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Biology of Human Tumors

Clinical Cancer Research

Noninvasive Diagnosis of Actionable Mutations by Deep Sequencing of Circulating Free DNA in Lung Cancer from Never-Smokers: A Proof-of-Concept Study from BioCAST/IFCT-1002

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Abstract

Purpose: Tumor somatic mutation analysis is part of the standard management of metastatic lung cancer. However, physicians often have to deal with small biopsies and consequently with challenging mutation testing. Circulating free DNA (cfDNA) is a promising tool for accessing the tumor genome as a liquid biopsy. Here, we evaluated next-generation sequencing (NGS) on cfDNA samples obtained from a consecutive series of patients for the screening of a range of clinically relevant mutations.

Experimental Design: A total of 107 plasma samples were collected from the BioCAST/IFCT-1002 lung cancer study (never-smokers cohort). Matched tumor DNA (tDNA) was obtained for 68 cases. Multiplex PCR-based assays were designed to target specific coding regions in *EGFR*, *KRAS*, *BRAF*, *ERBB2*, and *PI3KCA* genes, and amplicon sequencing was performed at deep coverage on the cfDNA/tDNA pairs using the NGS IonTorrent Personal Genome Machine Platform.

Results: CfDNA concentration in plasma was significantly associated with both stage and number of metastatic sites. In tDNA, 50 mutations (36 *EGFR*, 5 *ERBB2*, 4 *KRAS*, 3 *BRAF*, and 2 *PIK3CA*) were identified, of which 26 were detected in cfDNA. Sensitivity of the test was 58% (95% confidence interval, 43%–71%) and the estimated specificity was 87% (62%–96%).

Conclusion: These data demonstrate the feasibility and potential utility of mutation screening in cfDNA using IonTorrent NGS for the detection of a range of tumor biomarkers in patients with metastatic lung cancer. *Clin Cancer Res; 20(17); 4613–24. ©2014 AACR.*

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Translational Relevance

Obtaining a sufficient amount of tumor material for the analysis of somatic mutations in targetable genes can be challenging. Circulating free DNA (cfDNA) originating in the tumor has been proposed as a tool for liquid biopsy. However, the level of detection of tumor mutations in cfDNA is limited by the sensitivity of the detection method used. We have developed an IonTorrent-based deep-sequencing multiplex-PCR assay covering hotspot mutation regions of *EGFR*, *KRAS*, *BRAF*, *HER2/ERBB2*, and *PIK3CA*. Taking mutations found in tumor DNA as the reference, the sensitivity of our test was 58%, and the estimated specificity was 87%. We also found that cfDNA concentration in plasma was associated with both clinical stage and number of metastatic sites. This study shows the relevance of deep sequencing for the detection of tumor mutations in cfDNA.

Introduction

Somatic mutation analysis of known or potential druggable oncogenes has gradually become part of the routine practice in thoracic oncology (1, 2). In non–small cell lung cancer (NSCLC), the type of somatic mutations and the genes affected are related to smoking status. Indeed, lung cancers in never-smokers (LCINS) harbor a specific mutation profile characterized in particular by mutations in the *EGFR* tyrosine kinase domain (3). Thus, *EGFR* mutation status is now used to select patients eligible for a treatment with a tyrosine kinase inhibitor (4–7).

In thoracic oncology, tumor samples are often limited as they are mainly obtained through minimally invasive procedures such as bronchial (ultrasono-) endoscopy or CT-guided trans-mural punctures. Testing a wide range of molecular biomarkers in addition to pathologic diagnosis may thus be challenging because of the limited amount of material that can be extracted from these small samples (8, 9).

Circulating free DNA (cfDNA) are small double-stranded fragments of DNA found in plasma. In patients with cancer, the release of cfDNA in the bloodstream is thought to be related to apoptosis, necrosis, as well as active secretion from tumor cells (10). CfDNA recently attracted growing interest in oncology for multipurpose use (11). It has been considered as a prognostic and predictive biomarker (12, 13), and as a "liquid biopsy" to perform noninvasive testing for biomarker detection (14–20). However, the main challenge remains technical because, in many cases, tumor DNA (tDNA) may only represent a very small fraction of cfDNA (21, 22). The level of detection of tumor mutations in cfDNA is thus limited by the sensitivity of the detection method used.

Massive parallel sequencing comprises a new set of promising technologies in the field of cancer research and personalized medicine. Several studies showed that these technologies can be applied to the detection of somatic mutation and suggested that they can be used in clinical settings (23).

To date, no study has reported an inclusive multiplex analysis of relevant biomarkers in NSCLC from plasma cfDNA using massive parallel sequencing. Here, we used the IonTorrent Personal Genome Machine (PGM) for the deep sequencing of the most clinically relevant hotspot somatic mutations in tDNA and plasma cfDNA of never-smoker NSCLC patients.

Materials and Methods

Patients

Patients were consecutive cases recruited in the BioCAST/IFCT-1002 study (ClinicalTrials.gov identifier NCT01465854). BioCAST is a prospective, multicentric cohort sponsored by the French intergroup IFCT, designed to describe clinical, pathologic, and molecular epidemiology of LCINS in a French population. The detailed protocol is reported elsewhere (24). Briefly, newly diagnosed cases of NSCLC from self-declared nonsmokers were included and surveyed. All patients provided written informed consent for participation in the study and consent for blood and tumor samples collection. Overall, from December 2011 to January 2013, 384 patients were included in 75 active centers. IFCT ensured the quality insurance of data management according to international standards.

The present study was planned to be restricted to the 100 first consecutives cases of patients recruited with complete blood sample and tumor molecular analysis (up to May 2012).

Ethical approval

The BioCAST study was approved by the *Sud-Est IV* Ethics Committee of Lyon and by the National Advisory Committee on Information Processing in Health, Paris. Sample collection was declared to French Ministry of Superior Education and Research and to the National Committee for Ethic in Informatics. This ancillary study was also approved by the International Agency for Research on Cancer (IARC) Ethics Committee.

Tumor DNA samples and routine tumor biomarker analysis

tDNA samples were obtained from a network of genetic laboratories. This network was initiated by the French National Cancer Institute (INCa) to perform routine molecular analysis (2). The performance and concordance of sample processing and mutation screening methods across participating centers were previously investigated in blinded cross-validation studies (25). Standards for sample collection and processing used in each laboratory are described in Supplementary Table S1. Most of the paraffin-embedded samples were first microdissected. DNA was extracted using commercial kits.

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Blood samples and circulating free DNA isolation and quantification

All patients included in BioCAST underwent a blood sampling before receiving any cancer treatment. Samples were shipped at room temperature and within 24 hours from each center to the BioCAST central laboratory in Paris (*Centre d'étude du polymorphisme humain - Fondation Jean Dausset*). Blood samples were immediately processed upon receipt (mean time between delivery and processing: 19 ± 13 minutes) to isolate plasma. Plasma was isolated from EDTA tubes by centrifugation at 1,600 g during 10 minutes at 4°C. Plasmas were then aliquoted and stored at −80°C. Plasma samples were shipped to IARC according to international standards. CfDNA was extracted from aliquots (1 mL) of plasma using the QIAamp circulating nucleic acid kit (Qiagen) with the QIAvac 24 Plus vacuum manifold, following the manufacturer's instructions. CfDNA was quantified by picogreen.

Amplicon design and multiplex-PCR conditions

A set of 12 primers pairs was designed using Primer3 software (v4.0) with default parameters (26) to amplify sequences of 98 to 125 bp covering hotspot regions of *EGFR*, *BRAF*, *ERBB2* (*HER2*), *KRAS*, and *PIK3CA* (see Supplementary Table S2 for primer sequences and targeted regions). For amplicon production, 2 ng of DNA were used in multiplex PCR reactions (Supplementary Table S3) with the GoTaq HotStart DNA polymerase (Promega Corporation) and with the following program: 30 seconds at 94°C, 3 cycles of 30 seconds at 58.5°C, 30 seconds at 72°C, then, the annealing temperature was decreased from 0.5°C every 3 cycles until reaching 55.5°C; then 15 cycles of 30 seconds at 94°C, 45 seconds at 50°C, 30 seconds at 72°C, and a final extension of 7 minutes at 72°C.

Library preparation

One microliter of each PCR multiplex reaction was loaded on a gel to check levels of amplification and adjust for the quantity of each multiplex to be pooled by sample for equalizing multiplex representation. Multiplex pools were purified with Agencourt AMPure beads (Beckman Coulter Incorporated) and quantified by Qbit (Invitrogen Corporation). Library preparation was done using 100 ng of multiplex pools and the NEBNext End Repair Module (New England Biolabs) following the manufacturer's instructions. Individual barcodes (designed in-house and produced by Eurofins MWG Operon) were ligated to each multiplex pool for sequencing.

Next-generation sequencing with IonTorrent and variant calling

The libraries were sequenced with the IonTorrent PGM sequencer (Life Technologies) at deep coverage (aiming for 10,000X for plasma and 1,000X for tumors) using the Ion OneTouch 200 Template Kit v2 DL and Ion PGM Sequencing 200 Kit v2 with the 314 or 316 chip kits (all produced by Life Technologies), following the manufacturer's instructions. Six runs were performed to process all samples. For cfDNA, amplicons that failed to reach the targeted depth of coverage and were negative for an expected mutation (according to routine biomarker analysis of tDNA) were repeated in an independent run to obtain a minimum coverage of 10,000X.

The sequencing reads were aligned to the human reference hg19 genome with the IonTorrent Suite V3.4.2. The IonTorrent Variant Caller (ITVC) v3.4 was used for the detection of mutations in tDNA, setting a frequency above 5% for a variant to be called. Variant annotation was done with ANNOVAR (27). For the detection of ultra-low frequency variations in cfDNA, an in-house program was used to extract, from BAM files (Binary sequence Alignment/Map format), various statistics on read counts at the targeted hotspot positions. These statistics were used to filter variants based on three criteria: (i) minimum number of reads carrying the mutation ≥ 5 (a priori choice), (ii) variant allele frequency $\geq$ 0.2%, and (iii) variant reads strand orientation ratio $\geq$ 0.33 and ≤ 3 (*a priori* choice based on the range of strand bias values, 99% of 3,760 observations within this range, observed in plasma samples for reference alleles at positions of mutations reported in the COSMIC database within the analysed regions). To determine the minimum allele frequency threshold, we calculated the average variant allele frequencies (0.13%) for 272 non-hotspot mutations reported in the COSMIC database within the analyzed regions as an estimate of the background sequencing noise. Mutations were called if above this threshold. Mutations identified with these parameters were further checked by manual inspection of BAM files using the Integrative Genomics Viewer (IGV) 2.2 (Broad Institute, Cambridge, MA; ref. 28). Low allele frequency mutations (below 1.5%) in cfDNA were confirmed by independent PCR reactions and resequencing of the amplicons indicative of the presence of mutations.

Statistical analysis

Categorical variables are expressed in percent and compared with χ^2 tests if expected count in each category is at least 5. Plausibility of normal distribution assumption for continuous variables was assessed with the one-sample Kolmogorov–Smirnov test. Continuous variables are expressed in mean and SD or median and interquartile range (IQR) if non-normally distributed. Differences in distribution of continuous variables between two independent samples were assessed by Mann–Whitney *U* test, or Kruskal–Wallis one-way ANOVA when comparing more than two independent samples. Correlations between non-normal distributed variables were assessed with the Spearman correlation coefficient (rho).

Tests using plasma cfDNA were compared with tDNA analysis, which was considered as the reference. True positives were considered when both tDNA and cfDNA samples carried the same mutation and true negatives when both samples had no mutation; false positives were considered when a mutation was found in cfDNA

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but not in tDNA; and false negatives when a mutation was found in tDNA but not in cfDNA. Sensitivity and specificity were calculated as appropriate. In addition, we computed the mutation detection rate (n mutation detected in cfDNA/n mutation detected in tDNA) and concordance rate [(true positive + true negative)/n]. All analyses were performed with IBM SPSS Statistics version 20 (IBM Corp). All statistical tests were conducted two sided and a P value <0.05 was considered to indicate statistical significance. No correction of P values was performed in the course of multiple testing; however, results of all performed tests were thoroughly reported, allowing for an informal adjustment for multiplicity while reviewing the data (29). To express uncertainty of estimated probabilities, 95% Wilson confidence intervals (CI) were reported.

Results

cfDNA and clinical correlates

One hundred and seven patients were included in this study (Supplementary Fig. S1). CfDNA extraction failed in one sample. The main characteristics of the remaining 106 patients are shown in Table 1. Most of patients were women (88%) with adenocarcinoma (86%) and stage IV disease (75%). Characteristics of patients included in the molecular analysis ($n = 68$), and those with a stage IV disease ($n = 50$) are also shown in Table 1. These two subpopulations were similar to their respective complement populations except that adenocarcinomas were more frequently represented ($P = 0.035$ and 0.005, respectively).

Overall, the median cfDNA concentration in plasma samples was 67.1 ng/mL (IQR = 122.11 ng/mL). CfDNA concentration was not associated with the histologic subtype, tumor size ("T"), or node status ("N"). However, cfDNA concentrations were significantly higher in stage IV cases when compared with stages I to III (median concentration 92.3 ng/mL vs.34.7, respectively; $P = 0.002$), and in patients with higher number of metastatic sites (Table 2 and Supplementary Fig. S2). CfDNA concentrations were also significantly correlated, although modestly, both to stage and number of metastatic sites (Table 2).

Next-generation sequencing of tDNA and cfDNA with the IonTorrent PGM platform

Among the 107 patients recruited, 68 had both cfDNA and tDNA available for molecular analyses. The 68 matched cfDNA/tDNA samples were analyzed by deep sequencing (Supplementary Fig. S1). In tDNA, a hotspot mutation was found in 50 samples (74%) while 18 samples (26%) were wild-type at hotspot positions (Supplementary Table S4). Five mutations identified by next-generation sequencing (NGS) were not detected by routine clinical biomarker analyses. Among them, two had not been tested for the corresponding amplicons by routine clinical biomarker analyses, and three had variant allele frequencies ≤25% (which is the usual limit of level of detection by biomarker assays used in clinical settings). In addition, one tumor mutation expected from clinical testing was not confirmed by NGS. As shown in Fig. 1, mutations were mainly located in *EGFR* exon 19 (44%) and *EGFR* exon 21 (18%). No mutation was found in *PIK3CA* exon 20, *BRAF* exon 11, *KRAS* exon 3, and *ERBB2* exon 19. No sample was found to carry multiple mutations. Most mutations were insertions or deletions ($n = 29$; 58%). The median mutant allele frequency was 20% (±29%) in tDNA sample.

In cfDNA, seven of the 50 (14%) mutations expected from tDNA results were called by the manufacturer's ITVC. However, using the in-house program and filtering strategy described in the Materials and Methods section, 28 (56%) tumor mutations were found in cfDNA with allele frequencies ranging from 0.2% to 44% (median ± IQR = 2.0% ± 12%; Fig. 1 and Supplementary Table S4). Three cfDNA samples had hotspot mutations (two in *EGFR* and one in *PIK3CA*) that were not called in tDNA and were thus considered as false positives. However, examination of the tDNA BAM files with IGV showed that two of these mutations were present at allele frequencies below 5% (allele frequency threshold value used for variant calling in tDNA).

Mutation detection in cfDNA and clinical correlates

Results of the comparative analyses of cfDNA and tDNA, using tDNA as reference, are presented in Table 3. When evaluating each amplicon independently, sensitivity ranged from 55% for *EGFR* exon 19 to 100% for *EGFR* exon 18. Overall, considering all amplicons, the sensitivity was 58% (95% CI, 43%–71%), the mutation detection rate was 52%, and the concordance rate was 68%. We estimated the specificity of the assay by taking into account only cases that reached at least 10,000X of coverage for cfDNA and 50X in tDNA (Table 4). We obtained a specificity value of 86% for PI3KCA exon 9, 88% for *EGFR* exon19, 100% for other amplicons, and 87% (62%–96%) overall.

Although it did not reach statistical significance, there was a trend for a better detection rate of point mutations compared with insertions/deletions: in the 19 patients with a tDNA mutation but no mutation detected in cfDNA, six tDNA mutations (32%) were point mutations and 13 (68%) were insertions or deletions compared with 15 and 16, respectively, for true positives ($P = 0.376$). Among cfDNA-positive patients, no correlation was found between mutant allele frequency in plasma and mutant allele frequency in tumor (Spearman rho = −0.227, $P = 0.246$), nor between mutation concentration in plasma (computed as mutant allele frequency in cfDNA multiplied by cfDNA concentration in ng/mL) and mutant allele frequency in tumor (Spearman rho = −0,051, $P = 0.797$). In a *posthoc* analysis restricted to stage IV cases only ($n = 50$), the sensitivity was higher 65% (Supplementary Table S5) but we found no significant difference or correlation between the detection of mutation in cfDNA and disease stage or any other clinical variable (not shown).

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Table 1. Main characteristics of patients and samples included in the study

		All patients		Patients with paired samples analyzed by NGS		
		n = 106	%	*n* = 68	%	*P*[a]
Sex	Male	13	12.3%	10	14.7%	0.370[b]
	Female	93	87.7%	58	85.3%	
Age at diagnosis (mean ± SD)		68.7 ± 13.7		68.0 ± 13.6		0.498
Geographical origin	Africa	5	5.4%	4	6.5%	1.0[b,c]
	Europe	84	91.3%	56	90.3%	
	Asia	1	1.1%	1	1.6%	
	Caribbean	2	2.2%	1	1.6%	
	Missing	14		6		
Histology	SCC	8	7.5%	2	2.9%	0.035[d]
	ADC	91	85.8%	62	91.2%	
	LCC	4	3.8%	2	2.9%	
	Other[e]	3	2.8%	2	2.9%	
TTF1 Immunostaining	Negative	16	15.1%	7	10.3%	NC
	Positive	83	78.3%	56	82.4%	
	Not required	7	6.6%	5	7.4%	
tDNA sample origin	Tumor	68	64.2%	42	61.8%	NC
	Nodes	5	4.7%	3	4.4%	
	Metastasis	33	31.1%	23	33.8%	
tDNA sample type	Biopsy	95	89.6%	59	86.8%	0.321[b]
	Cytology	11	10.4%	9	13.2%	
Stage	Stage I	10	9.6%	6	9.0%	0.810[f]
	Stage II	3	2.9%	2	3.0%	
	Stage III	13	12.5%	9	13.4%	
	Stage IV	78	75.0%	50	74.6%	
	Missing[g]	2		1		
Number of metastatic sites	None	27	26.0%	18	26.5%	0.950
	1 site	33	31.7%	22	32.4%	
	2+ sites	44	42.3%	28	41.2%	
	Missing	2		—		

NOTE: A total of 106 were included, 68 had matched samples considered for NGS analysis.

Abbreviations: NC, not computed; SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.

[a]*P* value was computed between samples included in the NGS analysis ($n = 68$) and those which were excluded ($n = 38$; column not shown).

[b]Fisher exact test (two-sided); others are χ^2 tests (categorical) or Student (continuous).

[c]*P* value was computed between the European and the non-European categories.

[d]*P* value was computed between the adenocarcinoma and the non-adenocarcinoma categories.

[e]Includes one adenosquamous carcinoma and two sarcomatoid carcinomas.

[f]*P* value computed between the stage I to III and the stage IV categories.

[g]One was recorded as "nonmetastatic disease not otherwise specified" in the database.

Discussion

In this proof-of-concept study, we assessed the performance of the NGS IonTorrent PGM platform for the detection of tumor mutations in cfDNA using samples collected in conditions close to routine practice. Assay's sensitivity was 58% (26 mutations on 50 detected). CfDNA concentration was positively associated with tumor stage and with the number of metastatic sites, in agreement with other studies in lung and other cancer types (11–13). NGS for the detection of tumor mutation in cfDNA may thus be clinically meaningful because biomarker analysis is currently only useful for the selection of treatment in patients with lung cancer with metastatic disease.

To our knowledge, this study is the first to report the use of the Ion Torrent PGM for the detection of tumor mutations in cfDNA. The main advantage of this technology is that it

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Table 2. cfDNA concentrations according to histology and stage, and correlations between cfDNA concentration and the corresponding variable in all samples ($n = 106$)

		n	cfDNA concentration Median ± IQR (in ng/mL)	*P*	Spearman rho	$P_{correlation}$
Histology	Non-adenocarcinoma	15	50.2 ± 71.0	0.580[a]	+0.054	0.583
	Adenocarcinoma	91	68.4 ± 126.7			
Tumor "T" stage	T1	18	42.4 ± 152.1	0.195[b]	+0.057	0.578
	T2	36	70.4 ± 119.8			
	T3	16	40.7 ± 33.9			
	T4	29	81.0 ± 130.5			
	Missing (Tx)	7				
Node "N" stage	N0	29	70.0 ± 123.0	0.536[b]	−0.014	0.895
	N1	10	114.6 ± 339.8			
	N2	37	49.1 ± 52.0			
	N3	21	124.3 ±187.7			
	Missing (Nx)	9				
Metastatic "M" stage	M0	27	34.7 ± 25.0	0.001[a]	+0.313	0.001
	M1	78	92.3 ± 128.0			
	Missing	1				
Number of metastatic sites	0	27	34.7 ± 25.0	0.003[b]	+0.322	0.001
	1	33	69.7 ± 146.1			
	2+	44	103.0 ± 117.8			
	Missing	2				
TNM stage	Stage I–II	13	32.6 ± 170.9	0.002[b]	+0.327	0.001
	Stage III	13	34.7 ± 24.0			
	Stage IV	78	92.3 ± 127.9			
	Missing	2				
Overall		106	67.1 ± 122.1	—	—	—

[a]Mann–Whitney *U* test.
[b]Kruskal–Wallis one-way ANOVA test.

allows the simultaneous screening of a broad range of known hotspot mutations in a large number of samples in a cost and time effective way compatible with clinical practice. Deep sequencing (10,000X) allowed the detection of mutations with frequencies as low as 0.2% in cfDNA. However, the manufactured PGM variant caller was not designed to call low abundant mutations diluted in wild-type genomic DNA (30). We thus had to use an in-house variant calling strategy, which improved the overall sensitivity from 16% (ITVC approach for all amplicons together) to 58% (Supplementary Table S6). This limitation of the manufacturer variant caller should be addressed in the future by dedicated software to allow the implementation of the test in clinical settings. Accurate detection of insertions and deletions is another limitation of NGS technologies, including the IonTorrent PGM (31). Given that the expected deletions and insertions were of defined size and location, they could be unequivocally detected with IGV, even those with an allele frequency as low as 0.2%. However, we found a trend for a lower detection rate of insertions/deletions compared with point mutations. This may be due to the principle of the technology that may fail to properly align reads to the reference genome in the presence of insertions or deletions. Using a NGS technology that uses paired-end sequencing may improve the detection of insertions/deletions in this setting. However, this would need to be investigated in a dedicated study directly comparing such technology with IonTorrent.

Several studies have previously investigated the potential use of cfDNA for the noninvasive detection of diagnostic biomarkers in lung cancer using various methods (Table 5). However, most have focused only on one biomarker and included small series of patients. In addition, the majority has only included advanced stages (IIIB/IV). The reported sensitivity of these studies ranged from 36% to 100%, and the specificity from 80% to 100%. Our results are thus consistent with those previously published in the field. However, our method has the advantage to assess several biomarkers simultaneously and to be easily scalable to a higher number of biomarkers.

Only a handful of studies have used NGS technologies for the detection of tumor somatic mutations in body fluids (22, 23, 32–34). Narayan and colleagues, for example,

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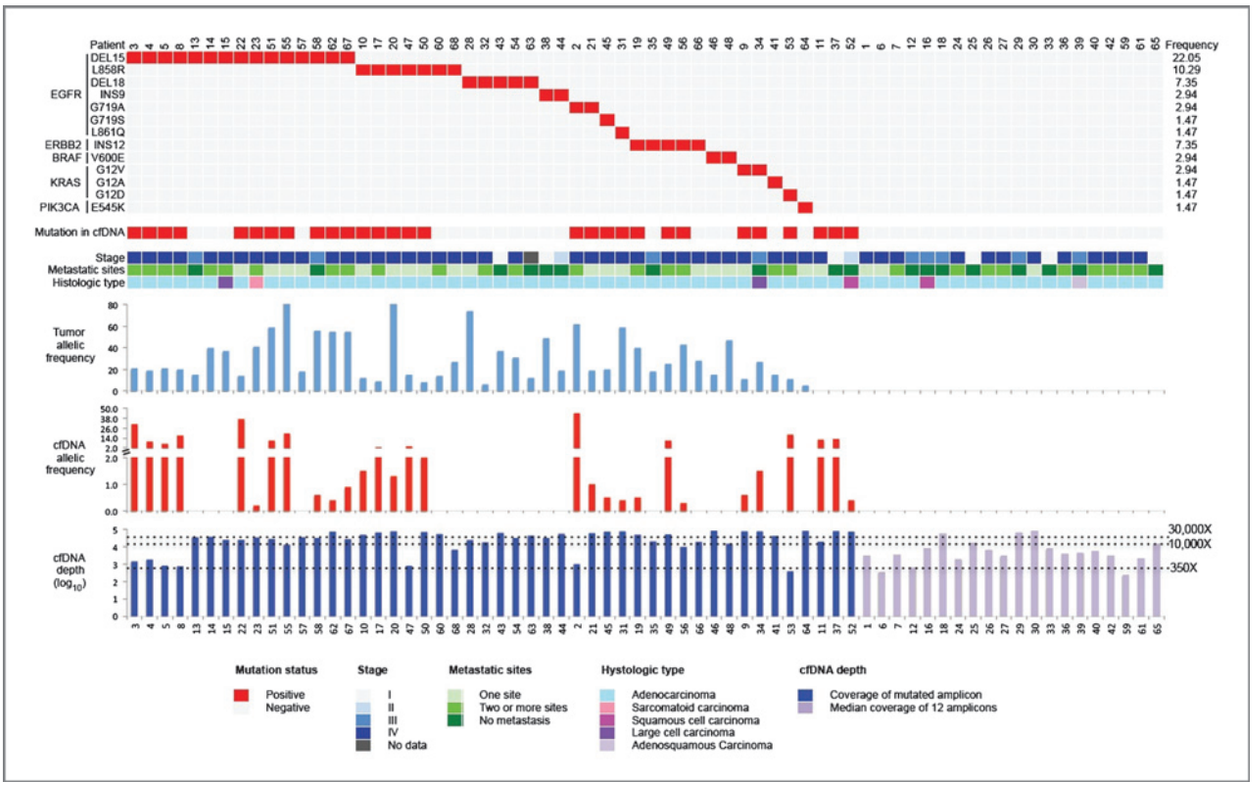

Figure 1. Summary results of NGS sequencing for all matched samples ($n = 68$). Top frequency chart, details of mutations found in tumors in each specific sample. (Red dot indicates mutated samples). Middle frequency chart, cfDNA samples positive for the tumor somatic mutation indicated in top panel. Bottom frequency chart, clinical characteristics of the tumors. Top bar graph, allelic frequency of mutation in tDNA. Middle bar graph, allelic frequency of mutation in cfDNA. Bottom bar graph, total coverage at the position of the mutation in cfDNA or median coverage of all amplicons in wild-type samples.

showed that a deep-sequencing approach can be an appropriate strategy for the detection of low abundant point mutations in surrogate tissues (32). Our work, assessing a larger panel of mutations, including both point mutations and insertions/deletions, and performed on a larger series of patients, further supports and extends the applicability of the NGS for the detection of aberrant genetic events in cfDNA.

In the present study, two *EGFR* and one *PIK3CA* hotspot mutations were detected in cfDNA but were not called in tDNA. Two of these mutations were found at a 4% allelic fraction in tDNA by manual inspection with IGV. We have set a 5% allelic fraction threshold for calling variant in tDNA to look for mutations representative of the tumor content. Results on these samples suggest that mutations poorly represented in tDNA can still be found in cfDNA. However, it is also possible that the analyzed tumor biopsy was not representative of the tumor content (sampling bias). Indeed, recent findings provide strong evidence that a tumor may host different subclonal populations carrying distinct somatic mutation profiles (35, 36). This point is particularly critical for the molecular analysis of small biopsies (36). Some studies have isolated circulating tumor cells to detect tumor-specific molecular alterations (37, 38). Although this approach would require less sensitive techniques for detecting relevant mutations, it may have the same drawback as biopsies because these cells may also represent a limited set of subclones of the tumor. Therefore, applying highly sensitive NGS-based techniques on cfDNA (that may come from different tumor subpopulations) may provide a better assessment of the spectrum of alterations present in the entire tumor. However, this hypothesis remains to be tested. Indeed intra-tumor heterogeneity about *EGFR* remains controversial, as a recent study suggested that heterogeneous distribution of EGFR mutations is extremely rare and may simply be artefacts due to differences in *EGFR* DNA sequences amplification (39).

In some samples, tumor mutations could not be detected in cfDNA even if the coverage obtained for the amplicons carrying the expected mutation was high (13 samples had above 30,000X coverage, including 5 samples above 60,000X). In other cases, some mutations were detected in cfDNA while the obtained coverage was low (between 400X and 800X). Thus, although high sensitivity allows the detection of low abundant mutations, parameters related to the tumor biology may be the most important factors influencing the capacity to detect tumor mutation in cfDNA. Our results show that metastatic stage is one major factor. Stage IV cases have

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Table 3. IonTorrent NGS test on cfDNA properties for each individual amplicon and overall, using tDNA for reference in all matched samples ($n = 68$)

		cfDNA+		cfDNA−			
	n	tDNA+	tDNA−	tDNA−	tDNA+	Mutation detection rate (sensitivity)	Concordance rate
PI3KCA exon 9	61		1	59	1	—	97%
PI3KCA exon 20	60			60		—	100%
EGFR exon 18	59	3		56		100%	100%
EGFR exon 19	59	11	2	37	9	55%	81%
EGFR exon 20	60			58	2	—	97%
EGFR exon 21	61	6		53	2	75%	97%
BRAF exon 11	60			60		—	100%
BRAF exon 15	61			58	3	—	95%
KRAS exon 2	59	3		55	1	75%	98%
KRAS exon 3	58			58		—	100%
ERBB2 exon 19	59			59		—	100%
ERBB2 exon 20	63	3		58	2	60%	97%
Overall (95%CI; all patients, at least one amplicon)	68	26	3	20	19	58% (43%; 71%)	68% (56%; 78%)
All 12 amplicons together (95% CI)	56	21	3	16	16	57% (41%; 71%)	66% (53%; 77%)

Abbreviations: cfDNA+, mutation in detected in cfDNA; cfDNA−, no mutation detected in cfDNA; tDNA+, mutation detected in tDNA; tDNA−, no mutation detected in tDNA.

higher concentration of cfDNA. These results suggest that a part of cfDNA may come from metastatic sites and that cfDNA reflects the tumor burden. However, the origin of cfDNA is still poorly understood and further studies would be needed to fully address this question.

A possible limitation of our study resides in suboptimal cfDNA samples. Indeed, the BioCAST study protocol allowed storage of blood samples at room temperature for up to 24 hours before plasma isolation. Thus, plasma DNA may have been contaminated with genomic DNA

Table 4. NGS test specificity estimate for each individual amplicon and overall, using tDNA for reference

		cfDNA+		cfDNA−		
	n	tDNA+	tDNA−	tDNA−	tDNA+	Specificity
PI3KCA exon 9	7		1	6		86%
PI3KCA exon 20	6			6		100%
EGFR exon 18	8	2		6		100%
EGFR exon 19	33	7	2	15	9	88%
EGFR exon 20	13			11	2	100%
EGFR exon 21	18	3		13	1	100%
BRAF exon 11	8			8		100%
BRAF exon 15	31			29	2	100%
KRAS exon 2	9	2		6	1	100%
KRAS exon 3	6			6		100%
ERBB2 exon 19	6			6		100%
ERBB2 exon 20	28	3		23	2	100%
Overall (95%CI; all patients, at least one amplicon)	51	17	2	13	19	87% (62%; 96%)

NOTE: This analysis was restricted to paired samples for which deep sequencing with the coverage reached at least 50X in tDNA and 10000X in cfDNA ($n = 51$).

Abbreviations: cfDNA+, mutation in detected in cfDNA; cfDNA−, no mutation detected in cfDNA; tDNA+, mutation detected in tDNA; tDNA−, no mutation detected in tDNA.

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Table 5. Main results of studies that have focused on cfDNA as a biomarker for noninvasive diagnosis of lung cancers

Reference	Comparison with tDNA	Biomarker tested	Stage	*N* paired sample	*N* mutated in tumor	Mutation diagnosis technique	Sen	Spe	Conc
Kimura et al. (41).	Y	EGFR	IV	42	8	Tissue: direct sequencing Blood: direct sequencing and Scorpion ARMS	75%	97%	93%
Rosell et al. (42).	Y	EGFR	IV	164	164	Taqman assay	59%	NC[a]	59%
Kuang et al. (17).	Y	EGFR T790M	IIIB/IV	43	30	Scorpion ARMS + direct sequencing	70%	85%	74%
Wang et al. (43)	Y	KRAS	IIIB/IV	273	30	RFLP	77%	95%	93%
Taniguchi et al. (16)	Y	EGFR	IV	44	44	BEAMing	72%	NC[a]	73%
Jian et al. (44)	N	EGFR	IIIB/IV	56	22	Taqman assay	—	—	
Nakamura et al. (14)	N	EGFR T790M	IIIB/IV	49	—	MPB-QP	—	—	
Goto et al. (19)	Y	EGFR	IIIB/IV	86	51	Scorpion ARMS	43%	100%	66%
Yam et al. (15)	Y	EGFR	III/IV	37	32	Taqman assay	100%	80%	97%
Narayan et al. (32)	Y	KRAS (EGFR, BRAF)	I–IV	21	6 (KRAS)	NGS (Illumina HiSeq2000)	100%	100%	100%
Nygaard et al. (45)	Y	KRAS	II to IV	10	10	Inhouse qPCR method	90%	NC[a]	90%
Zhao et al. (20)	Y	EGFR (L858R and del19)	I to IV	111	45	Mutant-enriched PCR	36%	95%	71%
Sakai et al. (46)	N	EGFR T790M	IV	75	21	SABER	—	—	
Mok et al. (47)	Y	EGFR (del19, L858R and G719X)	IIIB/IV	224	89	Allelic-specific cobas PCR	76%	96%	88%
Kim et al. (48)	Y	EGFR (L858R and del19)	III/IV	40	6	PNAClamp EGFR mutation Detection kit	55%	100%	88%
Douillard et al. (49)	Y	EGFR (del19, L858R, T790M)	III/IV	652	105	Scorpion ARMS	66%	100%	94%
Oxnard et al. (50)	Y	EGFR L858R/EGFR del19/KRAS G12C	—	23/23/31	12/9/14	Droplet Digital PCR	67%/67%/71%	100%/100%/100%	83%/87%/87%
IARC/IFCT	Y	EGFR, KRAS, BRAF, ERBB2, PI3KCA	I to IV	67	50	NGS (IonTorrent PGM)	58%	87%	68%

Abbreviations: NC, not computable; NR, not reported; SABER, highly sensitive single allele base extension reaction; RFLP, restriction fragment length polymorphism; MBP-QP, mutation-biased PCR quenching probe; Sen, sensitivity; Spe, specificity; Conc, concordance rate.

[a]All included are mutated.

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released from necrotic white blood cells, resulting in the dilution of tDNA in plasma, negatively affecting the detection of tumor mutations in cfDNA (10). This contamination with genomic DNA could also explain the absence of correlation between mutation allelic frequency in the tumor and mutation detection rate in cfDNA or mutation allelic frequency in plasma. Nonetheless, the detection rate obtained in these suboptimal conditions shows the potential of this approach in samples obtained in more controlled clinical settings. Indeed, plasma sample quality could be easily addressed in future cohorts by processing (centrifuging and freezing) samples within 3 hours after blood collection, which is achievable in routine clinical settings (40).

One of the strengths of our study is that the analyzed never-smoker population carries a high frequency of actionable mutations of various types that represent the most relevant driver mutations in the field of thoracic oncology. We were thus able to test our NGS IonTorrent PGM platform approach on a panel of deletions, insertions, and point mutations (12 hotspot regions targeting five genes) that have not been evaluated in other cfDNA-based studies.

In conclusion, we showed that targeted NGS with the IonTorrent platform for the detection of tumor mutations in cfDNA is applicable to clinical samples in lung cancer. The assay could therefore be a good alternative for initial molecular diagnosis when the obtained histologic sample is poor and could be used as a noninvasive test for assessing regularly the efficacy of targeted therapy by monitoring mutations in blood. Further studies on prospective validation cohorts are required to determine whether an optimized version of the assay may be applicable in routine clinical practice.

Disclosure of Potential Conflicts of Interest

S. Couraud is a consultant/advisory board member for Astra Zeneca, Boehringer Ingelheim, and Roche. G. Zalcman is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Pfizer, and Roche. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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References

1. Cardarella S, Ortiz TM, Joshi VA, Butaney M, Jackman DM, Kwiatkowski DJ, et al. The introduction of systematic genomic testing for patients with non-small-cell lung cancer. J Thorac Oncol 2012;7:1767–74.
2. Nowak F, Soria J-C, Calvo F. Tumour molecular profiling for deciding therapy-the French initiative. Nat Rev Clin Oncol 2012;9:479–86.
3. Couraud S, Zalcman G, Milleron B, Morin F, Souquet P-J. Lung cancer in never smokers–a review. Eur J Cancer 2012;48:1299–311.
4. Mok TS, Wu Y-L, Thongprasert S, Yang C-H, Chu D-T, Saijo N, et al. Gefitinib or carboplatin–paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361:947–57.
5. Zhou C, Wu Y-L, Chen G, Feng J, Liu X-Q, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol 2011;12:735–42.
6. Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012;13:239–46.
7. Yang JC-H, Shih J-Y, Su W-C, Hsia T-C, Tsai C-M, Ou S-HI, et al. Afatinib for patients with lung adenocarcinoma and epidermal growth factor receptor mutations (LUX-Lung 2): a phase 2 trial. Lancet Oncol 2012;13:539–48.
8. Thunnissen E, Kerr KM, Herth FJF, Lantuejoul S, Papotti M, Rintoul RC, et al. The challenge of NSCLC diagnosis and predictive analysis on small samples. Practical approach of a working group. Lung Cancer 2012;76:1–18.
9. Travis WD, Rekhtman N, Riley GJ, Geisinger KR, Asamura H, Brambilla E, et al. Pathologic diagnosis of advanced lung cancer based on small biopsies and cytology: a paradigm shift. J Thorac Oncol 2010;5:411–4.

Published OnlineFirst July 10, 2014; DOI: 10.1158/1078-0432.CCR-13-3063

10. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.
11. Perkins G, Yap TA, Pope L, Cassidy AM, Dukes JP, Riisnaes R, et al. Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. PLoS ONE 2012;7:e47020.
12. Dawson S-J, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin S-F, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;68:1199–209.
13. Lee YJ, Yoon K-A, Han J-Y, Kim HT, Yun T, Lee GK, et al. Circulating cell-free DNA in plasma of never smokers with advanced lung adenocarcinoma receiving gefitinib or standard chemotherapy as first-line therapy. Clin Cancer Res 2011;17:5179–87.
14. Nakamura T, Sueoka-Aragane N, Iwanaga K, Sato A, Komiya K, Abe T, et al. A noninvasive system for monitoring resistance to epidermal growth factor receptor tyrosine kinase inhibitors with plasma DNA. J Thorac Oncol 2011;6:1639–48.
15. Yam I, Lam DC-L, Chan K, Chung-Man Ho J, Ip M, Lam W-K, et al. EGFR array: uses in the detection of plasma EGFR mutations in non-small cell lung cancer patients. J Thorac Oncol 2012;7:1131–40.
16. Taniguchi K, Uchida J, Nishino K, Kumagai T, Okuyama T, Okami J, et al. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. Clin Cancer Res 2011;17:7808–15.
17. Kuang Y, Rogers A, Yeap BY, Wang L, Makrigiorgos M, Vetrand K, et al. Noninvasive detection of EGFR T790M in gefitinib or erlotinib resistant non-small cell lung cancer. Clin Cancer Res 2009;15:2630–6.
18. Ramirez JL, Sarries C, de Castro PL, Roig B, Queralt C, Escuin D, et al. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. Cancer Lett 2003;193:207–16.
19. Goto K, Ichinose Y, Ohe Y, Yamamoto N, Negoro S, Nishio K, et al. Epidermal growth factor receptor mutation status in circulating free DNA in serum: from IPASS, a phase III study of gefitinib or carboplatin/paclitaxel in non-small cell lung cancer. J Thorac Oncol 2012;7:115–21.
20. Zhao X, Han R-B, Zhao J, Wang J, Yang F, Zhong W, et al. Comparison of epidermal growth factor receptor mutation statuses in tissue and plasma in stage I-IV non-small cell lung cancer patients. Respiration 2013;85:119–25.
21. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.
22. Chan KCA, Jiang P, Zheng YWL, Liao GJW, Sun H, Wong J, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013;59:211–24.
23. Tran B, Brown AMK, Bedard PL, Winquist E, Goss GD, Hotte SJ, et al. Feasibility of real time next generation sequencing of cancer genes linked to drug response: results from a clinical trial. Int J Cancer 2013;132:1547–55.
24. Couraud S, Labonne S, Missy P, Morin F, Tran Qu, Deroy A, et al. [Lung cancer in never smokers: a French national cohort (BioCAST/IFCT-1002)]. Rev Mal Respir 2013;30:576–83.
25. Beau-Faller M, Blons H, Domerg C, Gajda D, Richard N, Escande F, et al. A multicenter blinded study evaluating EGFR and KRAS mutation testing methods in the clinical non-small cell lung cancer setting–IFCT/ERMETIC2 Project Part 1: Comparison of testing methods in 20 French molecular genetic National Cancer Institute platforms. J Mol Diagn 2014;16:45–55.
26. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3–new capabilities and interfaces. Nucleic Acids Res 2012;40:e115.
27. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010;38:e164.
28. Thorvaldsdóttir H, Robinson JT, Mesirov JP. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 2013;14:178–92.
29. Saville DJ. Multiple comparison procedures: the practical solution. American Statistician 1990;44:174–80.
30. Stead LF, Sutton KM, Taylor GR, Quirke P, Rabbitts P. Accurately identifying low-allelic fraction variants in single samples with next-generation sequencing: applications in tumor subclone resolution. Hum Mutat 2013;34:1432–8.
31. Bragg LM, Stone G, Butler MK, Hugenholtz P, Tyson GW. Shining a light on dark sequencing: characterising errors in Ion Torrent PGM data. PLoS Comput Biol 2013;9:e1003031.
32. Narayan A, Carriero NJ, Gettinger SN, Kluytenaar J, Kozak KR, Yock TI, et al. Ultrasensitive measurement of hotspot mutations in tumor DNA in blood using error-suppressed multiplexed deep sequencing. Cancer Res 2012;72:3492–8.
33. Murtaza M, Dawson S-J, Tsui DWY, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–12.
34. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med 2012;4:162ra154.
35. Fisher R, Pusztai L, Swanton C. Cancer heterogeneity: implications for targeted therapeutics. Br J Cancer 2013;108:479–85.
36. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012;366:883–92.
37. Pailler E, Adam J, Barthélémy A, Oulhen M, Auger N, Valent A, et al. Detection of circulating tumor cells harboring a unique ALK rearrangement in ALK-positive non-small-cell lung cancer. J Clin Oncol 2013;31:2273–81.
38. Maheswaran S, Sequist LV, Nagrath S, Ulkus L, Brannigan B, Collura CV, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366–77.
39. Yatabe Y, Matsuo K, Mitsudomi T. Heterogeneous distribution of EGFR mutations is extremely rare in lung adenocarcinoma. J Clin Oncol 2011;29:2972–7.
40. Vallée A, Marcq M, Bizieux A, Kouri CE, Lacroix H, Bennouna J, et al. Plasma is a better source of tumor-derived circulating cell-free DNA than serum for the detection of EGFR alterations in lung tumor patients. Lung Cancer 2013;82:373–4.
41. Kimura H, Suminoe M, Kasahara K, Sone T, Araya T, Tamori S, et al. Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA). Br J Cancer 2007;97:778–84.
42. Rosell R, Moran T, Queralt C, Porta R, Cardenal F, Camps C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009;361:958–67.
43. Wang S, An T, Wang J, Zhao J, Wang Z, Zhuo M, et al. Potential clinical significance of a plasma-based KRAS mutation analysis in patients with advanced non-small cell lung cancer. Clin Cancer Res 2010;16:1324–30.
44. Jian G, Songwen Z, Ling Z, Qinfang D, Jie Z, Liang T, et al. Prediction of epidermal growth factor receptor mutations in the plasma/pleural effusion to efficacy of gefitinib treatment in advanced non-small cell lung cancer. J Cancer Res Clin Oncol 2010;136:1341–7.
45. Nygaard AD, Garm Spindler K-L, Pallisgaard N, Andersen RF, Jakobsen A. The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. Lung Cancer 2013;79:312–7.
46. Sakai K, Horiike A, Irwin DL, Kudo K, Fujita Y, Tanimoto A, et al. Detection of epidermal growth factor receptor T790M mutation in plasma DNA from patients refractory to epidermal growth factor receptor tyrosine kinase inhibitor. Cancer Sci 2013;104:1198–204.
47. Mok TS, Wu YL, Lee JS, Yu C-J, Sriuranpong V, Wen W, et al. Detection of EGFR-activating mutations from plasma DNA as a potent predictor of survival outcomes in FASTACT 2: a randomized phase III study on intercalated combination of erlotinib (E) and chemotherapy (C). J Clin Oncol 31, 2013 (suppl; abstr 8021).

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48. Kim H-R, Lee SY, Hyun D-S, Lee MK, Lee H-K, Choi C-M, et al. Detection of EGFR mutations in circulating free DNA by PNA-mediated PCR clamping. J Exp Clin Cancer Res 2013; 32:50.
49. Douillard J-Y, Ostoros G, Cobo M, Ciuleanu T, McCormack R, Webster A, et al. First-line gefitinib in Caucasian EGFR mutation-positive NSCLC patients: a phase-IV, open-label, single-arm study. Br J Cancer 2014;110:55–62.
50. Oxnard GR, Paweletz CP, Kuang Y, Mach SL, O'Connell A, Messineo MM, et al. Noninvasive detection of response and resistance in EGFR mutant lung cancer using quantitative next-generation genotyping of cell-free plasma DNA. Clin Cancer Res 2014;20:1–8.

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Clinical Cancer Research

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