

Circulating DNA in diagnosis and monitoring *EGFR* gene mutations in advanced non-small cell lung cancer

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Contributions: (I) Conception and design: All authors; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: None; (V) Data analysis and interpretation: None; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) are current treatments for advanced non-small cell lung cancer (NSCLC) harboring activating *EGFR* gene mutations. Histological or cytological samples are the standard tumor materials for *EGFR* mutation analysis. However, the accessibility of tumor samples is not always possible and satisfactory in advanced NSCLC patients. Moreover, totality of EGFR mutated NSCLC patients will develop resistance to EGFR-TKIs. Repeat biopsies to study genetic evolution as a result of therapy are difficult, invasive and may be confounded by intra-tumor heterogeneity. Thus, exploring accurate and less invasive techniques to (I) diagnosis *EGFR* mutation if tissue is not available or not appropriate for molecular analysis and to (II) monitor EGFR-TKI treatment are needed. Circulating DNA fragments carrying tumor specific sequence alterations [circulating cell-free tumor DNA (cftDNA)] are found in the cell-free fraction of blood, representing a variable and generally small fraction of the total circulating DNA. cftDNA has a high degree of specificity to detect *EGFR* gene mutations in NSCLC. Studies have shown the feasibility of using cftDNA to diagnosis of EGFR activating gene mutations and also to monitor tumor dynamics in NSCLC patients treated with EGFR-TKIs. These evidences suggested that non-invasive techniques based on blood samples had a great potential in EGFR mutated NSCLC patients. In this review, we summarized these non-invasive approaches and relative scientific data now available, considering their possible applications in clinical practice of NSCLC treatment.

Keywords: Circulating DNA; epidermal growth factor receptor (EGFR); non-small cell lung cancer (NSCLC); tyrosine kinase inhibitor (TKI)-resistance

Submitted Aug 09, 2015. Accepted for publication Aug 11, 2015.

doi: 10.3978/j.issn.2218-6751.2015.08.09

View this article at: <http://dx.doi.org/10.3978/j.issn.2218-6751.2015.08.09>

Serum biomarkers for non-small cell lung cancer (NSCLC)

NSCLC is still the main cause of cancer related death in males and females across Western countries. It is commonly known that about 50% of NSCLC is diagnosed in advanced stage and for the majority of these patients, even if encouraging data regarding immunotherapy have been published, to date chemotherapy still represents the mainstay

of treatment and prognosis remains poor (1,2). However, approximately 15-20% of advanced NSCLC presents a targetable driver mutation, a condition that dramatically changes therapeutic perspectives and patient outcome (3-6).

Mutations in the gene encoding for the epidermal growth factor receptor (EGFR) represent the first driver mutations identified in NSCLC. The presence of the mutation implicates a receptor constitutively activated that continuously gives the cell input favoring proliferation

(7,8). In 90% of cases EGFR activating mutations are represented by exon 19 deletions and exon 21 L858R point mutations (9). It has been established that EGFR activating gene alterations are more common in patients with specific clinico-pathological characteristics, such as female, never smoker, Asiatic origin and adenocarcinoma histological subtype. *EGFR* mutations represent the most important factor for prediction of response to EGFR tyrosine kinase inhibitors (TKIs). In fact, they are associated with significant increase in response rate (approximately 70%) and improvement in progression free and overall survival (OS) (4,5,10). To date, TKI registered as first line therapy for patients with EGFR mutated NSCLC are gefitinib, erlotinib and afatinib and their toxicity profile is certainly more tolerable than standard chemotherapy. However, for drug prescription purpose, the presence of *EGFR* mutation needs to be demonstrated and therefore neoplastic tissue sample is always required.

Unfortunately, biopsies in lung cancer represent a criticism. Bronchoscopy and trans-thoracic biopsies are not well accepted by patients and the event that tumoral material is not sufficient or adequate for molecular analyses is not so infrequent (11). Bone biopsies are a critical issue because decalcification procedures interfere with molecular testing and results (12). Moreover, a single biopsy cannot reflect the clonal heterogeneity of the tumor, which could be present in a single tumor lesion (intratumoral heterogeneity) or between different sites of the tumor (intermetastatic heterogeneity) (13-15). Finally, biopsy procedures are not free from related risks (16). Recent advances in therapeutic management of patient with EGFR mutated NSCLC demonstrated the importance of identifying, after the progression to TKI, the molecular mechanisms of acquired resistance in order to continue, as long as possible, a tailored therapy based on the developed resistance alteration (17,18). This approach entails the repetition of a biopsy theoretically every time a patient experiences a progression of disease with a consequent increased discomfort for the patient who undergoes re-biopsy. Moreover, the re-biopsy after progression is not feasible when disease progression involves a body site that can be reached only with complicated surgical procedures (i.e., brain). All these considerations have given the research the incentive for the identification of more accessible and tolerated methodologies for molecular alteration identification.

Several attempts were done in order to identify reliable serum biomarkers for cancer. In the past, serum proteins,

such as for example carcinoma carcinoembryonic antigen (CEA), have been commonly used for diagnosis of different cancer but due to low specificity and sensibility their routine use is not recommended (19). Subsequently, the identification of circulating tumor cells (CTCs) in serum of patient with cancer seemed to represent the solution for cancer serum diagnosis and monitoring. However, several problems emerged regarding the best method for their isolation as different available devices, basing the selection on cells dimension or antigen expression, presented a moderate risk of false negatives (20). Recently, the attention moved to the possibility of isolation and analysis of cell-free tumor DNA (cftDNA) that, to date, represents the best candidate for identification and monitoring of molecular tumor-related alterations in blood of patients with cancer (21).

Fragments of circulating DNA were isolated in plasma many years ago (22). In particular, patients with cancers present higher levels of circulating DNA comparing to healthy volunteers because of the presence of tumoral counterpart, which express the same molecular abnormalities expressed by DNA of primitive mass (13). The elevated cellular turnover and consequent cellular necrosis and apoptosis cause a massive release of tumoral DNA into the bloodstream where it can be isolated and analyzed. Therefore, tumor size, localization and vascularity may influence cftDNA plasmatic levels. It is also possible that part of cftDNA comes from CTCs lysis (13). The analysis of cftDNA, defined as liquid biopsy, could be repeated every time needed and without any discomfort for patients. Moreover, the mutational analysis of cftDNA demonstrated a significantly better sensitivity if compared with CTCs one, establishing cftDNA as the best circulating source for molecular analysis (23). Information derived from liquid biopsy could be used in future for early cancer diagnosis, assessment of genetic determinants for targeted therapies, monitoring of tumor dynamics and early evaluation of tumor response, identification of resistance mechanisms (13).

In the last years, techniques for cftDNA analysis have been largely employed for identification of activating and resistance mutations in NSCLC EGFR mutated patients and the aim of this review is to discuss principal findings.

Circulating free tumor DNA and technologies for its detection

cftDNA could be a relevant biomarker to molecular

diagnosis and monitor treatment resistance, because of its sensitivity and specificity, but it really needs reproducible and standardized methods, both for the extraction and for its analyses.

Most of the published papers used conventional methods for the cftDNA extraction with commercially available kits for routine use, based on selective binding to a silica-based membrane for improved recovery of fragmented nucleic acids (i.e., Qiagen, Norgen). While the amount and the quality of cftDNA can deeply vary, high-analytical sensitivity and specificity techniques are required for its detection; moreover, a critical issue is to make a distinction and a choice between the importance and the clinical role of cftDNA quantification and mutation analysis. Because of it, many published studies applied a combined quantitative and qualitative analysis of cftDNA starting from surgery and during follow-up, founding that during follow-up, cftDNA levels decrease progressively, but rapidly increased when a relapse occurred, whereas specific mutations were detected only in relapsed patients (24). Dawson and colleagues analyzed the cftDNA of 30 metastatic breast cancer patients to monitor response to treatment. cftDNA was detected in 29/30 patients, showing that cftDNA levels have a dynamic range and the correlation with variations in tumor burden were better than did CA 15.3 serum biomarker or CTCs (25).

Regarding the mutation analysis of cftDNA, a large number of technologies is now available to analyze mutations in cftDNA, including automatic sequencing, real-time polymerase chain reaction (PCR) platforms, mass spectrometry (MS) genotyping, amplification protocols with magnetic beads in oil emulsions [beads, emulsion, amplification and magnetics (BEAMing)] and next-generation sequencing (NGS), digital PCR platforms (26-30). The sensitivity range of the available techniques varies from 15% to 0.01%, but one of the major gaps in this field is the lack of standardization of techniques, in order to understand how those techniques are cost-effective and reliable to fit clinical needs.

Among techniques most of them are able to detect mutant allele frequencies with a sensitivity of at least 2%, other, like cold-PCR, can reach somatic mutations at very low frequencies of 0.1-0.5%, and many genotyping approaches can be combined with it to analyze known mutations [i.e., MS-based matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) genotyping technologies] (31).

Real-time PCR

One of the widely used methods to detect known mutations

is the real-time PCR. The real-time PCR works with either TaqMan probes or TaqMan Detection Mutation Assay. TaqMan probes have a sensitivity detection limit of approximately 10% (32), otherwise, TaqMan Detection Mutation Assay is a competitive allele-specific TaqMan PCR technology, with high sensitivity and specificity because the mutant allele detection is based on an allele-specific primer, while an MGB blocker oligonucleotide suppresses the wild-type background and high sensitivity. Thanks to this mechanism of action, the TaqMan Detection Mutation Assay is able to detect as low as 0.1% mutant molecules in a background of wild type genomic DNA (Cancer Biomarker Research using castPCR™ Technology, AACR 2012). Real-time PCR can also work with Scorpion primers, a kind of bi-functional molecule in which a primer is covalently linked to the probe, with a fluorophore and a quencher. In the absence of the mutation, the quencher close to the fluorophore absorbs its fluorescence. During the Scorpion PCR reaction, the presence of a mutation separates the fluorophore and the quencher increasing the emitted fluorescence (http://www.premierbiosoft.com/tech_notes/Scorpion.html).

Most of the published studies adopted this technology for the analysis of cftDNA in lung cancer. In particular, results coming from analyses focused on the detection of the *EGFR* mutations in cftDNA of patients with *EGFR* mutated tumors showed a wide variability: the concordance ranges from 43% to 100% (23,33). Unfortunately, in some cases, mutations can be missed using Real Time technology, and therefore the results are inferior compared to more sensitive approaches.

Digital PCR

The digital PCR approach is based on the same principle of the real-time PCR, but while the real-time PCR works as a unique solution, the digital PCR is able to divide the amplification mix in several thousand of replicates. This partition permits the amplification and the analysis considering single spots, which means that the system is able to decrease the ration of cftDNA/germinal DNA, increasing sensitivity. Digital PCR can works on various principles, for example, silicon chips (Quant studio 3D, Life technologies, Carlsbad, CA, USA) or micro droplets (Bio-Rad Qx100, BioRad, Hercules, CA, USA). This kind of technology can theoretically increase the sensitivity to 1:100,000 molecules of cftDNA in a germinal DNA background (34). Disadvantage of this technology is the not

standardized threshold to establish the presence and the amount of mutations.

Beads, emulsion, amplification and magnetics (BEAMing)

Many other approaches, like BEAMing technology, are able to detect a very small amount of mutant DNA sequences in a larger pool of fragments containing wild-type DNA, in order of a single mutant allele in a background of 10,000 wild-type alleles, and it is able to enabling copy-number quantification (35). BEAMing is a sensitive method to detect known genetic mutations, even when at very low copy numbers. The technique is based on a combination of emulsion digital PCR and flow cytometry, with beads, emulsification, amplification and magnetics to achieve the necessary level of sensitivity. DNA sequences are amplified via emulsion PCR covalently bound to magnetic microbeads via streptavidin-biotin interactions; the PCR products generated in each emulsion droplet will remain physically affixed to the microbeads at the end of the reaction, allowing them to be easily separated and purified using a magnet, to determine the presence and number of known mutant variations. The wild-type or mutant DNA can be easily differentiated using flow cytometry. Unfortunately, the BEAMing workflow results complex limiting the feasibility and reproducibility of the technology.

Next-generation sequencing (NGS)

All the mentioned techniques are able to find only known mutations in samples, and this means that a patient need to have a tumor biopsy screened in advance to capture the mutational status, consequently, in terms of costs and standardization of the analysis, it is need to personalized a panel test for each patient. The analysis of cftDNA using NGS technology has recently demonstrated to offer increase detection sensitivity, showing also a good specificity in patients with advanced cancers (27). Published studies demonstrate that deeper sequencing of plasma DNA may allow the problem of clonal heterogeneity and selection (36).

Many NGS technologies are available to date, all of them produce short sequences from single molecules of DNA and it is compared to a reference sequence, allowing the sequencing of large portion of the genome. Selecting only a limited number of sequences of frequently mutated genes, it is easy to reach very deep cover- age of sequencing for candidate mutation loci. This allows the identification of mutated alleles even if highly diluted. Moreover, one of the

advantages of the NGS is that whole-genome sequencing of cftDNA can allow the identification of rearrangement and region of copy number aberrations, not detectable with other techniques (27).

Unfortunately, in terms of daily application into the clinic, the use of a NGS technique is still so far, the management of the data requires expert biologists in library preparation, a dedicated bioinformatics support is recommended to solve computational problems that occur during the project and it is an expensive technique.

Genotyping MS

A considerable number of technologies are available for the detection of mutations using MS, but nowadays, the MALDI-TOF MS has become the most used method. The genotyping method is able to distinguishing different alleles by the different masses of primer extension products. The experimental procedure is divided into three steps: amplification, primers extension reaction, transfer of the reaction product into a chip that contains a specific matrix, with two intermediate cleaning reactions, before detection of the extension products. At the end of the analysis, the peak spectrum resulting from MALDI-TOF MS analysis can be analyzed with software that traces back primer masses to assayed alleles. MALDI-TOF MS is relatively more expensive and time consuming than RT-PCR-based methods, but it is more suitable for the simultaneous analysis of multiple mutations. Sequenom is nowadays into clinical routine for the analysis of somatic mutations from FFPE tissue; one of the limitations of this method, common to other similar genotyping techniques, is that it only returns genotypic data. For this reason, analyses with more than one single nucleotide polymorphism (SNP), such as linkage disequilibrium or haplotype diversity, require the most likely haplotypes to be inferred.

cftDNA for identification of *EGFR* mutations in patients with NSCLC

To validate cftDNA analysis for *EGFR* mutations detection, results obtained in serum have been compared with the actual gold standard that is analysis on tissue from tumor biopsy. To our knowledge, the first authors that compared results from serum and paired tissue samples were Kimura and colleagues in 2006 (37). Even if paired samples were just 11, authors reported a 72.7% of concordance between serum and tissue. One year later, the same author published

another casistic of 42 patients were EGFR mutational status was consistent with tissue one in 92.9% of cases (38). In 2009, Yung *et al.* detected EGFR 19del and L858R in 17% and 26%, respectively, of 35 pre-therapy plasma samples by using digital PCR; when data were compared with results from tumor samples, overall serum analysis demonstrated very high sensitivity and specificity (92% and 100%, respectively) (26).

Other studies published and conducted on Asiatic populations, revealed high grade of specificity and moderate grade of sensitivity (39,40). Furthermore, authors observed a significant increase in sensitivity when only patients with advanced stage or poorly differentiated adenocarcinoma were evaluated. These data can be explained considering that overall tumor mass and aggressivity can influence levels of cftDNA and therefore the possibility of *EGFR* mutation detection.

The principal data regarding Caucasian patients were published by Weber and Douillard (41,42). Weber *et al.* analyzed pairs of diagnostic biopsy and plasma sample of 199 patients obtained prior commencing therapy with EGFR-TKI (41). The overall concordance between plasma and tissue was 179/199 (90%) and six mutations were present only in plasma sample but not in bioptic specimens suggesting a possible role of tumoral heterogeneity. Douillard and colleagues published data regarding patients enrolled in the phase IV study of gefitinib in Caucasian patients with advanced stage IV EGFR mutated NSCLC (42). All patients were centrally screened for *EGFR* mutation in tissue sample and matched baseline plasma samples were mandatory. Authors matched 652 tumor and plasma samples and concordance resulted 94.3%, sensitivity 65.7% and specificity 99.8%, concluding that, even if tumor remains the preferred source, plasma testing could be appropriate in patients without available tissue. This statement is based on the evidence that patients with EGFR mutated cftDNA presented a response rate similar to patient with EGFR mutated tissue.

Recently, Mok published results of analysis conducted on data from the FASTACT-2 study where patients were randomized to receive platinum-based chemotherapy plus sequential erlotinib or placebo (43). Authors matched 238 plasma and tissue samples and concordance was 88%, sensitivity 75% and specificity 96%. Similar to previous study, patients with EGFR-positive cftDNA treated with erlotinib presented a significantly better outcome than patients treated with placebo [progression-free survival (PFS) 13.1 *vs.* 6.0 months; $P < 0.0001$], while no difference

emerged between EGFR-negative cftDNA patients treated with erlotinib or placebo. These results enforce the role of cftDNA *EGFR* mutations as predictive factor for response to EGFR-TKI confirming they could represent a reliable surrogate of tissue determination.

Considering the high number of reports present in literature, two meta-analysis investigating the diagnostic value of cftDNA for *EGFR* mutations identification have been published and both included studies with paired tissue and plasma samples (44,45). Characteristics of the studies included in the two meta-analyses are summarized in *Table 1*. The first one considered results from 20 published studies of which all were conducted in Asia but one conducted in USA (44). Results showed a pooled sensitivity of 0.674 (95% CI: 0.517-0.800) and a pooled specificity of 0.935 (95% CI: 0.888-0.963). Positive and negative likelihood ratios were 10.307 (95% CI: 6.167-17.227) and 0.348 (95% CI: 0.226-0.537), respectively. The summary receiver operating characteristic (SROC) curve was generated and area under the curve (AUC) resulted 0.93 [0.90-0.95] indicating high diagnostic accuracy. The other meta-analysis considered 27 studies of which a consistent part already included in the previous one, five studies regarding Caucasian populations and five studies published in 2014 including ones by Douillard and Weber. Pooled sensitivity and specificity were 0.620 (95% CI: 0.513-0.716) and 0.959 (95% CI: 0.929-0.977), respectively and AUC was 0.91 (95% CI: 0.89-0.94). As previously reported, accuracy increased in patients with advanced stage disease (AUC 0.96, 95% CI: 0.94-0.97). The authors of both meta-analyses conclude in favor of the high diagnostic accuracy showed by cftDNA underlying the high specificity and non-invasivity that make it a useful tool for screening. However, some limitations have been described including the presence of heterogeneity between studies and the absence of a unique and specified time of blood collection that could have a significant impact as chemotherapy could influence EGFR status (66).

After publication of these meta-analysis, results of two relevant studies (ASSESS and IGNITE trials) investigating the utility of ctDNA in plasma for the detection of *EGFR* mutation were presented at European Lung Cancer Conference 2015 (67,68). Both are multicenter diagnostic studies evaluated the utility of ctDNA for *EGFR* mutation testing in a real-world setting (Europe and Japan in ASSESS and Asia-Pacific and Russia in IGNITE, respectively), having as primary objective the concordance between *EGFR* mutation status obtained via tissue or cytology and plasma-based testing (*Table 2*). Both studies have

Table 1 Characteristics of studies included in the two meta-analyses evaluating cftDNA in *EGFR* mutation detection

First author	Country	Year	Detection methods	Female (%)	Adenocarcinoma (%)	Ever smokers (%)	No. of samples	Sensitivity (%)	Specificity (%)
Kimura H (37)	Japan	2006	ARMS	37.3	85.2	NA	11	75	40
Kimura H (38)	Japan	2007	ARMS	33.3	73.8	66.7	42	75	97
He C (46)	China	2009	ME-PCR	36.6	75.4	53	18	89	100
Yung TK (26)	China	2009	Digital PCR	NA	NA	NA	29	100	94
Kuang Y (47)	USA	2009	ARMS	81.5	NA	NA	43	70	85
Bai H (48)	China	2009	DHPLC	46.5	74.3	44.8	230	97	92
Sriram KB (49)	Australia	2011	ME-PCR	33.9	56.3	93.7	64	50	100
Jiang B (50)	China	2011	ME sequencing	31	72.4	62.1	58	78	100
Taniguchi K (51)	Japan	2011	BEAMing	65.9	95.5	NA	44	73	0
Brevet M (52)	USA	2011	Sequenom	51.6	96.8	54.8	31	44	85
Goto K (33)	Japan	2012	AS-APEX	87.6	NA	9	86	43	100
Nakamura T (53)	Japan	2012	I-PCR-QPM	51.3	100	46.2	70	45	100
Hu C (54)	China	2012	HRM	50	58.3	45.8	24	100	0
Huang Z (55)	China	2012	DHPLC	46.7	78	41.4	822	64	85
Xu F (56)	China	2012	ARMS	39.2	84.3	NA	34	50	100
Yam I (57)	China	2012	AS-APEX	60	94.3	14.3	35	100	80
Jing CW (58)	China	2014	HRM	42.5	58.3	NA	120	64	97
Liu X (59)	China	2013	ARMS	34.9	98.8	54.7	86	68	100
Lv C (60)	China	2013	DHPLC	54.5	NA	45.5	6	0	100
Zhang H (61)	China	2013	MEL	43	75.6	51.2	86	68	100
Kim ST (62)	Korea	2013	PNA-LNA PCR clamp	38.6	70.2	56.1	57	66	93
Zhao X (39)	China	2013	ME-PCR	31.5	65.8	51.4	111	35	98
Kim HR (63)	Korea	2013	PNAClamp	NA	NA	NA	40	17	100
Li X (plasma) (64)	China	2014	ARMS	42.5	78	46.8	141	48	95
Li X (serum) (64)	China	2014	ARMS	44	79.6	43.5	108	40	96
Weber B (41)	Denmark	2014	Cobas EGFR blood test	49	95	91	196	61	96
Douillard JY (42)	Europe	2014	ARMS	NA	NA	NA	652	66	99
Wang S (65)	China	2014	ARMS	48.5	80.6	46.3	74	22	97

ARMS, amplification refractory mutation system; ME-PCR, mutant-enriched-PCR; DHPLC, denaturing high-performance liquid chromatography; ME-sequencing, Mutant-enriched sequencing; BEAMing, beads, emulsion, amplification and magnetics; AS-APEX, allele-specific arrayed primer extension; I-PCR-QPM, inhibiting-PCR-sequencing probe method; HRM, high-resolution melting; MEL, mutant-enriched liquid chip; PNA-LNA, peptide nucleic acid-locked nucleic acid; NA, not available

controversial results, probably in relation to heterogeneous methodologies used; in fact, if plasma samples were processed in central designated laboratories, nevertheless *EGFR* mutation testings on tissue were performed according to local practices and, sometimes, with low sensitive techniques. In ASSESS trial, 1,311 patients were enrolled

with data available on both tissue and plasma samples of 1,162. Considering overall results, the concordance obtained was 89.1%, with a sensitivity of 46%, specificity of 97.4%, positive predictive value (PPV) of 77.7% and negative predictive value (NPV) of 90.3%. Considering a subgroup with same methodology used in tissue and plasma,

Table 2 ASSESS and IGNITE trials

Parameter	ASSESS trial				IGNITE trial			
	Overall (n=1,162)		Same method (n=254)		Asian pacific patients (n=1,687)		Russian patients (n=894)	
	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI	n/N (%)	95% CI
Concordance	1,035/1,162 (89.1)	87.1-90.8	221/254 (87.0)	82.2-90.9	1,310/1,687 (77.7)	75.6-79.6	767/894 (85.8)	83.3-88.0
Sensitivity	87/189 (46.0)	38.8-53.4	25/56 (44.6)	31.3-58.5	343/692 (49.6)	45.8-53.4	33/109 (30.3)	21.8-39.8
Specificity	948/973 (97.4)	96.2-98.3	196/198 (99.0)	96.4-99.9	967/995 (97.2)	96.0-98.1	734/785 (93.5)	91.5-95.1
PPV	87/112 (77.7)	68.8-85.0	25/27 (92.6)	75.7-99.1	343/371 (92.5)	89.3-94.9	33/84 (39.3)	28.8-50.5
NPV	948/1,050 (90.3)	88.3-92.0	196/227 (86.3)	81.2-90.5	967/1,316 (73.5)	71.0-75.8	734/810 (90.6)	88.4-92.5

n, numerator value for each parameter; N, denominator value for each parameter; PPV, positive predictive value; NPV, negative predictive value.

in particular as Therascreen[®], results improve (concordance 94.9%, sensitivity of 72.7%, specificity 99.1%, PPV 94.1% and NPV 95%) and are similar those obtained in previous small experiences (42). In IGNITE trial, 3,382 patients were enrolled with data available on both tissue and plasma samples of 2,581. Results obtained (see Table 2) showed findings that need some clarification, in particular in Russian patients; in fact, PPV is low, the percentage of mutations in non-adenocarcinoma is higher than expected (about 10% of cases overall, with higher percentage in plasma than in tissue samples in Russian patients, 7.1% *vs.* 3.7%, respectively), as well as the percentage of rare mutations (15.5% and 26.7% in Russian adenocarcinoma and non-adenocarcinoma samples, respectively).

The role of *KRAS* mutations in patients with EGFR mutated NSCLC is still controversial. In fact, *EGFR* and *KRAS* mutations have always been considered mutually exclusive in lung cancer and *KRAS* mutations demonstrated a negative predicting effect for response to EGFR-TKI. However, recently studies demonstrating the coexistence of these molecular alterations on tissue samples were published (69). Authors observed that *KRAS* mutation did not preclude response to EGFR-TKI suggesting that the interaction between the two pathways may be more complex (69). Coexisting *EGFR* and *KRAS* mutations have been isolated also in plasma in some studies (62,70,71). Wang *et al.* reported EGFR/*KRAS* co-presence in five out of 120 patients who presented PFS and OS significantly inferior to patients harboring only *EGFR* mutation (70). The presence

of both mutations at diagnosis was reported also by Kim *et al.* in five out of 57 patients. However, in their experience, *KRAS* serum mutation did not influence prognosis (62). It is worth noting that advances in technologies for DNA molecular analysis could open new scenarios and the role of different mutations may be re-assessed.

Acquired resistance to EGFR-TKI and role of cftDNA

Unfortunately, acquired resistance is an inevitable process during therapy with EGFR-TKI and usually it develops after a median treatment period of 10-12 months (72). Molecular mechanisms underlying acquired resistance have been largely investigated and the occurrence of a second *EGFR* mutation in exon 20 (T790M) resulted the most frequent resistance-associated molecular alteration with a prevalence ranging from 49% to 63% (72,73). Other less frequent mechanisms of resistance are represented by *HER2* amplification (12-13% of cases), *MET* amplification (5-11%), *PIK3CA* mutations (about 5%) or *BRAF* mutations (1%) (73,74). A particular situation is represented by the emergence of a neoplastic clone with clinical and histological features consistent with small cell lung cancer (SCLC) that is reported in 3-14% of cases and implies a more aggressive behavior (72-74).

T790M was reported for the first time in 2005 and its presence increases receptor affinity for ATP that reduces TKI capability to bind EGFR translating in drug

inefficacy (75-77). The presence of a clone harboring T790M resistance mutation has been associated with indolent progression and favorable prognosis (78). In fact, Oxnard and colleagues evaluated T790M expression in patients with EGFR-TKI acquired resistance and found out that T790M was significantly more frequent in loco-regional sites of disease than in distant ones and associated with longer post-progression survival. On the contrary, patients without T790M were more likely to progress with new sites of disease in previously uninvolved organs and presented poorer performance status. Similar results have been reported by Oya and colleagues (79); 48% of patients presented T790M in the re-biopsy specimen that was significantly associated with more local than systemic disease progression. Different results were recently reported by Zheng *et al.* in a Chinese cohort of 117 patients; in fact, even if T790M prevalence (47%) in resistant patients and early onset are confirmed, authors showed that T790M patients presented significantly shorter OS (80).

The importance of the identification of the mechanism involved in acquired resistance is not only theoretical since the efficacy of next generation EGFR-TKI has been demonstrated. Recently, results from trials testing two new molecules AZD9291 and rociletinib have been published and show an impressive efficacy especially in T790M-positive patients, with response rate ranging between 59% and 61% and a median PFS ranging from 9.6 to 13.1 months after progression to first-line TKI (17,18). Similarly to what stated above, the T790M presence need to be demonstrated with re-biopsy after progression and frequently this could represent a limit in lung cancer patients. However, the feasibility of resistance monitoring by plasma DNA sequencing has been proved in several cancers, including EGFR mutated NSCLC [(36), Table 3]. In this study, authors evaluated the variation of mutant allele fractions associated with resistance to oncological treatment in patients with different cancers. Principal findings included the increase of mutations in *PIK3CA* after therapy with paclitaxel in breast cancer, increase of RB1 mutations after cisplatin in ovarian cancer and increase of T790M in patient with NSCLC EGFR positive treated with gefitinib. T790M was not detectable in plasma at the start of treatment and increased along with *NFkB1* and *p53* mutations.

Oxnard *et al.* reported on a series of nine EGFR mutated patients treated with first-line erlotinib and six of them exhibited T790M in plasma during treatment (81). Sorensen *et al.* described a group of 23 EGFR mutated

patients treated with erlotinib as second-line therapy and the presence of T790M was documented in nine patients as acquired resistance mechanism (82). In particular, authors identified a new response parameter, represented by the plasmatic response, a condition defined by the reduction or disappearance of EGFR activating mutation in plasma during TKI treatment. Reduction in *EGFR* mutations plasmatic levels can be demonstrated very early, as recently also reported by Marchetti *et al.*, that observed decreased levels starting from the 4th day of therapy with TKI (83). Several authors demonstrated that in patients that developed T790M-mediated acquired resistance, the level of plasmatic EGFR activating mutations started to increase along with the appearance of T790M (81,82,84). Interestingly, in all reports authors demonstrated that T790M was detectable in plasma several days (range: 15-344) before the evidence of disease progression per RECIST criteria. This observation is consistent with the hypothesis of the selection of a resistant neoplastic clone operated by EGFR-TKI, that grows until becomes clinically relevant. However, it should be noted that the presence of T790M in association with EGFR sensitizing mutations has been documented in pre-treatment tissue and plasmatic samples, suggesting that the resistance clone could be present since the beginning and reach the blood stream after the clonal expansion (85,91,92). The identification of T790M in patients TKI-naïve could have a significant impact as double-positive patients presented shorter PFS than patients positive only for activating mutations.

Dynamic evolution of *EGFR* mutation plasmatic levels has been confirmed from others authors. Nakamura *et al.* reported on a series of 49 patients diagnosed with adenocarcinoma of whom 19 with acquired resistance (86). They found that 53% of resistant patients were positive for T790M and observed that T790M was not detectable in non-responders since T790M appeared in plasma only in responsive patients supporting the theory of a clone selection. Marcq and colleagues described two cases of patients treated with EGFR-TKI (87). In one case activating mutation decreased in plasma and the subsequent increase at progression was associated with T790M appearance; in the other case the patient experienced a complete plasmatic response, with only EGFR activating mutation re-appearing at progression. Wang *et al.* retrospectively analyzed a series of 135 patients treated with EGFR-TKI and found out that patients with pre-TKI plasma sample positive for T790M had significantly inferior PFS and OS comparing with pre-TKI negative patients (85). Moreover, among

Table 3 List of studies evaluating *EGFR* gene activating and resistance mutations and their level modification

First author	Year	Methodic	No. of patients	EGFR determination	EGFR variation levels	T790M determination (timing)	T790M variation levels	Others
Murtaza M (36)	2013	Digital PCR	1	√	√	√ (R)	√	p53, NFKB1
Oxnard GR (81)	2014	dd-PCR	9	√	√	√ (R)	√	–
Sorensen BS (82)	2014	Cobas EGFR blood test	23	√	√	√ (R)	√	–
Marchetti A (83)	2015	Cobas EGFR blood test	57	√	√	–	–	–
Ahn MJ (84)	2015	dd-PCR	60	√	√	√ (R)	–	–
Wang Z (85)	2014	Digital PCR, ARMS	135	–	–	√ (D)	√	–
Nakamura T (86)	2011	MBP-PQ	49	–	–	√ (R)	√	–
Marcq M (87)	2014	ARMS	2	√	√	√ (R)	√	–
Piotrowska Z (88)	2015	BEAMing	12	√	√	√ (R)	√	–
Sequist LV (89)	2015	BEAMling	113	–	–	√ (R)	√	–
Thress KS (90)	2015	NGS, dd-PCR	19	√	√	√ (R)	√	EGFR C797S

dd-PCR, digital droplet-PCR; ARMS, amplification refractory mutation system; MBP-PQ, mutation-biased PCR quenching probe; BEAMing, beads, emulsion, amplification and magnetics; (R), at resistance; (D), at first diagnosis.

patients with pre-TKI positive sample, higher levels were associated with significantly shorter PFS. On the contrary, patients with increased quantity of T790M during TKI therapy presented better PFS and OS than patients with decreasing T790M levels. Interestingly, authors observed high plasmatic levels of MET amplification in patients with decreasing T790M suggesting that TKI pressure could select a MET-amplified tumoral clone responsible of earlier resistance. Similarly to what reported for EGFR activating mutations, also reduction in T790M plasmatic levels can be considered as early parameter of response. In fact, Sequist reported that plasmatic T790M positivity is a predictor of durable response in patients treated with rociletinib, a third generation EGFR-TKI, and that responding patients show decrease of circulating T790M during treatment. However, authors have noted that about 33% of patient with T790M negative plasma responded and that also non-responding patients' present level reduction during treatment, concluding that probably T790M is not always the dominant resistance driver (89).

Finally, as new third-generation TKI with high affinity for T790M positive receptor have been developed, mechanisms of acquired resistance to new TKI have been studied and identified (88). In a group of 12 re-biopsied patients resistant to rociletinib, Piotrowska and colleagues reported the disappearance of T790M in six patients (of whom two presented transformation to small cell histology) and EGFR amplification in three T790M-positive patients. Regarding

plasma analysis, they observed an increased in EGFR activating mutation during TKI therapy that was associated in some patients with T790M increase and in other patients with persistent T790M suppression. Similarly, Thress *et al.* analyzed plasmatic modifications of patients treated with AZD9291. Together with fluctuations of T790M circulating levels, the appearance of a new mutation C797S was documented as mechanisms of acquired resistance. In vitro studies have documented that this mutation impairs binding of TKI to EGFR thus inducing resistance (90).

Conclusions

Despite tissue biopsy still represents the gold standard for diagnosis, sophisticated technologies have permitted the isolation and identification of lung cancer related mutations in plasma opening new scenarios with a major impact in cancer patients management. Mutational analysis of cftDNA represents one of the most important recent breakthroughs in thoracic oncology. In fact, in certain situations, liquid biopsy could be an essential tool for clinicians because it gives the chance of a targeted therapy also in patients who cannot undergo invasive diagnostic procedures, due to comorbidities or the absence of biopsable tumor lesions. Moreover, liquid biopsy presents the advantages of a non-invasive technique that, without any discomfort, can be repeated every time needed during a patient therapeutic history. In particular, cftDNA analysis assumes a crucial

role for patients with EGFR mutated lung cancer, since they represent a group of patients receiving a huge benefit from targeted mutation identification, not only at diagnosis but also at the onset of acquired resistance, but for whom obtaining tissue sample is sometimes not feasible.

Several issues remain outstanding regarding the routine employment of cftDNA. First, many devices for cftDNA detection and analysis have been developed, characterized by a slight different spectrum of sensitivity and specificity. Data in literature are extremely heterogeneous from this point of view as different authors tested the reliability of different devices. Therefore, univocal conclusions cannot still be formulated and two meta-analyses were conducted to clarify the feasibility of plasmatic *EGFR* mutation detection. Many studies were included, even though conducted with different methods, and globally emerged that plasmatic molecular analysis of EGFR presents a high accuracy suggesting its possible employment when tissue is not available. The evidence that the predictive role of plasmatic *EGFR* mutation has been confirmed and is consistent with data obtain from tissue enforces the utility of plasmatic analysis for *EGFR* mutations detection lung cancer. However, diagnostic sensitivity and specificity are influenced also by plasmatic cftDNA levels that depend on cftDNA mechanisms of release and clearance. Moreover, it has been demonstrated that the levels of cftDNA are also determined by several tumor-related factor including tumor mass, stage of disease, vascularization, aggressivity and certainly other are unknown. These issues need to be clarified before cftDNA enter in current clinical practice.

In a minority of patients, the analysis on cftDNA permitted the isolation of *KRAS* mutation along with the presence of EGFR activating mutation. This is an element of particular interest, as these two alterations have been always considered mutually exclusive and only one report signaled their co-existence in tissue. This finding could be explained considering that plasmatic molecular characterization overcome the limit of tumoral heterogeneity and theoretically permit to identify mutations expressed by clones situated in different body sites. However, it should be considered that new technologies present higher sensitivity than previous ones and therefore could be able to detect molecular alterations expressed by limited number of tumoral cells opening new perspectives on tumor biology.

Finally, the application of cftDNA analysis in the field of acquired resistance to EGFR-TKI is of particular interest. In general, the profile of acquired resistance mechanisms

expressed in plasma is consistent to what revealed in tissue samples and T790M, which represent a predicting factor of response to third-generation TKI, emerged as the most frequent resistance mutation. The opportunity of obtaining molecular information avoiding serial re-biopsies permitted to explore the dynamic process leading to resistance. Different authors demonstrated that levels of EGFR activating mutation promptly decreased in plasma after the initiation of EGFR-TKI and that the occurrence of T790M is an early phenomenon that anticipates of several weeks the radiological progression. Again, modifications of T790M levels in response to third-generation EGFR-TKI have been described, even if predictive and prognostic impact is unclear. To date, these findings have not any clinical consequences. However, the efficacy of TKI-therapy modulation basing on fluctuations of plasmatic activating and resistance mutations levels deserved to be valued prospectively in the future and represent a promising research topic.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Bordi P, Del Re M, Danesi R, Tiseo M. Circulating DNA in diagnosis and monitoring *EGFR* gene mutations in advanced non-small cell lung cancer. *Transl Lung Cancer Res* 2015;4(5):584-597. doi: 10.3978/j.issn.2218-6751.2015.08.09