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Epidermal growth factor receptor mutation analysis in previously unanalyzed histology samples and cytology samples from the phase III Iressa Pan-ASia Study (IPASS)[☆]



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ABSTRACT

Objectives: Epidermal growth factor receptor (*EGFR*) mutation testing is standard practice after lung adenocarcinoma diagnosis, and provision of high-quality tumor tissue is ideal. However, there are knowledge gaps regarding the utility of cytology or low tumor content histology samples to establish *EGFR* mutation status, particularly with regard to the proportion of testing performed using these sample types, and the lack of an established link with efficacy of treatment.

Methods: The randomized phase III Iressa Pan-ASia Study (IPASS; ClinicalTrials.gov identifier NCT00322452) of first-line gefitinib versus chemotherapy analyzed samples meeting preplanned specifications ($n=437$ evaluable for *EGFR* mutation; $n=261$ mutation-positive). This supplementary analysis assessed tumor content and mutation status of histology ($n=99$) and cytology samples ($n=116$) which were previously unanalyzed due to sample quality, type, and tumor content (<100 cells). Objective response rate (ORR) and change in tumor size with gefitinib treatment were assessed.

Results: *EGFR* mutation testing was successful in 80% and 19% of previously unanalyzed histology and cytology samples, respectively. Mutations were detected in 54 tumors previously described as mutation-unknown (histology, $n=45$; cytology, $n=9$). ORRs in mutation-positive cytology (83%) and histology (74%) subgroups were consistent with previous analyses (71%). Tumor size decrease was consistent across previously analyzed and unanalyzed samples (all mutation subgroups), with less consistency across ORRs in mutation-negative cytology (16%) and histology (25%) subgroups versus the previous analysis (1%).

Conclusions: Histology samples with low tumor content and cytology samples can be used for *EGFR* mutation testing; patients whose mutation status was confirmed using these sample types achieved a response to treatment consistent with those confirmed using high-quality histology samples. Better sample quantity/quality can potentially reduce false-negative results.

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1. Introduction

Non-small-cell lung cancer (NSCLC) is traditionally treated using platinum-based chemotherapy [1,2]. Recently, the management of advanced lung adenocarcinoma has evolved, and use of molecular diagnosis to investigate driver mutations in tumor samples has become the most important step toward selecting the right agent for a patient's treatment [3].

The most established example is the use of epidermal growth factor receptor (*EGFR*) mutations as a predictive marker of tumor

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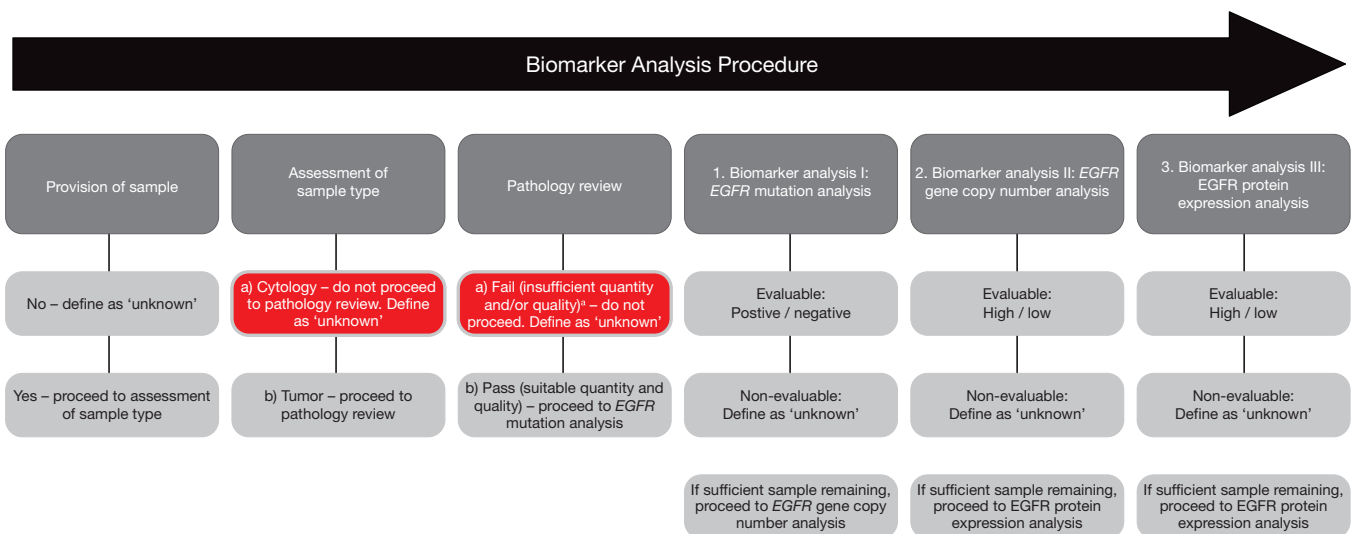
response to EGFR tyrosine kinase inhibitor (TKI) treatment. The first trial to confirm the utility of *EGFR* mutation as a predictor of anticancer efficacy was the Iressa Pan-ASia Study (IPASS), which investigated the outcomes of the overall study population ($n = 1217$) and subgroups (including those evaluable for *EGFR* mutation status [$n = 437$]) treated with gefitinib or carboplatin/paclitaxel [4,5]. IPASS demonstrated superior progression-free survival (PFS), objective response rate (ORR), symptom control, and quality of life with first-line gefitinib versus carboplatin/paclitaxel in patients with *EGFR* mutation-positive tumors. This finding was replicated in the smaller FIRST-Signal study [6]. Five additional phase III studies have subsequently reported significantly increased PFS with *EGFR*-TKIs (gefitinib, erlotinib, and afatinib) versus platinum-based chemotherapy in patients with *EGFR* mutation-positive tumors [7–11].

IPASS (overall population $n = 1217$) included exploratory objectives to investigate efficacy according to *EGFR* biomarker status (*EGFR* mutation, gene copy number, and protein expression) [4,5]. Collection of histology samples for biomarker analysis was not mandated; 85% of patients consented to donate their tumor. Samples were provided by 683/1217 patients (56%). Fukuoka et al. presented the IPASS exploratory biomarker data for 261 patients with *EGFR* mutation-positive tumors out of 437 evaluable patients (60%) [4].

The streamlined biomarker analysis process (Fig. 1) required all samples to meet stringent pre-specified thresholds for the number of tumor cells and sample quality/type, based on the higher cell requirements of fluorescent in situ hybridization (FISH) for gene copy number and immunohistochemistry (IHC) for protein expression. Prior to *EGFR* mutation analysis samples underwent central histopathological review, and samples were included in the biomarker analysis based on their quality, quantity, type, and tumor content (>100 cells) (Fig. 1). These criteria ensured quality results, reflecting the design of IPASS, determination of differential efficacy in biomarker positive/negative subgroups, limited data at the time regarding the predictive nature of the biomarkers, and extent of validation of the biomarker assays at the time IPASS was conducted (biomarker assays were not validated for cytology

samples at that time). This approach provided a definitive answer regarding patients who derived most benefit in the clinical setting. While appropriate to answer the questions posed by the IPASS protocol, the *EGFR* mutation analysis threshold stringency was higher than would be employed for the diagnosis of patients in daily practice. Since IPASS reported, laboratories have gained experience of using existing *EGFR* mutation detection techniques on a spectrum of samples with varying tumor content and sample quality. Small biopsies and cytology samples make up ~30–80% of available diagnostic material, depending on diagnostic practices between different hospitals and countries [12], therefore their successful testing is paramount to ensure this sizeable proportion of patients are given the opportunity to receive optimal treatment. The percentage of mutation testing that occurs using cytology samples can be very variable however, and is currently not consistent across institutions or countries [13]. Smouse et al's retrospective review of *EGFR* sequencing over a two year period at a US hospital noted that only 12/239 (5%) specimens tested for *EGFR* mutation were cytological in origin [13], with focus given to the testing of high-quality tumor tissue samples. Conversely, Hagiwara et al. recently noted that ~40% of samples submitted for *EGFR* mutation testing across three major commercial test centers in Japan were of cytological origin [14], further commenting that this high percentage highlights that cytological samples are indispensable for testing all patients with advanced NSCLC.

The aim of the current study was to investigate whether cytology/histology samples that were not included in the IPASS pre-planned exploratory biomarker analyses could be used successfully to define *EGFR* mutation status and predict which patients were more likely to respond to *EGFR*-TKI treatment. We describe data generated from pathology review and mutation analysis of the previously unanalyzed histology samples and previously unanalyzed cytology samples, with the aim of testing the outcome of patients with NSCLC as per the study protocol, but by looking at the full spectrum of samples that are available from this population of patients. These data will help to inform the most appropriate thresholds for further trials, as well as the utility of samples received by diagnostic laboratories on a daily basis.



*Histology samples that failed pathology review due to insufficient tumor material for biomarker analysis (<100 tumor cells) or poor quality (inadequate fixation or a sample where accurate diagnosis was not possible) and were therefore not included in the main IPASS biomarker analyses. Previously unanalyzed samples included cytology samples and any samples that did not pass pathology review (highlighted in red). *EGFR*, epidermal growth factor receptor; IPASS, Iressa Pan-ASia Study.

Fig. 1. The biomarker analysis process.

2. Materials and methods

2.1. Study design and patients

Full details of IPASS (ClinicalTrials.gov identifier NCT00322452) have been published previously [4,5]. Patients were eligible for inclusion into the study if they had histologically or cytologically confirmed stage IIIB or IV pulmonary adenocarcinoma (including bronchoalveolar carcinoma), were never-smokers (<100 cigarettes in their lifetime) or former light smokers (stopped smoking \geq 15 years previously and smoked \leq 10 pack-years), and had received no prior chemotherapy, biologic therapy, or immunologic therapy. Patients provided written informed consent with separate consent for the optional assessment of *EGFR* biomarkers. The study protocol was approved by independent ethics committees at each institution. Of 1217 randomized patients, 683 (56%) provided a sample for biomarker analysis. Tumor *EGFR* mutation status was evaluable for 437 patients (261 *EGFR* mutation-positive). Prior to *EGFR* mutation analysis samples underwent central histopathological review; only those considered suitable for the analysis of all exploratory biomarkers, including two methods requiring a specified cell number (*EGFR* gene amplification by FISH requiring 60 cells, and *EGFR* protein expression by IHC requiring 100 cells, for accurate scoring respectively), were included in the biomarker analysis (sample quality, type, and tumor content [$>$ 100 cells]) (Fig. 1). At the time of the original analysis, according to the protocol biomarker analyses were not performed for 215 samples: 116 cytology samples (biomarker analyses had not been validated for this sample type, as previously reported in the appendix of Fukuoka et al. [4]) and 99 histology samples (determined during pathology review not to meet pre-specified biomarker analysis thresholds regarding tumor content [$>$ 100 tumor cells] and sample quality/quantity [including samples with inadequate cellular morphology due to poor/inappropriate fixation]). The previously unanalyzed cytology and histology samples are the subject of this additional analysis.

The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation/Good Clinical Practice, applicable regulatory requirements, and AstraZeneca's policy on bioethics.

2.2. *EGFR* mutation analyses

EGFR mutation analyses were conducted at two central laboratories (Genzyme, Framingham, MA, USA and AstraZeneca Innovation Center China, Shanghai, China). *EGFR* mutation status of the previously unanalyzed samples was determined by analyzing paraffin-embedded archival histological and cytological cell blocks/smears. Sample tumor content was assessed (histopathological review) prior to categorization based on the number of tumor cells present; 0–9, 10–49, 50–99, and $>$ 100 cells. *EGFR* mutations were detected using an amplification mutation refractory system with *EGFR* mutation detection (Qiagen, Manchester, UK), as previously reported for IPASS [5]. Tumors were considered positive if \geq 1 of 29 *EGFR* mutations was detected.

2.3. Statistical analyses

Statistical analyses were performed by AstraZeneca. Owing to the small numbers of evaluable cytology and previously unanalyzed histology samples, formal statistical testing was not appropriate. The ORR with exact 95% (Clopper–Pearson) confidence intervals (CIs) was calculated for *EGFR* mutation-positive and -negative cytology samples and *EGFR* mutation-positive and -negative previously unanalyzed histology samples.

Percentage change in tumor size was presented graphically (waterfall plots), with each patient's maximum percentage

decrease in tumor size presented as a separate bar (largest increase to largest decrease).

3. Results

3.1. Patients

A total of 215 samples (99 histology; 116 cytology) were available but not analyzed in the main IPASS analysis (Fig. 2). Of the 99 histology samples, 79 (80%) were evaluable for *EGFR* mutations of which 45 (57%) were *EGFR* mutation-positive. Of these 45 patients with *EGFR* mutation-positive tumors, 27 (60%) had received gefitinib and 18 (40%) carboplatin/paclitaxel. Of the 116 cytology samples, 31 (19%) were evaluable for *EGFR* mutation of which nine (29%) were *EGFR* mutation-positive. Of these nine patients with *EGFR* mutation-positive tumors, six (67%) had received gefitinib and three (33%) carboplatin/paclitaxel. A total of 20 histology samples (20%) and 85 cytology samples (73%) were not evaluable for *EGFR* mutation status (insufficient DNA for mutation analysis or no material available for DNA extraction and subsequent analysis).

3.2. Analysis success and tumor cell number: cytology and histology samples that previously failed pathology review

Fig. 3 summarizes the number of evaluable and *EGFR* mutation-positive samples observed, according to tumor cell content. A total of 52 cytology samples (45%) had $<$ 100 tumor cells; eleven of these samples provided an evaluable *EGFR* mutation result, of which two (18%) were *EGFR* mutation-positive. A total of 64 cytology samples (55%) had $>$ 100 tumor cells; twenty of these samples provided an evaluable *EGFR* mutation result, of which seven (35%) were *EGFR* mutation-positive.

Data from the previously unanalyzed histology samples showed that 73 samples (74%) had $<$ 100 tumor cells, with 59 samples providing an evaluable *EGFR* mutation result; thirty (51%) were *EGFR* mutation-positive. A total of 26 histology samples (26%) had $>$ 100 tumor cells. These samples had previously been excluded from the main IPASS study on the basis that they did not meet the pre-specified thresholds regarding tumor content and sample quality/quantity (described in Section 2). Twenty samples provided an evaluable *EGFR* mutation result; 15 (75%) were *EGFR* mutation-positive.

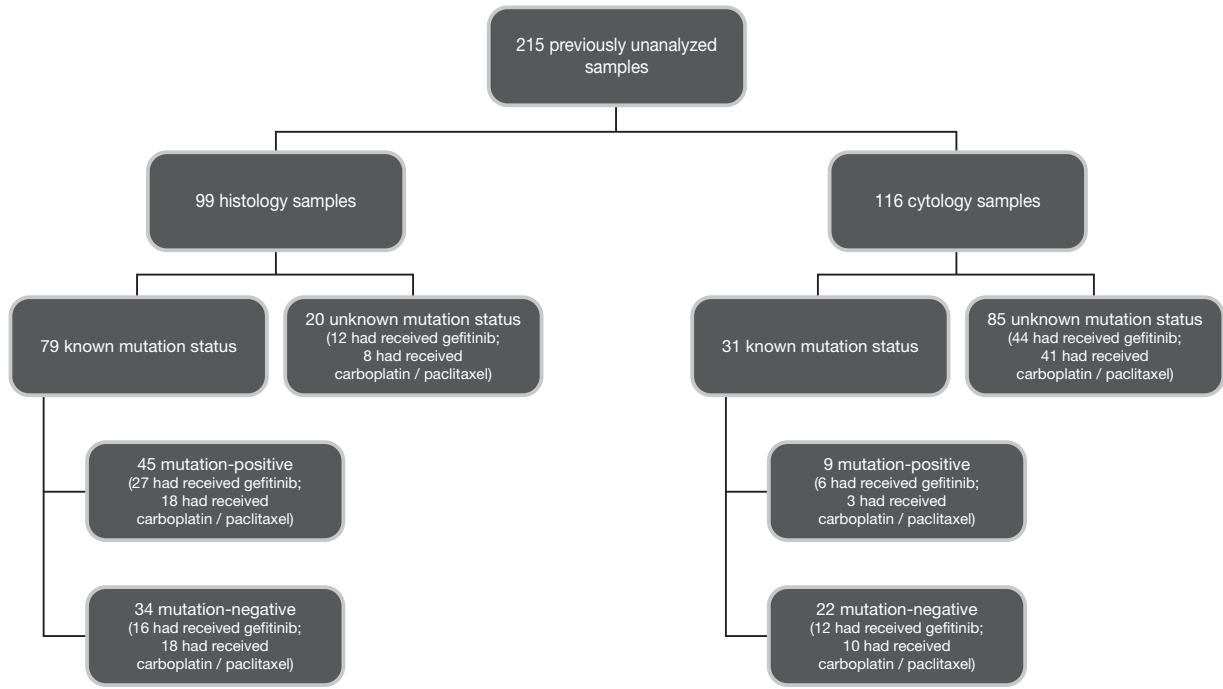
In total, therefore, *EGFR* mutation-positive tumors were detected in 54 patients which had previously been described as *EGFR* mutation-unknown.

3.3. Mutation subtype and frequency

Of the *EGFR* mutation-positive cytology samples, 5 (55.6%) were positive for exon 19 deletions and 4 (44.4%) were positive for exon 21 L858R. Of the *EGFR* mutation-positive histology samples, 22 (48.9%) were positive for exon 19 deletions, 18 (40%) for exon 21 L858R, and two (4.4%) for exon 18 G719S/A/C. A total of three samples were identified as having double mutations: two (4.4%) for exon 19 deletions and exon 21 L858R, and one sample (2.2%) for exon 18 G719S/A/C and exon 21 L861Q.

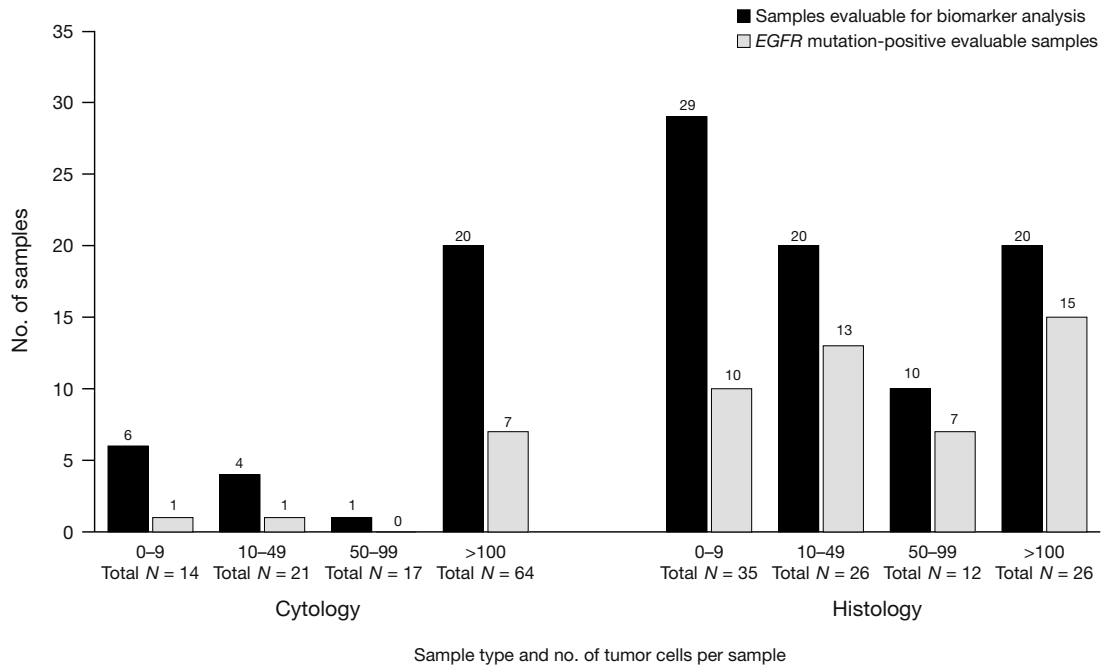
3.4. Efficacy

Data from the previously analyzed samples demonstrated the differential efficacy in terms of ORRs for patients with gefitinib, with 1% of patients ($n=1/100$) having an objective response in the *EGFR* mutation-negative subgroup, 43% ($n=167/386$) in the mutation-unknown subgroup, and 71% ($n=94/132$) in the mutation-positive subgroup [4,5]. Note that in the previous analysis, the *EGFR* mutation-unknown subgroup consisted of 386



Among the 105 patients for whom tumor *EGFR* mutation status was unknown, the main reasons for unknown *EGFR* mutation status were insufficient DNA for analysis, no material available for analysis, inadequate fixation, and patient diagnosis unable to be confirmed. *EGFR*, epidermal growth factor receptor.

Fig. 2. Sample disposition.



EGFR mutations were identified in both the previously unanalyzed histology and cytology samples, with a greater number of histology samples being evaluable for *EGFR* mutation status. *EGFR* mutation pick-up rate in the histology samples was also higher. *EGFR*, epidermal growth factor receptor.

Fig. 3. Tumor cell content of the previously unanalyzed histology and cytology samples (intent-to-treat population).

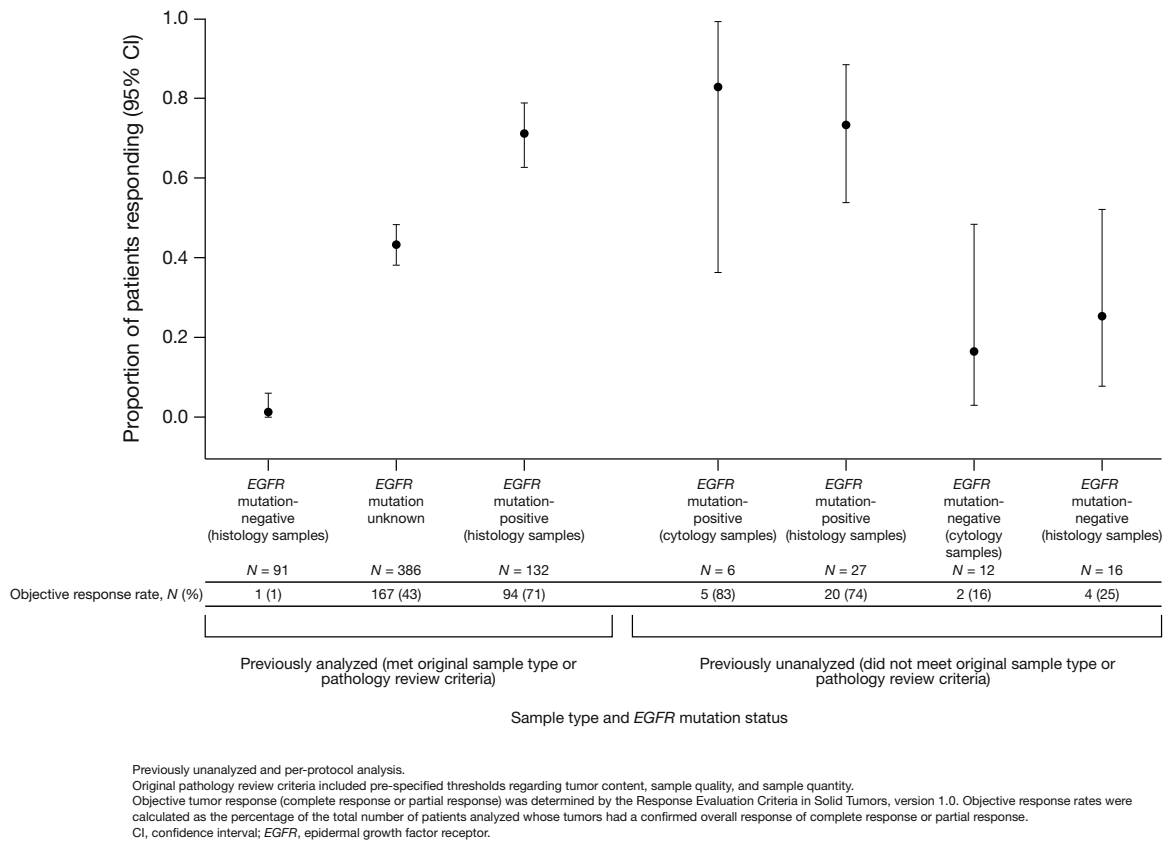


Fig. 4. Objective response rate for patients treated with gefitinib by sample type and EGFR mutation status (intent-to-treat population).

patients, including 61 patients described as not previously analyzed and who are described here.

Fig. 4 summarizes the ORR in the previously unanalyzed cytology and histology samples by EGFR mutation status for patients with gefitinib. The ORR in the EGFR mutation-positive subgroups by cytology and previously unanalyzed histology samples are consistent with the data from the previously determined EGFR mutation-positive subgroups: EGFR mutation-positive on the basis of cytology ORR 83% ($n = 5/6$), previously unanalyzed histology sample 74% ($n = 20/27$) versus 71% in the previous analysis. The ORR in the EGFR mutation-negative subgroups by cytology and previously unanalyzed histology samples are higher than those observed in the previously determined EGFR mutation-negative subgroups: EGFR mutation-negative on the basis of cytology 16% ($n = 2/12$), previously unanalyzed histology sample 25% ($n = 4/16$) versus 1% in the previous analysis.

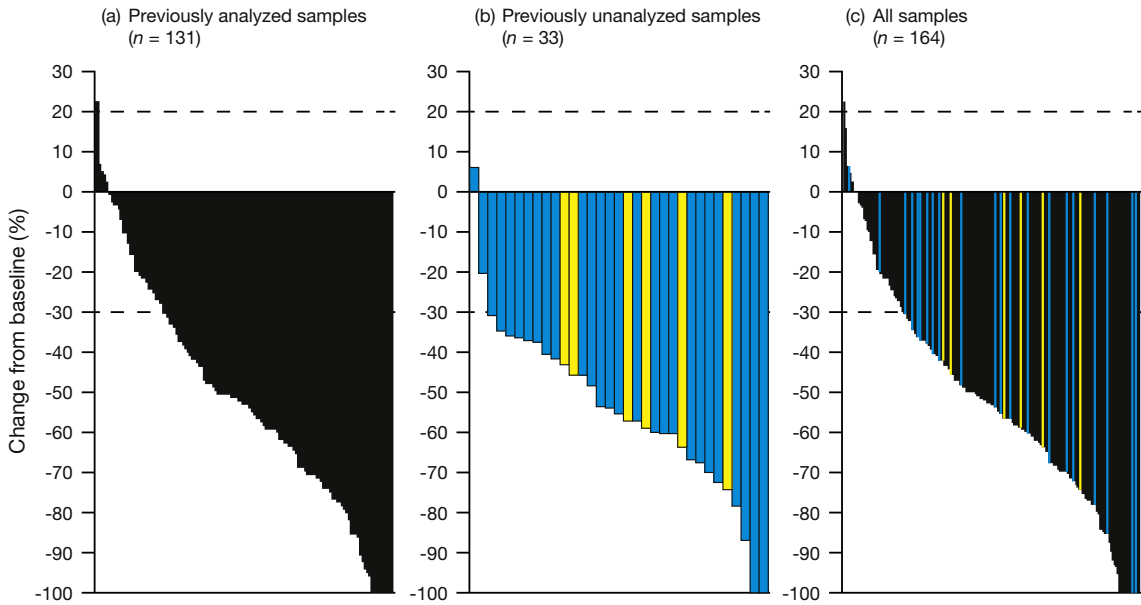
Tumor size reduction (percentage change from baseline) with gefitinib in the previously unanalyzed cytology and histology samples appears to be consistent with previously analyzed histology samples, for both EGFR mutation-positive (Fig. 5a and b) and -negative samples (Fig. 5d and e). The EGFR mutation-positive and -negative tumors from the updated analysis are evenly distributed throughout the waterfall plots of the previously analyzed histology samples (Fig. 5c and f, respectively). Maximum percentage change in tumor size from baseline for patients whose tumors were of unknown EGFR mutation status is shown in Fig. 6a (including previously analyzed samples, and cytology and low tumor content samples), Fig. 6b (previously unanalyzed samples highlighting those cytology and low tumor content tumor samples subsequently found to be EGFR mutation-positive), and Fig. 6c (previously unanalyzed samples highlighting those cytology and low tumor content tumor samples subsequently found to be EGFR mutation-negative).

4. Discussion and conclusions

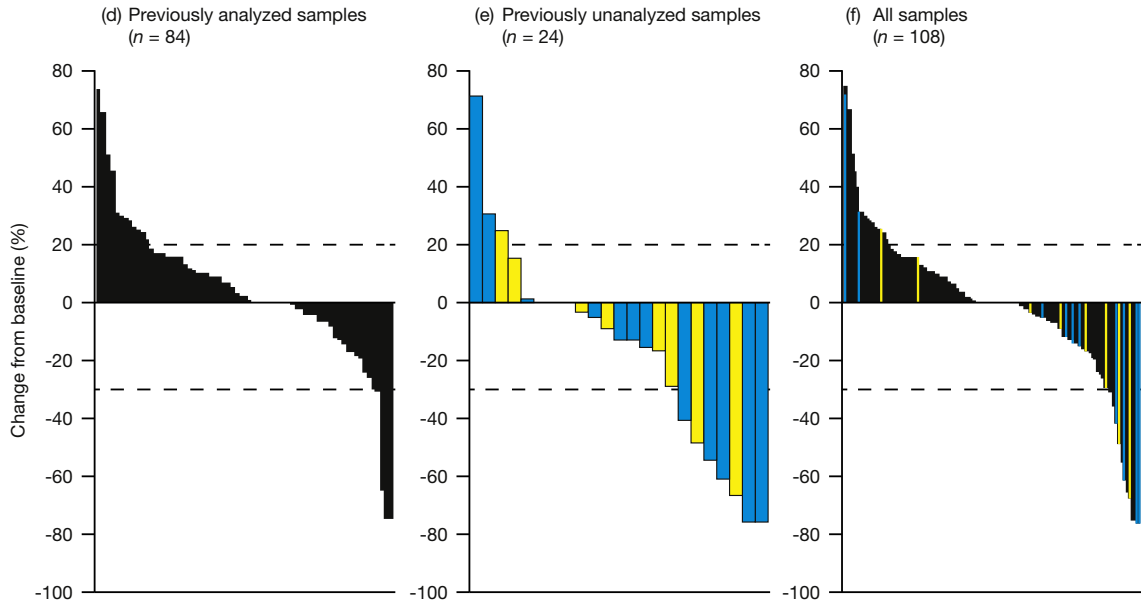
The results of IPASS clearly demonstrated the differential efficacy of EGFR-TKIs in the EGFR mutation-positive, -negative, and -unknown subgroups [4,5]. EGFR-TKIs are now recommended for the treatment of patients with EGFR mutation-positive tumors [15]. As a result of available data, accurate identification of patients who might benefit from EGFR-TKI therapy has become an important step in the treatment-decision pathway for advanced NSCLC [16].

This study shows that both histology and cytology samples used to diagnose NSCLC are suitable for the detection of EGFR mutations. This study demonstrates that where an EGFR mutation-positive result is observed, EGFR-TKI efficacy is consistent with that observed in the sample analysis according to the protocol, albeit with wider ORR CIs due to sample number. In both the cytology and previously unanalyzed histology subgroups, a higher response rate was observed in samples in which no EGFR mutation was detected compared with the 1% response rate in the previously analyzed histology samples in which no mutation was detected. While the EGFR mutation frequency is as expected in the previously unanalyzed histology samples, it was lower than expected in the cytology samples. Taken together, these two observations demonstrate that there are likely to be a number of false-negative results within the EGFR mutation-negative (or EGFR mutation-not-detected) subgroups in these previously unanalyzed samples, showing that the EGFR mutation-negative results are less robust than in the previously analyzed samples of good quality/quantity. This study therefore demonstrates that while high quality and high tumor content samples should be obtained and tested where possible, it is feasible to use low tumor content or cytology samples if these are the only sample available from the initial diagnosis of advanced NSCLC. Additionally, feedback from pathologists and molecular biologists on sample quality would help to minimize

EGFR mutation-positive



EGFR mutation-negative



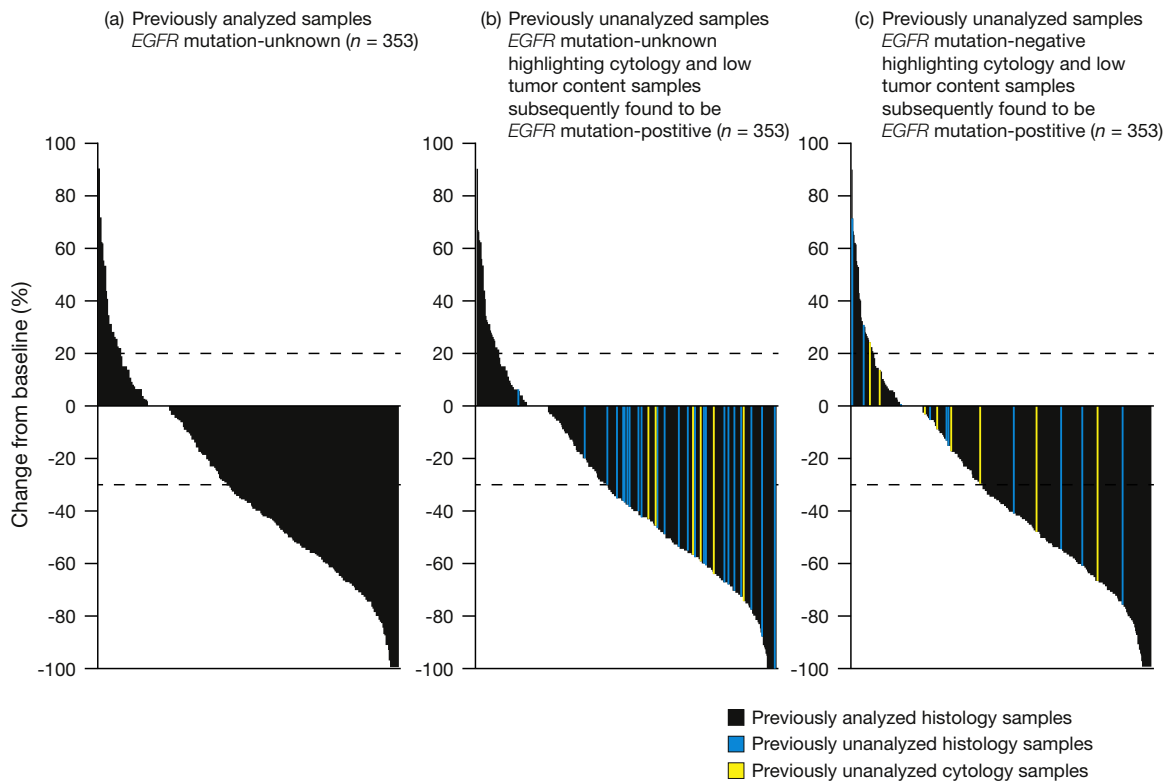
Previously analyzed tumor samples
 Previously unanalyzed tumor samples
 Previously unanalyzed cytology samples

The horizontal dashed lines at -30% and +20% represent the percentage change required for a response or progression of target lesions, respectively, according to Response Evaluation Criteria In Solid Tumors, version 1.0.

Only patients with a baseline and one evaluable post-baseline target lesion assessment are included. Plots do not include assessment of non-target or new lesions.

EGFR, epidermal growth factor receptor.

Fig. 5. Waterfall plots for maximum percentage change in tumor size from baseline in patients with EGFR mutation-positive tumors treated with gefitinib from (a) previously analyzed samples, (b) previously unanalyzed samples, and (c) all analyzed samples; and EGFR mutation-negative tumors treated with gefitinib from (d) previously analyzed samples, (e) previously unanalyzed samples, and (f) all analyzed samples.



The horizontal dashed line at 30% shrinkage represents the percentage change required for a response of target lesions according to Response Evaluation Criteria In Solid Tumors, version 1.0.

Only patients with a baseline and one evaluable post-baseline target lesion assessment are included. Plots do not include assessment of non-target or new lesions.

EGFR, epidermal growth factor receptor.

Fig. 6. Waterfall plots for maximum percentage change in tumor size from baseline in patients with tumors of unknown *EGFR* mutation status treated with gefitinib from (a) previously analyzed samples, (b) previously unanalyzed samples with *EGFR* mutation-positives from updated analysis, and (c) previously unanalyzed samples with *EGFR* mutation-negatives from updated analysis.

the costs of repeat testing and optimize the process of obtaining a quality result that the physician can take into consideration when making a treatment decision.

The importance of ensuring that samples are of sufficient quality/quantity has been confirmed in this study. The *EGFR* mutation frequency observed in the cytology samples implies that the pre-specified tumor content of 100 cells is still relevant within the clinical setting in order to avoid the issue of false-negative results in this sample type. In contrast, these data suggest that for histology sample analysis, it may be possible to reduce the criteria.

Several groups have released recommendations for *EGFR* mutation testing practices which include guidance on good quality/quantity samples, but little guidance on how laboratories should deal with low tumor content or cytology samples [17–20]. Any samples used for diagnosis of NSCLC (e.g. biopsy, resection, cytology) should be tested for *EGFR* mutation status provided the laboratory performing the analysis is confident in the result. This confidence will depend on the method used, laboratory expertise, and the quality/quantity of the samples, typically those that contain sufficient tumor material to obtain an accurate result, regardless of sample source. Testing of samples judged to be of low quality or low tumor content should be carried out using sensitive testing methods with or without a technique such as Laser Capture Microdissection (LCM), to enrich for the tumor cells. This technique was not attempted in IPASS, because while the technology is available in some institutions, it is not widely available and therefore not possible for all routine *EGFR* testing labs to employ. The Molecular

Assays in NSCLC Working Group highlighted that LCM may be used to facilitate accurate test results by increasing the ratio of tumor to normal tissue, which is particularly important for techniques such as direct sequencing, which requires samples with ≥ 50 –70% tumor cells for analysis [17]. However, the Working Group also noted that LCM can be laborious, and is unlikely to be acceptable for routine clinical sample analysis.

This analysis of previously unanalyzed samples from IPASS has shown that NSCLC samples of either low tumor cell content or cytological origin are suitable for the detection of *EGFR* mutation-positive disease. While consideration should be given to the individual capabilities of diagnostic laboratories, the testing of these additional samples may lead to an increase in the number of successful mutation results, enabling a greater number of patients to be accurately diagnosed, and receive the most effective and personalized therapy.

Role of the funding source

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Conflict of interest statement

J.C.-H. Yang has received advisory fees from AstraZeneca, Roche, Genentech, Pfizer, and Clovis, and has been an uncompensated advisor to Boehringer Ingelheim and Eli Lilly. Y.-L. Wu and K. Nakagawa have received speaker fees from AstraZeneca.

G. McWalter and R. McCormack are employees of AstraZeneca and hold shares in AstraZeneca. T.S. Mok has received research funding from AstraZeneca and advisory fees from AstraZeneca, Roche, Eli Lilly, Boehringer Ingelheim, Merck Serono, and Pfizer. M. Fukuoka, N. Saijo, V. Chan, and J. Kurnianda have no conflicts of interest to disclose.

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