EGFR Protein Expression in Non-Small Cell Lung Cancer Predicts Response to an EGFR Tyrosine Kinase Inhibitor – A Novel Antibody for Immunohistochemistry or AQUA Technology

Celine Mascaux*1, Murry W. Wynes*1, Yasufumi Kato1, Cindy Tran1, Bernadette Reyna Asuncion1, Jason M. Zhao1, Mark Gustavson4, Jim Ranger-Moore5, Fabien Gaire5, Jun Matsubayashi3, Toshitaka Nagao3, Koichi Yoshida2, Tatuso Ohira2, Norihiko Ikeda2, Fred R Hirsch1.

1Division of Medical Oncology and Pathology, University of Colorado Denver, Aurora, CO, USA
2Department of Thoracic Surgery, Tokyo Medical University Hospital, Tokyo, Japan
3Department of Anatomic Pathology, Tokyo Medical University Hospital, Tokyo, Japan
4HistoRX, Inc, Brandford, CT, USA
5Ventana Medical System, Inc, Tucson, AZ, USA

*Contributed equally

Correspondence Author:
Fred R Hirsch, MD, PhD
University of Colorado Denver; Division of Medical Oncology
12801 E. 17th Ave; Mail Stop 8177, Aurora, Colorado USA, 80045.
Tel: 303-724-3858; Fax: 303-724-0714

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Running Title: EGFR Protein expression predicts response to EGFR TKI
**Translational Relevance**

Several biomarkers are being evaluated in non-small cell lung cancer patients to predict response to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). Currently, EGFR protein expression is not recommended for predicting outcome to EGFR TKIs due to conflicting published results using immunohistochemistry (IHC) with antibodies detecting the extracellular domain of EGFR. The present study shows that AQUA technology and IHC assessments of EGFR protein expression with a specific antibody detecting the internal domain of EGFR predicts response to gefitinib. Furthermore, we show in the subgroup of patients with EGFR mutations that EGFR protein expression using this antibody discriminated responders from non-responders, which may be of practical clinical importance since a fraction of patients with mutated tumors do not respond to EGFR TKIs. These data call attention to the importance of the choice of method and the diagnostic antibody used to evaluate the predictive role of EGFR protein expression.
Abstract

Introduction: Epidermal growth factor receptor (EGFR) protein expression in non-small cell lung cancer (NSCLC) is not recommended for predicting response to EGFR tyrosine kinase inhibitors (TKIs) due to conflicting results, all using antibodies detecting EGFR external domain (ED). We tested the predictive value of EGFR protein expression for response to an EGFR TKI using an antibody that detects the intracellular domain (ID) and compared fluorescence-based Automated QUantitative Analysis (AQUA) technology to immunohistochemistry (IHC).

Methods: Specimens from 98 gefitinib-treated NSCLC Japanese patients were evaluated by IHC (n=98/98) and AQUA technology (n=70/98). EGFR ID- (5B7) and ED-specific antibodies (3C6 and 31G7) were compared.

Results: EGFR expression evaluated with 5B7 was significantly higher in responders versus non-responders to gefitinib both with IHC and with AQUA. ED-specific antibodies did not significantly predicted response. Using AQUA and ID-specific antibody resulted in the best prediction performance with a positive and negative predictive value (PPV/NPV) for responders of 50% and 87%, respectively. EGFR expression with ID-specific antibody and AQUA also predicted responders in EGFR mutated patients. Increased EGFR expression with the ID antibody associated with increased median PFS (11.7 months vs 5.0, Log-rank p=0.034) and OS (38.6 vs 14.9, p=0.040), from gefitinib therapy.

Conclusions: EGFR protein expression using an ID-specific antibody specifically predicts response to gefitinib in NSCLC patients, including in EGFR mutated patients, and increased PFS/OS from gefitinib. These data suggest that the choice of diagnostic antibody and methodology matters to predict response and outcome to specific therapies. The potential clinical application needs further validation.
**Introduction**

Personalized therapy with individualized biomarker analysis has come to lung cancer treatment. In patients with advanced non-small cell lung cancer (NSCLC), response to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) is significantly higher in females, never-smokers, patients with Asian ethnicity, adenocarcinoma histology and tumors harboring activating EGFR mutations (1). EGFR TKIs are shown to be superior to chemotherapy in first line therapy of advanced NSCLC patients with EGFR mutations (2-5). Thus, it is important to evaluate EGFR mutation status before choosing treatment of advanced NSCLC. However, EGFR mutation status alone may be insufficient to predict outcome as 17% - 38% of patients with mutations do not respond to EGFR TKIs in 1st line therapy (2, 6-9) and 58% - 84% in 2nd/3rd line therapy (10-12). Furthermore 1% - 7% of patients without mutations respond to EGFR TKIs in 1st line (2, 6-9) and 3% to 7% in 2nd/3rd line therapy (10-12). Several other biomarkers are being evaluated in NSCLC patients to predict response or resistance to EGFR TKIs. Current sensitive biomarkers include EGFR activating mutations, gene copy number and protein expression and resistant biomarkers include EGFR T790M and KRAS mutations, c-Met and IGF1R expression.

Currently, EGFR protein expression is not recommended for predicting outcome to EGFR TKIs due to controversial published results. Some studies showed a better outcome after EGFR TKIs treatment for tumors over-expressing the EGFR protein (10, 13, 14), while other studies did not (7, 11, 15). Inconsistent results from these studies could be due to insufficient sensitive methodology, lack of stringency in the assessments, cohort differences, or confounded by the concomitant administration of chemotherapy (15).
The mutations that predict response to EGFR TKIs are localized within the kinase domain, resulting in constitutive signaling of EGFR, which can therefore be inactivated by TKIs. The EGFR external domain (ED)-specific antibodies bind to receptors containing the ED and do not discriminate between active or inactive receptors, and may not even detect some truncated forms of the receptor that are constitutively active and potentially valid targets for EGFR TKIs. This may explain why assessment of protein expression using ED-specific antibodies is not a consistent predictor of response to EGFR TKIs.

A novel antibody, 5B7, specific for the intracellular domain (ID) of EGFR and directed against the epitope located at the suppressor of cytokine signaling 3 (SOCS3) protein binding site has been developed (16). An ID-specific antibody would also detect truncated forms of the receptor that are constitutively active. Therefore, using this antibody to quantify EGFR expression may more accurately predict response to EGFR TKIs. We have previously reported that EGFR expression evaluated by 5B7 does not predict survival in surgical NSCLC patients not treated with EGFR TKIs (17).

A concern regarding IHC analyses is the lack of standardization in procedures and assessment. IHC scoring is semi-quantitative, subjective and highly dependent on poorly controlled variables, e.g. intensity of staining or percentage of stained cells is highly dependent on the evaluator (18). For applicability in the clinical setting, new NSCLC testing paradigms must produce objective and reproducible results. The Automated QUantitative Analysis (AQUA) technology is an immunofluorescence-based technique allowing automated and quantitative analysis of protein expression providing objective and reproducible scores (19). Analysis of protein expression with AQUA scoring for other biomarkers in NSCLC has been shown to be a
compelling methodology for predicting prognosis and response to therapy in patients with NSCLC (20, 21).

The present study is the first to report the predictive value of a novel antibody, detecting the ID of EGFR for response to gefitinib in patients with NSCLC. We compared the predictive values of EGFR protein expression using this novel antibody versus two ED-specific antibodies, utilizing IHC and AQUA technology.

**Patients and Methods**

**Patients**

This study included 98 Japanese patients treated with gefitinib as monotherapy (250 mg per day) for their recurrent disease after having undergone curative pulmonary resection at the Tokyo Medical University Hospital between May 1995 and March 2008. Clinical characteristics are described previously in **Supplemental Table 1**. There were 46 (47%) males and 50 (51%) smokers. NSCLC was histologically confirmed on H&E stained sections according to the World Health Organization criteria (22) and consisted of 80 adenocarcinomas, 9 squamous cell carcinomas, 5 large cell carcinomas, and 4 other NSCLC. Pathological staging at the time of surgery using the TNM Classification of malignant tumors seventh edition (23) showed 14, 17, 10, 9, 36, 7 and 5 patients at stage IA, IB, IIA, IIB, IIIA, IIIB and IV, respectively.

Tumor response to gefitinib therapy was measured according to response evaluation criteria in solid tumors (RECIST) (24) as complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD) using computed tomography scanning. A confirmatory evaluation was repeated at least 4 weeks after the initial determination of response. During treatment with gefitinib, assessments were performed every 4 weeks for the first 4
months and then every 8 weeks until disease progression. Disease control rate ($DCR = CR + PR + SD$) was evaluated at 12 weeks, as the RECIST (24) recommends that the duration of SD should specify the minimal time interval required between two measurements for determination of SD. In the 98 patients, 10 (10%) received gefitinib as their 1st systemic anticancer therapy after recurrence, 43 (44%) received gefitinib as a 2nd and 45 (46%) as ≥3rd line therapy. The ORR was considered only for patients treated with gefitinib for at least four weeks. ORR were available for 94/98 patients. Overall survival (OS) and progression free survival (PFS) were defined as the time from start of gefitinib therapy to death or progression/death, respectively.

**Tissue microarrays**

TMA construction has been described previously (25-27). Seventy tumors (=70 patients) were included in the TMA. The specimens were fixed with 10% formalin and embedded them in paraffin, then stored at room temperature.

Three replicate core samples (1.2 mm) from the most representative tumor areas from the formalin-fixed paraffin embedded resected tumors were collected and the TMA was assembled using a tissue-arraying instrument (J.M and T.N, Department of Anatomic Pathology, Tokyo Medical University Hospital, Tokyo, Japan). Normal liver tissues were used for slide orientation purposes. After sectionning, the slides were stored at 4°C.

**EGFR IHC staining**

IHC was performed on the 98 patients. Serial 4-μm-thick tissue sections were cut from the TMA (N=70 pts) and from whole tumor section (N=28 pts). The slides were baked at 60°C overnight, then deparaffinized in xylene (Surgipath®, Richmond, IL) and rehydrated through a graded ethanol (Surgipath®, Richmond, IL) series. Unstained slides were barcoded for a standardized antibody-specific protocol and loaded into a Benchmark® XT automated stainer.
Antigen retrieval for EGFR 3C6 and EGFR 5B7 was performed with Cell Conditioning 1 for 60 minutes (VMSI, #950-124) and with Protease 2 (VMSI, #760-2019) for 16 minutes, respectively. Pre-diluted EGFR antibody was automatically dispensed and the slides were incubated at 37°C for 32 and 16 minutes, for 3C6 (VMSI, #790-2988) and 5B7 (VMSI, #790-4347), respectively. UltraView™ DAB detection kit (VMSI, #760-091) was used with an extra washing step. Slides were counterstained with Hematoxylin (VMSI, #760-2021) for 4 minutes, post-counterstained with Bluing (VMSI, #760-2037) for 4 minutes, washed with mild soapy water and then dehydrated in ethanol and xylene before applying coverslips. Positive (HT-29 cells) and negative (CAMA-1) control tissue slides from Dako PharmDx EGFR kit (Dako, Carpinteria, CA, #1494) were included. Additionally, as a negative control, non-immune antibody was substituted for the primary antibody on positive control tissue.

**IHC scoring methodology**

IHC scoring was performed by one pathologist (B.R.A) at the University of Colorado Cancer Center according to the University of Colorado hybrid (H)-scoring criteria (28). The H-score is derived by summing the products of the percentage of positive cells (0-100%) and the intensity (0-4) for that group, resulting in a final IHC score ranging from 0 to 400. The final score for each patient was taken as the core with the maximum H-score (29). One score was obtained for membrane staining only, as previously described (30) and a second score was obtained by counting both membranous and cytoplasmic staining. We scored both the membrane and the cytoplasm to be more comparable with AQUA technology evaluation, which quantifies both.

**AQUA Technology Staining**
AQUA technology staining was only applied to the subset of 70 tumors on the TMA due to tissue unavailability for the other 28 tumors. Unstained slides were baked at 60°C for 1 hour and then deparaffinized. Antigen retrieval for EGFR 31G7 (Zymed, San Francisco, CA, #28-0005) was carried out by incubating samples in Pepsin Solution Digest-All-3 (Invitrogen, Carlsbad, CA, #00-3009) at 37°C for 10 minutes. Antigen retrieval for EGFR 5B7 was done using a pressure cooker at 105°C for 20 minutes while using a 1:10 dilution of Tris-EDTA solution, pH 9.0 (Dako, #S2367). Using an automated stainer (Dako), EGFR protein expression was detected with Aquantiplex™ EGFR Quantification Assay Kit (HistoRx Inc. New Haven, CT, #AQ-EMT1-0001). The slides were blocked with Aquantiplex Blocking Buffer 1 for 5 minutes at room temperature and then rinsed with working buffer (1:10 diluted tris-buffered saline containing 0.05% Tween-20, pH 7.6, Dako, #S3006) for 5 minutes. Slides were blocked with a second blocking buffer, Aquantiplex Blocking Buffer 2, for 10 minutes at room temperature and rinsed with working buffer for 5 minutes. Aquantiplex Primary Antibody Cocktail, EGFR 31G7 (pre-diluted and included in kit) or EGFR 5B7 (0.1 ug/ml), in addition to anti-pan-cytokeratin antibodies were incubated on slides at room temperature for 1 hour. Slides were washed 3 times with working buffer for 5 minutes each and incubated in Aquantiplex Epithelial Mask Visualization Solution, containing Dako Envision and Alexa Fluor 555 goat anti-rabbit IgG (H+L) for 30 minutes in the dark at room temperature. For fluorescence amplification, slides were exposed, 10 minutes at room temperature, to Cy5-tyramide and a 1:50 solution of Aquantiplex EGFR Visualization Solution in Aquantiplex EGFR Signal Amplification Diluent, for. Slides were mounted using the Prolong Gold antifade reagent with 4’,6-diamidino-2-phenylindole (DAPI) solution (Invitrogen). In order to assess reproducibility between runs and batches, a control TMA slide was included in each run. The Pearson
The correlation coefficient was 0.936 with a slope at 0.9899, showing an excellent reproducibility between runs for the control tissue.

**AQUA scoring**

Automated image acquisition and analysis using AQUA technology has been described previously (19, 31). The finalized TMA slides were scanned with a PM-2000 microscopy system (HistoRx) and image data were analyzed with AQUAAnalysis™ software (HistoRx). AQUA technology allows measurement of protein concentration within subcellular compartments within tumor regions (19, 31, 32). To summarize, a series of high-resolution monochromatic images were captured with a PM-2000 microscope. For each histospot, images were obtained using the signal from the 4’,6-diamidino-2-phenylindole (DAPI), cytokeratin-Alexa 555 and EGFR-Cy5 channels. EGFR was measured using a channel with emission maximal above 620 nm to minimize tissue autofluorescence. Tumor was distinguished from stromal and nonstromal elements by creating an epithelial tumor ‘mask’ from the cytokeratin signal. Cytokeratin and DAPI were used to identify tumor-specific cytoplasm and nuclei (19, 32) using an unsupervised image analysis algorithm. The AQUA score in each subcellular compartment was calculated by dividing the EGFR compartment pixel intensities by the area of the compartment within which they were measured. AQUA scores were normalized to the exposure time and bit depth at which the images were captured, allowing comparison of scores collected with different exposure times. To stabilize variance over the wide fluorescence range and normalize distributions, scores were log₂ transformed for statistical analyses.

**DNA sequencing of EGFR mutations**

The methodology has been described in a previous publication (25).

**Statistical analysis**
Groups were compared by t-tests, choosing specific test and reported results were reported based on parallel testing for equality of variances. The optimal cut-points were determined using ROC curves by selecting the highest % of correct classification when both sensitivity and specificity are ≥ 50%. Sensitivity, specificity, likelihood ratios and area under the curves (AUC) were also calculated. Correlations between continuous variables were performed using Pearson’s correlation. Standard descriptive statistics and Kaplan-Meier survival curves were utilized. Differences in PFS were determined by the log-rank test. Association between the continuous scores and progression free survival were assessed with univariate Cox models and multivariate Cox models adjusted for smoking, histology and grade. Statistical significance was considered with a p value < 0.05. Statistical analyses were performed twice using three different softwares: SPSS for Windows Version 12.0 (SPSS Inc., Chicago, IL), Graph Prism Version 5.0 (GraphPad Software, La Jolla, CA) and SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

**Results**

**Evaluation of EGFR protein expression**

Among the 98 patients, the median H-scores for the membranous EGFR expression were 10 (range 0–400) and 180 (range 0–400) for ED and ID-specific antibodies, respectively. For the combined membranous + cytoplasmic staining, the median H-scores were 23 (range 0–280) and 200 (range 0–350) for ED and ID-specific antibodies, respectively. The distribution of EGFR expression in these patients are illustrated in the histograms in Figure 1.

On the 70 patient cohort on TMA, EGFR protein expression was determined both with IHC (70/70 evaluable) and AQUA technology (66/70 evaluable) using the 5B7 ID-specific antibody. We also evaluated, on the same cohort, EGFR expression with two different ED-
specific antibodies (3C6 and 31G7), that detect the same epitope of EGFR, with IHC (using 3C6, 70/70) and AQUA (using 31G7, 69/70), respectively. When using IHC, the median H-scores for the membranous EGFR expression were 10 (range 0–400) with ED-specific antibody and 205 (range 0–400) with ID-specific antibody, respectively. In the evaluation of the membrane-and-cytoplasm staining, the median H-scores were 10 (range 0–280) and 200 (range 0–350) for ED and ID. The median AQUA scores were 8.05 (range 6.80–11.58) and 11.68 (range 6.89–14.30), when using ED- and ID-specific antibodies, respectively.

Pearson correlation tests were performed between results according to the different antibodies, compartments and technologies (Supplemental Figure 1). The correlations were performed on 98 patients when including IHC evaluations only and on 70 when including AQUA technology. A strong correlation was found between IHC scores for the membranous versus membranous + cytoplasmic expression when using the 3C6 ED antibody (r=0.92) and the 5B7 ID antibody (r=0.7). There was also a strong correlation between the scores obtained by IHC and AQUA technologies, for ED-specific antibodies even if the antibodies used were different (3C6 versus 31G7) (r=0.73) and for the ID-specific antibodies (5B7 for both methods) (r=0.83). The weakest correlation was between the EGFR expression scores when comparing antibodies detecting the ED versus the ID whether by IHC (3C6 versus 5B7) when evaluating the membrane only (r=0.55), when evaluating membrane + cytoplasm (r=0.51) or when using AQUA technology (31G7 versus 5B7) (r=0.63). All of the above-discussed correlations were statistically significant (p<0.001).

As illustrated by the histograms (Figure 1), the expression level of EGFR was lower using ED-specific antibodies. Regardless of the antibody or technology used, both ED with IHC (3C6 antibody) and ED with AQUA technology (31G7 antibody), had lower EGFR scores when
compared to the ID-specific antibody 5B7. The lower levels of expression when using ED antibodies as compared to the ID antibody are illustrated in **Figure 2**, which displays representative samples by IHC and AQUA staining.

**Prediction of response to gefitinib by EGFR expression**

We analyzed the predictive value for response of EGFR expression by IHC based on the 94 patients evaluable for response. The level of EGFR expression using ED-specific antibodies with IHC was not significantly different between non-responders and responders to gefitinib whether evaluating membranous staining (mean H-score 31 versus 61, p=0.2230) or membranous + cytoplasmic staining (43 versus 76, p=0.1354). However, when using the ID-specific antibody, EGFR expression was significantly higher in gefitinib responders versus non-responders using IHC by scoring the membranous staining (156 versus 213, p=0.0214) and slightly more significantly when assessing membranous + cytoplasmic staining (168 versus 231, p=0.0013). EGFR expression assessed with the ID-specific antibody and IHC evaluating membranous + cytoplasmic staining predicted response with a PPV, NPV and accuracy of 42, 89 and 72%, respectively. The best cutpoint was 217 as determined by ROC curve analyses (Figure 3B).

We further compared the prediction of response of EGFR protein expression by using IHC versus AQUA in 70 patients (**Table 1**). The level of EGFR expression by IHC using ED-specific antibodies was not significantly different between responders and non-responders to gefitinib whether evaluating membranous staining (mean H-score 34 versus 72, non-responders versus responders, respectively, p=0.27) or membranous + cytoplasmic staining (43 versus 76, p=0.27). However, by using AQUA, it was borderline (8.2 versus 8.9, p=0.0581). When using the ID-specific antibody, EGFR expression was significantly higher in gefitinib responders.
versus non-responders using IHC and scoring membranous staining (185 versus 257, p=0.0063). Results were more significant for IHC when assessing membranous +cytoplasmic staining (179 versus 245, p=0.0026) and even more significant when assessing ID by AQUA analysis (11.2 versus 12.7, p=0.0017) (Scatter plots shown in Supplemental Figure 2).

To determine the highest accuracy for predicting response to gefitinib for each type of EGFR expression quantification, we performed ROC curve analysis (Table 2 and Figure 3A). When evaluating EGFR expression using ED-specific antibodies, the ROC curves did not significantly discriminate between responders and non-responders. When using ID-specific antibody, the ROC curves were able to significantly discriminate between responders to gefitinib and non-responders. Using the ID antibody, the AUC was highest when using the AQUA scoring (0.78, p=0.0018), intermediate when using IHC with evaluation of the membrane + cytoplasm staining (0.73, p=0.0078) and the lowest by IHC with evaluation of membrane-only staining (0.70, p=0.0289). The corresponding positive predictive values (PPV) for being responders were 38, 38.5 and 50%, the negative predictive values (NPV) were 85, 88 and 88% and the accuracies were 70, 69 and 80% when evaluating EGFR expression by IHC membrane-only (cut-point=235), IHC membrane-and-cytoplasm (cut-point=215) and AQUA scoring (cut-point=12.72), respectively.

**Association of EGFR protein expression with EGFR mutations analyses**

A statistically significant association was found between EGFR protein expression and mutation status, except when assessing ED by IHC. Tumors with EGFR mutations harbored a significantly higher level of EGFR protein expression when using an ID-specific antibody or an ED-specific antibody by AQUA (Table 3).

We further compared the level of EGFR protein expression of patients with tumors
harboring activating mutations and expected to be sensitive to EGFR TKIs (exon 19 and exon 21), versus other patients (EGFR wild type tumors and those harboring other EGFR mutations, ie exon 18 or 20). Activating EGFR mutations in the tumor were associated with a significantly higher level of EGFR protein expression, regardless of the method used. The most significant association was obtained when detecting the ID with mean scores 195 versus 152 for tumors with activating mutations or not, respectively (p=0.0412), 213 versus 156 (p=0.0006) and 12.19 versus 10.99 (p=0.0010) when using IHC membrane-only, IHC membrane + cytoplasm and AQUA technology, respectively. When detecting the ED, the level of EGFR expression was also significantly higher in mutated tumors but only with AQUA and not IHC: 50 versus 25, p=0.0912, 61 versus 40, p=0.1533 and 8.78 versus 8.09, p=0.0105, when using IHC membrane-only, IHC membrane + cytoplasm and AQUA technology, respectively.

**Prediction of response to gefitinib by EGFR mutation**

Mutation status predicted response (98 patients, 94 evaluable for response, Chi square test p value = 0.0106). The NPV, PPV and accuracy of EGFR mutation for predicting response on the 94 patients were respectively 91%, 33% and 52%.

**Prediction of response to gefitinib by EGFR expression in patients with EGFR mutations**

We further determined if EGFR expression could predict response in mutation status subgroups. The analyses could not be performed on wild type patients due to a low response rate. However, in EGFR mutated patients, the responders, as compared to the non-responders, had a significantly higher level of EGFR expression as revealed by ID-specific antibody staining with IHC and assessing membrane (Mean H-score 232 vs 172, p = 0.0274) or membrane + cytoplasm (246 vs 284, p = 0.0018) or with AQUA (12.68 vs 11.51, p=0.0109). EGFR expression assessed with the ID-specific antibody and AQUA technology predicted response with an NPV, PPV and
accuracy of 81%, 67% and 76% (AUC = 0.75). This was based on a cutpoint (12.51) which gave
the highest accuracy. In order to better predict the non-responders within mutated patients,
another cutpoint (11.6), which optimized the NPV and the sensitivity, was chosen. Doing so,
resulted in a NPV, PPV and accuracy of 93%, 50% and 66%, as 1/14 of responders highly
expressed EGFR.

**Outcome**

The median OS and PFS are reported in Supplemental table 1. We evaluated PFS and OS
first by Kaplan-Meier curves and log-rank analyses using the best cut-points derived from ROC
analysis and then by Cox proportional models evaluating EGFR expression as a continuous
variable. EGFR protein expression, regardless of the antibody used, compartment analyzed, or
technique used, did not significantly associate with PFS in the cohort of 70 patients using the
log-rank test. However, with the Cox model, EGFR expression evaluated with the ID-specific
antibody and AQUA technology was significantly associated with PFS in univariate (p=0.0406)
and multivariate analyses (p=0.0134). In the total cohort of 98 patients, increased EGFR
expression determined with the ID antibody and membranous + cytoplasmic evaluation
associated with increased median PFS (11.7 months vs 5.0, Log-rank p=0.034) and OS (38.6 vs
14.9, p=0.040). The association between, increased EGFR expression determined with the ID
antibody using membranous + cytoplasmic evaluation and increased PFS was confirmed by Cox
proportional models in univariate (p=0.0242) and multivariate (p=0.0151) analyses but the
association between EGFR expression and OS was not confirmed in the Cox proportional model.
In the subgroup of patients with EGFR mutations, EGFR expression evaluated with the ID-
specific antibody and scoring membrane + cytoplasm was also associated significantly with PFS.
in univariate (p=0.0323) and multivariate (p=0.0074) analyses and borderline for OS (p=0.0794 and 0.0666, respectively) using the Cox proportional hazards model.

PFS and OS Kaplan Meier curves are shown in Supplemental Figure 3 and 4, respectively.

**Discussion**

We assessed and compared the predictive role of the novel antibody, 5B7, which detects the ID of EGFR versus antibodies detecting its ED, utilizing two detection methods namely, IHC (evaluation of the membrane only versus evaluation of both the cytoplasm and the membrane) and the AQUA technology. EGFR expression levels were significantly correlated between the different antibodies/techniques. The level of EGFR expression using ED-specific antibodies was markedly lower as compared to those detecting the ID. EGFR expression by IHC using the ID-specific antibody was a significant predictor of response to gefitinib in this cohort of Japanese NSCLC patients and assessment of EGFR expression measured by AQUA technology using the ID antibody increased the predictive performance of the assay. In addition, EGFR expression using the ID-specific antibody predicted responders in the subgroup of mutated patients. Finally, high EGFR expression evaluated with the ID-specific antibody was associated with increased PFS and OS.

Although significantly associated to mutation status, EGFR expression determined by ED-specific antibodies did not significantly predict response to gefitinib. Thus, we found EGFR expression being predictive of response to gefitinib as using a novel ID-specific antibody. The importance of epitope variability has been shown previously (33). The 5B7 antibody for EGFR, which was predictive of response to gefitinib in our cohort of Japanese NSCLC patients, detects the SOCS3 binding epitope of the ID. When SOCS3 is bound to EGFR, it is thought to inhibit the downstream signaling cascade of the receptor (16). Therefore, it is hypothesized that when
SOCS3 is present, EGFR is inactive and 5B7 will not bind due to steric hindrance. When the 5B7 antibody binds the receptor on this particular epitope, it only does so when SOCS3 is not present and cannot inhibit the EGFR cascade. This may explain the enhanced ability of 5B7 mediated detection of EGFR to predict response to EGFR TKIs.

Another point is that previously published studies were using cut-off as low as 10% (10-14) of positive cells to define EGFR IHC positive tumor. When we performed the ROC analyses to determine the best cut-off to discriminate between responders and non-responders, the cut-off values were much higher than 10% of cells.

The measured levels of expression of EGFR was globally lower when assessing the ED versus the ID, many cases even being negative for the former and positive for the latter. The level of expression of EGFR using ED-specific antibodies in our study (median H-score of 10) was a little bit lower than observed in the BR21 study (median H-score 40 using Dako PharmDX kit) (34) and much lower than in the ISEL study (median H-score of 300 using 31G7 and median of 45% using Dako PharmDX kit) (28). These variable results strongly indicate the need for standardization of IHC procedure. However, the lower level of EGFR expression using ED-specific antibody compared to ID-specific antibody is consistent whether using IHC with 3C6 or AQUA technology with 31G7 to detect the ED. Thus, this is not antibody or technology related but more likely to the detection of ED versus ID. One hypothesis to explain this difference in EGFR expression level when detecting the receptor by ED or ID could be a cleavage of the ED. Truncated forms of the HER2 receptor with a basal cleavage of the ED has been largely reported and shown to keep and even increase the tyrosine kinase activity of the receptor (35-38). An amino-terminal truncation of EGFR extracellular domain has been reported (39, 40). This hypothesis requires further experimentation. Due to the fact that the ED truncated form is...
constitutively activated, EGFR TKIs are supposed to be an efficient treatment for tumors harboring these forms of EGFR. Therefore using an ID-specific antibody able to detect these ED truncated EGFR proteins is worthy of predicting response to EGFR TKIs. Conversely, as monoclonal therapeutic antibodies target the ED, the use of a diagnostic antibody detecting the expression of the ligand domain is required to predict any potential response to these therapeutic antibodies. Therefore, using an antibody detecting the ED might be a better choice to predict response to therapeutic monoclonal antibodies against EGFR and using an ID-specific antibody could be more accurate to predict response to EGFR TKIs, but this conclusion requires further validation and evidentiary support.

Previous publications on EGFR IHC were based on the evaluation of membrane staining exclusively (10, 13, 14, 30). We assessed membranous staining, membranous + cytoplasmic staining by IHC and compared it to membrane + cytoplasmic staining by AQUA technology. The evaluation of 5B7 by IHC was more significantly predictive of response to EGFR TKIs once cytoplasmic staining was integrated in the evaluation. This could reflect that the presence of a high turnover of the receptor in the cytoplasm indicating a higher activity of EGFR in the cell and a higher sensitivity to EGFR TKIs.

Finally, using AQUA technology to evaluate EGFR expression further improved the predictive performance as compared to IHC, even with the use ED-specific antibodies. However, we must be cautious in interpreting these last results because the ED-specific antibodies used to compare IHC and AQUA scoring were different (3C6 versus 31G1), but this bias is very likely minimal as the two antibodies detect the same epitope on the ED of the receptor. Using 5B7, AQUA technology improved PPV and accuracy by 10% and specificity by 17% as compared with IHC.
The need to standardize biomarker testing for clinical use led us to assess and compare AQUA technology to classical IHC. IHC provides information on protein expression and localization and has been the standard protein in situ assay. However, IHC scoring is semi-quantitative and subjective (41). TMAs provide a high-throughput method to analyze potential biomarkers on multiple samples but are limited by the pathologist’s ability to reproduce scores on a continuous scale, discriminate between subtle low-level staining differences, and accurately score expression within subcellular components. AQUA analysis is an immunofluorescence-based technique with automated and quantitative analysis of proteins, thus reducing the human variability occurring with IHC scoring. AQUA technology automatically measures protein expression in subcellular compartments (i.e. nuclear versus cytoplasmic), providing a continuous score in an accurate, reliable, and reproducible way. The remaining investigator based operations in the AQUA score acquisition are the selection of the area to be scanned and, after acquisition of the image, the cropping of artifactual features or exclusion of entire cores. The other steps of AQUA score acquisition are fully automated, therefore reducing the investigator based variability. Reported results in NSCLC support AQUA technology as a valid methodology for use (20, 21). Few other studies compared IHC versus AQUA scoring. Anagnostou et al (33) found that estrogen receptor (ES) evaluation by IHC and AQUA scoring were correlated and in close proportion (Spearman correlation coefficient at 0.69) to what we observed (0.732 and 0.827, respectively for ED- versus ID-specific). Analyses of TS expression in small cell lung cancer by IHC versus AQUA scoring showed a much lower, but significant correlation (r=0.25) (42). An analysis of HER2 expression in breast tumors showed a correlation at 0.704 between manual (four categories) versus automated acquisition of the score for protein expression (43). The prediction of the categories as determined by IHC or AQUA scoring were different. These
results demonstrated clearly the difficulty in manually translating a biological (continuous) marker into a nominal four-point scale. Even for a trained pathologist, accurate distinction between categories (e.g., histoscore of 2 versus 3) is difficult and potentially arbitrary. The lack of reproducibility of IHC scoring has been reported by many authors (44-46). The AQUA assay is standardized and the acquisition of the score is automated. Therefore the value of the cutpoint will be obtained with a high reproducibility. However in order to determine the optimal cutpoint, further validation studies must be performed in different cohorts.

Attention must be given to the specific characteristics of the cohort we studied when interpreting our data. The cohort was exclusively Asian (Japanese) and therefore may not translate into a non-Asian population. The treatment lines within the current cohort, 10% having received gefitinib as their 1st systemic anticancer therapy after relapse, 44% as 2nd and 45% as ≥3rd line therapy, were highly variable. Therefore, the interpretation of the outcome data in this cohort is difficult. There is a need for validation of the present study’s results in other cohorts and the applicability to a Caucasian cohort needs to be demonstrated.

In conclusion, this study shows that the evaluation of EGFR protein expression using a novel antibody detecting a specific epitope on the ID, which is only accessible on activated EGFR, can predict response and outcome to gefitinib using traditional IHC or AQUA technology. This data shed a new light on the potential use of EGFR protein expression as a biomarker for the prediction of response to EGFR TKIs. The current results also emphasize the importance of the choice of the methods to be used (e.g. type of antibody, compartment assessed, and detection technique) for the detection of EGFR protein, and in particular, the choice of the diagnostic antibody to evaluate EGFR expression relative to the therapeutic strategy, i.e. an ED-specific antibody may be better predicting response to anti-EGFR monoclonal antibodies.
whereas an ID-specific antibody may be better for EGFR TKIs response prediction. This hypothesis needs further validation. Interestingly, EGFR expression using the ID-specific antibody predicted responders in the subgroup of EGFR mutated patients. This could have a potential clinical application, as EGFR mutation might not be sufficient as stand alone procedure for discriminating responders versus non-responders to EGFR TKIs.
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Table 1: Association of EGFR expression with response status to gefitinib

<table>
<thead>
<tr>
<th>Methods of evaluation of EGFR expression</th>
<th>Statistics described</th>
<th>Response</th>
<th>p-value §</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific antibodies for the external domain of EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED IHC Membrane</td>
<td>N (missing) Mean (SEM)</td>
<td>52 (0) 33.94 (7.924)</td>
<td>15 (0) 72.00 (32.40)</td>
</tr>
<tr>
<td>ED IHC Membrane &amp; Cytoplasm</td>
<td>N (missing) Mean (SEM)</td>
<td>52 (0) 42.88 (8.39)</td>
<td>15 (0) 76.00 (27.51)</td>
</tr>
<tr>
<td>ED AQUA</td>
<td>N (missing) Mean (SEM)</td>
<td>51 (1) 8.26 (0.15)</td>
<td>15 (0) 8.90 (0.34)</td>
</tr>
<tr>
<td>Specific antibody for the internal domain of EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID IHC Membrane</td>
<td>N (missing) Mean (SEM)</td>
<td>52 (0) 184.6 (12.14)</td>
<td>15 (0) 257.3 (22.94)</td>
</tr>
<tr>
<td>ID IHC Membrane &amp; Cytoplasm</td>
<td>N (missing) Mean (SEM)</td>
<td>52 (0) 178.8 (10.35)</td>
<td>15 (0) 245.3 (16.38)</td>
</tr>
<tr>
<td>ID AQUA</td>
<td>N (missing) Mean (SEM)</td>
<td>50 (1) 11.22 (0.21)</td>
<td>13 (2) 12.68 (0.35)</td>
</tr>
</tbody>
</table>

Legend: § Independent, two-sample t-test for difference in mean score between responders and non-responders. Equivalence of variance was tested. In case of inequality of variance *, a t-test not assuming equality of variances was used. SEM, standard error of the mean; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease. In order to compare the performance of the techniques/antibodies to discriminate between responders and non-responders, this table displays the results using the 70 patients on which the 6 different evaluations were performed.
Table 2: Accuracy to predict response to gefitinib according to the method used to evaluate EGFR expression

<table>
<thead>
<tr>
<th>Methods of evaluation</th>
<th>Best cutoff</th>
<th>sensitivity</th>
<th>specificity</th>
<th>accuracy</th>
<th>PPV</th>
<th>NPV</th>
<th>LR</th>
<th>AC</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SUBSET OF 70 PATIENTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED IHC Membrane</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.54</td>
<td>0.6038</td>
</tr>
<tr>
<td>ED IHC Membrane &amp; Cytoplasm</td>
<td>25</td>
<td>53.3%</td>
<td>55.8%</td>
<td>55.2%</td>
<td>25.8%</td>
<td>80.6%</td>
<td>1.21</td>
<td>0.54</td>
<td>0.6197</td>
</tr>
<tr>
<td>ED AQUA</td>
<td>8.48</td>
<td>53.3%</td>
<td>68.6%</td>
<td>65.2%</td>
<td>36.4%</td>
<td>83.3%</td>
<td>1.70</td>
<td>0.66</td>
<td>0.0653</td>
</tr>
<tr>
<td><strong>Specific antibody for the internal domain of EGFR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID IHC Membrane</td>
<td>235</td>
<td>53.3%</td>
<td>75%</td>
<td>70.1%</td>
<td>38.1%</td>
<td>84.8%</td>
<td>2.13</td>
<td>0.70</td>
<td>0.0289</td>
</tr>
<tr>
<td>ID IHC Membrane &amp; Cytoplasm</td>
<td>215</td>
<td>66.7%</td>
<td>69.2%</td>
<td>68.7%</td>
<td>38.5%</td>
<td>87.8%</td>
<td>2.17</td>
<td>0.73</td>
<td>0.0078</td>
</tr>
<tr>
<td>ID AQUA</td>
<td>12.72</td>
<td>53.8%</td>
<td>86%</td>
<td>79.4%</td>
<td>50%</td>
<td>87.8%</td>
<td>3.85</td>
<td>0.78</td>
<td>0.0018</td>
</tr>
<tr>
<td><strong>WHOLE COHORT OF 98 PATIENTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED IHC Membrane</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.58</td>
<td>0.2643</td>
</tr>
<tr>
<td>ED IHC Membrane &amp; Cytoplasm</td>
<td>37.5</td>
<td>52.4%</td>
<td>64.4%</td>
<td>61.7%</td>
<td>29.7%</td>
<td>82.5%</td>
<td>1.47</td>
<td>0.59</td>
<td>0.1763</td>
</tr>
<tr>
<td><strong>Specific antibody for the internal domain of EGFR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID IHC Membrane</td>
<td>205</td>
<td>52.4%</td>
<td>69.9%</td>
<td>65.9%</td>
<td>33.3%</td>
<td>83.6%</td>
<td>1.74</td>
<td>0.64</td>
<td>0.0484</td>
</tr>
<tr>
<td>ID IHC Membrane &amp; Cytoplasm</td>
<td>217</td>
<td>66.7%</td>
<td>74.0%</td>
<td>72.3%</td>
<td>42.4%</td>
<td>88.5%</td>
<td>2.56</td>
<td>0.72</td>
<td>0.0016</td>
</tr>
</tbody>
</table>

Legend: PPV, positive predictive value; NPV, negative predictive value; NA, no accuracy for both a sensitivity and specificity of at least 50%; LR, likelihood ratio; AC, Area under the curve; p value, p value for the area under the curve. In order to compare the performance of the techniques/antibodies to discriminate responders versus responders, this table displays the results using the 70 patients on which the 6 different evaluations have been performed. In addition, data on the total cohort are reported for IHC.
Table 3: Association of EGFR expression with EGFR mutation

<table>
<thead>
<tr>
<th>Methods of evaluation of EGFR expression</th>
<th>Statistics described</th>
<th>Mutation</th>
<th>p-value §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mutated</td>
<td>Wild type</td>
</tr>
<tr>
<td>Specific antibodies for the external domain of EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED IHC Membrane</td>
<td>N Mean (SEM)</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.29 (10.79)</td>
<td>30.43 (8.77)</td>
</tr>
<tr>
<td>ED IHC Membrane &amp; Cytoplasm</td>
<td>N Mean (SEM)</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.20 (10.20)</td>
<td>46.16 (8.76)</td>
</tr>
<tr>
<td>ED AQUA</td>
<td>N Mean (SEM)</td>
<td>41</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.69 (0.18)</td>
<td>7.95 (0.18)</td>
</tr>
<tr>
<td>Specific antibody for the internal domain of EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID IHC Membrane</td>
<td>N Mean (SEM)</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>192.0 (12.79)</td>
<td>144.4 (14.83)</td>
</tr>
<tr>
<td>ID IHC Membrane &amp; Cytoplasm</td>
<td>N Mean (SEM)</td>
<td>56</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>205.3 (9.60)</td>
<td>163.8 (12.25)</td>
</tr>
<tr>
<td>ID AQUA</td>
<td>N Mean (SEM)</td>
<td>39</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.92 (0.22)</td>
<td>10.94 (0.30)</td>
</tr>
</tbody>
</table>

Legend: § Independent, two-sample t-test for difference in mean score between responders and non-responders. Equivalence of variance was tested. In case of inequality of variance *, a t-test not assuming equality of variances was used. SEM, standard error of the mean. The analyses were performed for on the total population for IHC and and on 70 patients for AQUA technology.
Figure Legends.

Figure 1. Histograms showing the distribution of EGFR protein expression using the ED- and ID-specific antibodies and the different technologies, IHC and AQUA. The distribution of the scores when using the antibody that is specific for the ID of EGFR, whether using IHC or AQUA technology, is normally distributed and higher compared with the expression of the ED that has a skewed distribution with a higher frequency of low scores. The distribution is shown on the total population for IHC and on 70 patients for AQUA technology. The number on the x-axis represents the bin center.

Figure 2. Examples of IHC and AQUA EGFR staining using the different antibodies

Each row is the same patient stained with the antibodies and technologies listed on the top. EGFR was detected with standard DAB (brown) in the IHC panels, while for AQUA, EGFR is detected with Cy5 (red). The merged AQUA panels are a combination of EGFR (Cy5), pan cytokeratin (green – Cy3), and nucleus (blue – DAPI). The patient’s EGFR H-scores for IHC and AQUA scores are: A) ED: IHC membrane-0, membrane with cytoplasm-0, AQUA-7.30; ID: membrane-0, membrane with cytoplasm-60, AQUA-8.85; B) ED: IHC membrane-10, membrane with cytoplasm-30, AQUA-7.97; ID: membrane-170, membrane with cytoplasm-160, AQUA-10.84; C) ED: membrane-190, membrane with cytoplasm-210, AQUA-10.48; ID: membrane-300, membrane with cytoplasm-270, AQUA-12.70; D) ED: membrane-290, membrane with cytoplasm-280, AQUA-10.98; ID: membrane-400, membrane with cytoplasm-350, AQUA-14.28.
Figure 3. Receiver operator characteristic curves showing the sensitivity vs (1-specificity) rate for the ED- and ID-specific antibodies and the different technologies. These figures illustrate the differences for the accuracy of predicting response for each method of assessing EGFR protein expression. The area under the curves are listed in table 2.
Legend Supplemental Figure

Supplemental Figure 1. Dot plots showing the correlations between the different methodological evaluations of EGFR protein expression. Pearson correlations were performed and the correlation coefficients (r), the confidence intervals for r and the p values are shown in the graph. The analyses were performed on the total population for IHC and on 70 patients for correlation including AQUA technology.

Supplemental Figure 2. Scatter plots showing the distribution of EGFR protein expression using the ED- and ID-specific antibodies and the different technologies (IHC and AQUA) for both responders and non-responders. The horizontal bar represents the mean and the error bars are the 95% CI. Refer to the text for the mean values and the comparisons of the means results.

Supplemental Figure 3. Kaplan Meier curves for progression-free survival from the start of gefitinib for both the ED- and ID-specific antibodies with both IHC and AQUA. The cut offs shown are based on the curves and are the ones that gave the best accuracy for predicting response. Log rank p values are shown in each graph.

Supplemental Figure 4. Kaplan Meier curves for overall survival from the start of gefitinib for both the ED- and ID-specific antibodies with both IHC and AQUA. The cut offs shown are based on the curves and are the ones that gave the best accuracy for predicting response. Log rank p values are shown in each graph.
Supplemental Figure 5. Scatter plots showing the distribution of EGFR protein expression using the ED- and ID-specific antibodies and the different technologies (IHC and AQUA) for both responders and non-responders in EGFR mutated patients. The horizontal bar represents the mean and the error bars are the 95% CI. Refer to the text for the mean values and the comparisons of the means results.
References


EGFR Protein Expression in Non-Small Cell Lung Cancer Predicts Response to an EGFR Tyrosine Kinase Inhibitor - A Novel Antibody for Immunohistochemistry or AQUA Technology

Celine Mascaux, Murry W. Wynes, Yasufumi Kato, et al.

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