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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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jclinpath-2013-201728).

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Received 7 May 2013
Revised 26 June 2013
Accepted 27 June 2013
Published Online First 25 July 2013

To cite: Liu X, Lu Y, Zhu G, *et al*. *J Clin Pathol* 2013;**66**:1065–1069.

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.

Acknowledgements We thank Changting Liu and Yanping Xu for their contribution in sample collection and preparation, Xiaolu Yin for her pathology support and Sunny Sun for her contribution to experimental data generation. We also thank Dr Yi Liu for participating in the initial study design discussion and help with sample collection. Editing assistance was provided by Sarah Lewis, from Complete Medical Communications, and funded by AstraZeneca.

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.

Funding This study was funded by AstraZeneca. Study design, EGFR mutation detection, data analysis, writing and decision to submit this manuscript for publication was supported by AstraZeneca.

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.

Ethics approval Ethics approval for this study was given by the Affiliated Hospital of Academy of Military Medical Science Institutional Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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Supplemental Appendix 1

MATERIALS AND METHODS

Sample collection and handling

All tumour tissue samples were formalin-fixed, paraffin-embedded (FFPE) diagnostic samples, archival or recently collected for recruitment to this study. All tissue samples went through pathological evaluation to confirm the diagnosis of non-small cell lung cancer (NSCLC) and to estimate the percentage of tumour cells. Owing to the time-consuming nature of macro-dissection, the limited availability of micro-dissections of tumours, and a readily available, sensitive method for epidermal growth factor receptor (EGFR) mutation testing, we did not include macro- or micro-dissection in tumour sample processing. Instead, we defined a cut-off of 2% tumour cell content as a sample quality check according to the minimum requirement of amplification refractory mutation system (ARMS) technology (about 1% analytical sensitivity). Samples below this threshold were rejected. Four to eight sections (5 μm thickness) of each qualifying tumour tissue sample were mounted on slides and used for DNA extraction.

For malignant pleural effusion (MPE) samples, 250–500 mL was collected from each patient, centrifuged at 1000 *g* for 10 min at room temperature within 1 h of sample collection. Ten mL (10 mL) of each supernatant was transferred into a new collection tube and stored at -80°C until ready to proceed to DNA extraction. The cell pellets were fixed using standard 10% formalin and embedded in paraffin to form the MPE cell block. The MPE cell blocks were pathologically evaluated in the same way as the tumour tissue samples to confirm the diagnosis and the percentage of tumour cells. The tumour cell content had to be ≥2% for the sample to be included in the mutation analysis step. Ten to 15 sections (4 μm thickness) from each qualifying MPE cell block sample were used for DNA extraction.

Plasma was prepared from 10 mL of ethylene diamine tetraacetic acid-anticoagulated peripheral whole blood by centrifugation for 10 min at 2500 *g* at 4°C within 1 h after collection, and then stored at -80°C until DNA extraction.

For tumour tissue and MPE cell block samples, an additional three sections (4 μm thickness) were prepared on slides for EGFR mutation detection using the mutant-specific immunohistochemistry (IHC) assay.

DNA extraction and quality check

The QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) was used for DNA extraction from tumour tissue samples and MPE cell block samples, as advised in the user manual. The QIAamp Circulating Nucleic Acid Kit (Qiagen) was used for DNA extraction from MPE supernatant and plasma, as advised in the user manual. Extracted DNA samples were quantified by real-time quantitative polymerase chain reaction (PCR) method using a commercial Taqman assay for the RNase P gene (Life Technologies, New York, USA). The concentration of each DNA sample was normalised to 0.4 ng/μL if the original concentration was >0.4 ng/μL.

EGFR mutation detection by ARMS method ADx-ARMS kit (Amoy Diagnostics, Xiamen, China), which has been approved by the Chinese State Food and Drug Administration for in vitro diagnostics use and has been used for scientific research,[1] was used for the EGFR mutation detection in this study. This kit can be used with multiple real-time PCR platforms, including Stratagene 3005P, with which our laboratory is equipped. Conversely, the Qiagen Scorpion ARMS TheraScreen kit can only be used with the RGQ real-time PCR platform, with which few laboratories in China are equipped. Before the application of this kit for the study, we carried out an evaluation study, using 115 cases of lung adenocarcinoma tumour FFPE samples (tumour content: 2–80%), to compare the ADx-ARMS kit with the Qiagen

Scorpion ARMS kit (a previous version that could be used with Stratagene 3005P and was used for EGFR mutation detection in IPASS and other studies), achieving a concordance rate of 96.5% (data not shown). This kit detects the 29 most common EGFR mutations so far described in lung cancer. All experiments were performed following the manufacturer's instructions. Briefly, 5 μL DNA was added to 45 μL of the PCR master mix for each assay, which contained PCR primers, fluorescent probes, PCR buffer and DNA polymerase. The PCR cycling conditions were: 5-min incubation at 95°C, followed by 15 cycles of 95°C for 25 s, 64°C for 20 s, 72°C for 20 s and then 31 cycles of 93°C for 25 s, 60°C for 35 s, 72°C for 20 s. Fluorescent signal was collected from FAM and HEX channels. Genotypes were determined according to threshold count (Ct) and/or change in Ct value, as indicated in the manufacturer's instructions.

EGFR mutation detection by Sanger sequencing

PCR was performed in a 25 μL reaction mix containing 1× AmpliTaq Gold® 360 Master Mix (Life Technologies), 200 μM of each primer and 5 μL of genomic DNA. Exons 18–21 of the EGFR gene were amplified using the following primers: EGFR exon 18 forward 5′GCTGAGGTGACCCTTGTCTCTGTGT3′, EGFR exon 18 reverse 5′ATACAGCTTGCAAGGACTCTGGGCT3′; EGFR exon 19 forward 5′CAGCATGTGGCACCATCTCACAAT3′, EGFR exon 19 reverse 5′AGACATGAGAAAAGGTGGGCCTGAG3′; EGFR exon 20 forward 5′GAAGCCACACTGACGTGCCTCTC3′, EGFR exon 20 reverse 5′GCTCCTTATCTCCCCTCCCCGTAT3′; EGFR exon 21 forward 5′ATCTGTCCCTCACAGCAGGGTCTTC3′, EGFR exon 21 reverse 5′GCAGCCTGGTCCCTGGTGTC3′. The PCR cycling conditions were: 10-min incubation at 95°C, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s and then a final

incubation at 72°C for 10 min. The resulting PCR products were digested with ExoSAP-IT reagent (Affymetrix, Cleveland, Ohio, USA), and then sequenced in forward and reverse directions with BigDye Terminator Kit (Life Technologies) and an ABI 3730XL DNA analyser (Life Technologies) following the manufacturer's instructions. The sequencing data were analysed for mutations after assembly and quality calling with SeqScape sequence analysis software (version 2.5; Life Technologies).

EGFR mutation detection by IHC

Specificity of EGFR L858R mutant-specific (clone 43B2, CST3197; Cell Signaling Technology, Inc., Beverly, MA, USA) and EGFR E746-A750del specific (clone 6B6, CST2085; Cell Signaling Technology, Inc.) antibodies was confirmed by cross IHC staining on NSCLC cell lines with known EGFR mutations (L858R or E746-A750del). FFPE tumour tissue or MPE cell block samples were sectioned (4 μm thickness) and mounted onto coated slides. The slides were dewaxed and rehydrated before antigen retrieval was performed in PT Link (Dako, Carpinteria, California, USA) at 95°C for 15 min in high pH retrieval buffer (K8004; Dako). Endogenous peroxidase activity was blocked with hydrogen peroxide (S2023; Dako) for 5 min at room temperature. Sections were then incubated separately with total EGFR (CST4267; Cell Signaling Technology, Inc.), EGFR (L858R specific, clone 43B2, CST3197; Cell Signaling Technology, Inc.) and EGFR (E746-A750del specific, clone 6B6, CST2085; Cell Signaling Technology, Inc.) antibodies for 1 h at room temperature. Immunocomplexes were detected by incubation with anti-rabbit (K4003; Dako) horseradish peroxidase-labelled polymer and were detected by treatment with diaminobenzidine (K3468; Dako) for 10 min followed by rinsing in tap water. The sections were then counter-stained, dehydrated and cleared in Leica XL autostainer (Leica Biosystems, Illinois, USA), and finally sealed in the ClearVue automated coverslipper (Thermo Scientific, Boston, USA).

Normal immunoglobulin G (X9003; Dako) from the same species of primary antibody was diluted to match the concentration of the primary antibody and used as the negative control.

The IHC intensity was scored following the criteria of Tsai et al:[2] 0 if tumour cells had a complete absence of staining or faint staining intensity of <10%; 1+ if >10% of the tumour cells had faint staining; 2+ if the tumour cells had moderate staining; and 3+ if the tumour cells had strong staining. An expression score of 1+, 2+ and 3+ was considered positive for immunoreactivity. Cases with no EGFR expression (total EGFR negative) were excluded from data analysis in the study.

Quality assurance

Two well-trained senior biologists with molecular genetics expertise executed ARMS and Sanger sequencing experiments together and analysed the data independently. A third senior molecular genetics expert combined the data interpretation together and addressed any discrepancy between the two analysers through three persons' meeting and discussion.
A well-trained technician executed the mutant-specific IHC experiments. Two senior pathologists performed staining interpretation independently and any discrepancy was addressed through discussion between the two pathologists. Different samples and methods were executed blindly before final combination by a senior researcher of the study.

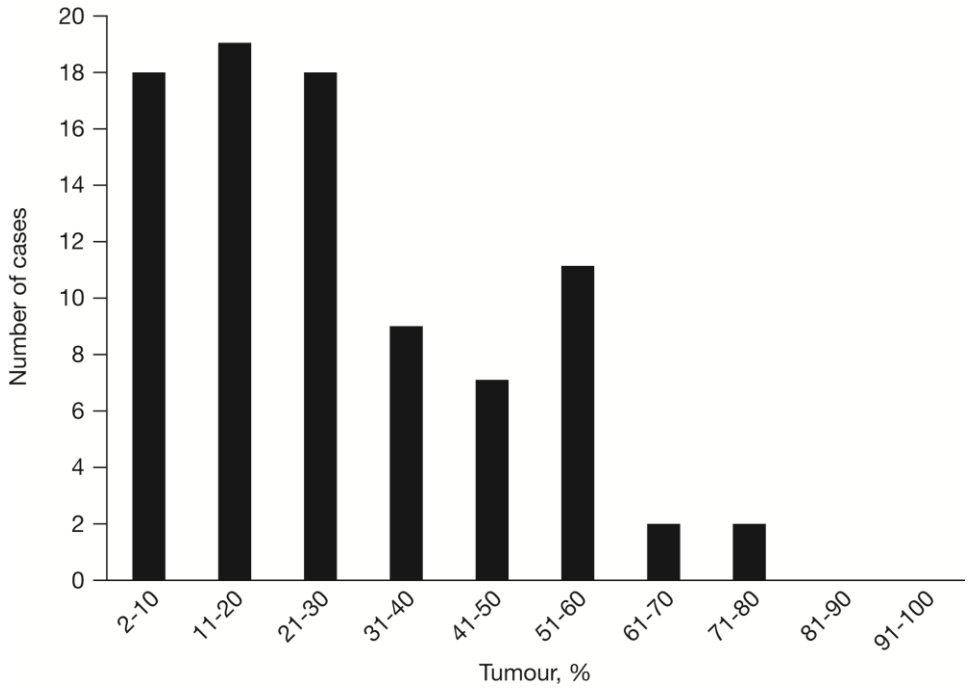
RESULTS

Sample characteristics

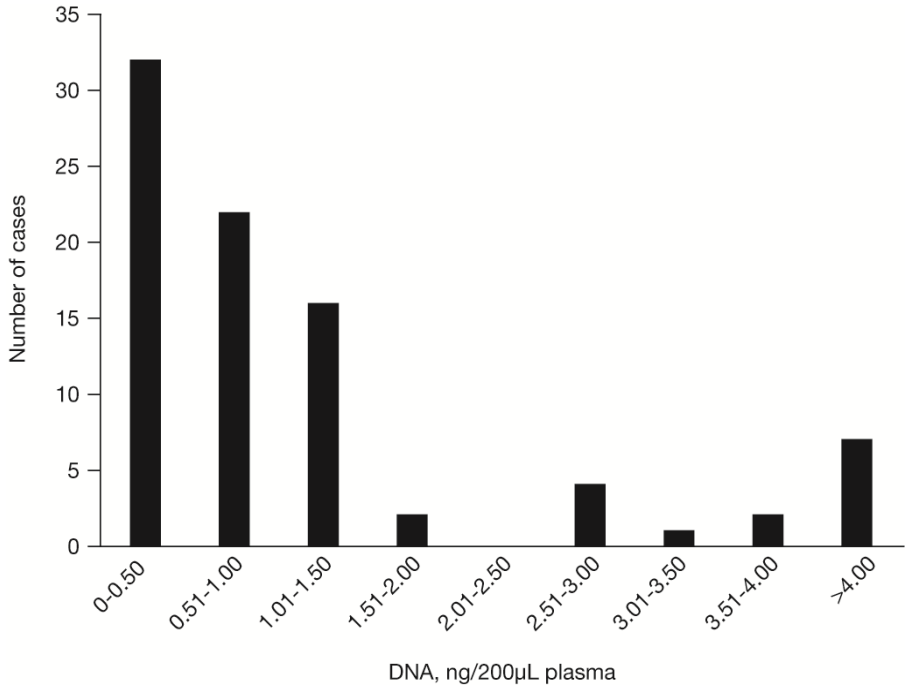
The tumour tissue samples were obtained from surgery of the primary lung tumour (n=4), small biopsy of the primary lung tumour (n=25), biopsy of a metastatic lymph node (n=48) and biopsy of metastatic loci other than a lymph node (n=9). Of the 86 patients who provided adequate tumour tissue samples (tumour content distribution is illustrated in Figure 1), 31

also provided MPE samples. Twenty-one of these MPE cell block samples passed pathology review and were included for EGFR mutation analyses, together with their supernatant counterparts. A further six patients whose MPE cell block samples passed the quality check but who had inadequate tumour tissue samples were included for EGFR mutation analyses for MPE samples only. The concentration of five tumour DNA samples, four MPE supernatant DNA samples and 21 plasma DNA samples was <0.4 ng/μL but >0.2 ng/μL; the concentration of seven tumour DNA samples, two MPE block DNA samples, two MPE supernatant DNA samples and 28 plasma DNA samples was <0.2 ng/μL. The overall yield of amplifiable cfDNA from 86 plasma samples is illustrated in Figure 2.

Supplemental Appendix Figure 1 Tumour content in the 86 tumour tissue samples.



Supplemental Appendix Figure 2 Yield of amplifiable cell-free DNA from 86 plasma samples.



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