HER-2/Neu Alterations in Non-Small Cell Lung Cancer: A Comprehensive Evaluation by Real Time Reverse Transcription-PCR, Fluorescence in Situ Hybridization, and Immunohistochemistry

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ABSTRACT

Purpose: Amplification and/or overexpression of HER2/neu have been documented in many types of epithelial tumor, and HER2/neu evaluation is now gaining importance, because this mechanisms of disease can be inhibited in vivo using humanized monoclonal antibodies. The main purpose of our investigation includes the evaluation of the prevalence of HER2/neu alterations in non-small cell lung cancer (NSCLC) at different molecular levels.

Experimental Design: We performed a comprehensive investigation of HER2/neu alterations in a series of 115 NSCLC, using fluorescence in situ hybridization (FISH), real time reverse transcription (RT)-PCR, and immunohistochemistry.

Results: HER2/neu immunoreactivity was detected in 26 of 115 of specimens (23%), with 5 carcinomas (4%) showing intense staining. Real time RT-PCR demonstrated HER2/neu mRNA in all samples analyzed, with levels above normal in 54 of 115 of carcinomas (47%). FISH documented HER2/neu gene amplification in 9 of 41 carcinomas (22%).

Conclusions: These results demonstrate that HER2/neu alterations occur in NSCLC, albeit with significantly different prevalence depending on the technical assay used for the assessment. It is therefore likely that inhibitory monoclonal antibodies will be appropriate in the treatment of a subgroup of NSCLC patients. The results suggest that other mechanisms unrelated to gene amplification could be responsible for HER2/neu mRNA or protein overexpression.

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide with a still increasing incidence. Despite decades of research, prognosis is still poor, and patient survival depends mainly from early diagnosis. Identification of specific gene abnormalities that may be targeted by novel therapies, based on the recently accumulated molecular knowledge pertaining cancer pathogenesis and progression, appears the most potentially rewarding approach.

The HER2/neu (also known as c-erbB-2) oncogene is activated in several human tumors (1–12), correlates to neoplastic transformation and progression (13–18), and is linked to shortened patients’ survival (2) and chemoresistance (7, 19).

More importantly, it is now possible to target HER-2/neu with a humanized monoclonal antibody (Trastuzumab), which inhibits neoplastic cell proliferation both in vitro and in vivo (11, 18) and strengthens receptor chemosensitivity (19). The use of Trastuzumab significantly increases survival for patients with advanced, metastatic breast tumors (20).

In NSCLC,3 the HER-2/neu gene activation prevalence, its prognostic role, and possible therapeutic implications are still under study (21, 22).

HER-2/neu protein overexpression may occur in all major histotypes of NSCLC, mainly in adenocarcinomas, with a highly variable percentage of immunoreactivity ranging from 5 to 80% (23–34). A shorter patients’ overall survival in adenocarcinomas with HER-2/neu overexpression (14, 23) has not been confirmed in all studies (32).

HER-2/neu amplification seems to be a rare event in

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; FISH, fluorescent in situ hybridization; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry.
NSCLC (21, 27, 35, 36) more commonly seen in adenocarcinomas (36), possibly correlated either to cancer invasiveness or metastatization (25).

Very few data exist about HER-2/neu mRNA evaluation in lung cancer. High mRNA levels investigated by RNA dot blot and Northern blot analysis were demonstrated only in NSCLC with the highest expression levels detected in adenocarcinomas (36). Recently, increased HER2/neu mRNA levels, together with epidermal growth factor receptor levels, were linked to poor patient prognosis (37).

Despite the controversial data in the literature, based mainly on IHC, lung cancer patients are currently enrolled in clinical trials for treatment with monoclonal antibodies against HER2/neu (for review, see Ref. 38).

In this study, we comprehensively investigated a large series of NSCLC with the purpose to document HER2/neu alterations in these carcinomas. Specifically, we have used FISH, real time RT-PCR, and IHC to evaluate gene amplification, gene transcription, and protein expression in NSCLC.

The purpose of the investigation is 2-fold: (a) to document the prevalence of HER2/neu alterations in NSCLC at different molecular levels; and (b) to evaluate the applied techniques for their potential clinical application.

**MATERIALS AND METHODS**

**Patients and Tissue Specimens.** The study group comprised all of the patients who underwent surgical resection for NSCLC in 1995 and 1996 at the Department of Surgery, University of Pisa, with available frozen tumor samples; 115 patients were included. Clinical and pathological characteristics of patients are detailed in Table 1. For each case, fresh macroscopically normal tissue samples (taken as far as possible from patients are detailed in Table 1. For each case, fresh macroscopically normal tissue samples were fixed both in Amex ™ and 10% buffered neutral formalin and embedded in paraffin. In all tumor specimens, the amount of neoplastic area was ≥80% of tumor sample, confirmed by histological examination. Similarly, all macroscopically nonneoplastic samples were histologically judged to be benign.

Standard 5-μm tissue sections were cut and stained with H&E and Alcian PAS and examined by light microscopy. Histopathological diagnosis was carried out according to the WHO classification of lung and pleural tumors (1999); pathological stage was determined according to the Tumor-Node-Metastasis staging system (2001).

**IHC.** IHC was carried out on 5-μm tissue sections using the standard avidin-biotin-peroxidase complex method with the HER-2/neu DAKO polyclonal antiserum (DAKO, Glostrup, Denmark).

For each batch, a breast carcinoma with amplified HER-2/neu gene and strong immunoreactivity for HER-2/neu was used as positive control to ensure the quality.

Each slide was independently evaluated by two pathologists (M. F. and S. B.) unaware of tumor clinicopathological characteristics. The evaluation of HER-2/neu immunoreactivity was performed according to the DAKO protocol for the HerceptTest, with minor modifications. Only membrane staining was considered positive, whereas cytoplasmic staining was considered nonspecific. In each case, the intensity (weak, moderate, or strong) and pattern (incomplete or complete) of membrane staining and percentage of neoplastic immunoreactive cells (cut-off of 10%) were evaluated. Tumors were scored as follows: score 0: no appreciable staining or staining in <10% neoplastic cells; score 1+: tumors with faint/barely appreciable incomplete membranous staining in >10% neoplastic cells; score 2+: tumors with weak to moderate complete membrane staining or containing >10% neoplastic cells; score 3+: tumors with moderate to complete membrane staining or containing >10% neoplastic cells with moderate incomplete basolateral membrane immunostaining; score 3+: strong immunoreactivity of the entire membrane in >10% neoplastic cells or containing >10% neoplastic cells with strong basolateral incomplete membrane immunoreactivity. Tumors classified as 0 or 1+ were considered “negative,” and those scored as 2+ or 3+ were classified as “positive.”

**Real time RT-PCR.** To measure HER-2/neu transcripts in these tumors, we used real time quantitative RT-PCR based on TaqMan methodology, using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), as described previously (39, 40).

Specificity validation of probes used in the present study was based on the quantification of HER-2/neu mRNA levels in two cellular lines, MCF-7 and SKBR-3. As reported previously (41), HER-2/neu mRNA levels were overexpressed in SKBR-3 cell line compared with those of the calibrator MCF-7.

SKBR-3 transcripts levels were ~97-fold higher than

**Table 1 Clinical and pathological characteristics of the patients**

<table>
<thead>
<tr>
<th>Sex</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>107 (92)</td>
</tr>
<tr>
<td>Male</td>
<td>7 (8)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Mean: 61.2 yrs, range: 42–75</td>
<td></td>
</tr>
<tr>
<td>Smoking habits</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>59 (51)</td>
</tr>
<tr>
<td>Former smoker</td>
<td>35 (30)</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Tumor histotype</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>45 (39)</td>
</tr>
<tr>
<td>AC</td>
<td>57 (50)</td>
</tr>
<tr>
<td>LCC</td>
<td>13 (11)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>19 (16)</td>
</tr>
<tr>
<td>T2</td>
<td>86 (75)</td>
</tr>
<tr>
<td>T3</td>
<td>10 (9)</td>
</tr>
<tr>
<td>N0</td>
<td>62 (54)</td>
</tr>
<tr>
<td>N1</td>
<td>23 (20)</td>
</tr>
<tr>
<td>N2</td>
<td>30 (26)</td>
</tr>
<tr>
<td>Stage grouping</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>62 (54)</td>
</tr>
<tr>
<td>Stage II</td>
<td>15 (13)</td>
</tr>
<tr>
<td>Stage III</td>
<td>38 (33)</td>
</tr>
</tbody>
</table>

a Smoking habits were not available in 15 patients.
b SCC, squamous cell carcinoma; AC, adenocarcinoma; LCC, large cell carcinoma.
MCF-7 HER-2/neu levels (range: 80–110n). We used the MCF-7 and SKBR-3 cell lines as calibrator and reproducibility control, respectively, in every assay plate with the unknown samples.

**Dual Color FISH.** Forty-one NSCLCs were analyzed by FISH analysis, Tumor blocks were cut in 2.5-μm sections, applied to silanized slides, and treated using a modified procedure of the standard Oncor (Oncor Resnova, Gaithersburg; MS) protocol. The paraffin-embedded sections were dewaxed by adding Xylene (100%) for 30 min at 37°C for two times and dehydrated in ethanol (100%) for 30 min at room temperature. The sections were treated using pretreatment powder (20% in SSC2×) supplied by Oncor at 45°C for 15 min. After rinsing in SSC2× the tissues were digested using RNase (50 μg/ml in SSC2×) for 15 min at 37°C and rinsed with SSC2× and then pepsin buffer (0.1 M HCl in distillate water with 0.05 μg/ml pepsin) at 37°C. After rinsing (SSC2×), the slides were dehydrated by ethanol series (70, 90, and 100%), air dried, denatured in a solution of 70% formamide in SSC2× (pH 7) for 12 min, and then dehydrated in 70, 80, and 95% ice cold ethanol for 2 min each. On each slide, an area of 18 × 18 mm² was simultaneously hybridized overnight with digoxigenin-labeled HER2/neu and biotinylated α-satellite chromosome 17 probes (Oncor Resnova, Gaithersburg, MS) according to manufacturer’s recommendations. The HER2/neu probe was detected by FITC antidigoxigenin, and α-satellite chromosome 17 probe was detected by Texas Red-labeled avidin (Oncor Resnova, Gaithersburg, MS). Slides were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride hydrate (Sigma) and then visualized using an Olympus microscope equipped for 4,6-diamidino-2-phenylindole dihydrochloride hydrate and FITC/tetramethylrhodamine isothiocyanate epifluorescence optics.

**Scoring Criteria.** At least 100 well-defined nuclei were scored for each slide. FISH evaluation was performed only on tissue sections with uniform hybridization; partially or totally overlapping nuclei were not evaluated.

HER-2/neu status in tumors was scored as follows: (a) single copy: up to four specific signals of both HER-2/neu and chromosome 17 centromeric probes. This accounts for those cells that are disomic for HER-2/neu gene but which are in G2 phase of the cell cycle; and (b) polysomy: more than four specific signals of both HER-2/neu and chromosome 17 centromeric probes. Amplification was assessed calculating the ratio of the average number of HER-2/neu spots/nucleus by the average number of chromosome 17 centromere spots/nucleus. Amplification was further classified as low-level amplification (3–5 HER-2/neu spots per chromosome 17 centromere) and high-level amplification (>5 HER-2/neu spots per chromosome 17 centromere). In cases of evident amplification (>10–20 spots/nucleus), a precise signals enumeration was often not possible because of overlapping fluorescence in the signal cluster.

**Statistical Analysis.** Statistical significance of the relationships between clinicopathological data and IHC and molecular data were assessed by the Fisher exact test. The differences were considered statistically significant when Ps were <0.05.

**RESULTS**

**IHC.** HER-2/neu immunoreactivity (3+/2+) was detected in 26 of 115 cancers (23%). Five tumors (4%) showed strong HER2/neu immunoreactivity (3+); one case showed moderate complete membrane staining in the majority of neoplastic cells and displayed strong basolateral incomplete immunoreactivity in >10% neoplastic cells, and therefore, it was classified as 3+. Twenty-one cases (18%) were moderately immunoreactive (2+); four of these cases were scored as 2+ because of the presence of >10% neoplastic cells with moderate incomplete basolateral membrane immunostaining. Eighty-nine cases (77%) were considered negative; 67 tumors were classified as score 0 (58%) and 22 cases as score 1+ (19%). Four of the five strongly immunoreactive (3+) tumors were adenocarcinomas. Examples are shown in Fig. 1.

**Real Time RT-PCR.** Twenty specimens of nonneoplastic lung tissue were used as control to determine HER-2/neu mRNA basal levels. All samples expressed detectable levels of HER-2/neu mRNA with highest value at 1.42. Only tumors with mRNA values > 1.42 were considered as HER-2/neu overexpressers.

Compared with nonneoplastic tissue levels, HER-2/neu mRNA overexpression was detected in 54 of the 115 tumors (47%) under study.

Sixty-one cases (53%) showed HER-2/neu mRNA levels within the range of nonneoplastic lung specimens. HER-2/neu mRNA levels are shown in Table 2.

**Dual Color FISH.** HER-2/neu gene alterations were found in 9 of the 41 tumors (22%) investigated. Specifically, one carcinoma (2%) displayed polysomy, 5 cases (12%) showed low-level gene amplification, and 3 tumors (7%) had high-level gene amplification. Four of five low amplified cases showed heterogeneity because only a subset of nuclei had a ratio of fluorescent signals >2. All of the other tumors displayed homogeneous results. Fig. 2 depicts two samples showing single copy and high-level gene amplification of HER-2/neu gene, respectively.

**Comparative Analysis and Clinicopathological Correlation.** A statistically significant association between HER-2/neu immunoreactivity and HER-2/neu mRNA levels (P = 0.0135) is documented, although 44 cases displayed discordant results (Table 3).

The most common type of discrepancy (67%) related to tumors showing higher levels of HER-2/neu mRNA without a concomitant increase in protein expression. Less frequently, some carcinomas (13%) displayed HER-2/neu immunoreactivity unaccompanied by increased mRNA levels.

All five cases with strong HER-2/neu immunoreactivity displayed elevated mRNA levels (P < 0.0001). HER-2/neu mRNA levels and HER-2/neu immunoreactivity scores were higher in adenocarcinomas (P < 0.0001).

Gene amplification documented by FISH was always accompanied by increased mRNA levels (Table 4). In two carcinomas, however, low-level gene amplification was associated with faint HER-2/neu immunoreactivity (1+) and therefore classified as negative for protein expression.

No statistically significant correlations were found between HER-2/neu mRNA levels, HER-2/neu immunoreactivity, and
the other clinicopathological parameters under study, including smoking history, tumor grade, and stage.

DISCUSSION

The results of our investigation document HER2/neu alterations at different molecular levels in a significant number of NSCLC. Indeed, immunohistochemical analysis revealed an overall HER-2/neu protein overexpression prevalence of 23%, of which 4% comprised the strongly positive cancers. In turn, real time RT-PCR demonstrated HER-2/neu mRNA levels above the nonneoplastic controls in 47% of the tumors. Our study is the first to include a real time RT-PCR assay in a comparative investigation aimed to evaluate the prevalence of HER-2/neu alterations in this type of tumor.

FISH analysis confirmed that gene amplification occurs in lung cancer, although its exact prevalence cannot be estimated here because only a subgroup of patients in our series had material available for this study.

Our results document that a spectrum of HER-2/neu activation levels occurs, most clearly seen with real time RT-PCR analysis, which provides precise, quantitative data. At one end of this spectrum, it is possible to identify cases with high-level gene amplification accompanied by extremely elevated mRNA overexpression and strong protein immunoreactivity. At the other end, there are cases that display HER-2/neu alterations documented only with one technique, frequently at borderline levels.

Given these results, it is apparent that the way techniques used to assess HER-2/neu activation are applied in the clinics and results interpreted will have a profound influence on current and future clinical trials in lung cancer.

To this respect, important lessons can be learned from the experience gained in breast cancer. The results of comparative studies have demonstrated that an excellent correlation exists between strongly immunoreactive (3+) breast cancers and FISH documented gene amplification (42, 43). For the moderately immunoreactive cases (2+), the correlation with gene amplification is dramatically reduced (43). Furthermore, it is well documented that the HER-2/neu receptor may be overexpressed in the absence of gene amplification (3, 41, 44). Pauletti et al. (45) have carefully evaluated the use of Western, Southern, and Northern blots, together with IHC and FISH analysis, to detect HER-2/neu alterations in breast cancer. This group concluded that FISH is more sensitive and accurate than the solid matrix techniques (45).

The HER-2/neu status in the patients reported thus far has been mainly assessed by IHC (20), but recent data indicate that HER-2/neu alterations caused by gene amplification, evidenced by FISH analysis, has a much stronger correlation with patient response to treatment than the overall immunohistochemical evaluation (42).

The data of a carefully conducted comparative study, including routine IHC, quantitative IHC using an iodine-125-labeled HER-2/neu antibody, and FISH also support the advantages of FISH analysis in breast cancer for routine diagnostic laboratories (46). These investigators point out that conventional IHC, using HercepTest, does not detect one-third of the tumors displaying increased HER-2/neu by quantitative IHC. Arguably,

Table 2  HER-2/neu mRNA levels in NSCLC

<table>
<thead>
<tr>
<th>HER2/neu mRNA levels</th>
<th>No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.42n</td>
<td>61 (53)</td>
</tr>
<tr>
<td>1.42-2n</td>
<td>9 (8)</td>
</tr>
<tr>
<td>2-3n</td>
<td>31 (27)</td>
</tr>
<tr>
<td>3-4n</td>
<td>3 (3)</td>
</tr>
<tr>
<td>4-10n</td>
<td>8 (7)</td>
</tr>
<tr>
<td>&gt;10n</td>
<td>3 (3)</td>
</tr>
</tbody>
</table>

Fig. 1 HER-2/neu patterns of immunoreactivity in NSCLC. Examples of strong complete (A) and weak incomplete (B) membrane immunostaining.
only the final data based on patients outcome will provide definitive guidance.

In lung carcinoma, the data pertaining HER-2/neu status has been evaluated mainly by immunohistochemical techniques (14, 23, 26, 27, 30, 33, 34), with few studies using FISH to detect HER-2/neu gene amplification (47–50). It has been claimed that HER-2/neu gene amplification is rare in NSCLC (38), which more commonly display polysomy, but these conclusion are based mostly on studies performed on cell lines (47, 49). In primary lung cancers, Shiraishi et al. (48) found HER-2/neu gene amplification in 1 of 51 tumors, and Heinmoller et al. (50) reported the preliminary finding of a multinational trial, including 378 patients screened by FISH with a 2% prevalence of gene amplification (34). Hirsh et al. (34) found 2 of 51 (4%) NSCLCs showing high-level HER-2/neu amplification. We analyzed 41 cases by FISH; of these, 3 (7%) were highly amplifed, and 6 (15%) showed low level of gene amplification, displaying, however, elevated mRNA levels and/or HER-2/neu immunoreactivity. These results and the data reported in the literature document that genuine HER-2/neu gene amplification occurs in lung cancer, although with a prevalence lower than in breast cancer. The differences among our and data reported previously could be attributable to the lack of a clear definition of gene amplification by FISH in literature. We considered gene amplification when gene/chromosome signal ratio is $>2$, as reported by Hirsh et al. (34); in addition, we underline that in the three cases highly amplified, the exact ratio between HER-2/neu and chromosome 17 centromeric probes cannot be determined because of the elevated number of HER-2/neu gene copy, as depicted in Fig. 2.

Table 3  Relationship between HER-2/neu mRNA levels and protein expression

<table>
<thead>
<tr>
<th>HER-2/neu mRNA levels</th>
<th>HER2/neu immunoreactivity</th>
<th>Total No. of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of cases (%)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>Negative 8 (13)</td>
<td>61 (53)</td>
</tr>
<tr>
<td>Overexpressed</td>
<td>Positive 18 (33)</td>
<td>54 (47)</td>
</tr>
<tr>
<td>Total</td>
<td>26 (33)</td>
<td>115 (100)</td>
</tr>
</tbody>
</table>

Table 4  HER-2/neu gene alterations in NSCLC

<table>
<thead>
<tr>
<th>FISH</th>
<th>HER-2/neu immunoreactivity</th>
<th>HER-2/neu mRNA levels</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification</td>
<td>Negative 11</td>
<td>Normal 21</td>
<td></td>
</tr>
<tr>
<td>Polysomy</td>
<td>Positive 0</td>
<td>Overexpressed 11</td>
<td></td>
</tr>
<tr>
<td>Low-level amplification</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>High-level amplification</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>21</td>
<td>20</td>
</tr>
</tbody>
</table>

In lung carcinoma, the data pertaining HER-2/neu status has been evaluated mainly by immunohistochemical techniques (14, 23, 26, 27, 30, 33, 34), with few studies using FISH to detect HER-2/neu gene amplification (47–50). It has been claimed that HER-2/neu gene amplification is rare in NSCLC (38), which more commonly display polysomy, but these conclusion are based mostly on studies performed on cell lines (47, 49). In primary lung cancers, Shiraishi et al. (48) found HER-2/neu gene amplification in 1 of 51 tumors, and Heinmoller et al. (50) reported the preliminary finding of a multinational trial, including 378 patients screened by FISH with a 2% prevalence of gene amplification (34). Hirsh et al. (34) found 2 of 51 (4%) NSCLCs showing high-level HER-2/neu amplification. We analyzed 41 cases by FISH; of these, 3 (7%) were highly amplified, and 6 (15%) showed low level of gene amplification, displaying, however, elevated mRNA levels and/or HER-2/neu immunoreactivity. These results and the data reported in the literature document that genuine HER-2/neu gene amplification occurs in lung cancer, although with a prevalence lower than in breast cancer. The differences among our and data reported previously could be attributable to the lack of a clear definition of gene amplification by FISH in literature. We considered gene amplification when gene/chromosome signal ratio is $>2$, as reported by Hirsh et al. (34); in addition, we underline that in the three cases highly amplified, the exact ratio between HER-2/neu and chromosome 17 centromeric probes cannot be determined because of the elevated number of HER-2/neu gene copy, as depicted in Fig. 2.

The data on mRNA in NSCLC are limited: Schneider et al. (36) reported high expression levels in all seven NSCLC cell lines, with the highest levels being present in adenocarcinomas.

In a study including both cell lines and primary NSCLC, a $>2$-fold increase in HER-2/neu mRNA has been detected in 30% of tumors (51). In addition, these authors also confirmed the clustering of HER-2/neu overexpression among lung adenocarcinomas.

Recently, Brabender et al. (37) have evaluated epidermal growth factor receptor and HER-2/neu in a series of NSCLC using a real time RT-PCR assay, documenting their overexpression in 34 and 35% of patients, respectively. This study did not provide immunohistochemical and FISH results for comparative
analyses. Survival evaluation, however, demonstrated a significant link between the overexpression of these receptors and poor patient prognosis.

Our results of real time RT-PCR analysis document the feasibility of this technique to detect HER-2/neu mRNA even in the normal lung parenchyma, albeit at very low expression levels. Furthermore, HER-2/neu mRNA levels above the normal controls were present in almost half of the tumors investigated. Forty percent of tumors displayed a 2-fold increase, 10% of the carcinomas showed a 4-fold increase, and 4% of cases had at least a 10-fold increase in HER-2/neu mRNA levels. The cases with the highest HER-2/neu expression levels displayed gene amplification and immunohistochemical positivity, and in fact, all FISH-positive cases were included among the high mRNA expressers. However, a limited number of cases (13%) displayed HER-2/neu immunoreactivity without a corresponding increase in mRNA levels. Because mRNA degradation could be excluded, because of the presence of appropriate real time amplification plots and thresholds values, this could be a consequence of the fact that HER-2/neu mRNA and its product have different half-life periods. Alternatively, antigenic loss in formalin-fixed, paraffin-embedded material may explain the negative immunohistochemical results.

Real time RT-PCR is now considered the preferable technique to quantify gene expression in human tumors because it provides several advantages relative to other techniques. Real time RT-PCR makes RNA quantitation more precise and reproducible, because it is based on the threshold cycle values established in the early exponential phase of the PCR reaction (52). It has a high sample throughput capacity, a wide dynamic range, and does not require post-PCR sample handling, thereby avoiding problems related to carryover.

Indeed, real time RT-PCR has been proposed as a major alternative to FISH and IHC for HER-2/neu analysis, based on a study on 134 breast tumor samples (53).

The data of our study suggest that this alternative may also provide robust and powerful results in NSCLC. The real time RT-PCR assay could be easily performed on minute tissue specimens, and the results could be obtained within hours, therefore allowing the timely enrolment of patients in clinical trials. Furthermore, the test can be largely automated with elevated sample processing capacity. Compared with FISH or IHC, the only disadvantage relates to the RNA extraction from human tumor specimens with the inherent, unavoidable, cellular heterogeneity. Such heterogeneity is relevant to the analysis of neoplastic cell population versus nonneoplastic, inflammatory, and stromal cellular components. Furthermore, intratumoral heterogeneity may also be significant. To partially solve this disadvantage, multiple small samples from the same tumor could be evaluated by real time RT-PCR, and the histological examination of tissues can help in assuring that a large percentage of the specimens comprises tumor cells.

In this study, to assure the specificity of results, the quantitative assessment of HER-2/neu mRNA levels was carried on tissue samples with neoplastic components comprising ≥80% of the sample.

The ongoing clinical trials evaluating Trastuzumab in lung cancer use different eligibility criteria for patient enrolment; all trials include the immunohistochemical evaluation, but the type of positivity required varies, starting at 1+ (38). Considering the data of our investigation, it seems likely that the interpretation of the results of these trials would greatly benefit from the addition of other types of testing HER-2/neu activation.

Because lung carcinoma is the first cause of death for cancer in Western countries, even a small percentage of patients responding to the monoclonal antibodies inhibitory therapy would be significant and the identification of the group of responders of crucial importance.

ACKNOWLEDGMENTS

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HER-2/Neu Alterations in Non-Small Cell Lung Cancer: A Comprehensive Evaluation by Real Time Reverse Transcription-PCR, Fluorescence in Situ Hybridization, and Immunohistochemistry

Caterina Pellegrini, Monica Falleni, Antonio Marchetti, et al.


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