Reproducibility of Immunohistochemical Scoring for Epidermal Growth Factor Receptor Expression in Non–Small Cell Lung Cancer

Round Robin Test

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Conclusions.—After appropriate training, assessing EGFR expression by this immunohistochemistry-based method allowed a highly reproducible allocation of non–small cell lung cancers into clinically relevant high– or low–EGFR expression groups.

EGFR Round Robin Test in NSCLC—Rüschoff et al

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Cetuximab is a therapeutic monoclonal antibody targeting the epidermal growth factor receptor (EGFR) protein. The randomized phase III First-Line Erbitux in Lung Cancer (FLEX) study showed that the addition of cetuximab to first-line cisplatin and vinorelbine chemotherapy statistically significantly improved overall survival compared with chemotherapy alone (hazard ratio [HR], 0.871; 95% confidence interval [CI], 0.762–0.996; P = .04) in patients with EGFR-expressing advanced non–small cell lung cancer (NSCLC). In determining patient eligibility for the FLEX study, EGFR expression was assessed prospectively prior to entry, by immunohistochemistry, using the Dako (Glostrup, Denmark) pharmDx kit. During this process, and following training in a central facility, a single pathologist in 1 of 4 regional centers in Germany, Brazil, Poland, or Hong Kong scored the tumor of each screened patient for membrane-staining intensity (on a scale of 0–3) and the fraction of cells staining at each intensity.

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Prospectively collected FLEX study EGFR immunohistochemistry data were subsequently used to investigate whether tumor EGFR expression level was predictive of the efficacy of chemotherapy plus cetuximab. Using an approach previously described in NSCLC studies, the expression data were first used to generate an immunohistochemistry score for each tumor on a continuous scale of 0–300. Subpopulation treatment effect pattern plot analysis of response rate was then used to select a discriminatory immunohistochemistry score threshold of 200 allowing the outcome-based classification of patients into subgroups with low and high tumor EGFR expression. Evaluation of overall survival, the FLEX study primary endpoint, according to the treatment arm in the low– and high–EGFR expression groups demonstrated a clear benefit for the addition of cetuximab to chemotherapy in the high–EGFR expression group (HR, 0.73; 95% CI, 0.58–0.93; P = .01). No such benefit was seen in the low–EGFR expression group (HR, 0.99; 95% CI, 0.84–1.16; P = .88). The benefit in the high–EGFR expression group was apparent for both major histology–defined subgroups: patients with squamous cell carcinoma (HR, 0.62; 95% CI, 0.43–0.88) and patients with adenocarcinoma (HR, 0.74; 95% CI, 0.48–1.14). A treatment interaction test indicated a predictive value of EGFR expression level in patients with advanced NSCLC receiving chemotherapy plus cetuximab (P = .04). There was no clinically meaningful difference in the safety profile between the high– and low–EGFR expression groups, suggesting an improved benefit/risk ratio for patients in the high EGFR expression group compared with the overall intention-to-treat population.

The data from the FLEX immunohistochemistry analysis therefore suggested that high EGFR expression may be a disease-related biomarker with clinical application in relation to predicting which patients with advanced NSCLC will be most likely to benefit substantially from the addition of cetuximab to first-line platinum–based chemotherapy. To evaluate the level of interobserver reproducibility of the EGFR immunohistochemistry scoring system in NSCLC according to the discriminating threshold score of 200, an international round robin test (RRT) was initiated.

**MATERIALS AND METHODS**

**RRT Process**

The RRT was carried out in 2 separate phases, with both parts 1 and 2 investigating interobserver variability in the classification of tumors into low– and high–EGFR expression groups according to a threshold score of 200 on an immunohistochemistry scale of 0 to 300. Round robin test participants (raters) were, in each case, lung cancer pathology specialists. In both parts 1 and 2, serial sections of the tissue microarrays used in the analyses were stained in a central reference laboratory (Targos Advance AG, Kassel, Germany) using the Dako EGFR pharmDx kit and Dako autostainer, according to the pharmDx kit protocol. In addition, one tissue microarray section was stained with hematoxylin and eosin and used as a tissue guide. Individual tissue microarray sections stained for EGFR were subsequently provided to individual participants for evaluation of tumor expression levels. Part 1, including 14 raters from 12 countries, was primarily a feasibility study to investigate factors that might have impacted on the level of interobserver variability. In this phase, study participants did not receive any specific training. Following the completion of part 1, and informed by the outcome, a face-to-face meeting with the 10 raters from 7 countries who would be participating in part 2 was used to align practice in relation to the process and scoring technique. Part 2 therefore represented the main RRT evaluation of interobserver variability of the immunohistochemistry score.

**NSCLC Specimens**

For both parts 1 and 2, a formalin-fixed, paraffin–embedded tissue microarray block including 50 anonymized NSCLC specimens as single cores was provided by Provitro GmbH (Berlin, Germany). The tissue microarray blocks for each part of the RRT contained different tumor samples. All specimens were obtained from surgery performed for diagnostic/therapeutic purposes, in accordance with German ethics regulations. All patients providing tissue had given signed informed consent for their anonymized samples to be used in third-party research projects.

**Staining, Reference, and RRT Evaluation of Tissue Microarrays**

After sections of the tissue microarray had been stained for EGFR expression, 3 pathologists who were board certified in human pathology (J.R., P.M., and S.S.) participated in a histopathologic reference evaluation for EGFR expression. In the first instance, and based on an overall assessment of the staining quality and tissue availability across the sections, potentially evaluable tumor cores were identified according to predefined criteria, which included the presence of invasive tumor tissue in each serial section and the absence of major staining artifacts. Subsequently, in a preliminary nonsystematic assessment of EGFR expression level, 30 of these cores were selected for evaluation in the RRT. The aim was to include in this group cores showing staining that in relation to the EGFR immunohistochemistry threshold score of 200 were negative (immunohistochemistry score clearly <200), positive (immunohistochemistry score clearly >200), and equivocal (staining intermediate between clear negative and clear positive). Approximately 10 specimens in each expression subcategory, including both adenocarcinomas and squamous cell carcinomas, were to be selected for evaluation by the RRT participants.

For each of the 30 selected specimens, reference immunohistochemistry scores on a scale of 0 to 300 were then defined systematically in a consolidative process between the 3 reference laboratory pathologists according to the following formula: EGFR immunohistochemistry score = 1 × (% cells staining weakly [1+] + 2 × (% cells staining moderately [2+] + 3 × (% cells staining strongly [3+]). Only linear intercellular staining of tumor cells was to be considered as positive in this evaluation, with membrane staining evaluated independent of the completeness. Staining intensity was to be defined in both parts 1 and 2 according to a “magnification rule,” the aim of which was to use physical magnification. The data from the FLEX immunohistochemistry analysis therefore suggested that high EGFR expression may be a disease-related biomarker with clinical application in relation to predicting which patients with advanced NSCLC...
The sample size for the present study was determined on the basis of feasibility considerations. No power calculations were performed and this analysis reports descriptive statistics. All data processing, summarizing and analyses were performed using SAS (SAS Institute, Cary, North Carolina), Version 9.1.3, or R (www.r-project.org; last accessed October 15, 2012), version 2.10.1. If a participant rated a tumor sample as not evaluable, and if this assessment was confirmed by the reference laboratory, this specimen was not taken into consideration in the calculation of agreement measures. If the rater’s assessment of a specimen not being evaluable was not confirmed by the reference laboratory, this was scored as a discrepancy between the rater and the reference laboratory.

Concordance With Respect to the Reference Evaluation.— For each tumor sample, the per-specimen concordance rate was calculated as the proportion of specimens with a classification that was identical to that of the reference evaluation. The overall concordance rate was calculated as the mean of the per-rater concordance rates. χ2 coefficients were applied using the method described by Cohen.9 For each test center rater, the χ coefficient comparing the rater’s classifications with the results of the reference evaluation was calculated. In addition to these measures, the distribution of the differences of each rater’s immunohistochemistry score values from those of the reference evaluation was visualized using box plots.

Concordance Between Test Centers.—For every pair of test center raters, the relative proportion of specimens with identical evaluation results was calculated. Specimens categorized as not evaluable confirmed by the reference laboratory for at least 1 of the 2 raters were not considered in the calculation. Pairwise concordance rates were subsequently calculated. For every pair of test center raters, χ coefficients were also calculated.

RESULTS

Part 1

In the 1st feasibility study, 14 RRT participants evaluated EGFR expression of 30 tumors in consecutive tissue microarray sections. Thirteen of 420 tissue microarray cores were judged as not evaluable by participants. Reevaluation of the respective tissue microarray sections of study participants by the reference laboratory confirmed this assessment in 10 cases, with 3 cores considered evaluable on reassessment. After dichotomization of EGFR immunohistochemistry scores into cases with high and low EGFR expression, concordance rates between raters were calculated. The distributions of concordance rates of RRT participants were similar both when individual raters were compared with the reference evaluation and when all rater pairs were compared with each other. In general, the concordance rates of individual participants did not differ markedly, with the first to third quartile ranging from 73.3% to 80.0% for rater versus reference evaluations (Table 1). The overall concordance rate, defined as the mean of the per-rater concordance rates with respect to the reference evaluation, was 75.6%. The mean χ coefficient for rater versus reference evaluation was 0.522 (first to third quartile, 0.485–0.600). Figure 2, A, shows the distributions of the differences of individual raters’ scores from those of the reference evaluation.

Tumors that were at the time of selection of study samples classified by reference evaluation as clearly negative or clearly positive with respect to the cutoff of 200 showed high mean per-specimen concordance rates of 98.1% and 88.7%, respectively. In tumors with an equivocal EGFR immunohistochemistry score according to the reference evaluation, the mean concordance rate was 53.2%.

Factors Potentially Affecting Reproducibility of Immunohistochemical Scoring in Part 1

Following the completion of part 1, factors that had impacted on the reproducibility of EGFR scoring were identified, taken into consideration in the design and conduct of the second phase of the analysis, and discussed with the RRT part 2 participants at a face-to-face meeting prior to their analysis. Factors identified and modifications to the procedures used in part 1 are detailed in Table 2. These included inconsistent application of the magnification rule for scoring staining intensity with regard to both the process and the microscope equipment used.

Part 2

Ten RRT participants evaluated EGFR expression in 30 different NSCLC tumors in 10 consecutive tissue microarray sections. Six of 300 tissue microarray cores were judged as not evaluable by participants, with these assessments confirmed by the reference laboratory in each case. When individual raters were compared with the reference evaluation the concordance rates of RRT participants were slightly higher than when all rater pairs were compared with each other. In general, the concordance rates of individual raters did not differ markedly, with the first to third quartile ranging from 90.0% to 93.1% for rater versus reference evaluations (Table 1). The overall concordance rate in part 2 was 90.9%. The mean χ coefficient for rater versus reference evaluation was 0.812 (first to third quartile, 0.795–0.861). Figure 2, B, shows the distributions of the differences of individual part 2 raters’ scores from those of the reference evaluation. Although the scores of some raters tended to be slightly higher or lower than those of the reference evaluation, the variability of the differences in terms of their interquartile ranges was similar across the participants. Comparison between these distributions for part 1 (Figure 2, A) and part 2 (Figure 2, B) indicates that there was a much lower degree of difference in part 2 compared with part 1 in relation to scores determined by the individual raters compared with the reference evaluation.
Figure 1. Assessment of epidermal growth factor receptor (EGFR) antibody staining intensity (EGFR pharmDx kit) according to the level of magnification at which membrane staining is visible (magnification rule). A and B, Tumor with 3+ membrane staining, visible using a ×5 objective lens (A) and confirmed using a ×10 objective lens (B). C and D, Tumor showing moderate membrane staining at low power (C, ×10 objective lens) with confirmed intercellular linear staining at higher magnification (D, ×20 objective lens). E and F, Membrane staining at 1+ intensity (E, ×20 objective lens) with high magnification required for unequivocal scoring of linear intercellular staining (F, ×40 objective lens). Images were extracted from larger images of non–small cell lung cancer tissues stained with EGFR antibody. The eyepiece lens in all instances was ×10.
Tumors that were, at the time of selection of study samples, classified by reference evaluation as clearly negative or clearly positive with respect to the cutoff of 200 showed very high mean per-specimen concordance rates of 99.1% and 95.0%, respectively (Table 1). In tumors with an equivocal EGFR immunohistochemistry score according to the reference evaluation, the mean concordance rate was 79.9%. Tumors with a reference EGFR immunohistochemistry score of lower than 200 and of 200 or higher showed mean concordance rates of 94.7% and 85.6%, respectively. Tumors with a reference EGFR immunohistochemistry score clearly below or above the cutoff (≤200 or ≥250) were categorized with almost perfect mean concordance rates of 98.0% and 98.3%, respectively. Specimens with a reference EGFR immunohistochemistry score around the cutoff (200–250) showed a mean concordance rate of 73.7%.

**COMMENT**

The identification of predictive biomarkers permitting the tailoring of therapy to those patients most likely to benefit substantially is increasingly viewed as a realistic and necessary step in the clinical development of existing and novel therapeutic agents.\(^5\)–\(^7\) The FLEX study showed that in patients with advanced NSCLC, high EGFR expression is a tumor biomarker that can predict survival benefit associated with the addition of cetuximab to first-line chemotherapy, and that consequently, assessment of tumor EGFR expression in this setting may offer a personalized chemotherapy, and that consequently, assessment of tumor EGFR expression in this setting may offer a personalized

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**Table 1. Interobserver Agreement in Parts 1 and 2 of the Round Robin Test**

<table>
<thead>
<tr>
<th>Agreement per rater(^a)</th>
<th>Part 1</th>
<th>Mean</th>
<th>Q1–Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall concordance, % (\kappa) coefficient</td>
<td>14</td>
<td>75.6, 0.522</td>
<td>73.3–80.0, 0.485–0.600</td>
</tr>
<tr>
<td>Raters</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Overall concordance, % (\kappa) coefficient</td>
<td>10</td>
<td>90.9, 0.812</td>
<td>90.0–93.1, 0.795–0.861</td>
</tr>
</tbody>
</table>

**Concordance per specimen, %\(^b\)**

<table>
<thead>
<tr>
<th>Part 2</th>
<th>All samples</th>
<th>Overall concordance rate</th>
<th>EGFR score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17</td>
<td>94.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>85.6</td>
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<tr>
<th>EGFR expression category(^c)</th>
<th>Overall concordance, %</th>
<th>Q1–Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>11</td>
<td>99.1</td>
</tr>
<tr>
<td>Equivocal</td>
<td>9</td>
<td>75.9</td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>95.0</td>
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<table>
<thead>
<tr>
<th>EGFR immunohistochemistry score</th>
<th>Overall concordance, %</th>
<th>Q1–Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–&lt;150</td>
<td>15</td>
<td>98.0</td>
</tr>
<tr>
<td>150–&lt;250</td>
<td>9</td>
<td>73.7</td>
</tr>
<tr>
<td>250–300</td>
<td>6</td>
<td>98.3</td>
</tr>
</tbody>
</table>

Abbreviations: EGFR, epidermal growth factor receptor; Q1–Q3, first to third quartile.

\(^a\) Raters versus reference laboratory.

\(^b\) In subcategories, according to reference laboratory classification.

\(^c\) Categories relate to preliminary nonsystematic reference laboratory assessments according to the threshold immunohistochemistry score of 200 (clearly ≥200 = positive; clearly <200 = negative).
Overall, the EGFR immunohistochemistry scoring in part 2 demonstrated a high interobserver agreement, with an overall concordance rate of 90.9% and a mean κ coefficient of 0.812. Also, as shown in Figure 2, the variability in the differences between the rater’s scores and those of the reference evaluation was reduced in comparison to the feasibility study. Of particular importance, tumors with an immunohistochemistry score of lower than 200 and of 200 or higher showed mean concordance rates in part 2 of 94.7% and 85.6% respectively, suggesting that the characterization of patients into clinically important high- and low-EGFR expression groups based on a discriminatory threshold score of 200 was possible with a high degree of reproducibility. When considering specimens with immunohistochemistry scores clearly above or below this cut point (≥250 or <150), mean concordance per specimen with respect to discrimination into high- or low-EGFR expression groups relative to the threshold of 200 was near perfect at 98.3% and 98.0%, respectively. For samples with immunohistochemistry scores around the cut point (150–<250), as the defined range includes the threshold, small variations in observer assessment would be expected more often to result in differences in the subsequent classification of specimens into high- or low-expression groups. Therefore, the mean concordance rate of 73.7% for this intermediate-score group in part 2 of the RRT reflects in practice a good level of reproducibility.

Immunohistochemistry is a well-established and widely used low-cost technique that does not require specialist assay equipment. As such, it is an ideal system on which to base a predictive biomarker that can be used to inform routine clinical decisions. Moreover, as immunohistochemistry is only semiquantitative, with higher protein concentrations leveling off to maximal staining intensity of 3+ and lower protein concentrations tending towards staining intensities of 0 or 1+, a 2-tier, threshold-based approach for characterizing expression level is optimal.13 Indeed, immunohistochemistry assessment of the expression level of tumor-associated genes is already established as an integral component of existing predictive tests of clinical relevance in the treatment of breast and gastric cancer.5,14,15 In breast cancer, guidelines recommend the use of fluorescence in situ hybridization analysis of HER2 copy number status to inform treatment decisions in relation to trastuzumab therapy for patients whose tumors are initially scored as equivocal for HER2 expression by immunohistochemistry (2+).16 In the case of EGFR, although as might be expected associations have been reported between increased EGFR copy number and high levels of gene expression in tumors,5,17 a recent analysis of patients included in the phase III FLEX study did not demonstrate a predictive value for EGFR copy number in relation to the efficacy of chemotherapy plus cetuximab therapy.18 These findings suggest that EGFR fluorescence in situ hybridization may not be of benefit as an adjunct to EGFR immunohistochemistry with respect to tailoring the administration of cetuximab in the first-line treatment of NSCLC.

### Table 2. Factors Potentially Affecting Reproducibility of Epidermal Growth Factor Receptor Immunohistochemistry Scoring Identifed in Round Robin Test Part 1

<table>
<thead>
<tr>
<th>Factor</th>
<th>Modification of Process</th>
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<tr>
<td>Raters initially inspecting samples at high magnification, preventing a subsequent unbiased assessment at low magnification. Some raters scored 3+ staining using a ×2.5 objective lens, others using a ×5 objective lens. There was some quantitative variation in the staining pattern on the tissue microarray slides provided to the raters compared with the reference slide. Raters were not made aware of the statistical effect of scoring a sample not evaluable.</td>
<td>Raters instructed to first define the percentage of cells staining at 3+ at low magnification before moving to higher magnifications. All raters were instructed to use a ×5 objective lens to finally score 3+ staining. A new quality control step was introduced to ensure that all stained tissue microarray cores from test slides were comparable to the tissue microarray cores from the slide that was used for the reference evaluation. Raters were informed that if they scored a sample not evaluable and this diagnosis was not confirmed by reference evaluation, it was considered as a disagreement with the reference evaluation in the statistical analysis.</td>
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</table>

**Figure 2.** Distribution of differences in the epidermal growth factor receptor (EGFR) immunohistochemistry score from reference evaluation per rater in part 1 (A) and part 2 (B). The upper and lower boundaries of each box plot represent the 25th and 75th percentiles, respectively; horizontal lines within the box denote the median values. Whiskers extend to the last observation within 1.5 times the interquartile range from the boundaries (values outside this range represented by o). Abbreviation: RRT, round robin test.
The current analysis demonstrates the feasibility of using an immunohistochemistry score threshold score of 200 to reliably characterize patients with advanced NSCLC into high- or low–EGFR expression groups. Although further studies will perhaps be informative with regard to other potential sources of variability, such as tissue handling, it follows from the current analysis that practical considerations in relation to interobserver reproducibility of immunohistochemistry scoring should not preclude the use of the EGFR expression level, and in particular an EGFR immunohistochemistry score of 200 or higher, as a predictive biomarker for the selection of patients who would be more likely to derive a substantial survival benefit from first-line treatment with chemotherapy plus cetuximab.

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