



Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejancer.com



Review

What do we need to make circulating tumour DNA (ctDNA) a routine diagnostic test in lung cancer?



Reyes Bernabé ^{a,b}, Nicholas Hickson ^c, Andrew Wallace ^c,
Fiona Helen Blackhall ^{a,d,*}

^a Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester, UK

^b Medical Oncology Department, Hospital Virgen Del Rocío, Seville, Spain

^c Manchester Centre for Genomic Medicine, St. Mary's Hospital, Manchester, UK

^d Division of Molecular and Clinical Cancer Sciences, University of Manchester, Manchester, UK

Received 16 January 2017; received in revised form 31 March 2017; accepted 27 April 2017

Available online 10 June 2017

KEYWORDS

Epidermal growth
factor receptor
mutation;
Circulating tumour
DNA;
Liquid biopsy

Abstract The gold standard test for detection of epidermal growth factor receptor (EGFR) mutation is to genotype somatic DNA extracted from a tissue biopsy or cytology specimen. Yet, in at least 20% of patients this is not possible for various reasons including insufficient availability of neoplastic tissue, lack of fitness of the available tissue for a biopsy or that a biopsy is not technically feasible. Consequently, there has been intense investigation of circulating tumour DNA (ctDNA), released into the plasma fraction of blood from cancer cells during apoptosis/necrosis, as a minimally invasive ‘liquid biopsy’ and surrogate for cancer tissue. In 2014, the license for the EGFR tyrosine kinase inhibitor (EGFR-TKI), gefitinib, was updated to allow the use of plasma to determine EGFR mutation status in patients where tissue was not available. Then in 2016 the United States Food and Drug Administration (US FDA) approved the first companion diagnostic plasma EGFR test. Herein, we review the evidence for ctDNA as a diagnostic in patients with non-small cell lung cancer (NSCLC) and describe steps needed to incorporate such ‘liquid biopsies’ into everyday routine practice.

© 2017 Elsevier Ltd. All rights reserved.

* Corresponding author. Fax: +44 (0)161 446 3299.

E-mail address: Fiona.Blackhall@christie.nhs.uk (F.H. Blackhall).

1. Introduction

Therapeutic decisions for patients with non-squamous, non-small cell lung cancer (NSCLC) depend on the presence or absence of sensitising mutations in the epidermal growth factor receptor (EGFR) [1]. When an activating tyrosine kinase domain mutation is present, treatment with an EGFR tyrosine kinase inhibitor (EGFR-TKI) -gefitinib, erlotinib, afatinib – is superior to chemotherapy [2] but not when the mutation is absent [3]. Subsequently, progression on a first-line EGFR-TKI is associated with a resistance mutation (p.T790M) in around 50% of patients [4] that predicts for response to the third generation EGFR-TKI, osimertinib. [5]. The gold standard test for EGFR mutation is to genotype somatic DNA extracted from a tissue biopsy or cytology specimen [6,7]. Audit data from the United Kingdom has demonstrated that in the treatment naïve setting this is problematic in at least 20% of patients [8] and attributed to various reasons including insufficient availability of neoplastic tissue, lack of fitness of available for a biopsy or that a biopsy is not technically feasible [9]. In the setting of progression on an EGFR-TKI, a further limitation to the reliance on a biopsy or cytology specimen is that of tumour evolution and heterogeneity, whereby the presence of resistance gatekeeper mutation T790M may be missed in a biopsy of a single metastatic site [10]. Circulating tumour DNA (ctDNA) is released into the plasma fraction of blood from cancer cells undergoing apoptosis or necrosis and is a minimally invasive ‘liquid biopsy’ [7]. In 2014, the license for gefitinib was updated to state ‘If a tumour sample is not evaluable, then ctDNA obtained from a blood (plasma) sample may be used’ based on data for the therascreen EGFR RGQ PCR Kit [11]. In 2016, the first companion diagnostic plasma EGFR test to be

approved was the Cobas EGFR mutation testing platform [12]. Herein, we review the evidence for ctDNA as a routine molecular diagnostic test in NSCLC, in treatment naïve patients and in the context of progression on a first-line EGFR-TKI, with a focus on the steps required for routine implementation.

2. When is a molecular diagnostic test ready for the clinic?

An evaluation framework for emerging genetic technologies/tests, the well known ACCE framework (Fig. 1) describes a process for the development and introduction of a new molecular diagnostic test. This encompasses analytical performance and validation (A), clinical validation (C), clinical utility (C) and consideration of the ethical, legal and social implications of the test (E) [13]. These criteria were developed for germline DNA and equally apply to tests for somatic mutations including those detected in ctDNA. *Analytical performance and validation* refers to the optimisation and technical performance of the test including sensitivity, specificity, limits of detection and the influence of different sample processing conditions. *Clinical validation* includes the procedures and operational standards from the point of sample collection through DNA processing/extraction and clinical performance; concordance of the test with the gold standard test, if applicable, as well as sensitivity, specificity, positive and negative predictive value. *Clinical utility* refers to the value of the test for an individual patient and specifically whether the test and any subsequent interventions lead to an improved health outcome. The risks, benefits, social, *ethical, legal and cost implications* are also integral to the assessment of clinical utility. Finally, for a predictive test for therapy selection there must be a licensed indication and the new test should have

The ACCE framework

A nalytical performance and validation	technical performance - sensitivity, specificity, limit of detection, sample processing
C linical validation	operational standards from sample collection through DNA processing to clinical performance (sensitivity, specificity etc) compared to the ‘gold standard’ test
C linical utility	value of the test for the individual – does the test & subsequent intervention(s) lead to an improved health outcome ?
E thical, legal & social implications of the test	risks, benefits & cost implications

Fig. 1. When is a DNA test ready for the clinic? (Adapted from Haddow JE PG. ACCE: a model process for evaluating data on emerging genetic tests. In: Human Genome Epidemiology: A Scientific Foundation for Using Genetic Information to Improve Health and Prevent Disease. New York: Oxford University Press; 2003. p. 217–33) [13].

advantages over the gold standard test where one exists. The advantage can be in one or more aspects of test performance e.g. sensitivity, specificity, acceptability, speed or cost and can be limited to a subgroup of patients. The above aspects can be considered as two main questions for clinical implementation:

1. Is ctDNA reliable for determining the presence or absence of EGFR mutation?
2. Does the test result from ctDNA have the same clinical benefit as that from tissue?

3. Is ctDNA reliable for determining the presence or absence of EGFR mutation?

The reliability of the ctDNA test starts with sample processing as suboptimal ctDNA quality and quantity will impair test performance. It is now established that extraction from plasma compared to serum provides a higher yield [14–16]. Storage of blood at non-ambient temperatures for long periods increases lymphocyte haemolysis that ‘swamps’ ctDNA with germline DNA and reduces sensitivity [17,18]. Plasma can be separated and frozen within 1 h from phlebotomy to avoid this but is not ‘clinic friendly’. Proprietary blood collection tubes stabilise lymphocytes, prevent lysis at ambient temperature and accommodate delay in processing for several days [19]. Conventional methods of DNA extraction do not efficiently isolate the full range of ctDNA because

the DNA size profile differs markedly from that of nuclear DNA. Large ctDNA fragments of several kilobases (kb) in size are believed to arise from necrosis whereas small fragments (180–540 bp in size) are thought to originate from apoptosis [20]. A technical comparison of four proprietary kits demonstrated superior extraction efficiency and recovery of small fragments for the Qiagen QIAamp CNA kit [21]. Using this kit the sensitivity of the same plasma genotyping method for EGFR improved from 17% to 52% [22].

A plethora of genotyping methods to detect EGFR mutations in blood have been evaluated (Table 1). In four meta-analyses that collectively included more than 3000 patients and 30 studies, the specificities of EGFR testing ranged from 88 to 97% and sensitivities from 62 to 67.5% for ctDNA with tissue as reference [23–26]. The studies were highly heterogeneous with respect to the patients included, were retrospective, used different protocols for blood processing and DNA extraction, and comprised at least 13 different genotyping methods. While higher specificity than sensitivity was relatively consistent, independent of the method used, variable results for the same method were obtained in these and other analyses calling the reliability of ctDNA for routine clinical use to be questioned. For example, sensitivities reported for denaturing high-performance liquid chromatography (DHPLC) and amplification-refractory mutation system (ARMS) ranged from 63.5% [27] to 81.2% [28] and 43.1% [29] to 70%, respectively

Table 1
Genotyping Methods assessed for detection of EGFR mutation in ctDNA.

Genotyping method (Abbreviation)	Comment	p.T790M detection	Reference
Scorpion Amplification Refractory Mutation System (ARMS)	Real time PCR	Yes	[54]
High Resolution Melting (HRM)	PCR		[23–26]
Mutant Enriched Liquid chip (MEL)			[23–26]
Peptide Nucleic Acid (PNA)-mediated PCR clamping (PNA)	PCR		[23–26]
Denaturing High-Performance Liquid Chromatography (DHPLC)	PCR	Yes	[54]
Peptide Nucleic Acid-Locked Nucleic Acid PCR (PNA-LNA)	PCR		[23–26]
Mutant Enriched PCR (ME-PCR)	PCR		[23–26]
Allele-Specific Arrayed Primer Extension (AS–Apex)	Minisequencing		[23–26]
Digital Polymerase Chain Reaction (dPCR)	Digital PCR	Yes	[54]
Beads, emulsion, amplification, and magnetics (BEAMing)	Digital PCR		[38]
Mass spectrometry genotyping (MSGa)	Mass spectrometry		[23–26]
Restriction fragment length polymorphism (RFLP)	PCR		[23–26]
Pyrosequencing	Pyrosequencing		[23–26]
Sequenom	Agena. Mass spectrometry		[55]
Cobas EGFR blood test	Real time PCR		[44]
Qiagen Therascreen® EGFR RGQ PCR kit	ARMS based	Yes	[22,33,41]
DxS EGFR mutation test kit for research use only	ARMS based		[29]
Multiplex 5' nuclease real-time PCR (Taqman) – PNA clamp	Real time PCR		[43]
Peptide nucleic acid-zip nucleic acid polymerase chain reaction clamp method (PNA-ZNA PCR clamp)	PCR		[22,49]
BEAMing digital PCR (BEAMing dPCR)	Digital PCR	Yes	[33,38]
Roche Cobas® EGFR Mutation Test	Real time PCR	Yes	[22,33]
Roche Cobas EGFR Mutation Test v2	FDA approved		[12]
Short footprint mutation enrichment next generation sequencing (NGS)	NGS		[37]
Droplet Digital™ PCR	Digital PCR	Yes	[33]
Cycleave®	Real time PCR		[22]

[30]. ARMS compared to non-ARMS-based methods was less sensitive as per one meta-analysis [26], more sensitive than DHPLC and mutant-enriched polymerase chain reaction (ME-PCR) according to another study [31] and of similar sensitivity (65%) to peptide nucleic acid-clamp in a further study [22,32]. These discrepancies were most likely due to heterogeneous clinical populations, sample processing and differences in laboratory practice.

In an effort to reduce such confounders, there was a study involving 56 centres on newly diagnosed patients with locally advanced or metastatic treatment naïve advanced NSCLC; ASSESS was a large, non-interventional study that enrolled 1311 patients (881 in Europe) with advanced NSCLC from 56 centres in Europe and Japan [22] to evaluate ‘real-world’ diagnostic performance of various plasma ctDNA diagnostics compared to tissue/cytology based testing. The latter was performed according to local practice and plasma testing was centralised in seven designated laboratories with a standard protocol for ctDNA processing and a prespecified protocol (one of five methods) for genotyping. The concordance of EGFR mutation status in tissue/cytology samples versus plasma ctDNA was 89%, specificity was 97%, and sensitivity was 46%. Of the five methods used for plasma testing the Qiagen therascreen EGFR RGQ PCR kit and the Roche cobas EGFR Mutation test were more sensitive (73% and 75% respectively).

There have been few direct comparisons of different genotyping methods on the same samples. The aforementioned studies were conducted in the ‘pre-T790M’ era with a focus on sensitising mutations. Thress *et al.* [33] provide results of a study designed to compare four genotyping methods for sensitising mutations and T790M on the same samples. In this study, there was variation in technical performance according to the precise EGFR mutation with sensitivities of 78%–100% for the commonest sensitising exon 19 deletions and the p.L858R mutation; and sensitivities of 29%–71% for the p.T790M resistance mutation. Overall the Cobas EGFR mutation test and Beads, Emulsion, Amplification and Magnetics type digital polymerase chain reaction (BEAMing dPCR) were most sensitive [33]. The Cobas EGFR mutation v2 testing platform was approved based on preclinical performance data and clinical data submitted from the ENSURE [34], Aspiration [35] and AURA2 trials [36]. Other tests continue to evolve. A short footprint mutation enrichment next generation sequencing method demonstrated sensitivities of 93% for p.T790M, 100% for p.L858R and 87% for exon 19 deletions in plasma ctDNA [37]. In a similar analysis with BEAMing dPCR sensitivities of 70%, 82% and 86% were obtained for plasma for p.T790M, exon 19 deletions and p.L858R mutations respectively [38].

It can be concluded from studies conducted in treatment naïve patients and in the context of

progression on an EGFR-TKI that ctDNA can reliably detect an EGFR mutation with methods that are now available for plasma processing, ctDNA extraction and genotyping. Using the tissue test as the reference, the plasma ctDNA test is less sensitive in both the treatment naïve and progression settings. For this reason, current licenses for EGFR-TKIs and for the Cobas method state that a tissue biopsy should be considered where possible when the plasma result is negative. Although the Cobas assay is approved there are other methods currently, and in the future that may have comparable and improved sensitivity. Also, some rare mutations are not covered in the Cobas panel. With the increasing use of third generation EGFR-TKIs and potential for other resistance mutations to emerge plasma genotyping technologies will need to evolve in parallel. As a case in point, acquisition of a p.C797S mutation is demonstrated to confer acquired resistance to osimertinib, and this mutation is currently not tested for within the Cobas EGFR mutation testing platform [12,39].

4. Does the ctDNA test have the same clinical benefit as the tissue-based test?

Independent of the method used to establish the presence of an EGFR mutation, evidence of clinical utility and health outcome benefit is required for approval by payers and regulators [13,40]. Ideally results from randomised studies that include test negative and positive populations allocated to different treatments are needed although the license update for gefitinib to be prescribed using a ctDNA result in the absence of a tissue result came from the single cohort IFUM study [41]. With respect to randomised trials the IPASS (gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia) trial [5] included a pre-planned, exploratory analysis in a subgroup of 233 Japanese patients. A false negative result in blood was obtained in 57% of cases, but there were no false positive results for plasma with tissue as the reference. Among patients ctDNA positive for an EGFR mutation the progression-free survival (PFS) was significantly longer and the response rate (RR) higher for gefitinib compared to chemotherapy [29], mirroring the results previously obtained for the tissue positive population [42]. Similarly, in the EURTAC trial in a prespecified analysis of 76 patients with both positive plasma and tissue for EGFR mutation only erlotinib treatment was an independent predictor of longer PFS in multivariate analysis (HR, 0.41 [95% CI, 0.23–0.74]; $p = 0.003$) [43]. The First-line Asian Sequential Tarceva and Chemotherapy Trial (FASTACT-2) trial [44] assessed the efficacy of erlotinib or placebo intercalated with gemcitabine and platinum chemotherapy followed by maintenance erlotinib or placebo. A preplanned, exploratory analysis in 238

patients for whom both tumour tissue and blood samples were available demonstrated a significantly longer median PFS of 13.1 months for erlotinib intercalated with chemotherapy compared to 6 months for treatment with placebo and chemotherapy (HR 0.22, 95% CI 0.14–0.33, $p < 0.0001$). Together the results for first-line EGFR-TKI therapy in patients with positive ctDNA for EGFR mutation infer the same clinical utility as for tissue derived EGFR mutation status. With respect to the United States Food and Drug Administration (US FDA) approval of EGFR Cobas v2 as a plasma companion diagnostic, the evidence for clinical utility and equivalent health benefit of the plasma test was bridged from the clinical outcomes based on tissue diagnosis of EGFR mutation. Accompanying plasma samples were analysed and the clinical outcome inferred from the tissue-based result. As outlined earlier with respect to the lower sensitivity of plasma testing compared to tissue testing it is important to note that in the case of a plasma negative result but a tumour-positive result the plasma result is non-informative for clinical utility. This is best illustrated in treatment naïve patients by the results for the LUX LUNG 3 and 6 trials [16] in which all patients were mutation-positive in tissue and a substantial proportion were plasma-negative but still benefited from the EGFR-TKI (Table 2); reinforcing why tissue testing is still required in patients who are plasma-negative. Similarly, in the context of progression on an EGFR-TKI, the response rates and survival in patients with a positive plasma ctDNA test for p.T790M who were treated with a third generation EGFR-TKI mirror those with a positive p. T790M tissue test and plasma-negative, tissue-positive patients are again uninformative for clinical utility [5,37,38].

The question then is what about tissue-negative, plasma-positive patients? In the treatment naïve setting, data is lacking for outcomes of patients with positive plasma and negative tissue as most studies have excluded tissue-negative patients from analysis. In the progression setting, and using tissue as a reference, a lower specificity for plasma testing has been reported meaning a higher ‘false positive’ rate for plasma. Yet, the clinical outcomes of patients with p.T790M positive plasma but negative tissue are the same as those obtained for patients with both positive plasma and tissue. The tissue result is therefore misleading and a ‘false negative’ most likely due to tumour heterogeneity. Interestingly, patients with negative plasma and positive tumour for p.T790M were noted to have inferior response rates and survival on treatment with osimertinib when compared to patients with positive plasma [38]. The plasma ctDNA result may provide a superior overall ‘picture’ of the mutational burden of the tumour on progression. Clinical outcome on treatment with the appropriate targeted therapy is the most relevant reference for further development of ctDNA tests [38].

5. What else do we need for routine implementation of ctDNA testing?

The approvals for the use of EGFR-TKIs on the basis of EGFR mutation status obtained from ctDNA [11,12] open the door for long awaited, less invasive and logistically easier ‘liquid’ biopsies in place of tissue biopsies. Methods to optimise blood sample processing and efficiency of ctDNA extraction alongside technologies with better analytical and clinical performance are

Table 2

Results for clinical outcomes of patients according to plasma EGFR mutation status in randomised trials of EGFR-TKIs versus chemotherapy and the IFUM phase IV study.

Study [ref] (TKI)	Method (see Table 1 for abbreviations)	ctDNA test performance (tissue as reference)			Clinical outcome for TKI versus chemotherapy (CT)			
					Tissue +		Plasma +	
		Specificity (PPV)	Sensitivity (NPV)	Concordance	RR %	PFS (Hazard ratio)	RR %	FS (Hazard ratio)
Studies evaluating first-line EGFR-TKIs								
IPASS [29] (G)	DxS ARMS	100%	43.1%	66.3%	69 versus 48.5%	0.70	75 versus 64%	0.29*
IFUM [41] (G)	Therascreen RGQ	99.8% (98.6%)	65.7% (93.8%)	94.3%	60	NA	77	NA
EURTAC [43] (E)	Taqman	100%	78%	72.7%	65.1 versus 16.1%	0.34	No difference	0.36**
FASTACT-2 [44] (E) (note CT ± E)	Cobas blood	96% (94%)	75% (85%)	88%	—	0.25	66.vs24.2%***	0.22
LUX-LUNG 3 [16] (A)	Therascreen 29	—	—	60.5%	—	—	—	0.35
LUX-LUNG 6 [16] (A)								0.25
ENSURE [12,34] (E)	Cobas v2	(96.8%)	(81.4%)	77%	62.7 versus 33.6%	0.34	NA	NA
AURA3 [5,12] (O)	Cobas v2	—	—	—	71% versus 31%	0.3	77% versus 39%	0.42

Abbreviations: TKI: tyrosine kinase inhibitor, PPV: positive predictive value, NPV: negative predictive value, G: gefitinib, E: erlotinib, A: afatinib, O: osimertinib, RR: response rate, PFS: progression free survival (median months) HR: Hazard Ratio, NA: not assessed (single arm study), NS: not stated, *significant interaction test for biomarker p value < 0.05 , **E independent predictor in multivariate analysis, ***based on cycle 3 ctDNA EGFR mutation status.

now available. However, a ‘best’ test and ‘optimal’ criteria are not established. The specificity should be as high as possible, approaching 99–100%, in order to avoid giving an ineffective treatment (i.e. an EGFR-TKI to a patient with a false positive result). The sensitivity should also be as high as possible but a lower sensitivity of approximately 65% can still be clinically useful provided the treating clinician understands the probability of a false negative result. Currently, ctDNA testing is complementary to tissue testing.

Laboratories delivering ctDNA tests will first need to conduct a validation or verification for implementation of a laboratory-developed test or commercial diagnostic, respectively. A verification process ensures that ‘the test is being performed correctly’ [45]. Ideally the laboratory should have: (1) previous experience in the analysis of tumour tissue and preferably, germline and circulating free DNA; (2) accreditation as a Medical Laboratory to ISO15189 or equivalent; (3) conduct internal validation quality assurance (QA), maintain on-going validation and verification of the ctDNA test and develop a clinical test report that can be easily interpreted by the treating clinician [EMQN]; (4) participate in external QA programmes [e.g. EMQN] to identify process errors [46]; (5) analyse a sufficiently large number of cases per year to monitor the clinical utility and health economic impact of the test and metrics such as turnaround times compared to tissue testing. Collection and analysis of outcomes of patients with a negative result; how often a biopsy is attempted, the result, and their outcomes will also be relevant for health economic analyses. Clinical characteristics such as poor performance status and comorbidity that might occur more frequently in patients for whom biopsy is not feasible should be factored into outcomes [38]. Further data on the influence of disease distribution, tumour burden and heterogeneity; and factors that increase the chance of a false negative result will help to refine clinical diagnostic algorithms and guidelines.

Currently two clinical scenarios are proposed for a ctDNA test; in newly diagnosed patients in whom tissue is not available and in patients on progression on a first-line EGFR-TKI. In newly diagnosed patients, more data are needed to determine the proportion who have a plasma positive test in the absence of tissue to test. In the latter context in the AURA3 trial of osimertinib versus chemotherapy 23% of patients with a negative T790M tissue test had a positive plasma test [47]. This result reinforces inadequacy of reliance on tissue alone in the progression setting and the clinical algorithm of first plasma testing, then only if negative to perform a biopsy for a tissue test [38].

A scenario for ongoing investigation is whether an individual’s treatment can be tailored more precisely according to the type, level and dynamic change of the mutation in the circulation. The presence of p.L858R in blood [43], the level of mutation [48], persistence of

mutation after commencing treatment [49] and emergence of p.T790M on first-line treatment [50] have been associated with poorer outcomes. The possibility to monitor serially for p.T790M also raises several questions. Does p.T790M emerge prior to radiological progression? When and how often should serial monitoring be performed? Does switching therapy on detection of p.T790M prior to radiological progression improve outcome? Can serial plasma EGFR monitoring replace conventional imaging of disease status? Finally, there is potential to assay DNA in urine with results comparable to those derived from blood [37].

While there has been considerable progress in establishing liquid biopsies for assessment of EGFR mutation, future development would be enhanced by standards for reporting on predictive biomarkers from liquid biopsy. Although STARD [51], REMARK [52], CONSORT [53] and ACCE guidelines [13] exist they do not encompass all the data and evidence required. Anecdotally more recent publications have provided levels of detail required by regulatory authorities (FDA, NICE) with that of Reckamp *et al.* (2016) an exemplar [37]. Such standards would facilitate more accurate cross study comparisons of technologies and outcomes.

To conclude, the ACCE framework mandates that a new test should have advantage over the gold standard [13]. The ease and convenience of a blood test compared to a biopsy is clear. Increased use of ctDNA testing where clinically appropriate will widen access to molecular testing, reduce risks from invasive biopsy, improve patient experience, ease pressure on interventional resource and in the case of p.T790M detection identify more patients who can benefit from a third generation EGFR-TKI than tissue biopsy alone. The turnaround time of ctDNA testing appears to be shorter than for tissue [37] and as technology continues to evolve plasma (and/or urine) could replace tissue for diagnosis of EGFR mutation. To this end the next step is to implement ctDNA into everyday practice to fully realise the potential for liquid biopsy as a diagnostic tool for precision medicine.

Conflict of interest statement

FB has received consulting fees or honoraria from Astra Zeneca, Pfizer, Boehringer-Ingelheim, Medivation and MSD. AW received speaker services from Astra-Zeneca, Merck-Serono and Boehringer-Ingelheim.

Role of the funding source

Dr R. Bernabé was funded by Spanish Society of Medical Oncology (SEOM) translational research fellowship grant. The research was supported by Experimental Cancer Medicine Centres (ECMC) Network (C480\A15578) and the National Institute for

Health Research (NIHR) Christie Clinical Research Facility. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

References

- [1] Novello S, Barlesi F, Califano R, Cufer T, Ekman S, Levra MG, et al., ESMO Guidelines Committee. Metastatic non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol Off J Eur Soc Med Oncol* 2016 Sep;27(Suppl. 5):v1–27.
- [2] Greenhalgh J, Dwan K, Boland A, Bates V, Vecchio F, Dundar Y, et al. First-line treatment of advanced epidermal growth factor receptor (EGFR) mutation positive non-squamous non-small cell lung cancer. *Cochrane Database Syst Rev* 2016 May 25;5:CD010383.
- [3] Lee J-K, Hahn S, Kim D-W, Suh KJ, Keam B, Kim TM, et al. Epidermal growth factor receptor tyrosine kinase inhibitors vs conventional chemotherapy in non-small cell lung cancer harboring wild-type epidermal growth factor receptor: a meta-analysis. *JAMA* 2014 Apr 9;311(14):1430–7.
- [4] Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011 Mar 23;3(75):75ra26.
- [5] Mok TS, Wu Y-L, Ahn M-J, Garassino MC, Kim HR, Ramalingam SS, et al., AURA3 Investigators. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. *N Engl J Med* 2017 Feb 16;376(7):629–40.
- [6] Leighl NB, Rekhman N, Biermann WA, Huang J, Mino-Kenudson M, Ramalingam SS, et al. Molecular testing for selection of patients with lung cancer for epidermal growth factor receptor and anaplastic lymphoma kinase tyrosine kinase inhibitors: American Society of Clinical Oncology endorsement of the College of American Pathologists/Internat. *J Clin Oncol* 2014 Nov 10;32(32):3673–9.
- [7] Tan DSW, Yom SS, Tsao MS, Pass HI, Kelly K, Peled N, et al. The international association for the study of lung cancer consensus statement on optimizing management of EGFR mutation-positive non-small cell lung cancer: status in 2016. *J Thorac Oncol* 2016 Jul;11(7):946–63.
- [8] [Internet] National lung cancer audit report. 2012. Available from: <http://content.digital.nhs.uk/catalogue/PUB09183/clin-audi-supp-prog-lung-nlca-lap-2012-rep.pdf>.
- [9] Fenizia F, De Luca A, Pasquale R, Sacco A, Forgione L, Lambiase M, et al. EGFR mutations in lung cancer: from tissue testing to liquid biopsy. *Future Oncol* 2015;11(11):1611–23.
- [10] Campo M, Gerber D, Gainor JF, Heist RS, Temel JS, Shaw AT, et al. Acquired resistance to first-line afatinib and the challenges of prearranged progression biopsies. *J Thorac Oncol* 2016 Nov;11(11):2022–6.
- [11] Iressa product information [Internet]. [cited 2017 Jan 11]. Available from: www.ema.europa.eu/docs/en_GB/document_library/WC500036358.pdf.
- [12] Cobas® EGFR mutation test v2 [Internet]. [cited 2017 Jan 11]. Available from: http://www.accessdata.fda.gov/cdrh_docs/pdf15/P150047c.pdf.
- [13] Haddow JEPG. ACCE: a model process for evaluating data on emerging genetic tests. In: *Human genome epidemiology: a scientific foundation for using genetic information to improve health and prevent disease*. New York: Oxford University Press; 2003. p. 217–33.
- [14] Board RE, Ellison G, Orr MCM, Kemsley KR, McWalter G, Blockley LY, et al. Detection of BRAF mutations in the tumour and serum of patients enrolled in the AZD6244 (ARRY-142886) advanced melanoma phase II study. *Br J Cancer* 2009 Nov 17;101(10):1724–30.
- [15] Hung ECW, Chiu RWK, Lo YMD. Detection of circulating fetal nucleic acids: a review of methods and applications. *J Clin Pathol* 2009 Apr;62(4):308–13.
- [16] Wu Y-L, Sequist LV, Hu C-P, Feng J, Lu S, Huang Y, et al. EGFR mutation detection in circulating cell-free DNA of lung adenocarcinoma patients: analysis of LUX-Lung 3 and 6. *Br J Cancer* 2017 Jan 17;116(2):175–85.
- [17] Jung M, Klotz S, Lewandowski M, Fleischhacker M, Jung K. Changes in concentration of DNA in serum and plasma during storage of blood samples. *Clin Chem* 2003 Jun;49(6 Pt 1):1028–9.
- [18] Hidestrand M, Stokowski R, Song K, Oliphant A, Deavers J, Goetsch M, et al. Influence of temperature during transportation on cell-free DNA analysis. *Fetal Diagn Ther* 2012;31(2):122–8.
- [19] Norton SE, Lechner JM, Williams T, Fernando MR. A stabilizing reagent prevents cell-free DNA contamination by cellular DNA in plasma during blood sample storage and shipping as determined by digital PCR. *Clin Biochem* 2013 Oct;46(15):1561–5.
- [20] Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001 Feb 15;61(4):1659–65.
- [21] Devonshire AS, Whale AS, Gutteridge A, Jones G, Cowen S, Foy CA, et al. Towards standardisation of cell-free DNA measurement in plasma: controls for extraction efficiency, fragment size bias and quantification. *Anal Bioanal Chem* 2014 Oct;406(26):6499–512.
- [22] Reck M, Hagiwara K, Han B, Tjuland S, Grohé C, Yokoi T, et al. ctDNA determination of EGFR mutation status in European and Japanese patients with advanced NSCLC: the ASSESS study. *J Thorac Oncol* 2016 Oct;11(10):1682–9.
- [23] Qiu M, Wang J, Xu Y, Ding X, Li M, Jiang F, et al. Circulating tumor DNA is effective for the detection of EGFR mutation in non-small cell lung cancer: a meta-analysis. *Cancer Epidemiol Biomark Prev* 2015 Jan;24(1):206–12.
- [24] Li Z, Zhang Y, Bao W, Jiang C. Insufficiency of peripheral blood as a substitute tissue for detecting EGFR mutations in lung cancer: a meta-analysis. *Target Oncol* 2014 Dec;9(4):381–8.
- [25] Luo J, Shen L, Zheng D. Diagnostic value of circulating free DNA for the detection of EGFR mutation status in NSCLC: a systematic review and meta-analysis. *Sci Rep* 2014 Sep 9;4:6269.
- [26] Wu Y, Liu H, Shi X, Song Y. Can EGFR mutations in plasma or serum be predictive markers of non-small-cell lung cancer? A meta-analysis. *Lung Cancer* 2015 Jun;88(3):246–53.
- [27] Huang Z, Wang Z, Bai H, Wu M, An T, Zhao J, et al. The detection of EGFR mutation status in plasma is reproducible and can dynamically predict the efficacy of EGFR-TKI. *Thorac Cancer* 2012 Nov;3(4):334–40.
- [28] Bai H, Mao L, Wang HS, Zhao J, Yang L, An TT, et al. Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small-cell lung cancer. *J Clin Oncol* 2009 Jun 1;27(16):2653–9.
- [29] 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 Jan;7(1):115–21.
- [30] 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 Apr 15;15(8):2630–6.
- [31] Xu F, Wu J, Xue C, Zhao Y, Jiang W, Lin L, et al. Comparison of different methods for detecting epidermal growth factor receptor mutations in peripheral blood and tumor tissue of non-small cell

- lung cancer as a predictor of response to gefitinib. *Onco Targets Ther* 2012;5:439–47.
- [32] Pasquale R, Fenizia F, Esposito Abate R, Sacco A, Esposito C, Forgione L, et al. Assessment of high-sensitive methods for the detection of EGFR mutations in circulating free tumor DNA from NSCLC patients. *Pharmacogenomics* 2015;16(10):1135–48.
- [33] Thress KS, Brant R, Carr TH, Dearden S, Jenkins S, Brown H, et al. EGFR mutation detection in ctDNA from NSCLC patient plasma: a cross-platform comparison of leading technologies to support the clinical development of AZD9291. *Lung Cancer* 2015 Dec;90(3):509–15.
- [34] Wu Y-L, Zhou C, Liang C-K, Wu G, Liu X, Zhong Z, et al. First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. *Ann Oncol Off J Eur Soc Med Oncol* 2015 Sep;26(9):1883–9.
- [35] Park K, Yu C-J, Kim S-W, Lin M-C, Sriuranpong V, Tsai C-M, et al. First-line erlotinib therapy until and beyond response evaluation criteria in solid tumors progression in Asian patients with epidermal growth factor receptor mutation-positive non-small-cell lung cancer: the ASPIRATION study. *JAMA Oncol* 2016 Mar;2(3):305–12.
- [36] Yang J, Ramalingam SS, Jänne PA, Cantarini M, Mitsudomi T. LBA2_PR: osimertinib (AZD9291) in pre-treated pts with T790M-positive advanced NSCLC: updated Phase 1 (P1) and pooled Phase 2 (P2) results. *J Thorac Oncol* 2016 Apr;11(4 Suppl.):S152–3.
- [37] Reckamp KL, Melnikova VO, Karlovich C, Sequist LV, Camidge DR, Wakelee H, et al. Highly sensitive and quantitative test platform for detection of NSCLC EGFR mutations in urine and plasma. *J Thorac Oncol* 2016 Oct;11(10):1690–700.
- [38] Oxnard GR, Thress KS, Alden RS, Lawrance R, Paweletz CP, Cantarini M, et al. Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer. *J Clin Oncol* 2016 Oct 1;34(28):3375–82.
- [39] Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. *Nat Med* 2015 Jun;21(6):560–2.
- [40] EGFR-TK mutation testing in adults with locally advanced or metastatic non-small-cell lung cancer | 9-related-nice-guidance | Guidance and guidelines | NICE [Internet]. Available from: <https://www.nice.org.uk/guidance/dg9/chapter/9-related-nice-guidance>.
- [41] 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 Jan 7;110(1):55–62.
- [42] Fukuoka M, Wu Y-L, Thongprasert S, Sunpawaravong P, Leong S-S, Sriuranpong V, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). *J Clin Oncol* 2011 Jul 20;29(21):2866–74.
- [43] Karachaliou N, Mayo-de las Casas C, Queralt C, de Aguirre I, Melloni B, Cardenal F, et al., Spanish Lung Cancer Group. Association of EGFR L858R mutation in circulating free DNA with survival in the EURTAC trial. *JAMA Oncol* 2015 May;1(2):149–57.
- [44] Mok T, Wu Y-L, Lee JS, Yu C-J, Sriuranpong V, Sandoval-Tan J, et al. Detection and dynamic changes of EGFR mutations from circulating tumor DNA as a predictor of survival outcomes in NSCLC patients treated with first-line intercalated erlotinib and chemotherapy. *Clin Cancer Res* 2015 Jul 15;21(14):3196–203.
- [45] Mattocks CJ, Morris MA, Matthijs G, Swinnen E, Corveleyn A, Dequeker E, et al., EuroGentest Validation Group. A standardized framework for the validation and verification of clinical molecular genetic tests. *Eur J Hum Genet* 2010 Dec;18(12):1276–88.
- [46] Patton S, Normanno N, Blackhall F, Murray S, Kerr KM, Dietel M, et al. Assessing standardization of molecular testing for non-small-cell lung cancer: results of a worldwide external quality assessment (EQA) scheme for EGFR mutation testing. *Br J Cancer* 2014 Jul 15;111(2):413–20.
- [47] Mok TS, Wu Y-L, Ahn M-J, Garassino MC, Kim HR, Ramalingam SS, et al., AURA3 Investigators. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. *N Engl J Med* 2017;376(7):629–40.
- [48] 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 Aug 1;17(15):5179–87.
- [49] Tseng J-S, Yang T-Y, Tsai C-R, Chen K-C, Hsu K-H, Tsai M-H, et al. Dynamic plasma EGFR mutation status as a predictor of EGFR-TKI efficacy in patients with EGFR-mutant lung adenocarcinoma. *J Thorac Oncol* 2015 Apr;10(4):603–10.
- [50] Soria J, Kim S, Wu Y, Nakagawa K, Yang J, Ahn M, et al. Gefitinib/chemotherapy vs chemotherapy in EGFR mutation-positive NSCLC resistant to first-line gefitinib: IMPRESS T790M subgroup analysis. In: IASLC world lung conference; 2015.
- [51] Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig L, et al., STARD Group. STARD 2015: an updated list of essential items for reporting diagnostic accuracy studies. *BMJ* 2015 Oct;28(351):h5527.
- [52] McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics. Reporting recommendations for tumour MARKer prognostic studies (REMARK). *Eur J Cancer* 2005 Aug;41(12):1690–6.
- [53] Schulz KF, Altman DG, Moher D, CONSORT Group. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Ann Intern Med* 2010 Jun 1;152(11):726–32.
- [54] Wang Z, Chen R, Wang S, Zhong J, Wu M, Zhao J, et al. Quantification and dynamic monitoring of EGFR T790M in plasma cell-free DNA by digital PCR for prognosis of EGFR-TKI treatment in advanced NSCLC. *PLoS One* 2014;9(11):e110780.
- [55] 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 Sep;104(9):1198–204.