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Development of a gene panel for next-generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients

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Background: When tumour tissue is unavailable, cell-free DNA (cfDNA) can serve as a surrogate for genetic analyses. Because mutated alleles in cfDNA are usually below 1%, next-generation sequencing (NGS) must be narrowed to target only clinically relevant genes. In this proof-of-concept study, we developed a panel to use in ultra-deep sequencing to identify such mutations in cfDNA.

Methods: Our panel ('SiRe') covers 568 mutations in six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT* and *PDGFRα*) involved in non-small-cell lung cancer (NSCLC), gastrointestinal stromal tumour, colorectal carcinoma and melanoma. We evaluated the panel performance in three steps. First, we analysed its analytical sensitivity on cell line DNA and by using an artificial reference standard with multiple mutations in different genes. Second, we analysed cfDNA from cancer patients at presentation ($n=42$), treatment response ($n=12$) and tumour progression ($n=11$); all patients had paired tumour tissue and cfDNA previously genotyped with a Taqman-derived assay (TDA). Third, we tested blood samples prospectively collected from NSCLC patients ($n=79$) to assess the performance of SiRe in clinical practice.

Results: SiRe had a high analytical performance and a 0.01% lower limit of detection. In the retrospective series, SiRe detected 40 *EGFR*, 11 *KRAS*, 1 *NRAS* and 5 *BRAF* mutations (96.8% concordance with TDA). In the baseline samples, SiRe had 100% specificity and 79% sensitivity relative to tumour tissue. Finally, in the prospective series, SiRe detected 8.7% (4/46) of *EGFR* mutations at baseline and 42.9% (9/21) of *EGFR* p.T790M in patients at tumour progression.

Conclusions: SiRe is a feasible NGS panel for cfDNA analysis in clinical practice.

Precision medicine, coupled with the tissue-based assessment of biomarkers predictive of treatment outcome, has transformed pathology practice (Papadopoulos *et al*, 2006). *RAS* and *BRAF* mutation testing in colorectal cancer (CRC; Di Nicolantonio *et al*, 2008; Lièvre *et al*, 2008), *EGFR* in non-small-cell lung cancer (NSCLC; Lynch *et al*, 2004) *BRAF* in melanoma (Chapman *et al*, 2011) and *cKIT* and *PDGFRα* in gastrointestinal stromal tumours (GIST; Antonescu, 2008) has added a genotypic element to the

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phenotypic diagnostics of solid tumours. However, tumour tissue is not always available or may be insufficient for molecular testing, especially when cancer is diagnosed at advanced stages on small biopsy specimens. On other occasions, due to tumour location or small size, tissue sampling can be challenging and risky, particularly in extensively treated patients. As an alternative to cancer tissue, predictive biomarkers can be non-invasively assessed in cell-free DNA (cfDNA; Schwarzenbach *et al*, 2011; Crowley *et al*, 2013).

Using a Taqman-derived assay (TDA) we previously identified *EGFR* mutations in NSCLC (Karachaliou *et al*, 2015) and *BRAF* mutations in melanoma patients (Gonzalez-Cao *et al*, 2015) with a specificity of 100% and with sensitivities of 69% and 78%, respectively. One of the factors contributing to this high sensitivity was the concomitant analysis, in each patient, of serum- and plasma-derived cfDNA (Karachaliou *et al*, 2015; Gonzalez-Cao *et al*, 2015). This performance may be further improved by next-generation sequencing (NGS), which can be multiplexed across several genes to cover less common and even novel variants (Malapelle *et al*, 2016a). Large gene panels or whole-exome approaches to screen for a large number of genomic regions may not be easily implemented in cfDNA analysis (Cancer Genome Atlas Research Network, 2014). Conversely, small NGS panels tailored to target a limited number of actionable genes can be an effective tool in daily clinical practice (Paweletz *et al*, 2016). This strategy, known as 'ultra-deep sequencing', can significantly increase sensitivity, which is essential considering that circulating tumour DNA represents only a small fraction ($<0.5\%$) of the total cfDNA (Mead *et al*, 2011) in most patients with solid tumours. Since the low threshold levels of mutant alleles required to detect clinically relevant alterations may easily lead to false-positive results (van Dijk *et al*, 2014), implementation of the ultra-deep sequencing of cfDNA in the clinical setting must be validated in terms of blood collection, cfDNA extraction, automated library preparation, sequencing and variant calling (Gargis *et al*, 2012; Malapelle *et al*, 2016c).

In this proof-of-concept study, we report the development, performance evaluation and implementation in a clinical setting of a narrow gene panel that targets 568 clinically relevant mutations in six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT* and *PDGFRα*) involved in non *S*mall cell lung cancer, gastro*I*ntestinal stromal tumour, metastatic colo*R*ectal carcinoma and m*E*lanoma (whose acronym is SiRe). This panel has a high sensitivity and specificity and enables the detection and quantification of mutations in cfDNA purified from the plasma and serum of patients with different types of solid tumours.

MATERIALS AND METHODS

Design of the SiRe panel. The Ion AmpliSeq Designer suite v5.3.1 with hg19 was used as reference genome to develop a customised panel targeting six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT* and *PDGFRα*) that are associated with treatment outcome in NSCLC, GIST, CRC and metastatic melanoma (Lynch *et al*, 2004; Antonescu, 2008; Di Nicolantonio *et al*, 2008; Lièvre *et al*, 2008; Chapman *et al*, 2011). A single primer pool leading to the selection of 42 amplicons (ranging from 125 to 175 bp) enabled us to cover all COSMIC annotated mutations ($n=568$) in the selected exons of the target genes. The complete reference range of SiRe is reported in Supplementary Material (Supplementary Table S1). The amplicon design (available on request) covering 5.2 kb of genomic DNA was optimised for the simultaneous analysis of 16 samples with the 316v2 chip (Thermofisher, Foster City, CA, USA) on a Personal Genome Machine Torrent (Thermofisher).

Study design, patients and samples. The panel performance was evaluated in three steps (Figure 1). First, the analytical sensitivity of the assay was assessed on DNA from two cell lines and by using an artificial reference standard with multiple mutations in different genes. Second, clinical sensitivity and specificity was determined using archival cfDNA from 63 cancer patients (Table 1) with paired tumour tissue, previously genotyped with a TDA. As exploratory analysis, to confirm that our NGS approach cover the mutations in *cKit* and *PDGFRα* genes, two GIST samples (bloods and tissues) were tested with SiRe and the relative data are reported only in Supplementary Material. Third, the performance of the panel in daily clinical practice was assessed using blood samples prospectively collected from patients with advanced NSCLC. Written informed consent was obtained from all patients and documented in accordance with the general authorisation to process personal data for scientific research purposes from 'The Italian Data Protection Authority' (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/).

DNA purification. DNA from the two cell lines was isolated using the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Circulating-free DNA was purified as follows: 15 ml blood was withdrawn from patients and collected in Vacutainer tubes (BD, Plymouth, UK). Plasma and serum were isolated by centrifugation twice at 2300 r.p.m. for 10 min. The supernatant (serum or plasma) was aliquoted and used immediately for cfDNA isolation or stored at $-80\,^{\circ}C$. Cell-free DNA was purified from serum and plasma for each patient (1.2 ml). In the rare instances that the volume of the serum and plasma sample obtained from a patient was between 1 and 1.2 ml, PBS up to 1.2 ml was added to the samples, which were then purified using the QIAsymphony robot (Qiagen) and the QIAsymphony DSPVirus/Pathogen Midi Kit, according to the manufacturer's instructions, and cfDNA was eluted in a final volume of 30 μl. Since correct preanalytical handling of blood specimens is crucial to maintain the sample informative, the process was standardised (in terms of blood collection, sample centrifugation and cfDNA extraction) in the Department of Public Health of the University of Naples Federico II, and all procedures were performed in-house by a nurse belonging to the laboratory staff.

Sample sequencing. We analysed the serum and plasma cfDNAs of each patient enrolled in the study. Libraries were constructed and purified on the Ion Chef (Thermofisher), and eight samples (corresponding to 4 patients) were added per run. Library generation was as follows: 6 μl of cfDNA were dispensed on Ion Code plates and amplified using Ion AmpliSeq DL8 (Thermofisher). We used 22 cycles for cfDNA amplification and 6 cycles for library reamplification after barcoding, under the thermal conditions defined by the manufacturer. Purified libraries derived from eight cfDNA samples were diluted to 60 pM and combined with eight additional cfDNA-derived libraries to obtain a 16 Ion Code pooled library. The two-pooled libraries were re-loaded into the Ion Chef instrument, and templates were prepared using the Ion PGM Hi-Q IC Kit (Thermofisher). Finally, templates were loaded into the 316v2 chip and sequenced on PGM.

Data analysis. Signal processing and base calling were carried out using the default base-caller parameters on Torrent Suite [v.5.0.2] and coverage analysis was performed using SiRe designed bed files with coverage plug-in (v.5.0.2.0). BAM files were visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman,MT, USA). Variants were automatically annotated using variant caller plug-in (v.5.0.2.1) at specific optimised parameters of the SiRe

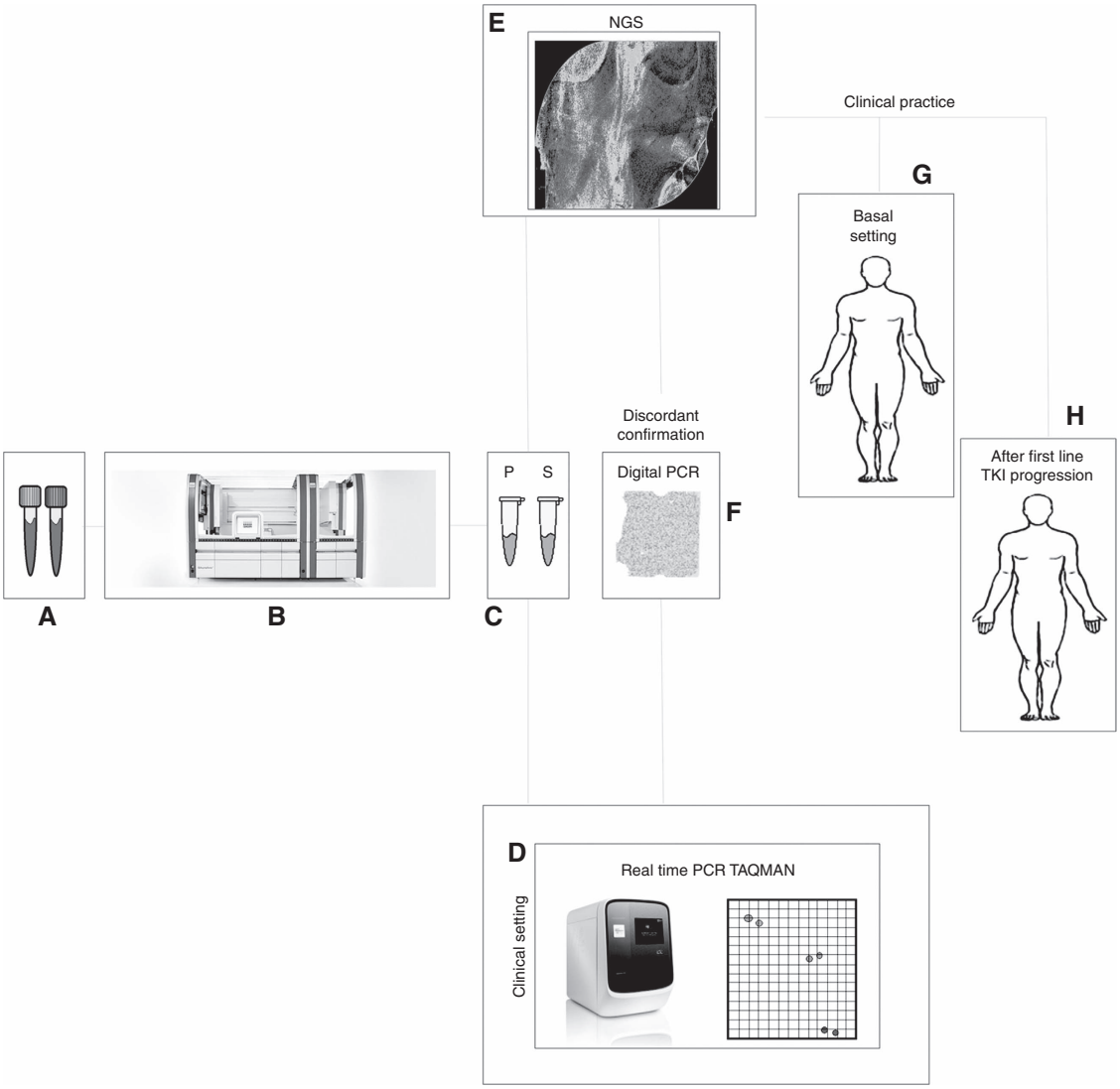

Figure 1. Study design. cfDNAs (**A**) extracted with the QIAsymphony virus/pathogen kit (**B**) from paired (P) plasma and (S) serum (**C**) samples were analysed by quantitative 5′-nuclease TaqMan PCR (**D**) and by the NGS SiRe panel (**E**). Any discordance between the two techniques was evaluated by dPCR (**F**). After preclinical validation, the SiRe panel was applied in clinical practice in cases in which tissues were not available to select patients for TKI treatment, at baseline (**G**), and to evaluate the selection of resistant clones after disease progression (**H**).

panel (Supplementary Table S2). In particular, only variants with $\geqslant$5X allele coverage and a quality score $\geqslant$20, within an amplicon that covered at least 1000X alleles, were called, and the frequency of each mutant allele was recorded.

Preclinical assessment. Genomic DNA from the HCC827 (*EGFR* p.E746-A750del; *KRAS* wt) and A549 (*EGFR* wt; *KRAS* p.G12S) cell lines was used to assess analytical performance. Both cell lines were obtained from the National Research Council/Institute of Experimental Endocrinology and Oncology on courtesy of Dr Pierlorenzo Pallante (Naples, Italy). The analytical sensitivity of the assay for point mutation and indel detection was determined by diluting DNA from the appropriate mutated cell line (A549 for point mutations and HCC827 for indels) into increasing concentrations of DNA from the appropriate wt cell line (HCC827 for point mutations and A549 for indels). DNA dilutions ranged between 1 : 10 and 1 : 10 000, which correspond to allelic fractions from 1 : 20 to 1 : 20 000 of the mutated allele (both cell lines are heterozygous). Each dilution was analysed in duplicate to estimate inter-run assay reproducibility, and the library obtained from each dilution was sequenced twice to evaluate intra-run assay reproducibility. In addition, customised Horizon Diagnostics Multiplex gDNA reference standard, with mutation in *EGFR* (p.E746_A750del and p.G719S), *KRAS* (p.G12D), *NRAS* (p.Q61L) and *BRAF* (p.V600E), each of them at three different dilution points (1, 0.5 and 0.1%), were assessed to provide stronger evidence on SiRe analytical performance.

Clinical validation. We determined the specificity and sensitivity of our assay by analysing archival serum and plasma cfDNA from 40 cancer patients at presentation attending the Quiron Dexeus University Hospital (33 NSCLC, 2 CRC and 5 metastatic melanoma) with paired tumour tissue. In addition, we tested archival serum and plasma cfDNAs from 12 responder patients and 11 patients at the time of tumour progression after treatment (18 NSCLC, 2 CRC and 3 metastatic melanoma; Table 1). All of the 63 cfDNA samples and tumour tissues had previously been genotyped for *EGFR*, *KRAS*, *NRAS* and *BRAF* mutations using a TDA (Gonzalez-Cao *et al*, 2015; Karachaliou *et al*, 2015). In the case of tumour tissues, genotyping had been confirmed by standard

PCR followed by Sanger sequencing. Cases showing discordance between the NGS SiRe panel and the TDA were further investigated by digital PCR (dPCR) on a QuantStudio 3D Digital PCR System platform (Thermofisher) as previously described (Malapelle *et al*, 2016b).

Performance of the SiRe panel in prospective clinical samples. To evaluate the performance of the SiRe panel in the clinical setting, we prospectively genotyped 79 advanced NSCLC patients (37 men and 42 women; mean age: 65 years) using blood samples collected at the Department of Public Health of the University of Naples Federico II. According to the European Medicines Agency guidelines, mutations related to *EGFR* disease were tested in patients when tissue was not available at presentation ($n=46$), or at tumour progression ($n=33$) in patients previously treated with erlotinib ($n=14$), gefitinib ($n=14$) or afatinib ($n=5$) in the attempt to detect the emergence of resistance secondary mutations. In 21 of the 33 cases with tumour progression, first-line TKI administration had been based on the demonstration of an *EGFR* mutation in tissue, whereas in the remaining 12/33 cases, TKI treatment had been administrated in second line without evidence of *EGFR* mutations.

Table 1. Characteristics of the patients included in the retrospective (left) and prospective (right) clinical validation of the SiRe panel

Clinical characteristics	Retrospective validation ($N=63$)	Prospective validation ($N=79$)
Age		
<29–60	22 (34.92%)	22 (27.85%)
<61–80	25 (39.68%)	57 (72.15%)
Unknown	16 (25.40%)	
Sex		
Male	24 (38.10%)	37 (46.84%)
Female	24 (38.10%)	42 (53.16%)
Unknown	15 (23.80%)	
Smoking status		
Never smokers	11 (17.46%)	38 (48.10%)
Ex-smokers	9 (14.30%)	29 (36.70%)
Smokers	5 (7.93%)	6 (7.60%)
Unknown	38 (60.31%)	6 (7.60%)
Type of tumour		
Lung	51 (80.95%)	79 (100%)
Colorectal carcinoma	4 (6.35%)	
Metastatic melanoma	8 (12.70%)	
Stage		
IIIB–IV	48 (76.20%)	79 (100%)
Unknown	15 (23.80%)	
Histology		
Adenocarcinoma	35 (55.55%)	79 (100%)
Large cell carcinoma	1 (1.60%)	
Undifferentiated carcinoma	4 (6.35%)	
Metastatic melanoma	8 (12.70%)	
Unknown	15 (23.80%)	
Somatic alterations		
EGFR mutations	32 (50.79%)	25 (31.65%)
KRAS mutations	15 (23.80%)	
BRAF mutations	7 (11.11%)	
NRAS mutations	1 (1.60%)	
No mutations	8 (12.70%)	
Type of sample		
Pretreatment	40 (63.50%)	46 (58.23%)
Response evaluation	12 (19.04%)	33(41.77%)
TKIs	8 (66.70%)	33 (41.77%)
Chemotherapy	4 (33.30%)	
Progressive disease	11 (17.46%)	
TKIs	9 (81.81%)	
Chemotherapy	2 (18.19%)	

Abbreviation: TKIs = thyrosine kinase inhibitors.

RESULTS

Panel design and preclinical performance evaluation. The SiRe panel was designed to cover 568 clinically relevant mutations in six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT* and *PDGFRα*) involved in NSCLC, GIST, CRC and metastatic melanoma (see Supplementary Table S1). The panel was intended for use in cfDNA purified from patients with advanced cancer. On cell line derived DNA, the SiRe panel detected the *EGFR* deletion p.E746_A750del and the *KRAS* point mutation p.G12S at a level as low as one copy of the mutated allele in a background of 20 000 copies of wild-type alleles (0.005% mutated allele fraction), with 100% of intra- and inter-run reproducibility. In addition, regarding the results obtained on multiplex gDNA reference standard (Horizon Diagnostics), p.E746_A750del and p.G719S point mutation in *EGFR*, p.G12D mutation in *KRAS* exon 2, p.Q61L mutation in *NRAS* exon 3 and p.V600E mutation in *BRAF* exon 15 were correctly identified for each different dilution point.

This high analytical performance was achieved thanks to the use of optimised parameters set in variant caller plug-in (v.5.0.2.1) which detected low abundant mutated alleles with a specificity of 100% (see Supplementary Table S2).

Clinical sensitivity and specificity of the SiRe panel in cfDNA samples. The retrospective series of cfDNAs (Supplementary Table S3) was constituted by 126 paired serum and plasma samples from 63 patients. In each run, up to 16 paired serum and plasma samples from eight patients on 316v2 were processed. Run median output was 257Mbases, median read length was 124 bp, mean read depth was 2821 × and coverage uniformity was 97%. Technical performance data relative to each processed sample are reported in Supplementary Material (Supplementary Table S4). When the 63 samples were tested with the SiRe panel, the cfDNA of all eight patients with wild-type tumour tissue was negative (specificity 100%, CI 64.6-100%). In the remaining 55 patients with *EGFR, KRAS, NRAS* or *BRAF* mutations in tumour tissue, the SiRe panel detected the same mutation in the serum and/or plasma cfDNA in 46 cases (sensitivity 83.6%, CI 67.3–94.3%; Table 2).

Comparison of the SiRe panel with a TDA in cfDNA samples. We compared the performance of the SiRe panel for mutation analysis in cfDNA with that of a previously reported TDA (Karachaliou *et al*, 2015; Gonzalez-Cao *et al*, 2015) in 63 samples: (i) the 40 cfDNA samples obtained at presentation mentioned above; (ii) archival serum and plasma cfDNAs from 12 patients in response to different types of antitumour drugs; and 11 patients mutations in the cfDNA of 46 of 63 patients. The test was positive in both serum and plasma cfDNA in 35 patients (76.1%), positive in plasma but not in serum in 5 patients (10.9%), and positive in

Table 2. Concordance of Taqman-derived assay (TDA) and the SiRe panel NGS in retrospective serum and plasma cfDNA samples

	TDA (cfDNA)		
SiRe panel (cfDNA)	**Mut +**	**Mut −**	**Total**
Mut +	42	4	46
Mut −	0	17	17
Total	42	21	63

Abbreviations: cfDNA = cell-free DNA; NGS = next-generation sequencing.

serum but not in plasma in 6 patients (13%). An *EGFR* sensitising mutation and the p.T790M resistance mutation were detected simultaneously in 10 patients at progression to *EGFR* TKIs.

As reported in Table 2, there was a high concordance (Cohen's Kappa 0.85) between the results obtained with the NGS SiRe panel and the TDA, although the performance of the SiRe was slightly better. All 42 patients with mutation-positive cfDNA at TDA were also positive with the SiRe panel, and the 17 negative samples with the panel were also negative at TDA. In addition, NGS detected mutations in the cfDNA of four patients, whereas TDA did not. The mutations in these four patients appeared also in paired tumour tissue. One was a p.L597R mutation in *BRAF* not covered by the TDA, and was confirmed by dPCR (Supplementary Figure S2). The remaining three mutations were a p.L861Q mutation in *EGFR* and two *KRAS* mutations, p.G12C and p.G12A. Both TDA and NGS using the SiRe panel enable quantification of the mutated alleles (Figure 2). There was a significant correlation in the levels of serum cfDNA between the two techniques ($r = 0.64$). In contrast, correlation was lower in the case of plasma ($r = 0.35$), but improved significantly when three outlier samples were removed ($r = 0.61$).

Evaluation of the SiRe panel for prospective analysis of clinical samples. The performance of the SiRe panel in the clinical setting was evaluated by prospectively testing the serum and plasma cfDNA of patients with advanced NSCLC for whom no tissue was available in order to select them for TKI treatment. Seventy-nine patients were tested, 46 at presentation and 33 at the time of tumour progression after first-line TKI treatment (Table 1). The NGS procedure was adequate for variant calling in the 79 cfDNA paired serum and plasma samples. The run metrics parameters were not dissimilar from those of the retrospective samples. In fact, in prospective cfDNA samples, the median output was 210Mbases, the median read length 125.57 bp, the mean read depth 3385.45 and coverage uniformity 97.49%. Among the 46 patients analysed at baseline (Supplementary Table S5), we detected four *EGFR* mutations (8.7%), one point mutation in exon 18 (p.G719A), two deletions in exon 19 (both p.E746_A750delELREA) and one insertion in exon 20 (p.H773-V774insH). In all four patients, the mutant alleles were detected in both serum and plasma cfDNA and were confirmed by digital PCR (data not shown).

Regarding samples at progression (Supplementary Table S6), the SiRe panel did not detect mutations in 12 patients, whose tissues had been identified as *EGFR* wild type in biopsies at presentation. In contrast, among the 21 patients *EGFR* positive in baseline tissue, the SiRe panel confirmed the same mutation in cfDNA in 19 cases (Table 3). Thus, sensitivity and specificity in this cohort of patients at progression were within the range of those observed in the retrospective cohort. Interestingly, in 9 of those 19 cases (47%), we observed the emergence of the *EGFR* p.T790M mutation in addition to the original *EGFR* activating mutation. The appearance of *EGFR* p.T790M mutation in relation to TKIs treatment regimen was reported in Figure 3. Of the 28 mutations (sensitising + p.T790M) detected, 10 (35.70%) were present in both serum and plasma, 7 (25%) in plasma alone and 11 (39.3%) in serum alone. All mutations detected by the SiRe panel at progression were confirmed by dPCR.

DISCUSSION

In this proof-of-concept study, we demonstrate that the performance of ultra-deep sequencing using a narrow NGS panel on Ion Torrent PGM is excellent, and that this procedure can be used for the routine testing of relevant tumour mutations in cfDNA. The high sensitivity (90.5%) and analytical specificity (100%) of this panel equal or even surpass those of such other procedures as real-time PCR-based methods. Unlike earlier NGS applications that cover large genomic regions (Cancer Genome Atlas Research Network, 2014), our small gene panel (5.2 kb) focuses on biomarkers that are currently used in the clinical setting.

The ultra-deep sequencing procedure reported herein has various advantages. In fact, using a single panel, we were able to detect up to 568 relevant mutations in six genes (*EGFR, KRAS, NRAS, BRAF, cKIT* and *PDGFRα*). These mutations included less common, but actionable variants such as the *BRAF* p.L597R mutation in melanoma (case #38 in Supplementary Table S3). Sequencing with the SiRe panel was more efficient than real-time PCR target techniques in detecting deletions ($n = 2$) and point mutations ($n = 6$) on cfDNA samples. In addition, NGS *per se* is a time-effective procedure for analysing large numbers of samples, thereby optimising the work flow in molecular pathology laboratories (Malapelle *et al*, 2016a). With our procedure, different types of samples (DNA from tumour tissues and cfDNAs from biological fluids) from patients affected by different types of diseases (e.g., NSCLC, GIST, CRC and melanoma) can be processed simultaneously. Consequently, sample batching is more effective and does not require a minimum number of a given tumour type. As a result, turnaround time (TAT) can be as short as three working days, as recommended by international guidelines (Lindeman *et al*, 2013). The recently developed Ion Chef automated library preparation station, which has a better procedure reproducibility and standardisation than manual procedures, also contributes to the short TAT (Malapelle *et al*, 2016a).

The Ion Torrent PGM protocols, panels and variant caller do not detect low abundant mutations diluted in a large amount of WT DNA. Therefore, we used several in-house strategies specifically tailored to cfDNA. Firstly, we reduced the number of genes and exons *vs* commercially available tests, and we modified the thresholds for variant calling, in particular all the variants with $\geqslant$5X allele coverage and a quality score $\geqslant$20, within an amplicon that covered at least 1000X alleles, were called (Supplementary Table S2).

We also adapted the Ion Chef template preparation protocol by pooling two 16-sample libraries in each run. Thus, using this well standardised procedure, we were able to sequence simultaneously up to 32 paired plasma/serum samples in less than 3 h on the PGM, with a consequent reduction in the total consumable cost. In a previous study (Malapelle *et al*, 2016a) we showed that by using a commercially available 22 gene panel (AmpliSeq Colon and Lung Cancer Panel) on the Ion Torrent PGM, the consumable cost was €196 per sample. Using the modified protocol that we developed in this current study the cost per sample was lowered to 98 euro for simultaneously analysis of six different genes. This is comparable with the cost of the most commercially available Real Time PCR based kits.

The simultaneous analysis of paired plasma/serum samples is a crucial feature of this new procedure since the sensitivity of somatic mutation analysis in cfDNA increases when serum and plasma are analysed together (Gonzalez-Cao *et al*, 2015; Karachaliou *et al*, 2015). Our results are in agreement with this finding. In fact, of the 89 patients found to carry mutations in cfDNA, 58 (65.17%) were positive in both serum and plasma, 15 (16.85%) in plasma alone and 16 (17.98%) in serum alone.

From the technical point of view, even when sequencing 16 samples simultaneously in a run, the SiRe panel had optimal run metrics in our daily clinical practice in terms of both mean depth reads and uniformity of coverage, which resulted in a high assay sensitivity in cfDNA *vs* tumour tissue (90.5%) and a specificity of 100%. This is a very high degree of concordance, particularly given the 91.7% concordance between paired surgical resection and cytological samples (Sun *et al*, 2013). Thanks to the high sensitivity of our assay, the *EGFR* mutational rate of 8.7% that we identified in NSCLC patients prospectively tested on cfDNA at baseline is in

keeping with previous data on tissue samples (Malapelle *et al*, 2013). Similarly, the frequency of the *EGFR* p.T790M mutation, which was detected in the cfDNA of 9 of 19 (47.4%) patients progressing after TKI treatment ($n=5$ gefitinib, $n=3$ afatinib, $n=1$ erlotinib), is in line with data obtained on tissues samples collected after disease progression (Karachaliou *et al*, 2015).

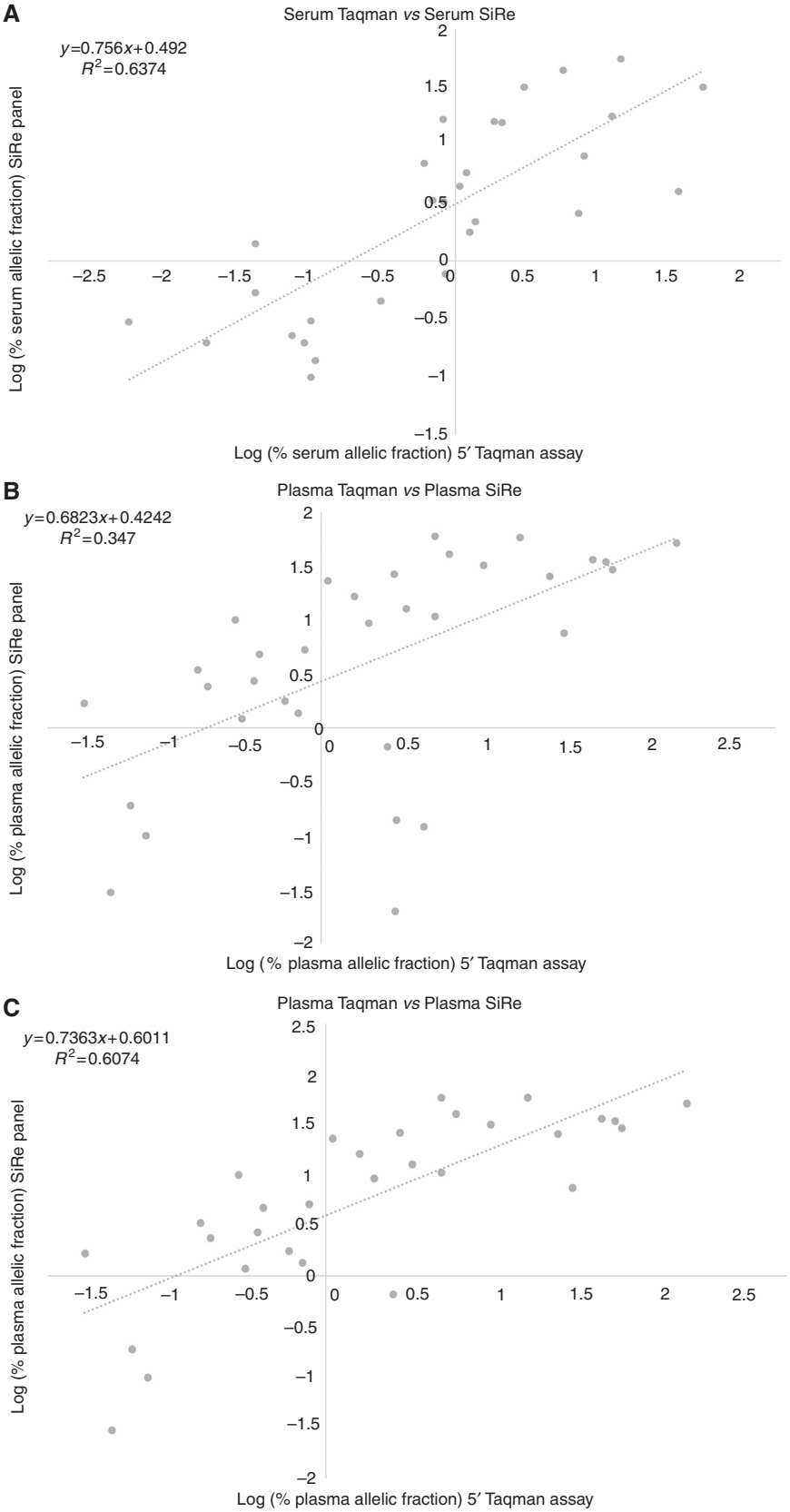

Figure 2. Quantification of mutated allele fractions. Comparison of the quantification of mutated allele fractions by Taqman Derived Assay *vs* SiRe NGS in serum (**A**) and plasma (**B**) cfDNA. In the case of plasma, three outliers were removed and results re-plotted (**C**).

The performance of our methodology compares favorably with that of NGS for mutational analysis in the blood of cancer patients. An Ion Torrent-derived sequencing of five genes in cfDNA purified from never smoking lung cancer patients achieved a modest 58% sensitivity and 87% specificity (Couraud *et al*, 2014). An analysis of 23 amplicons in five genes using cfDNA from breast cancer patients identified 10 mutations but missed 6 identified by droplet digital PCR (Guttery *et al*, 2015). When restricted to *EGFR*, deep sequencing achieved 61–80% sensitivity and 94–98% specificity in advanced NSCLC (Uchida *et al*, 2015). The 90.5% sensitivity of our assay also exceeds the 77% recently reported when NSCLC plasma-derived cfDNA was analysed on an Illumina NGS platform with a panel covering amplicons of 11 clinically relevant genes (Paweletz *et al*, 2016). Despite the variations inherent to the platforms used, such as the library preparation and the longer TAT (6 days), the Illumina-based NGS approach featured similar run metrics and analytical parameters as our assay, which supports the use of ultra-deep sequencing in the clinical setting (Paweletz *et al*, 2016). It is conceivable that the higher sensitivity achieved by our panel is due not only to technical differences but also to the simultaneous testing of serum and plasma in each patient.

Besides being an alternative to molecular diagnosis at presentation when tumour tissue is not available, liquid biopsy is also a noninvasive test with which to monitor response to targeted therapy and to detect the emergence of resistance mutations in genes such as *EGFR* (Sundaresan *et al*, 2016) and *ESR1* (Chu *et al*, 2016). Monitoring would consist in quantifying the mutant allelic fractions in cfDNA over time, which can be reliably assessed by our NGS assay. The SiRe panel detects the appearance of resistance mutations such as *EGFR* p.T790M (Figure 3). Finally, the non-synonymous mutation burden correlates with a good response to immunotherapy in NSCLC (Rizvi *et al*, 2015) and other tumours, and NGS has been proposed as a tool with which to design customised immunotherapies that target common driver mutations (Nielsen *et al*, 2016). Our panel, which covers several exons in frequently mutated genes, can be useful also in this setting.

In conclusion, we have developed and translate in clinical setting an NGS assay based on a narrow gene panel. The assay

Table 3. Comparison of the mutational status in FFPE tumour tissue at presentation with the results of the SiRe panel in archival cfDNA purified from serum and plasma baseline ($n = 42$, left) and at response or after tumour progression ($n = 23$, right)

	At presentation			At response or progression		
	TDA + Sanger (FFPE tumour tissue)			TDA + Sanger (FFPE tumour tissue)		
SiRe panel (cfDNA)	**Mut +**	**Mut −**	**Total**	**Mut +**	**Mut −**	**Total**
Mut +	28	0	28	18	0	18
Mut −	5	7	12	4	1	5
Total	33	7	40	22	1	23

Abbreviations: cfDNA=cell-free DNA; FFPE = formalin fixed paraffin embedded; TDA=Taqman-derived assay.

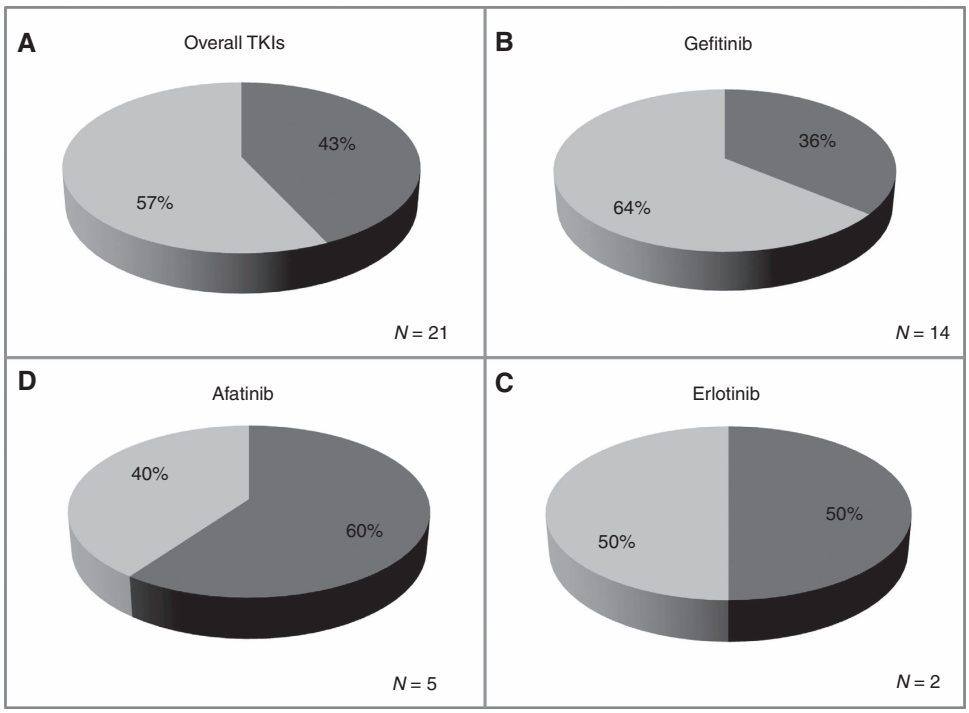

Figure 3. Frequency of the *EGFR* p.T790M mutation (green: T790M −; red T790M +) after progression to thyrosine kinase inhibitors (TKIs) in the serum and plasma cfDNA of EGFR-mutated patients evaluated with SiRe panel NGS. A full colour version of this figure is available at the *British Journal of Cancer* journal online.

detects relevant mutations in cfDNA purified from the serum and plasma of patients with the tumours most commonly tested for molecular alterations (such as NSCLC, CRC and metastatic melanoma). The SiRe panel has excellent sensitivity and specificity, and is hence suitable for testing blood samples in the clinical setting. Finally, it enables the application of NGS on a prospective basis in daily molecular predictive pathology practice, particularly when tumour tissue is not available, and is a tool with which to monitor disease course.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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