The diagnostic accuracy of pleural effusion and plasma samples versus tumour tissue for detection of EGFR mutation in patients with advanced non-small cell lung cancer: comparison of methodologies

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ABSTRACT

Aims To evaluate the suitability of malignant pleural effusion (MPE) and plasma as surrogate samples for epidermal growth factor receptor (EGFR) mutation detection, and compare three different detection methods.

Methods Matched tissue and plasma samples were collected from patients with advanced non-small cell lung cancer (NSCLC) (stage IIIB/IV adenocarcinoma/adenosquamous carcinoma), with matched MPE samples collected from a subgroup. DNA was extracted from tissue, MPE cell block, MPE supernatant and plasma before mutation detection by amplification refractory mutation system (ARMS) (all samples), Sanger sequencing and mutant-specific immunohistochemistry (IHC) (tissue and MPE cell blocks only).

Results Sensitivity of MPE cell block, MPE supernatant and plasma versus tissue: 81.8% (9/11), 63.6% (7/11) and 67.5% (27/40); specificity was 80.0% (8/10), 100% (10/10) and 100% (46/46), respectively. Sensitivity of Sanger sequencing versus ARMS: 81.8% (27/33) for tissue, 40% (4/10) for MPE cell blocks; specificity was 100% (36/36 and 12/12) for both. Sensitivity of mutant-specific IHC versus ARMS: 54.8% (17/31) for tissue, 50.0% (6/12) for MPE cell blocks; specificity was 97.1% (34/35) and 100% (14/14), respectively.

Conclusions MPE and plasma are valid surrogates for NSCLC tumour EGFR mutation detection when tissue is not available. ARMS is most suitable for mutation detection in tissue and MPE cell blocks; however, mutant-specific IHC could be a complementary method when DNA-based molecular testing is unavailable.

INTRODUCTION

Targeted epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, are approved for patients with EGFR mutation-positive advanced non-small cell lung cancer (aNSCLC),[1 2] and EGFR-activating mutations are an accepted molecular biomarker to predict EGFR TKI clinical efficacy.

Clinical application of EGFR mutation testing has progressed significantly, although sample availability remains a challenge. Surgery or biopsy tumour samples are preferred for optimal EGFR mutation detection, but are not always available. For example, 10–50% of patients with aNSCLC experience malignant pleural effusion (MPE),[3–5] for whom pathological diagnosis may rely exclusively on finding cancer cells in the MPE.[6] However, data detailing the sensitivity/specificity of testing MPE samples for EGFR mutations are limited.[7 8] Although currently restricted to the research setting,[9–15] peripheral blood containing circulating-free DNA (cfDNA) from cancer cells[7 8 16] may be an alternative.[9–16]

EGFR mutation testing methodology presents another challenge due to the number of different methodologies available.[8 17] Among these, the widely available, novel mutation-detecting Sanger sequencing method has become established as the 'gold standard'.[18] However, its limitations include low sensitivity, requirement for high-quality tumour samples, longer turn-around time and high reliance on staff experience.[19 20] To overcome these disadvantages, targeted PCR-based methods (eg, amplification refractory mutation system (ARMS)) have been developed and are becoming widely accepted clinically, although they are unable to detect novel mutations. Alternatively, mutant-specific immunohistochemistry (IHC) has the potential to be more easily integrated in pathology laboratories, requires a small amount of material, eliminates the need for DNA and has a very rapid turn-around time, in addition to providing a quantitative assessment of mutation-positive cells, which other methods cannot do. To date, mutant-specific antibodies have been developed to detect the two most common EGFR mutations: an exon 19 deletion (E746-A750del) and the L858R mutation in exon 21,[21] but this method shows inconsistent sensitivity/specificity.[22–29]

We collected matched tissue, MPE and plasma samples from each patient to evaluate the potential of MPE and plasma as surrogate samples for EGFR mutation detection, and compared three different mutation detection technologies: ARMS, Sanger sequencing and mutant-specific IHC. This article focuses on sensitivity/specificity data.

MATERIALS AND METHODS

Patients and samples

Patients with advanced lung adenocarcinoma/adenosquamous carcinoma who provided informed consent at one centre in Beijing, China, from January 2008 to March 2012, were recruited. The study was approved by the hospital institutional ethics committee. Collection of tumour tissue sample and matched plasma was mandatory; collection of a matched pleural effusion sample was

optional but encouraged. Further details relating to tissue, MPE and plasma sampling and handling, including DNA extraction and quality assurance, are provided in online supplementary appendix 1.

EGFR mutation detection

Three methods were used to detect EGFR mutations in the different types of samples. ARMS was used for all four sample types: tumour tissue, MPE cell block, MPE supernatant and plasma. Sanger sequencing and mutant-specific IHC were used for tumour tissue and MPE cell block samples only. Based on previous evaluation and availability of appropriate instruments, the ADx-ARMS kit was used for this study rather than the Qiagen Scorpion ARMS kit. We defined a cut-off of 2% tumour cell content as a sample quality check according to the minimum requirement of ARMS technology (about 1% analytical sensitivity). Samples below this threshold were rejected. Further details are provided in online supplementary appendix 1.

RESULTS

Patient characteristics

In total, 86/124 patients provided both adequate tissue samples (≥2% tumour cells) and sufficient plasma samples to be included in the mutation analyses (table 1 and see online supplementary appendix 1).

EGFR mutation status detected by ARMS

All samples included in the current analyses were evaluable using ARMS. EGFR mutation detection rates were 46.5% (40/86) in tumour tissue samples, 48.1% (13/27) in MPE cell block samples, 33.3% (9/27) in MPE supernatant samples and 31.4% (27/86) in plasma samples.

Table 1 Clinicopathological characteristics of patients and samples (n=86)

Characteristic	Value
Age, years	
Median	55
Range	28–81
Sex, n (%)	
Male	56 (65)
Female	30 (35)
Smoking history, n (%)	
Never smoker	39 (45)
Former smoker	10 (12)
Current smoker	37 (43)
Histology, n (%)	
Adenocarcinoma	85 (99)
Adenosquamous carcinoma	1 (1)
Disease stage, n (%)	
IIIB	4 (5)
IV	82 (95)
Sample collection, n (%)	
Tumour tissue sample	86 (100)
Matched pleural effusion	21 (24)
Unmatched pleural effusion	6 (7)
Matched plasma	86 (100)

EGFR mutation status detected by Sanger sequencing

A number of samples failed Sanger sequencing: 19.8% (17/86) of tumour tissue samples and 18.5% (5/27) of MPE cell block samples. EGFR mutation-positive rates were reported in successful analyses for 39.1% (27/69) of tumour tissue samples and 18.1% (4/22) of MPE cell block samples.

EGFR mutation status detected by mutant-specific IHC

Totally, 24.4% (21/86) of tumour tissue samples and 3.7% (1/27) of MPE cell block samples did not show total EGFR expression and were excluded from the mutation detection using IHC. Among the remaining total EGFR-expressed samples, EGFR mutation positive rates were 27.7% (18/65) in tumour tissue samples and 26.9% (7/26) in MPE cell block samples.

Comparison of different sample types using ARMS

Tumour tissue versus MPE samples

Twenty-one patients provided adequate tumour tissue samples, matched adequate MPE cell block samples and matched MPE supernatant samples. Compared with tumour tissue samples, the sensitivity and specificity of MPE cell block samples for EGFR mutation detection were 81.8% and 80.0%, respectively (table 2A);

Table 2 Comparison of EGFR mutation status detected by ARMS in different sample types

A. Tumour tissue versus MPE cell block

	Tumour tissue		
MPE cell block	**+**	**–**	**Total**
+	9	2	11
–	2	8	10
Total	11	10	21

NPV: 8/10 (80.0%); PPV: 9/11 (81.8%).

B. Tumour tissue versus MPE supernatant

	Tumour tissue		
MPE supernatant	**+**	**–**	**Total**
+	7	0	7
–	4	10	14
Total	11	10	21

NPV: 10/14 (71.4%); PPV: 7/7 (100.0%).

C. MPE cell block versus MPE supernatant

	MPE cell block		
MPE supernatant	**+**	**–**	**Total**
+	9	0	9
–	4	14	18
Total	13	14	27

NPV: 14/18 (77.8%); PPV: 9/9 (100.0%).

D. Tumour tissue versus plasma

	Tumour tissue		
Plasma	**+**	**–**	**Total**
+	27	0	27
–	13	46	59
Total	40	46	86

NPV: 46/59 (79.0%); PPV: 27/27 (100.0%).

ARMS, amplification refractory mutation system; EGFR, epidermal growth factor receptor; MPE, malignant pleural effusion; NPV, negative predictive value; PPV, positive predictive value.

concordance was 81% (17/21). Compared with tumour tissue samples, the sensitivity and specificity of MPE supernatant samples were 63.6% (7/11) and 100% (10/10), respectively (table 2B); concordance was 81% (17/21).

MPE cell block versus MPE supernatant samples

In the 27 patients who provided adequate MPE cell block samples and MPE supernatant samples, the EGFR mutation status concordance rate between MPE cell block and MPE supernatant samples was 85.2% (23/27). Compared with MPE cell block samples, the sensitivity and specificity of MPE supernatant for EGFR mutation detection were 69.2% (9/13) and 100% (14/14), respectively (table 2C).

Tumour tissue versus plasma samples

Compared with tumour tissue samples, the sensitivity and specificity of plasma for EGFR mutation detection were 67.5% (27/40) and 100% (46/46), respectively (table 2D); concordance was 84.9% (73/86).

Comparison of three different methods for EGFR mutation detection

Although the success (pass) rate with ARMS was 100% for tumour tissue samples and MPE cell block samples, the success rate with Sanger sequencing was 80.2% (69/86) and 81.5% (22/27), respectively. After total EGFR expression assessment by IHC, only 75.6% (65/86) of tumour tissue samples and 96.3% (26/27) of MPE cell block samples were judged adequate for mutant-specific IHC testing.

Sixty-nine tumour tissue samples were successfully tested by both ARMS and Sanger sequencing. Compared with ARMS, the sensitivity and specificity of Sanger sequencing for mutation detection were 81.8% (27/33) and 100% (36/36), respectively (table 3A); concordance was 91.3% (63/69). Twenty-two MPE cell block samples were successfully tested by both ARMS and Sanger sequencing. Compared with ARMS, the sensitivity and specificity of Sanger sequencing for EGFR mutation detection were 40% (4/10) and 100% (12/12), respectively (table 3B); concordance was 72.7% (16/22).

Sixty-six tumour tissue samples were successfully tested by both ARMS and mutant-specific IHC. Compared with ARMS, the sensitivity and specificity of IHC for mutation detection were 54.8% (17/31) and 97.1% (34/35), respectively (table 3C); concordance was 77.3% (51/66). Among the IHC mutation-positive cases, the ratio of E19 del:L858R was 10 : 7, which was similar to the 19 : 13 ratio for ARMS mutation-positive cases. Twenty-six MPE cell block samples were successfully tested by both ARMS and IHC. Compared with ARMS, the sensitivity and specificity of IHC for mutation detection were 50% (6/12) and 100% (14/14), respectively (table 3D); concordance was 76.9% (20/26).

Table 3 Comparison of EGFR mutation status detected by different methods in tumour tissue and MPE cell block samples

A. ARMS versus Sanger sequencing for tumour tissue samples

	ARMS		
Sanger sequencing	**+**	**–**	**Total**
+	27	0	27
–	6	36	42
Total	33	36	69

NPV: 36/42 (85.7%); PPV: 27/27 (100.0%).

B. ARMS versus Sanger sequencing for MPE cell block samples

	ARMS		
Sanger sequencing	**+**	**–**	**Total**
+	4	0	4
–	6	12	18
Total	10	12	22

NPV: 12/18 (66.7%); PPV: 4/4 (100.0%).

C. ARMS versus mutant-specific IHC for tumour tissue samples

	ARMS		
IHC	**+**	**–**	**Total**
+	17	1	18
–	14	34	48
Total	31	35	66

NPV: 34/48 (70.8%); PPV: 17/18 (94.4%).

D. ARMS versus mutant-specific IHC for MPE cell block samples

	ARMS		
IHC	**+**	**–**	**Total**
+	6	0	6
–	6	14	20
Total	12	14	26

NPV: 14/20 (75.0%); PPV: 6/6 (100.0%).

ARMS, amplification refractory mutation system; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; MPE, malignant pleural effusion; NPV, negative predictive value; PPV, positive predictive value.

DISCUSSION

Using the sensitive ARMS method, we detected EGFR mutations in 46.5% of tumour tissue samples, 48.1% of MPE cell block samples, 33.3% of MPE supernatant samples and 31.4% of plasma samples. Compared with tumour tissue, the sensitivity and specificity were 81.8% and 80.0% for MPE cell blocks and 63.6% and 100% for MPE supernatant, respectively. EGFR mutations can be detected in MPE from patients with NSCLC, with various mutation-positive rates, and EGFR mutation status between MPE and paired tissue could be different.[30–34] However, to date, there have been limited reports of the sensitivity and specificity of MPE for EGFR mutation detection defined by comparison against matched tumour tissue.[35] From our current study, the sensitivity of MPE cell blocks for EGFR mutation detection was 81.8%, potentially high enough for clinical adoption if tissue is unavailable. In two cases, EGFR mutation was detected in the MPE cell block but not in the tumour tissue by ARMS. As the other techniques confirmed these two cases as EGFR mutation-positive, the EGFR mutation-positive rate was 52.4% (11/21) for both tumour tissue and MPE, further strengthening the argument for the use of MPE cell block samples. We also showed that, although the specificity of the MPE supernatant was 100% versus MPE cell block and tumour tissue, the sensitivity of MPE supernatant was 69.2% versus MPE cell block and 63.6% versus tumour tissue. Therefore, if an adequate MPE cell block or tumour sample is available, MPE supernatant is not recommended for mutation testing.

Plasma cfDNA is generally increased in patients with lung cancer, but with significant interpatient variability.[36] Also, cfDNA tends to be fragmented, with DNA fragments <200 bp,[37 38] and it can be contaminated with wild-type cfDNA. Therefore, reliable extraction of cfDNA extraction prior to sensitive methodology to amplify relatively short DNA fragments is essential. cfDNA EGFR mutation detection rates range from 36% to 92% versus paired NSCLC tumour

tissue.[7 9–11 15 39] Using ARMS, we found a 31.4% EGFR mutation-positive rate in plasma cfDNA, with sensitivity of 67.5% and specificity of 100%. The sensitivity from our study was similar to the 70% reported by Kuang *et al*[11] who used ARMS and WAVE/Surveyor methods for plasma cfDNA EGFR mutation detection in patients with aNSCLC, but was much higher than the 43.1% reported by Goto *et al*[7] who also used an older version of the ARMS method to detect EGFR mutations in serum cfDNA in a similar population. The use of a different ARMS method and serum samples may account for differences between our study and that of Goto *et al*[7]

The positive predictive value from our study of cfDNA was 100%, consistent with Goto *et al*[7] using ARMS, and that reported by Yung *et al*[12] using digital PCR, suggesting that the positive mutation results detected by ARMS and/or digital PCR using cfDNA are highly predictive of a mutation-positive tumour sample. This is essential when considering the potential use of cfDNA, only when tumour tissue is not available. However, because the negative predictive value of cfDNA was only 78% in our study, it is not always possible to detect tumour EGFR mutations using this sample type and tumour tissue should be used if available. While the success (pass) rate of ARMS was 100% for tumour tissue and MPE cell blocks, success rates of Sanger sequencing were 80.2% and 81.5%, and success rates of mutant-specific IHC were 75.6% and 96.3%, respectively.

Sanger sequencing failed to detect ~20% of mutation-positive tumour samples (by ARMS) and 60% of mutation-positive MPE cell block samples; ~20% of samples also failed sequencing, demonstrating the inadequacy of Sanger methodology to detect EGFR mutations in some clinical samples. Sanger sequencing did not detect any novel mutation that would not have been detected using ARMS (which detects >90% of all EGFR mutations). Analysis of the yield of amplifiable DNA and tumour content and their relationship with the performance of Sanger sequencing revealed that 95% of samples that failed DNA sequencing (9/17) had DNA <0.4 ng/μL. Among these, only 25% passed sequencing (3/12), whereas in samples with DNA ≥0.4 ng/μL, 89% passed (66/74). In clinical formalin-fixed, paraffin-embedded samples, low yield of amplifiable DNA is therefore a major factor leading to failure of EGFR mutation detection with Sanger sequencing. When we raised the tumour content cut-off from 1% to 20%, the sensitivity of EGFR mutation detection with Sanger sequencing versus ARMS was 92.3% (24/26); and specificity remained at 100% (27/27), highlighting that when employing Sanger sequencing, samples must be of sufficient quality/quantity, with sufficient DNA for amplification.

Consistent with previous reports, our data showed that the mutant-specific IHC method was highly specific.[22–29] However, it detected considerably fewer mutants than ARMS, and even fewer than Sanger sequencing for tumour tissue, but marginally more than Sanger sequencing for MPE cell block samples. Sensitivity of IHC in this study is considerably lower than in most previously reported studies.[22–29] Possible explanations include: (1) the significant portion of archival tumour tissue samples, which could compromise EGFR protein detection and (2) variations in sample processing and IHC assay conditions. Nevertheless, considering the high specificity and wide availability of IHC in hospitals, this method should be encouraged if DNA-based molecular testing is not available.

Limitations of this study include the relatively small sample size, especially of MPE samples, and lack of clinical response data. Further investigations involving a greater number of samples with correlative clinical outcomes would also be a useful supplement.

In conclusion, MPE and plasma are valid surrogates for NSCLC tumour EGFR mutation detection when tissue is not available. ARMS is most suitable for mutation detection in tissue and MPE cell blocks; however, mutant-specific IHC could be a complementary method when DNA-based molecular testing is unavailable.

Take-home messages

- EGFR mutations in NSCLC predict treatment outcomes and guide patient selection for EGFR TKI therapy.
- Several established and emerging methods exist for the determination of EGFR mutations, most notably Sanger sequencing, ARMS and mutant-specific IHC.
- Determination of the sensitivity and specificity of these methods using paired tumour tissue and MPE or plasma samples revealed that MPE and plasma samples are valid surrogates for NSCLC tumour EGFR mutation detection when tissue is not available.
- ARMS is most suitable for mutation detection in tissue and MPE cell blocks; however, mutant-specific IHC could be a complementary method when DNA-based molecular testing is unavailable.

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Contributors All authors participated in the study design, collection, analysis and interpretation of data, in the writing of the manuscript and in the decision to submit the manuscript for publication. All authors reviewed the draft manuscript, and read and approved the final version for submission. XL and YL contributed equally to the study.

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Competing interests Guanshan Zhu, Yachao Lu, Li Zheng and Qunsheng Ji are employees of AstraZeneca. Gillian Ellison and Rose McCormack are employees of AstraZeneca and hold shares in AstraZeneca. Xiaoqing Liu, Yao Lei, Haifeng Qin and Chuanhao Tang have no conflicts of interest to disclose.

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REFERENCES

1 Peters S, Adjei AA, Gridelli C, *et al*. Metastatic non-small-cell lung cancer (NSCLC): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2012;23(Suppl 7):vii56–64.
2 Azzoli CG, Temin S, Aliff T, *et al*. 2011 Focused Update of 2009 American Society of Clinical Oncology Clinical Practice Guideline Update on Chemotherapy for Stage IV Non-Small-Cell Lung Cancer. *J Clin Oncol* 2011;29:3825–31.
3 Zhang X, Zhao Y, Wang M, *et al*. Detection and comparison of epidermal growth factor receptor mutations in cells and fluid of malignant pleural effusion in non-small cell lung cancer. *Lung Cancer* 2008;60:175–82.
4 Fenton KN, Richardson JD. Diagnosis and management of malignant pleural effusions. *Am J Surg* 1995;170:69–74.

5 Sekine I, Sumi M, Saijo N. Local control of regional and metastatic lesions and indication for systemic chemotherapy in patients with non-small cell lung cancer. *Oncologist* 2008;13(Suppl 1):21–7.
6 Antony VB, Loddenkemper R, Astoul P, *et al*. Management of malignant pleural effusions. *Eur Respir J* 2001;18:402–19.
7 Goto K, Ichinose Y, Ohe Y, *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.
8 Goto K, Satouchi M, Ishii G, *et al*. An evaluation study of *EGFR* mutation tests utilized for non-small-cell lung cancer in the diagnostic setting. *Ann Oncol* 2012;23:2914–19.
9 Bai H, Mao L, Wang HS, *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;27:2653–9.
10 Brevet M, Johnson ML, Azzoli CG, *et al*. Detection of EGFR mutations in plasma DNA from lung cancer patients by mass spectrometry genotyping is predictive of tumor EGFR status and response to EGFR inhibitors. *Lung Cancer* 2011;73:96–102.
11 Kuang Y, Rogers A, Yeap BY, *et al*. Noninvasive detection of EGFR T790M in gefitinib or erlotinib resistant non-small cell lung cancer. *Clin Cancer Res* 2009;15:2630–6.
12 Yung TK, Chan KC, Mok TS, *et al*. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin Cancer Res* 2009;15:2076–84.
13 Mack PC, Holland WS, Burich RA, *et al*. EGFR mutations detected in plasma are associated with patient outcomes in erlotinib plus docetaxel-treated non-small cell lung cancer. *J Thorac Oncol* 2009;4:1466–72.
14 Nakamura T, Sueoka-Aragane N, Iwanaga K, *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 Zhao X, Han RB, Zhao J, *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.
16 Schwarzenbach H, Hoon DS, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat Rev Cancer* 2011;11:426–37.
17 Angulo B, Conde E, Suarez-Gauthier A, *et al*. A comparison of EGFR mutation testing methods in lung carcinoma: direct sequencing, real-time PCR and immunohistochemistry. *PLoS One* 2012;7:e43842.
18 Bakker E. Is the DNA sequence the gold standard in genetic testing? Quality of molecular genetic tests assessed. *Clin Chem* 2006;52:557–8.
19 Ogino S, Kawasaki T, Brahmandam M, *et al*. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. *J Mol Diagn* 2005;7:413–21.
20 Li J, Wang L, Mamon H, *et al*. Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. *Nat Med* 2008;14:579–84.
21 Yu J, Kane S, Wu J, *et al*. Mutation-specific antibodies for the detection of EGFR mutations in non-small-cell lung cancer. *Clin Cancer Res* 2009;15:3023–8.
22 Simonetti S, Molina MA, Queralt C, *et al*. Detection of EGFR mutations with mutation-specific antibodies in stage IV non-small-cell lung cancer. *J Transl Med* 2010;8:135.
23 Kozu Y, Tsuta K, Kohno T, *et al*. The usefulness of mutation-specific antibodies in detecting epidermal growth factor receptor mutations and in predicting response to tyrosine kinase inhibitor therapy in lung adenocarcinoma. *Lung Cancer* 2011;73:45–50.
24 Ilie MI, Hofman V, Bonnetaud C, *et al*. Usefulness of tissue microarrays for assessment of protein expression, gene copy number and mutational status of EGFR in lung adenocarcinoma. *Virchows Arch* 2010;457:483–95.
25 Kato Y, Peled N, Wynes MW, *et al*. Novel epidermal growth factor receptor mutation-specific antibodies for non-small cell lung cancer: immunohistochemistry as a possible screening method for epidermal growth factor receptor mutations. *J Thorac Oncol* 2010;5:1551–8.
26 Brevet M, Arcila M, Ladanyi M. Assessment of *EGFR* mutation status in lung adenocarcinoma by immunohistochemistry using antibodies specific to the two major forms of mutant *EGFR*. *J Mol Diagn* 2010;12:169–76.
27 Tsai T-H, Wu S-G, Chang Y-L, *et al*. Effusion immunocytochemistry as an alternative approach for the selection of first-line targeted therapy in advanced lung adenocarcinoma. *J Thorac Oncol* 2012;7:993–1000.
28 Wu SG, Chang YL, Lin JW, *et al*. Including total EGFR staining in scoring improves EGFR mutations detection by mutation-specific antibodies and EGFR TKIs response prediction. *PLoS One* 2011;6:e23303.
29 Hasanovic A, Ang D, Moreira AL, *et al*. Use of mutation specific antibodies to detect EGFR status in small biopsy and cytology specimens of lung adenocarcinoma. *Lung Cancer* 2012;77:299–305.
30 Kimura H, Fujiwara Y, Sone T, *et al*. High sensitivity detection of epidermal growth factor receptor mutations in the pleural effusion of non-small cell lung cancer patients. *Cancer Sci* 2006;97:642–8.
31 Kimura H, Fujiwara Y, Sone T, *et al*. *EGFR* mutation status in tumour-derived DNA from pleural effusion fluid is a practical basis for predicting the response to gefitinib. *Br J Cancer* 2006;95:1390–5.
32 Soh J, Toyooka S, Aoe K, *et al*. Usefulness of *EGFR* mutation screening in pleural fluid to predict the clinical outcome of gefitinib treated patients with lung cancer. *Int J Cancer* 2006;119:2353–8.
33 Wu SG, Gow CH, Yu CJ, *et al*. Frequent epidermal growth factor receptor gene mutations in malignant pleural effusion of lung adenocarcinoma. *Eur Respir J* 2008;32:924–30.
34 Buttitta F, Felicioni L, Del GM, *et al*. Effective assessment of egfr mutation status in bronchoalveolar lavage and pleural fluids by next-generation sequencing. *Clin Cancer Res* 2013;19:691–8.
35 Han HS, Lim SN, An JY, *et al*. Detection of *EGFR* mutation status in lung adenocarcinoma specimens with different proportions of tumor cells using two methods of differential sensitivity. *J Thorac Oncol* 2012;7:355–64.
36 Jahr S, Hentze H, Englisch S, *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;61:1659–65.
37 Suzuki N, Kamataki A, Yamaki J, *et al*. Characterization of circulating DNA in healthy human plasma. *Clin Chim Acta* 2008;387:55–8.
38 Yuan H, Zhu ZZ, Lu Y, *et al*. A modified extraction method of circulating free DNA for epidermal growth factor receptor mutation analysis. *Yonsei Med J* 2012;53:132–7.
39 Hirsch FR, Bunn PA Jr. EGFR testing in lung cancer is ready for prime time. *Lancet Oncol* 2009;10:432–3.