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 cells9–16 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)       |

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

| A. Tumour tissue versus MPE cell block |
|---------------------------------------|
| MEP cell block                        |
| +                                     |
| -                                     |
| Total                                 |
| +                                     |
| Tumour tissue                         |
| Total                                 |
| NPV: 8/10 (80.0%); PPV: 9/11 (81.8%)  |

| B. Tumour tissue versus MPE supernatant|
|---------------------------------------|
| MEP supernatant                       |
| +                                     |
| -                                     |
| Total                                 |
| +                                     |
| Tumour tissue                         |
| Total                                 |
| NPV: 10/14 (71.4%); PPV: 7/11 (81.8%) |

| C. MPE cell block versus MPE supernatant|
|----------------------------------------|
| MEP cell block                         |
| MEP supernatant                        |
| +                                     |
| -                                     |
| Total                                 |
| +                                     |
| Tumour tissue                         |
| Total                                 |
| NPV: 14/18 (77.8%); PPV: 9/9 (100.0%)  |

| D. Tumour tissue versus plasma         |
|---------------------------------------|
| Plasma                                |
| +                                     |
| -                                     |
| Total                                 |
| +                                     |
| Tumour tissue                         |
| Total                                 |
| 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.
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).

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.10–14 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 samples.39–41

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

| Method Comparison | ARMS | Sanger sequencing | Total |
|-------------------|------|-------------------|-------|
| **A. ARMS versus Sanger sequencing for tumour tissue samples** | | | |
| + | 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** | | | |
| + | 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** | | | |
| + | 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** | | | |
| + | 6 | 0 | 6 |
| − | 6 | 14 | 20 |
| Total | 12 | 14 | 26 |
| NPV: 14/20 (75.0%); PPV: 6/6 (100.0%). |
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. Xiaojing Liu, Yao Lei, Haileng 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.

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