Microdroplet Digital PCR

Detection and Quantitation of Biomarkers in Archived Tissue and Serial Plasma Samples in Patients with Lung Cancer

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Introduction: There is much interest in the use of noninvasive biomarkers in the management of lung cancer, particularly with respect to early diagnosis and monitoring the response to intervention. Cell-free tumor DNA in patients with cancer has been shown to hold potential as a noninvasive biomarker, in which the response to treatment may be evaluated using a blood test only. Multiple technologies have been suggested as being appropriate to measure cell-free tumor DNA. Microdroplet digital polymerase chain reaction (mdPCR) has a number of attributes that suggest it may be a useful tool for detecting clinically relevant genetic events. It offers precise and accurate quantitation of mutant alleles, including rare variants.

Methods: We evaluate the performance of mdPCR in the analysis of DNA extracted from reference standards, tumor biopsies, and patient plasma.

Results: The potential of mdPCR to detect clinically relevant mutations is demonstrated, in both formalin-fixed paraffin-embedded material and plasma. Furthermore, we show that mdPCR can be used to track changes in peripheral blood biomarkers in response to treatment and to detect the emergence of drug-resistant clones.

Conclusions: MdPCR has potential as a tool to detect and quantify tumor-derived mutational events in cell-free DNA from patients with lung cancer.

Key Words: Droplet digital polymerase chain reaction, Circulating DNA, Liquid biopsy.

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Lung cancer is characterized by a grossly altered genomic landscape.[1] A number of mutations occur at high prevalence in lung cancer, including mutations in *EGFR* and *KRAS*,[2] and these are now routinely evaluated on diagnosis as part of a standard clinical assessment.

It is possible to detect tumor DNA noninvasively in peripheral blood (cell-free tumor DNA [ctDNA]). This was originally inferred by the measurement of total cell-free DNA (cfDNA) and the demonstration that patients with cancer tended to have higher cfDNA.[3] Latterly, a number of groups have demonstrated that it is possible to detect specific mutational events, or circulating nucleic acid biomarkers, in lung and other solid organ malignancies.[4]

The ability to detect tumor-specific DNA in the peripheral blood raises the possibility of applying so-called "liquid biopsies" to the routine management of patients with lung cancer. The potential advantages are both broad and significant. The ability to specifically detect mutational events in a diagnostic specimen will facilitate longitudinal studies in which peripheral blood is resampled at intervals to assess the mutant allele "load." CtDNA could be used to monitor the response to therapeutic intervention and to detect relapse before clinic-radiological manifestations.[5] Further possibilities include the potential to direct therapeutic decisions based on the analysis of ctDNA. This may include the identification of subclonal populations with mutations that confer resistance to specific therapeutic agents; or the decision to withdraw or alter a therapeutic strategy based on an incremental rise in ctDNA despite treatment.[4,6]

A number of platforms have been suggested for this approach, each with specific attributes.[7,8] Digital polymerase chain reaction (PCR) has a number of characteristics that make it particularly attractive for the analysis of ctDNA.[9] Digital PCR relies on the ability of PCR to detect a single copy of a locus (mutant allele) of interest if present in a reaction volume. The quantitation relies on a DNA sample being diluted so that partitioned digital PCR reactions (in microdroplets or reaction wells) may or may not contain the locus of interest. The absolute number of copies of a particular locus in a given sample of DNA can then be directly estimated from the number of positive droplets. In a number of studies, including those in which circulating biomarkers have been analyzed,

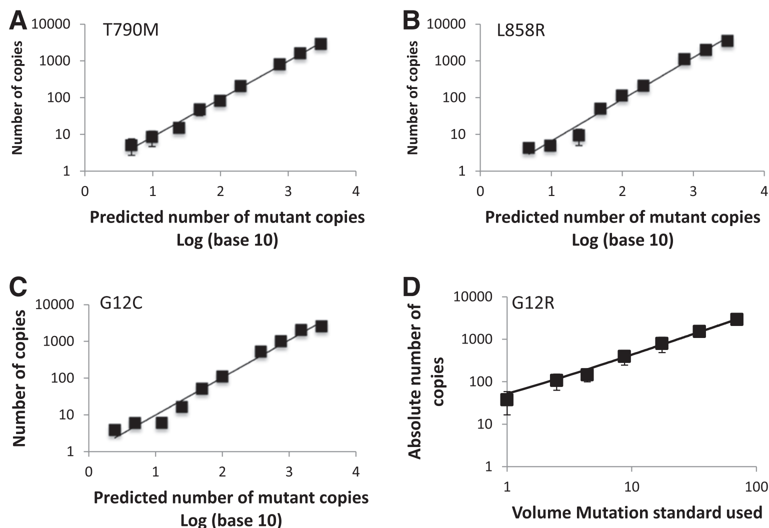

FIGURE 1. Validation of probes using reference DNA. The absolute number of copies of the *EGFR* locus was estimated in cell line DNA from cell lines known to have specific genotypes—H1975 (WT *KRAS*, mut *EGFR*); H23 (mut *KRAS*—G12C, WT *EGFR*). For each locus (*A*–*C*), increasing concentrations of mutant DNA from a cell line were assayed in a consistent background of 3000 copies of wild-type DNA. Experiments were performed in triplicate and are presented as the mean ± SD. For G12R (*D*) and *EGFR* exon 19 deletion, increasing volumes of a commercially available reference standard was used to establish efficacy of detection in an excess of WT molecules.

digital PCR has been demonstrated to be more precise and accurate than quantitative PCR, particularly when template DNA is dilute.[10,11]

Microdroplet digital PCR (MdPCR) is a form of digital PCR in which the "partitions" are microdroplets. In each single reaction up to 15,000 to 20,000 microdroplets are created.[12] The protocol is simple, economic, and it uses readily available, standard, fluorescently labelled locus-specific probes. Together with the general advantages of digital PCR, these attributes make mdPCR ideally suited to the analysis of ctDNA, in which the target is generally present at a low mutant allele frequency (MAF) in an excess of wild-type cfDNA.

In this study, we demonstrate that mdPCR can be used to detect and quantify ctDNA with high specificity in plasma from patients with lung cancer. We further demonstrate that it can be used to detect and quantify mutations in diagnostic specimens, track the response to treatment, and detect the

TABLE 1. Experiments to Establish Threshold Values for a Positive Assay

Patient	Genotype		Test Probe	+mut	+wt	MAF	Threshold for +ve
	EGFR	KRAS					
8	WT	G12C	EGFREx19del	0	1585	0	≥1
23	WT	WT	EGFREx19del	0	3997	0	≥1
31	WT	WT	EGFREx19del	0	726	0	≥1
33	WT	WT	EGFREx19del	0	1068	0	≥1
8	WT	G12C*	EGFRT790M	0	179	0	>1
12	WT	WT	EGFRT790M	0	544	0	>1
17	WT	G12V*	EGFRT790M	0	960	0	>1
23	WT	WT	EGFRT790M	1	4649	0.0002	>1
29	WT	G12A	EGFRT790M	1	2746	0.0004	>1
31	WT	WT	EGFRT790M	0	904	0	>1
33	WT	WT	EGFRT790M	1	576	0.002	>1
12	WT	WT	KRAS G12C	0	216	0	>1
23	WT	WT	KRAS G12C	1	4486	0.0002	>1
31	WT	WT	KRAS G12C	1	1224	0.0008	>1
32	WT	WT	KRAS G12C	0	585	0	>1
12	WT	WT	KRAS G12R	0	602	0	≥1
23	WT	WT	KRAS G12R	0	5559	0	≥1
31	WT	WT	KRAS G12R	0	738	0	≥1
32	WT	WT	KRAS G12R	0	88	0	≥1
33	WT	WT	KRAS G12R	0	572	0	≥1

Establishing probe specificity. The primer/probes were tested against the equivalent of 500 µl of plasma DNA extracted from enrollment (pretreatment) bloods. Patients were selected if their diagnostic biopsy was genotyped as negative for the corresponding mutation. * indicates lower volume plasma used.

MAF, mutant allele frequency; WT, wild type .

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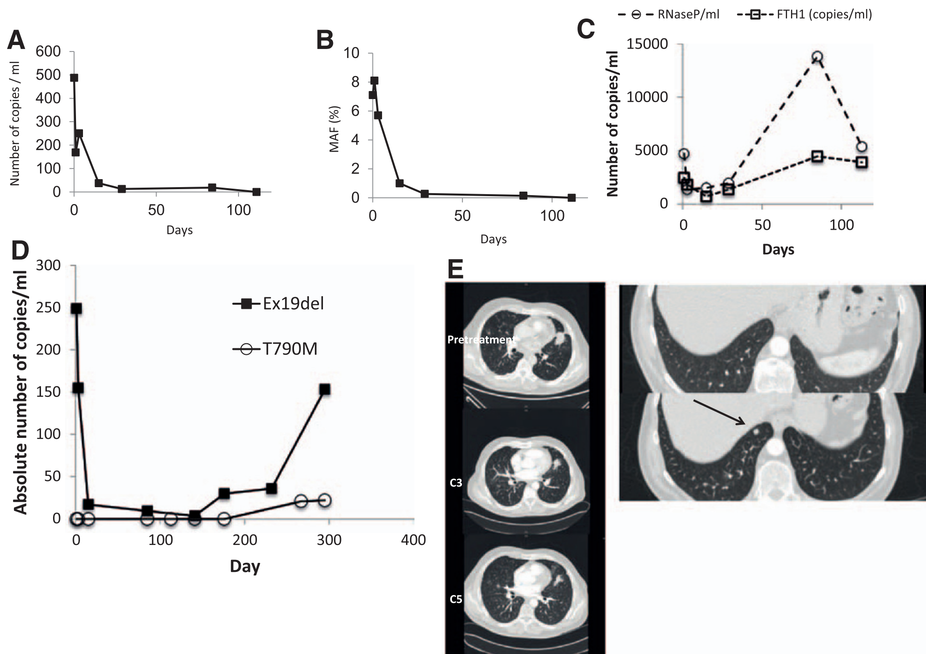

FIGURE 2. Patient 11. *A*, *B*, Analysis of serial plasma samples demonstrates a rapid fall in absolute numbers of *EGFR* exon 19 mutational events and MAF. *C*, The absolute numbers of copies of two reference autosomal loci are also shown and are noninformative when compared with the specific assay. *D*, By month 6, the exon 19 deletion was again detectable, and by month 10 T790M was detected for the first time in the peripheral blood. Serial CT scans on commencing treatment, after cycle 3 and on progression are shown (*E*) and demonstrate an initial partial response of the assumed primary (open arrow). This lesion did not increase in size but new pulmonary nodules appeared consistent with progressive disease. MAF, mutant allele frequency; CT, computed tomography.

emergence of resistance-conferring mutations in the peripheral blood.

PATIENTS AND METHODS

Subjects and Clinical Specimens

Patients with stage IIIb/IV adenocarcinoma of the lung were recruited from the lung oncology clinic at our UK cancer center. Three patients who had mutations in *EGFR* or *KRAS*, and in whom serial blood samples were available, were selected for the analysis.

Serial blood samples were collected and plasma prepared as per the National Institutes of Health guidelines.[13] Aliquots were stored at −80°C until further processing. CfDNA was extracted from frozen plasma using the QIAamp Circulating Nucleic Acid kit (Qiagen, Manchester, UK).

DNA was extracted from formalin-fixed paraffin-embedded blocks and cell lines using the DNeasy Blood and Tissue Kit (Qiagen). For control DNA, including positive controls for *EGFR* exon 19 deletion (ex19del) and *KRAS* G12R, a commercially available reference standard was used (Horizon Diagnostics, Cambridge, UK).

Microdroplet Digital PCR

Details of mdPCR are published and summarized in the Supplementary Information (Supplemental Digital Content 1, http://links.lww.com/JTO/A703). All reactions were prepared using the ddPCR Supermix for probes (BioRad, Hertfordshire, UK). Details of cycling conditions are documented in Supplementary Information (Supplemental Digital Content 1, http://links.lww.com/JTO/A703).

Results were "read" using the QX100 optical reader and processed using QuantaSoft (BioRad) software. Each droplet was scored as positive or negative based on the relative fluorescence intensity in a FAM or VIC/HEX channel. The number of positive microdroplets was then recorded. The detail of scoring of microdroplets is in the Supplementary Information (Supplemental Digital Content 1, http://links.lww.com/JTO/A703).

Absolute concentrations of DNA for each sample were estimated using mdPCR assays for reference genes RNaseP (*RPPH1*) and *FTH1*.

Primers/Probesets

All assays used were purchased directly from BioRad except for RNaseP (gene *RPPH1*), *FTH1*, and *EGFR* exon 19 deletion (Life Technologies, Grand Island, NY). Of note the Life Technologies' exon 19 deletion probe (Assay Reference Hs00000228_mu; Life Technologies) detects 19 separate exon 19 deletions from c.2235 to c.2258 including all of the commonly reported variants. Details of all probesets used are in the Supplementary Information (Supplemental Digital Content 1, http://links.lww.com/JTO/A703).

RESULTS

First, we tested the ability of mdPCR to detect and quantify specific mutant alleles in an excess of wild-type DNA. Clinically relevant mutant alleles were tested including activating *EGFR* mutations: ex19del and L858R, the resistance-conferring mutation *EGFR* T790M, and various activating mutations in *KRAS* including G12R and G12C. In each case, the expected linear relationship between the number of copies predicted and the number of copies detected was observed (Fig. 1).

If an assay is to be used to detect rare variants in plasma, then a key performance variable is the false-positive rate in plasma-derived DNA. Patients with genotyped tumors that were negative for specific mutations were chosen as "negative" controls. For each patient, it was impossible to be certain a priori that the specific mutation of interest was truly absent in

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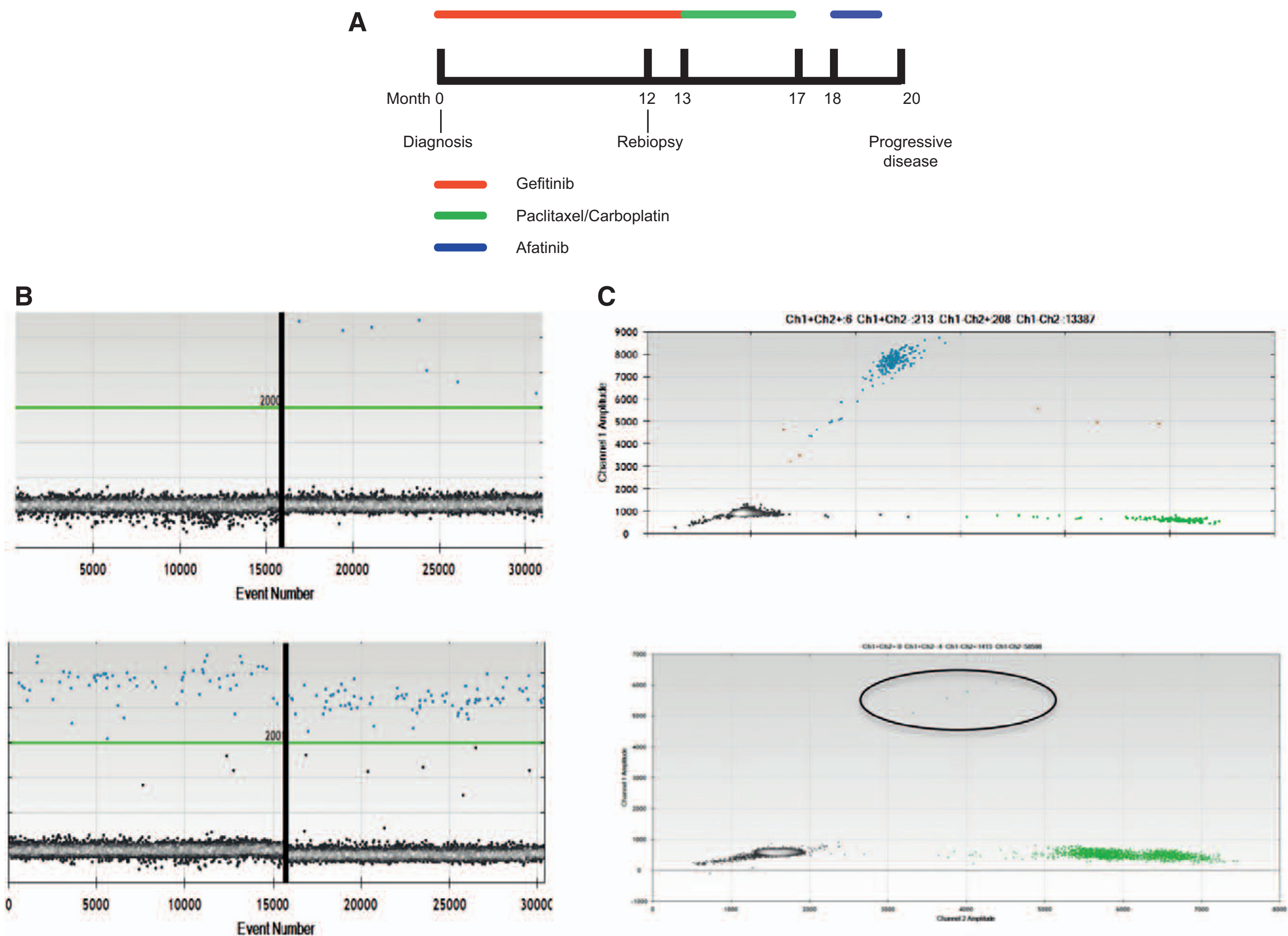

FIGURE 3. Patient 5. This patient's treatment course is illustrated. He was recruited to the study in month 17 after discontinuing paclitaxel and carboplatin (*A*). *EGFR* ex19del re-emerged in the peripheral blood a month later. For this assay, separate FAM-labelled probes for the mutation (*upper panel*) and wild type (*lower panel*) were used in separate reactions (Life Technologies, EGFR_6240_wt/mu) (*B*). Subsequent analysis of a rebiopsy using mdPCR (*C*) demonstrated a T790M mutation at a MAF of 17% (*upper panel*) and this mutation was detected in the peripheral blood at month 20 to 2 months after afatinib was discontinued. Of note, the T790M mutation was also detected at month 19, but at a very low MAF. These data illustrate how circulating biomarkers may be useful to guide treatment decisions in patients on complex therapeutic regimes. In (*C*) two differently labeled probes are tested in a single reaction (BioRad), one to detect the mutation (FAM label—blue) and the other to detect the wild-type allele (HEX label—green). mdPCR, microdroplet digital polymerase chain reaction; MAF, mutant allele frequency.

either the primary tumor or the peripheral blood. In each case, a threshold value for the limit of detection of the assay was established that depended on the number of positive events detected per 500 µl of plasma in a series of control plasmas. For ex19del, no positive events were detected in control samples. A positive result was therefore defined as any result with more than one positive microdroplet per 500 µl. For *KRAS* G12R, *KRAS* G12C, and *EGFR* T790M, a maximum of 0, 1, and 1 events were detected per 500 µl of plasma respectively. The performance characteristics of each assay are summarized in Table 1.

We proceeded to test the performance of these assays in patients with lung cancer that had been genotyped as part of their clinical standard-of-care and shown to have mutations in *KRAS* or *EGFR*. Three patient cases are presented to illustrate including a patient with an activating mutation in *KRAS* and two with *EGFR* mutations.

Case 1

Patient 11 presented with advanced non–small-cell lung cancer (adenocarcinoma, T3N3M1b) with metastases to bone and brain. Genotyping revealed an activating deletion in *EGFR* exon 19 (18 base pairs deletion, c.2240–2257 del18). Erlotinib was commenced and resulted in a marked clinical improvement and a partial response on Response Evaluation Criteria in Solid Tumors criteria. DNA was extracted from both the diagnostic biopsy specimen and serial plasma samples (Fig. 2*A*–*C*). The deletion was readily detected in the diagnostic biopsy using mdPCR at a MAF of 33% (Supplementary Figure 1, Supplemental Digital Content 2, http://links.lww.com/JTO/A704). Serial testing for the T790M mutation was negative until month 11, although an increase in the exon 19 deletion was noted in month 6. The patient had no symptoms suggestive of relapse, but subsequent imaging confirmed progressive disease (Fig. 2*D*, *E*).

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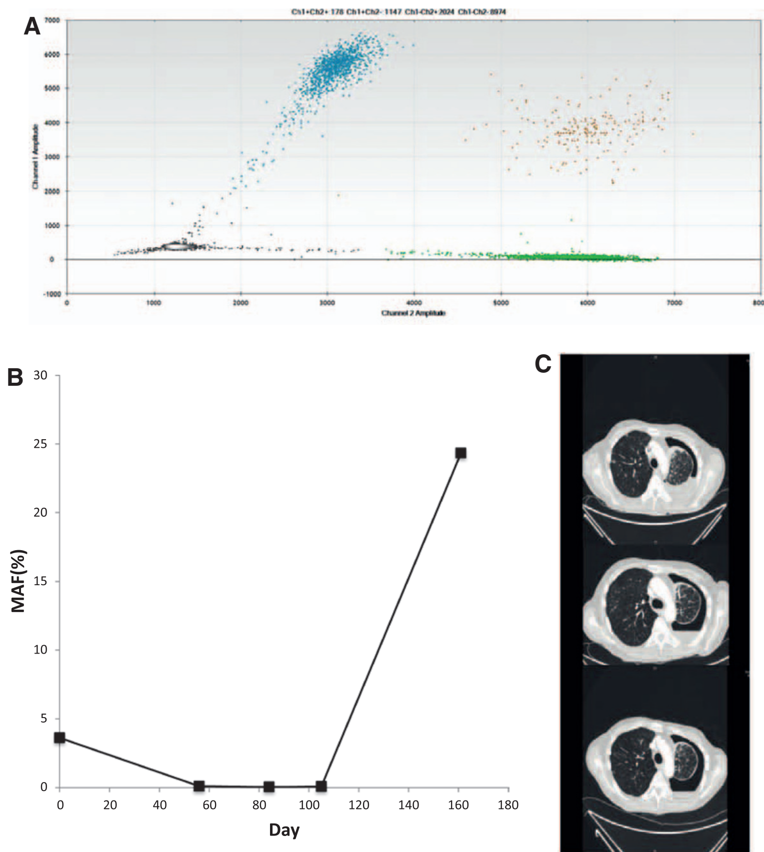

FIGURE 4. Patient 16 presented with stage IV lung cancer diagnosed from pleural fluid cytology and was clinically genotyped as having the *KRAS* G12R mutation. DNA extracted from pleural fluid cells was reanalyzed using the mdPCR protocol giving a MAF of 28% (*A*). Serial plasma samples from patient 16 were analyzed and a clear initial biomarker response to treatment demonstrated, with subsequent dramatic progression (*B*). Interestingly, the patient had been stable postchemotherapy at least until day 133, to a rapid clinical progression reflecting the biomarker rise, although there was not overt radiological progression. mdPCR, microdroplet digital polymerase chain reaction; MAF, mutant allele frequency.

Case 2

Patient 5 presented with advanced non–small-cell lung cancer with bone metastases (adenocarcinoma, T4N3M1b). An activating mutation in exon 19 of *EGFR* (c.2235_2249 del15) was detected on the diagnostic endobronchial biopsy. Gefitinib was prescribed with initial effect, but there was clinical and radiological progression by month 13. This prompted a rebiopsy of a cervical lymph node and second-line treatment (carboplatin/paclitaxel), which had to be discontinued after 3 months because of toxicity (Fig. 3). At this point (month 17), the patient was enrolled in the current study. Third-line afatinib was introduced at month 18 but discontinued because of clinical progression.

The ex19del was undetectable in the first available plasma at month 17 after four cycles of carboplatin/paclitaxel, but after only 1 month off chemotherapy was detectable at a MAF of 7% (Fig. 3*B*). T790M was detected in the rebiopsy and in the peripheral blood after clinical progression (month 20) (Fig. 3*C*).

Case 3

Patient 16 presented with advanced non–small-cell lung cancer (TXN0M1b) diagnosed on pleural fluid cytology and associated with an activating *KRAS* G12R mutation. Treatment was initiated (four cycles of pemetrexed and carboplatin) with an apparent clinical improvement and stable disease on Response Evaluation Criteria in Solid Tumors criteria. However, 9 weeks after the final cycle of chemotherapy, the patient progressed clinically although not radiologically. He was not well enough for further treatment.

DNA extracted from the pleural specimen was assayed using mdPCR for the *KRAS* G12R mutation (Fig. 4). Serial plasma analysis demonstrated an initial fall in both absolute numbers of copies of the mutation detected and MAF with treatment. The re-emergence of the mutation at a significantly higher MAF correlated with marked clinical progression.

DISCUSSION

Lung cancer is a disease that generally presents late and for which many treatments are of limited efficacy. Standard follow-up consists of clinical and radiological monitoring. The noninvasive monitoring of tumor burden and specific mutational events is attractive to both clinicians and patients. In this study, we address the ability to monitor the response to intervention and the progression of disease on or off therapy using an inexpensive and simple protocol to perform surveillance of known mutations.

We show that mdPCR, using readily available fluorescently labelled probes, can be used to detect and accurately quantify mutational events in DNA derived from archived biopsies and plasma. We show that it can be used to track the response to both targeted therapy and more traditional chemotherapy. Finally, we show that relapse/progression can be detected noninvasively and that the mechanism of resistance can be inferred by the detection of resistance-causing mutations in the peripheral blood. These data illustrate how real-time treatment decisions may be informed by circulating biomarkers. The work is consistent with that of others showing

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that serial analysis of tumor biomarkers in plasma may have an important role in the clinic.[4–6]

In case 1, the re-emergence of the *EGFR* exon 19 deletion and the T790M resistance-conferring mutation was detected before any symptoms and before any evidence of radiological progression. Of note, subsequent imaging investigations showed that the presumed index lesion had not progressed, but new nodules had appeared. In case 2, T790M was detected after 1 month off second-line paclitaxel/carboplatin chemotherapy and again after disease progression on afatinib.

The role of ctDNA biomarkers in the management of lung cancer requires further exploration. In addition to the potential for documenting treatment response and the early detection relapse or progression, numerous other questions are raised. For example, these cases raise the issue of the relationship between ctDNA biomarkers and the degree of radiological response (partial versus complete) or the clinical implications of biomarker persistence throughout various targeted and nontargeted therapeutic strategies.

Multiple platforms have been proposed for the analysis of ctDNA, all with different attributes. Next generation sequencing-based protocols have important advantages in terms of sequencing power and the high level multiplexing possible, meaning that genomic hotspots can be readily sequenced in an unbiased manner; but they are more complex protocols and are still relatively costly. There are a number of locus-specific PCR/probe-based approaches in which preselected mutational events are interrogated.[8,14]

MdPCR is a simple protocol with easily interpretable results that may be ideally suited to tracking specific mutations in serial blood samples. Although this article was in preparation, Oxnard et al.[14] reported a similar approach in a larger series using custom-designed primers/probes with different performance characteristics. For example, the "off-the-shelf" *EGFR*19del probe used in this study may well be more specific. Primer/probe selection for optimum specificity will be key to any translation of this approach to the clinic.

These studies, showing similar results and efficacy in different populations, suggest that mdPCR-based analysis of ctDNA has the potential to aid rational clinical decision making in lung cancer patients with known mutational genotypes and that prospective validation trials of this approach are warranted.

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