**c. 2375T > C**	**0.6**	**4**
26	**19**	**Deletion** (E746_A750delELREA)	**55.39**	**402**
27	**19**	**Deletion** (E746_A750delELREA)	**6.18**	**59**
28	**19**	**Deletion** (E746_A750delELREA)	**3.4**	**3**
	19	**P733L**	**1.74**	
29	**21**	**c. 2573T > G**	**0.76**	**23**
30	**19**	**Deletion** (E746_A750delELREA)	**3.19**	**35**
	19	**P733L**	**1.13**	
	20	**c. 2318A ≥ G**	**0.58**	
31	**19**	**Deletion** (E746_A750delELREA)	**1.4**	**81**
	20	**R766H**	**1.2**	
	20	**c. 2327G > A, c. 2375T > C**	**0.75, 0.55**	
32	**19**	**Deletion** (E746_A750delELREA)	**2.4**	
	19	**P735S G696E**	**1.2**	
	18	**L703P**	**1.01**	
	21	**P848L**	**0.86**	
34	**19**	**Deletion** (E746_A750delELREA)	**0.65**	
	21,19	**c. 2573T > c, c. 2281G > A**	**0.55, 1.21**	
36	**19**	**Deletion** (E746_A750delELREA)	**2.65**	
	19	**c. 2281G ≥ A**	**1.17**	
37	**19** **18**	**Deletion** (E746_A750delELREA) c. 2123A > G	**2.65** **0.83**	**90**
38	**19**	**Deletion** (E746_A750delELREA)	**6.11**	**32**
39	**19**	**Deletion** (E746_A750delELREA)	**5.83**	**11**
	20	**T790M**	**0.83**	

(cont'd...)

Table 2. ***(Continued)***

Patient ID	Exon	Mutations	Percentage of mutations detected using NGS	No. of CTCs counted
	18	**N700D**	**0.69**	
40	**19**	**Deletion** (E746_A750delELREA) **L841P**	**19.67** **1.4**	**5**
43	**19**	**Deletion** (E746_A750delELREA)	**2.43**	**41**
	21	**V843L**	**0.53**	
53	**19**	**Deletion** (E746_A750delELREA)	**8.47**	
54	**19**	**Deletion** (E746_A750delELREA)	**1.11**	
55	**19**	**Deletion** (E746_A750delELREA)	**14.34**	
56	**19** **20**	**Deletion** (E746_A750delELREA) **R776H**	**3.26** **1.34**	**300**
57	**19**	**Deletion** (E746_A750delELREA)	**32.64**	**115**
58	**19**	**Deletion** (E746_A750delELREA)	**22.85**	**505**
59	**19**	**Deletion** (E746_A750delELREA)	**1.66**	**52**

Abbreviations: WT: Wild-type (negative for an *EGFR* mutation); ND: Not done; NR: No result; *EGFR*: Epidermal growth factor receptor; CTCs: Circulating tumor cells; NGS: Next-generation sequencing. The bold values represent mutations/deletions on exons 18-21 of the EGFR gene.

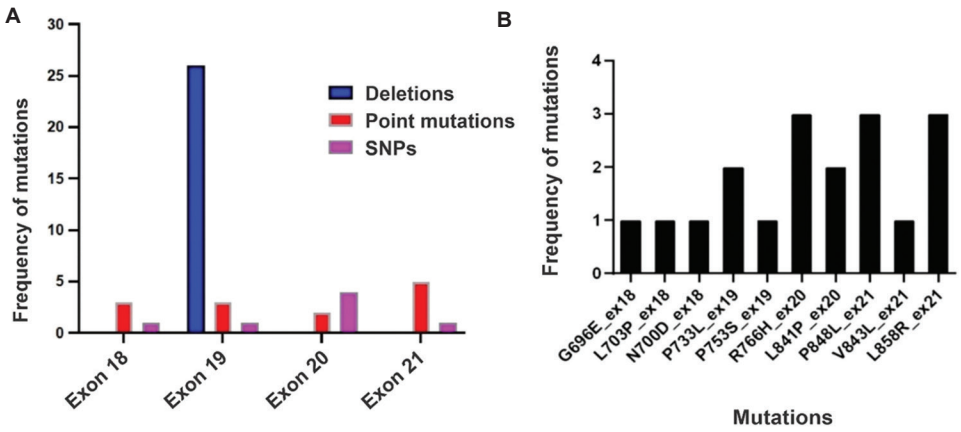


Figure 6. Number of mutations and single nucleotide polymorphism obtained in exons 18, 19, 20, and 21 of epithelial cell adhesion molecule (EpCAM)-positive circulating tumor cells (CTCs) obtained from patients with non-small cell lung cancer (NSCLC). A, frequency of aberrations on exons 18 – 21 of EpCAM-positive CTCs obtained from NSCLC patients; B, Frequency of point mutations on the exons.

from previously reported incidences of *EGFR* mutations in exons 18 – 21 among Caucasian patients with NSCLC, with most of these studies reporting an incidence between 10% and 40%.[48,49] The disparity in results may be attributed to the sample matrix used, as this study used CTCs, whereas most other studies used tissue biopsies. CTCs represent a culmination of events occurring at the initiation of the malignancy and during metastasis, whereas tissue biopsies represent molecular events only occurring at the primary site of a malignancy.[50] Second, most published works employed PCR for the analysis of mutations (e.g., amplification refractory mutation analysis, restriction fragment polymorphism mutant allele detection, and locked PCR clamping, all of which look for specific mutations).[51] Here, NGS had been used to scan the whole *EGFR* gene for mutations. Mao *et al.*[52] evaluated the frequency of *EGFR* mutations in 21,324 patients with NSCLC admitted to oncology clinics in China using PCR, Sanger sequencing, and NGS and reported that most of the mutations were detected using NGS (71%), whereas 45% and 35% of them were detected using Sanger sequencing and qPCR, respectively. The use of NGS in this Chinese study reported *EGFR* mutation rates that were very similar to those observed in the current, small-scale, cohort.

Furthermore, it has been reported that NGS has a relatively high false discovery rate of between 0.1% and 1%.[53] Overestimation of mutations by NGS has been linked to

sequence artifacts attributed, at least in part, to the low quality of DNA, the clonal amplification of DNA strands, and/or the need for a relatively high number of PCR cycles due to the low quantity of starting material, as well as chemical modifications that occur during the NGS workflow.[54] However, in this study, CTCs were analyzed for mutations at a read depth of ×10,000, which has been recommended as best practice in the literature.[55,56] Therefore, we believe this reduces the probability of methodological errors. In contrast, some studies have suggested that because CTCs comprise a very small percentage of the total tumor mass, any mutation detected with sufficient coverage should be recognized as a potentially important clinical variant.[55] The incidence of *EGFR* mutations using the CTC + NGS matrix will be investigated in further studies using a microfluidic device (designed by our group) capable of single-cell RNASeq.

Table 3. Patients with mixed *EGFR* mutations

Patient No.	Exon	Mutations
28	19	**Deletion (E746_A750delELREA), P733L**
30	19	**Deletion (E746_A750delELREA), P733L, L841P**
31	19 20	**Deletion (E746_A750delELREA)** **R776H**
32	19 21 18	**Deletion (E746_A750delELREA), P753S** **P848L** **G696E, L703P**
39	19 20 18	**Deletion (E746_A750delELREA)** **T790M** **N700D**
43	19 21	**Deletion (E746_A750delELREA)** **V843I**
17	19 21	**Deletion (E746_A750delELREA)** **L858R**
40	19 20	**Deletion (E746_A750delELREA)** **L841P, P848L**
56	20	**R766H**

Abbreviation: *EGFR*: Epidermal growth factor receptor. The bold values represent mutations/deletions on exons 18-21 of the EGFR gene.

The results from mutational profiling of the CTCs are generally consistent with the exon 18 – 21 mutational profile of patients with NSCLC using tumor biopsy. For example, 86.7% of CTC-enriched samples analyzed in this study had a deletion in exon 19 (E746_A750delELREA), which is consistent with published literature reporting that around 80% – 90% of NSCLC patients with an *EGFR* mutation have either this deletion or an exon 21 L858R mutation.[2,8,12,28] However, the frequency of mixed mutations was much higher in the current study (30%) than that in others, with Caucasian patients having reported frequencies of 5% – 7% for mixed mutations.[56-58] The difference in frequency rates may be attributed to the relatively small sample size of the present study or, as described earlier, the disparity in analysis techniques. To the best of our knowledge, the only study that has employed NGS for mutational analysis of *EGFR* mutations in CTCs obtained from NSCLC reported an incidence of 13% for mixed mutation for a cohort of 37 patients enrolled for the study,[59] which was higher than that reported in other studies using PCR-based mutation analysis on tumor biopsies but was still lower than that observed in the current study.[51,52]

The higher incidence reported in our study than in the study by Marchetti *et al.*[59] may be due to the difference in isolation technique for CTCs. The latter used CELL SEARCH for isolating CTCs, whereas the present study used a novel immunomagnetic microfluidic device. CELL SEARCH technique has been associated with a CTC yield of ≤60% and a purity of around 50%.[40] Perhaps the CTCs isolated were not totally representative of the molecular events in the malignant environment, which may have resulted in an under reporting of mutations present. Preliminary validation studies on the yield and purity of the device in this study using cell lines expressing varying levels of EpCAM spiked in media and blood have shown that the device isolates EpCAM-positive cells with a yield of ≥65% and purity of ≥95% (unpublished data from our laboratory). The yield and purity of CTCs isolated using the device may have contributed to the increased mixed mutation incidence reported in the current study.

Table 4. Rare *EGFR* single nucleotide variants identified in patient CTC-enriched samples using NGS

Patient ID	Nomenclature (SNV)	Exon	RS no.	Amino acid	Comments
31,34	**c. 2375T>C**	**20**	**132563568**	**L792P**	Pathogenic mutation of somatic origin[10,31,60]
24	**c. 2389T>A**	**20**	**1057519861**	**C797S**	Pathogenic mutation of somatic origin associated with drug resistance[10,31,62]
31	**c. 2327G>A**	**20**	**483352806**	**R509H**	Germline origin of uncertain significance[31,60,62]
30	**c. 2318A>G**	**20**	**121913432**	**H506R**	Somatic likely pathogenic[60]
37	**c. 2123A>G**	**18**	**144932466**	**K708R**	Somatic likely pathogenic[63,65]
34	**c. 2281G>A**	**19**	**121913418**	**D761N**	Somatic likely pathogenic[57,62-64]
29	**c. 2573T>G**	**21**	**121434568**	**L591R**	Somatic associated with a drug response[31,60,61]

Abbreviations: CTC: Circulating tumor cell; *EGFR*: Epidermal growth factor receptor; NGS: Next-generation sequencing. The bold values represent mutations/deletions on exons 18-21 of the EGFR gene.

Table 5. Comparison of mutations detected in CTCs and tumor biopsies

Patient ID	Exon EGFR CTC	*EGFR* mutation in CTCs	Percentage of mutations detected using NGS	Exon *EGFR* biopsy	*EGFR* mutation tumor biopsy	Matched (Yes or No)
1		**WT**			**WT**	Yes
3		**WT**			**WT**	Yes
8		**WT**			**WT**	Yes
9	**21**	**L858R**			**WT**	No
10		**WT**			**WT**	Yes
11		**WT**			**WT**	Yes
13	**19**	**Deletion** (E746_A750delELREA)			**WT**	No
14		**WT**			**WT**	Yes
15	**19**	**Deletion** (E746_A750delELREA)			**WT**	No
16	**19**	**Deletion** (E746_A750delELREA)			**WT**	No
17	**19**	**Deletion** (E746_A750delELREA)		20	**Exon 20 insertion**	No
	21	**L858R**				
19	**19**	**Deletion** (E746_A750delELREA)			**WT**	No
20	**21**	**L858R**			**WT**	No
21		**WT**			**WT**	Yes
22		**WT**			**WT**	Yes
24	**19** **20**	**Deletion** (E746_A750delELREA), c. 2389T>A	**2.01** **1.3**		**WT**	No
25	**20**	**c. 2375T>C**	**0.6**		**WT**	No
26	**19**	**Deletion** (E746_A750delELREA)	**55.39**		**WT**	No
27	**19**	**Deletion** (E746_A750delELREA)	**6.18**	19	**Exon 19 del**	Yes
28	**19**	**Deletion** (E746_A750delELREA)	**3.4**		**WT**	No
	19	**P733L**	**1.74**			
29	**21**	**c. 2573T>c**	**0.76**		**WT**	No
30	**19**	**Deletion** (E746_A750delELREA)	**3.19**		**WT**	No
	19	**P733L**	**1.13**			
	20	**c2318A≥G**	**0.58**			
31	**19** **20**	**Deletion** (E746_A750delELREA) c. 2375T>c, c. 2327>G	**1.4** **0.55, 0.75**		**WT**	No
	20	**R766H**	**1.2**			
	20	**c2441T≥A**	**0.75**			
32	**19**	**Deletion** (E746_A750delELREA)	**2.4**		**WT**	No
	19	**P735S**	**1.2**			
	18	**L703P**	**1.01**			
	21	**P848L**	**0.86**			
34	**19** **19**	**Deletion** (E746_A750delELREA) c. 2281G>A	**0.65** **0.58**		**WT**	No
	21	**c2573T≥c**	**0.55**			
36	**19**	**Deletion** (E746_A750delELREA)	**2.65**		**WT**	No
	19	**c2281G≥A**	**1.17**			
37	**19** **18**	**Deletion** (E746_A750delELREA) c. 2123A>G	**2.65** **0.83**		**WT**	No
38	**19**	**Deletion** (E746_A750delELREA)	**6.11**		**WT**	No

(cont'd...)

Table 5. ***(Continued)***

Patient ID	Exon EGFR CTC	*EGFR* mutation in CTCs	Percentage of mutations detected using NGS	Exon *EGFR* biopsy	EGFR mutation tumor biopsy	Matched (Yes or No)
39	**19**	**Deletion** (E746_A750delELREA)	**5.83**		**WT**	No
	20	**T790M**	**0.83**			
	18	**N700D**	**0.69**			
40	**19** **20**	**Deletion** (E746_A750delELREA) L841P	**19.67** **1.4**	21	**L858R**	No
43	**19**	**Deletion** (E746_A750delELREA)	**2.43**		**WT**	No
	21	**V843L**	**0.53**			
53	**19**	**Deletion** (E746_A750delELREA)	**8.47**		**WT**	No
54	**19**	**Deletion** (E746_A750delELREA)	**1.11**		**WT**	No
55	**19**	**Deletion** (E746_A750delELREA)	**14.34**		**WT**	No
56	**19** **20**	**Deletion** (E746_A750delELREA) R766H	**3.26** **0.92**		**WT**	No
57	**19**	**Deletion** (E746_A750delELREA)	**32.64**		**WT**	No
58	**19**	**Deletion** (E746_A750delELREA)	**22.85**		**WT**	No
59	**19**	**Deletion** (E746_A750delELREA)	**1.66**	21	**L858R**	No

Abbreviations: WT: Wild-type (no mutation detected); CTCs: Circulating tumor cells; *EGFR*: Epidermal growth factor receptor; NGS: Next-generation sequencing. The bold values represent mutations/deletions on exons 18-21 of the EGFR gene.

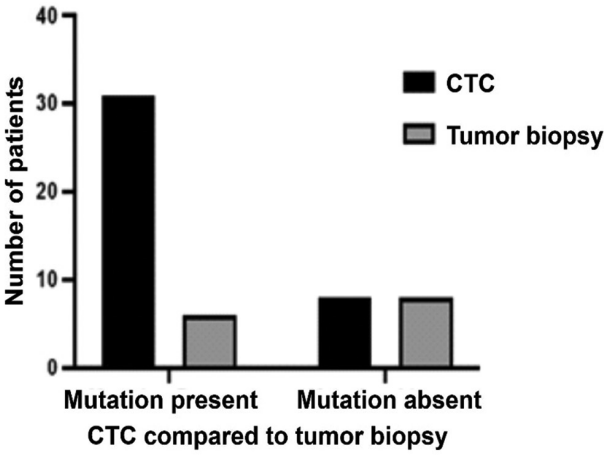


Figure 7. Epidermal growth factor receptor (*EGFR*) mutations present and/or absent in matched circulating tumor cells (CTCs) and tumor biopsy samples. Statistical analysis from Fisher's exact test showing that the CTC + next-generation sequencing (NGS) sampling technique detected significantly more mutations ($P = 0.0173$) than cobas® EGFR mutation analysis of the matched tumor biopsy. In addition, the likelihood of detecting mutations in *EGFR* from the CTC + NGS matrix was 1.855 (Fisher's exact test).

Rare mutations detected in the present study (e.g., L792P, C797S, H506R, and L591R) have been reported previously in NSCLC with clinical correlations. L591R has been associated with drug response to gefitinib,[31,60-63] and C795S mutation has been linked to resistance to osimertinib.[31,62] D761N mutation has been reported in biopsies of NSCLC and colon and prostate cancers, and has also been associated with partial response to erlotinib.[57,62-64] A K708R mutation has been reported in ovarian cancers where it has been associated with abnormal phosphorylation of AKT and ERK.[62] It has also been reported once in a Chinese study[65] but never in Caucasian populations; thus, the response of this mutation to TKIs is not clear. These patient-specific point mutations will potentially offer new insights into treatment responses and should be analyzed in a large cohort.

The marked disparity in the number of mutations detected in CTCs (78.95%) when compared to matched tumor biopsies (13.3%) reported in the current study differs from that published in other studies. Most of these studies report a similarity in the number of mutations in matched CTC and tumor biopsy samples,[38,39,46] whereas some studies have reported a lower number of mutations in CTC samples than in matched tumor biopsy samples.[37,66,67] However, both groups of studies highlighted above only analyzed CTCs for *EGFR* mutations in patients whose tumor biopsy samples were positive for a mutation. Conversely, the current study analyzed samples "blindly," i.e., CTC samples were analyzed for mutations from all patients before the mutational status of the biopsy was known, rather than only focusing on patients with a biopsy containing mutations.

The discordance in the type of mutations observed in three of the four patients who had a mutation detected in both CTCs and biopsies was similar to that observed in

the two previous studies.[66,67] Both these studies proposed that the discordance in mutations between CTCs and tumor biopsies may be due to the heterogeneity of tumors as discussed above. The apparent discordance in mutations observed in CTCs and tumor biopsies will need to be further investigated using approaches for CTC isolation and downstream analysis that properly define mutations derived from single cells or multiple cell clones.

5. Conclusion

The results obtained from this study suggest that the new microfluidic device described can be used to isolate CTCs for downstream mutational analysis of *EGFR* mutations. The device was able to isolate EpCAM-expressing PC-9 cell lines and EpCAM-positive CTCs from media and blood, respectively, with the isolated EpCAM-positive cells having been successfully analyzed for mutations in the *EGFR* gene. The mutational profile obtained from CTCs for the recruited patients has positive clinical implications as it indicates that a single blood draw may be able to provide a snapshot of molecular events in a malignancy from initiation to metastasis. However, the current study is limited by the sample size. Hence, it is our intention to carry out further studies in a larger cohort to better evaluate the utility of this technology and make the necessary modifications for translation into clinical practice. Furthermore, we intend to analyze different methods for the genomic analysis of CTCs to define mutations in single cells.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: John Greenman, Micheal J. Lind
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Methodology: Nkeiruka O. Ogidi, John Greenman
Writing – original draft: Nkeiruka O. Ogidi
Writing – review & editing: Micheal J. Lind, John Greenman

Ethics approval and consent to participate

The study adhered to the REMARK guidelines. It was conducted at the Queens Centre for Oncology and Hematology, Castle Hill Hospital, Hull, in collaboration with the Centres for Biomedicine and Clinical Sciences at the University of Hull, United Kingdom. The North East-Newcastle & North Tyneside Local Research Ethics Committee approved this study (REC13/NE/0242). In accordance with the Declaration of Helsinki, informed consent was obtained in written form from all participants.

Consent for publication

Consent in written form was obtained from participants in the study to publish their data.

Availability of data

The data from this study can be obtained on request from the senior author, Prof J. Greenman.

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Appendix

Appendix A1. Consent Form

Title: Developing an evidence-based system to facilitate the predictive assessment and optimization of older adults under investigation

Hull and East Yorkshire Hospitals NHS
NHS Trust

UNIVERSITY OF Hull

PATIENT CONSENT FORM

Peripheral blood detection of EGFR status in lung cancer patients

Name of Researcher: <insert name>

Chief Investigator: Professor M. J. Lind

Patient Study Number:

Please INITIAL boxes; do not tick

1. I confirm that I have read and understood the information sheet (Version 1.2 15/03/2017) for the above study. ☐
2. I confirm that I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
3. I understand that my participation is voluntary and that I am free to withdraw from the study at any time without giving any reason, and without my medical care or legal rights being affected. ☐
4. I give permission for a 10 ml blood sample to be taken for research purposes. ☐

   For selected individuals only:
   a. I give permission for an additional 5 ml blood sample to be taken for research purposes. ☐
5. I give permission, if I am given the tablet treatment, for a further 10 ml blood sample to be taken if the treatment stops working. ☐
6. I agree to my sample being sent to the University of Hull for the analysis to be performed. ☐
7. I agree to my medical records being looked at for research purposes by an authorised member of the research team to record my EGFR status results. ☐

(cont'd...)

Appendix A1. (*Continued*)

8. I understand that to enable the study to be properly monitored and regulated, relevant sections of my medical records and data collected during the study may be looked at by members of the research team, and individuals from the Sponsor (Hull and East Yorkshire Hospitals NHS Trust) or possibly from regulatory authorities. I give permission for these individuals to have access to my medical records to view sections that are relevant to my taking part in this study. ☐

9. I understand that the information will be used for medical research only and that I will not be identified in any way in the analysis and reporting of the results. ☐

10. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers. ☐

11. I agree to my General Practitioner being informed of my participation in the study. ☐

12. I agree to take part in the above research study. ☐

.....	.....	.....
Name of patient (BLOCK CAPITALS)	Signature	Date

.....	.....	.....
Name of person taking consent (if different from researcher) (BLOCK CAPITALS)	Signature	Date

.....	.....	.....
Name of researcher (BLOCK CAPITALS)	Signature	Date

When completed: 1 copy for patient, 1 copy to be kept in medical notes, 1 original for researcher site file.