HER2 Status in Non-Small Cell Lung Cancer: Results from Patient Screening for Enrollment to a Phase II Study of Herceptin

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ABSTRACT

Purpose: For the first time a large number (563) of non-small cell lung cancer (NSCLC) samples was used to compare three different technologies for the assessment of HER2 status. Fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) were used for tumor tissue samples, and ELISA for serum samples. The results were compared with other tumor entities, mainly breast.

Experimental Design: Samples (563) from patients suffering from primary advanced or metastatic NSCLC were evaluated.

Results: HER2 overexpression was demonstrated using IHC in 20% (83 of 410) of the specimens, whereas 2% (7 of 378) were positive by FISH and 6% (31 of 511) showed elevated serum HER2 levels (>15 ng/ml) by ELISA. Sixty-six specimens were positive by IHC only and 13 by ELISA only, whereas none of the specimens was positive only by FISH. Concordance between all of the techniques was seen for only 3 specimens. Of 7 IHC 3+ specimens, 4 showed gene amplification by FISH, and 3 were positive by ELISA (>15 ng/ml), whereas of 76 IHC 2+ cases only 2 were amplified by FISH, and 4 were positive by ELISA. HER2 positivity by at least one of the three techniques was most common in adenocarcinomas, at 29% (42 of 143).

Conclusion: Gene amplification and HER2 protein overexpression at the 3+ level appear to be uncommon in NSCLC. The concordance between FISH and IHC 3+ disease was good in this study, in addition, ELISA would have detected several patients without IHC/FISH-positive disease.

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INTRODUCTION

Lung cancer is the major cause of cancer-related death in North America and Europe. NSCLC² comprises ~75% of lung cancers, and the majority of patients have locally advanced stage III or metastatic stage IV disease at diagnosis (1). For many patients, successful treatment remains elusive, because advanced tumors are often not operable, and may also be resistant to tolerable doses of radiotherapy and chemotherapy.

The HER-2 proto-oncogene (HER2/neu/c-erbB-2) encodes a transmembrane tyrosine kinase receptor that shares homology with the epidermal growth factor receptor family (2). HER2 gene amplification and receptor overexpression have been associated with adverse tumor characteristics and poor prognosis in a range of tumor types (3–5). The humanized anti-HER2 monoclonal antibody, trastuzumab (Herceptin), the first oncogene-targeted drug for solid tumor therapy, has efficacy only in HER2-positive tumors, i.e., those with amplification/overexpression (6). HER2 overexpression has been reported in approximately 10–15% of NSCLCs, with a reported incidence as high as 30% in adenocarcinoma (7, 8). This overexpression is associated with poor prognosis and shortened survival (9–11).

The increasing importance of HER2 status determination in the clinical management of cancer patients means that consistent and accurate evaluation is crucial (12, 13), and the reliable assessment of HER2 status is a prerequisite for successful individualized, risk-adapted therapy with Herceptin. False-positive results may lead to inappropriate medical decisions, causing patients to undergo unnecessary therapy. Conversely, false-negative results may deprive a patient of potentially beneficial therapy.

Various analytes related to HER2 amplification/overexpression, including DNA, mRNA, and receptor protein, can be used in different assays. Of all of the diagnostic techniques, IHC and FISH are both practicable and effective. FISH has the advantage of being highly sensitive, specific, and standardized, and can be used on small tissue samples. It has been approved by the United States Food and Drug Administration to measure HER2 gene amplification for prognostic assessment of node-negative breast cancer patients who are at risk of recurrent disease. FISH also appears to be capable of overcoming the many inherent technical and interpretative limitations of other techniques. Because the definition of positivity is based on distinct signals in a cell, FISH is potentially less subjective than IHC. FISH can also evaluate the number of HER2 gene copies relative to chromosome 17 number, which allows gene amplification to be distinguished from chromosomal polyploidy. However, only a limited number of pathology laboratories are capable of using FISH.

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² The abbreviations used are: NSCLC, non-small cell lung cancer; HER, human epidermal growth factor receptor; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; ECD, extracellular domain.
equipped to assess HER2 status by FISH. It is also relatively time consuming and expensive compared with IHC, which is the most widely used assay available at present for the assessment of HER2 status.

IHC is a simple and rapid procedure, requiring few reagents. Because the expertise to perform the technique is more widely available, IHC is more readily accepted in pathology laboratories. However, the sensitivity and specificity of IHC can vary considerably depending on the antibody used (14, 15). Furthermore, tissue fixation, tissue processing, and antigen retrieval can affect HER2 antigen levels and the intensity of immunostaining (16). Standardization of methodology and interpretation, as well as the inclusion of validated controls are mandatory for IHC HER2 testing. The commercially available HercepTest meets these criteria and is approved by the Food and Drug Administration for this purpose.

FISH and IHC assess the level of HER2 gene amplification and HER2 protein overexpression, respectively, directly in tumor cells, whereas retaining the characteristic morphology of the tissue studied. In contrast, the ELISA evaluates the ECD of HER2, which is shed from tumor cells into the blood. If sufficiently predictive, ELISA would be an ideal technique for the evaluation of HER2 status, because it is rapid and economical, and the use of blood samples means that no tissue sample is necessary.

It was the aim of this study to define the HER2 status in NSCLC patients and to compare three different techniques (IHC, FISH, and ELISA), as part of screening for a multicenter, controlled phase II study.

MATERIALS AND METHODS

Study Population. A total of 563 advanced and/or metastatic stage IIIB and IV NSCLC patients were examined for HER2 status at the Institute of Pathology, Klinikum Kassel, Germany, submitted from a total of 38 international centers on a referral basis. Specimens were histologically typed according to WHO recommendations (17) by the local pathologists. The typing was confirmed by one senior pathologist (C. G.) at the Institute of Pathology. All of the tumors were examined as part of a multicenter, open-label, randomized, controlled Phase II study of Herceptin (trastuzumab) in combination with chemotherapy, in patients with advanced and/or metastatic NSCLC with HER2 overexpression/amplification. HER2 overexpression/amplification was defined as a score of 2+/3+ by IHC, and/or gene amplification by FISH and/or a serum HER2 ECD concentration >15.0 ng/ml by ELISA. Specimens for IHC and FISH were paraffin embedded and fixed for 18–24 h in neutral-buffered formalin. Tissue blocks were sectioned at 4–5 μm. Areas of invasive tumor were located using a serial section stained with H&E.

Twenty-seven of the tumor samples did not contain any or contained few tumor cells. FISH was repeated for 29 samples, but evaluation was not possible, probably because of sample preparation. Serum samples were hemolyzed, viscous, or the tube was broken on arrival in 26 cases. The results of these samples were not included in the data analysis (Fig. 1).

Depending on the available material, all of the possible assays were performed simultaneously. In some cases only slides or a tissue block was available, whereas in other cases only serum was available. Therefore, only those cases were considered for the comparison of the methods where all of the three results could be obtained.

IHC. HER2 overexpression was quantified using the HercepTest (DAKO, Hamburg, Germany). Tissue sections were deparaffinized in two 5-min changes of xylene and were rehydrated through alcohols to distilled water. Subsequently, sections were subjected to heat-induced epitope retrieval by immersing the slides in DAKO Epitope Retrieval Solution (0.01M) preheated to 98°C, and then incubated at 98°C for a total of 40 min, followed by a 20-min cool-down period at room temperature. Slides were incubated with peroxidase blocking reagent for 5 min, followed by 30-min incubation at room temperature with the primary rabbit polyclonal antibody to HER2 oncoprotein. The antibody was localized by incubating slides with

![Fig. 1](Overview of samples received for analysis.)
DAKO Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat antirabbit immunoglobulins) for 30 min. Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin. Each tissue specimen was also stained using the negative reagent control to verify the specific labeling of the target antigen by the primary antibody. A control slide consisting of three pelleted, formalin-fixed, paraffin-embedded human breast cell lines with staining intensity scores of 0, 1+, and 3+ (supplied with the HercepTest kit) was included in each staining run.

As defined in the HercepTest kit guide, scores of 0 or 1+ were considered negative for HER2 overexpression, 2+ was weak positive, and 3+ was strong positive. To qualify for 2+ scoring, complete membrane staining of >10% of tumor cells at a moderate intensity had to be observed. In the majority of 3+ cases, at least 80% of the tumor cells are stained, and staining is intense and membranous (18). All of the samples were scored by C. G. In doubtful cases consensus was obtained between C. G. and J. R.

**FISH.** FISH analysis was performed using the PathVysion HER-2 DNA Probe Kit (Vysis, Stuttgart, Germany) according to the manufacturer’s protocol (19). Sections of formalin-fixed, paraffin-embedded tissue were baked at 60°C overnight on microscope slides. After deparaffinization with xylene and dehydration with an isopropanol series, the slides were pre-treated chemically (pretreatment solution) and enzymatically (protease digestion) to remove DNA-blocking proteins. A hybridization solution containing direct-labeled DNA probes complementary to HER2 and chromosome 17 was applied to the tissue sections. After denaturing for 2 min at 72°C the tissue sections were incubated for 14–18 h at 37°C. Unannealed probe was removed using 20× SSC at 72°C, followed by 2× SSC containing 0.3% NP40, also at 72°C. Nuclei were counterstained with 4’,6-diamidino-2-phenylindol dihydrochloride.

Slides from amplified and nonamplified control tissue provided by Vysis were included in each run.

**HER2** and centromere 17 signals were counted for 60 nuclei per tumor specimen. Overlapping nuclei were excluded from analysis. HER2 gene amplification was defined as a HER2: chromosome 17 ratio >2.0 using the Vysis probe. Analysis and photography were performed using a fluorescence microscope (Zeiss, Jena, Germany) equipped with a triple band pass filter for simultaneous detection of Spectrum Green, Spectrum Orange, and 4’,6-diamidino-2-phenylindol dihydrochloride.

**ELISA.** Serum HER2 ECD levels were determined using the human HER-2/neu quantitative ELISA kit from Oncogene (Cambridge, MA). In brief, specimen and controls were diluted with sample diluent, and 100 µl of the dilution was added in duplicate to the microplate wells and incubated for 3 h at 37°C. After washing the plate, 100 µl of detector antibody was added to all of the wells except the substrate blank well. The plate was incubated for 1 h at 37°C, washed, and 100 µl of working conjugate was added to all of the wells except the substrate blank. An additional 30-min incubation at 37°C was followed by washing and the addition of a further 100 µl of working substrate. After the final incubation for 45 min at 37°C, 100 µl of stop solution was added, and the absorbance was measured at 490 nm. HER2 standards were used in a range from 0 to 35 ng/ml. The concentrations of three HER2 controls provided by Oncogene and three in-house controls (serum samples with known HER2 concentrations covering the working range) were determined during each run for validation of the assay. Samples showing a HER2 concentration >15.0 ng/ml were considered positive.

**Statistical Analysis.** The concordance between the methods was assessed by the Fisher’s exact test using JMP software (SAS Institute, Heidelberg, Germany).

**RESULTS**

A total of 563 specimens was analyzed using at least one of the three test platforms. A total of 108 (19%) of these specimens were HER2 positive by at least one technique (Table 1).

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC only</td>
<td>73</td>
</tr>
<tr>
<td>FISH only</td>
<td>0</td>
</tr>
<tr>
<td>ELISA only</td>
<td>23</td>
</tr>
<tr>
<td>IHC + FISH</td>
<td>3</td>
</tr>
<tr>
<td>IHC + ELISA</td>
<td>4</td>
</tr>
<tr>
<td>FISH + ELISA</td>
<td>1</td>
</tr>
<tr>
<td>IHC + FISH + ELISA</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
</tr>
</tbody>
</table>

Table 2 HER2 results by IHC, FISH, and ELISA

A. IHC

<table>
<thead>
<tr>
<th></th>
<th>No expression</th>
<th>Overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 410</td>
<td>241</td>
<td>86</td>
</tr>
<tr>
<td>58.8%</td>
<td>21.0%</td>
<td>18.5%</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

B. FISH

<table>
<thead>
<tr>
<th></th>
<th>No amplification</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 378</td>
<td>371</td>
<td>2</td>
</tr>
<tr>
<td>98.1%</td>
<td>1.3%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

C. ELISA

<table>
<thead>
<tr>
<th></th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 511</td>
<td>480</td>
<td>16</td>
</tr>
<tr>
<td>93.9</td>
<td>3.1%</td>
<td>2.9%</td>
</tr>
</tbody>
</table>

Oncogene and three in-house controls (serum samples with known HER2 concentrations covering the working range) were included in each run. For 5 of the IHC 2+ specimens no ELISA result was available and for another 2 IHC 2+ specimens no FISH result was available. Of the 76 IHC 2+ specimens, only 2 showed amplification, and 4 were positive by ELISA. Of the 327 negative cases, 86 demonstrated 1+ staining, and 241 showed no HER2 expression.
FISH. Seven (2%) of 378 samples showed HER2 gene amplification with a HER2:CEP17 ratio ranging from 2.1 to 5.9 (Table 2). Of these 7 FISH-positive samples, 1 tumor demonstrated 1+ staining, and 2 demonstrated 2+ staining (Table 3). Four samples with a HER2:CEP 17 ratio of ≥2.0 showed clear overexpression of the HER2 protein with a score of 3+. Four of the amplified specimens were also positive by ELISA.

ELISA. Serum HER2 ECD levels >15 ng/mL were found in 31 of 511 samples (6%; median 19.6 ng/mL; mean 22.5 ng/mL; Table 2). The serum HER2 ECD concentration in NSCLC patients ranged from 0 to 47.4 ng/mL. Patients with positive ELISA result tend to have higher serum HER2 levels compared with IHC-positive patients.

Comparison of Results of All of the Techniques. All three of the techniques were performed on a total of 339 samples (Table 3). Twenty percent (67 of 332) of the tumors without gene amplification showed positive immunostaining exhibiting a score of 2+, whereas <1% (2 of 332) of the FISH-negative specimens demonstrated a clear 3+ staining. In the FISH-positive group the overall concordance with positive results in the other two techniques was 43% compared with 4% in the IHC-positive group. In the 2+ group the positive concordance was 3% for FISH and 5% for ELISA, compared with 57% and 43%, respectively, in the 3+ group. Of the 339 samples, 66 were positive by IHC only (two of these were 3+) and 13 by ELISA only. None of the samples was positive only by FISH. Three specimens were positive by all of the three techniques.

The 2 × 2 presentation indicates that a 2+/3+ IHC score is highly likely to correspond with a positive FISH result (P = 0.0005; Table 3). Similarly, HER2 amplification is correlated with elevated HER2 serum levels (P = 0.0003). There is no significant correlation between HER2 protein overexpression determined by IHC and elevated HER2 serum levels.

The predictive value of a positive ELISA result was 33% for IHC (7 of 21) and 19% for FISH (4 of 21), whereas the predictive value of a negative ELISA result was 79% (252 of 318) for IHC and 99% for FISH (332 of 339).

Tumor Type and IHC, FISH, and ELISA. The 563 tumors that were evaluated consisted of 143 adenocarcinomas, 80 squamous cell carcinomas, 44 uncharacterized large cell carcinomas, and 296 NSCLC not otherwise specified.

When IHC scores of 2+ and 3+ were classified as HER2 receptor positive, the incidence of positivity was highest for adenocarcinomas (29%), followed by squamous cell carcinomas (18%), NSCLC (17%), and large cell carcinomas (11%; Table 4). Adenocarcinomas were also more frequently positive by FISH than squamous cell carcinomas and large cell carcinomas (4% versus 1% versus 2%, respectively). Serum HER2 ECD levels of >15 ng/mL were evident in 10 (7%) adenocarcinomas, 3 (4%) squamous cell carcinomas, 3 (7%) large cell carcinomas, and 3 (7%) NSCLC. Of 25 HER2-positive adenocarcinomas of known grade, 20 (80%) were poorly differentiated (G3). Four amplified tumors were adenocarcinomas with lower-grade differentiation (grade II and III).

Overall, 29% of the evaluated adenocarcinomas were HER2 positive by at least one of the three techniques. However, amplification of the HER2 gene could be demonstrated in only 2% of all tumors, whereas 20% were HER2 positive by IHC and 6% by ELISA.
DISCUSSION

Studies in which IHC and FISH have been performed on the same breast cancer samples have shown that these assays reliably demonstrate HER2 overexpression and amplification, respectively (16, 20, 21). Clear HER2 protein overexpression is generally detected only in tumors showing HER2 gene amplification (22–24). The differences between the results of these studies performed on breast cancer samples and the current NSCLC study are primarily in the number of samples that were intermediate-positive by IHC (2+) but negative by FISH. Chromosome 17 polysomy could account for this, because it can cause protein overexpression, whereas not qualifying for amplification. In breast cancer, increased receptor expression without genetic alteration has been observed in approximately 3–7% of cases, probably because of transcriptional or post-translational activation (25–27), although Xing et al. (28) have reported a frequency of chromosome 17 polysomy of up to 21%. However, aneuploidy has been observed in 81% of NSCLC tumors (29).

Conversely, rare cases show HER2 gene amplification without protein overexpression (13, 20). In the current study, only 1 sample showing HER2 gene amplification was considered to be negative by IHC. Similar results have been reported for breast cancer (22, 30).

Formalin-fixed, paraffin-embedded tumor tissue samples are appropriate for the current FISH and IHC assays. Other methods of tissue fixation, such as Bouin’s, can adversely affect reactivity (16). Because all of the samples were submitted on a referral basis, the effects of tissue fixation and processing cannot be excluded as a cause of discrepancies.

The literature data with regard to the usefulness of the Herceptest to determine the HER2 overexpression. In this study, shed antigen did not correlate with the results of cellular assays. This is in contrast to breast cancer, where, for example, Colomer et al. (36) report a 73% concordance between IHC overexpression and elevated circulating HER2. In this lung study, ELISA did not seem to describe the same patient population as the cellular methods, which means that if cellular positivity correlates with Herceptin response, ELISA would not be reliable for selecting patients for Herceptin treatment. However, technically it has the advantage of not being dependent on the availability of tissue, an aspect that is even more important in patients with NSCLC as compared with breast cancer.

In summary, relatively low levels of expression have been detected with these state-of-the-art assays. The rate of HER2-positive disease (defined as both IHC 3+ and FISH positive) is much lower in NSCLC than in breast cancer, which suggests that if lung cancers respond to Herceptin in the same way as breast cancers, only a few patients might benefit from Herceptin therapy. Preliminary results of the randomized Phase II study of gemcitabine/cisplatin alone and with Herceptin, for which the screening described in this study was performed, confirm this observation and indicate that the small subgroup of patients with HER2 3+ and/or gene amplification who received Herceptin did relatively well based on their response rate and median progression-free survival, whereas patients with 2+/FISH-negative disease did not appear to benefit (39). However, restricting a NSCLC study population to HER2-positive disease (IHC 3+ or FISH positive) does not seem practical, because many patients would have to be screened. Such a study could be best pursued in a multicenter set up.

All of the cases that showed a 3+ staining by IHC also demonstrated gene amplification by FISH. However, in tumors
with moderate (2+) overexpression. IHC staining did not correlate with FISH results. Thus, IHC screening for 3+ disease and confirmation of moderate (2+) IHC overexpression by FISH might be an effective strategy for selecting patients with NSCLC for Herceptin treatment, as described for patients with breast cancer.

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Appendix

The multicenter study included the following investigators and sites: Australia: M. Boyer, Royal Prince Alfred Hospital, Camperdown; J. Mckendrick, Box Hill Hospital, Victoria; K. Pittman, The Queen Elizabeth Hospital, Woodville; D. Rischin, Peter McCallum Cancer Institute, Melbourne; R. Snyder, St. Vincents Hospital, Fitzroy; Brazil: C. H. Barrios, Hospital Sao Lucas Da Pucrs, Porto Alegre; Canada: C. Butts, Cross Cancer Institute, Edmonton; G. Goss, Ottawa Regional Cancer Centre, Ottawa; V. Hirsh, Royal Victoria Hospital, Montreal; A. Jones, BC Cancer Centre, Vancouver; W. Kocha, London Regional Cancer Centre, London; F. Laberge, Laval Hospital, Ste. Foy; F. Shepherd, Princess Margaret Hospital, Toronto; D. Soulie, Notre-Dame Hospital, Montreal; China: T. Mok, Prince of Wales Hospital, Hong Kong; France: J-J. Lafitte, Hospital Calmette, Lille; B. Lebeau, Hospital St. Antoine, Paris; G. Zalcman, Institut Curie, Paris; L. Thiberville, Hospital Charles Nicolle, Rouen; Germany: U. Gatzemeier, Krankenhaus Grosshansdorf, Grosshansdorf; C. Manegold, Thoraxklinik der LVA Herforder Kreisklinikum, Essen; S. T., and Ashfaq, R. Laboratory assessment of the status of Her-2/neu in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res., 54: 2771–2777, 1994.

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