Predicting gefitinib responsiveness in lung cancer by fluorescence in situ hybridization/chromogenic in situ hybridization analysis of EGFR and HER2 in biopsy and cytology specimens

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Abstract

In non–small cell lung cancer (NSCLC), epidermal growth factor receptor (EGFR) mutational analysis is an excellent predictor of responsiveness to treatment with tyrosine kinase inhibitors, such as gefitinib. In up to 80% of NSCLCs, cytologic samples or endoscopic biopsies are the only specimens available for molecular analysis, but PCR amplification of DNA from small fixed and paraffin-embedded samples may create artifactual mutations. Fluorescence in situ hybridization (FISH) of EGFR and HER2 has been proposed as an alternative method of analysis. This project aimed to determine the optimal scoring method for FISH or chromogenic in situ hybridization (CISH) assays when analyzing small NSCLC samples to predict response. FISH or CISH analysis of EGFR and HER2 genes was done on 42 small samples derived from NSCLC patients treated with gefitinib. EGFR mutational analysis was done after quantity and quality controls of DNA. In seven of seven cases, a balanced increase in EGFR gene and chromosome 7 number was found to correlate with the presence of specific EGFR mutations. In addition, seven of seven cases with balanced EGFR/HER2 polysomy and two of three cases with balanced EGFR/HER2 trisomy responded to gefitinib (75% of responders). Instead, the EGFR mutations predicted only 7 of 12 (58%) of gefitinib-responsive patients. When only endoscopic biopsies or cytologic specimens are available, we propose using FISH/CISH for EGFR and HER2 as the test of choice for selecting patients for treatment with gefitinib and to consider as negative predictive factor the absence of EGFR/HER2 gene gain.

Introduction

Epidermal growth factor receptor (EGFR) is a prototypical member of the EGFR family that also includes HER2, HER3, and HER4. HER2 and HER3 are the preferential partners of EFGR in the process of heterodimerization and activation (1). EGFR is implicated in the development and progression of non–small cell lung cancer (NSCLC; ref. 1). Tyrosine kinase inhibitors (TKI), which selectively block the growth-stimulating effect of EGFR, such as gefitinib (ZD 1839 or Iressa) and erlotinib (OSI 774 or Tarceva), have been developed (2–4). These small molecules compete with and prevent the binding of ATP at its binding region within the EGFR tyrosine kinase domain, thereby inhibiting tyrosine phosphorylation and signaling events implicated in cell cycle progression, apoptosis, angiogenesis, and metastasis (5).

Several patient characteristics, such as never smokers, Asian ethnicity, female gender, and a histologic diagnosis of adenocarcinoma, are associated with increased responsiveness to EGFR TKIs (6, 7). Several approaches, including immunohistochemistry and gene mutation or copy number enumeration, have been tested to quantify EGFR protein or gene in tumor specimens to predict response to treatment. Immunohistochemistry is inadequate for discriminating responders from nonresponders, drawbacks, including the heterogeneity of anti-EGFR reagents and immunohistochemical methodologic approaches, and the diversity of immunohistochemical scoring methods (8). Somatic mutations in the EGFR kinase domain are correlated with gefitinib sensitivity (9–11). However, at the time of diagnosis, only about 20% to 25% of lung cancers can potentially be cured primarily by surgery, and samples from fine-needle aspiration or endoscopic biopsies of the lesion are the only specimens available for diagnosis and molecular analysis. Small samples may be inadequate to mutational studies because these analyses require significant amounts of tumor cell DNA to avoid contamination from unmutated wild-type DNA of normal cells and...
EGFR/HER2 Gene Analysis in Lung Cancer

Table 1. Type of specimens analyzed and in situ hybridization analysis of 42 cases of NSCLC

<table>
<thead>
<tr>
<th></th>
<th>FISH</th>
<th>CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE histologic specimens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical samples</td>
<td>13 (31)</td>
<td>13</td>
</tr>
<tr>
<td>Endoscopic bronchial biopsies</td>
<td>20 (48)</td>
<td>20</td>
</tr>
<tr>
<td>Cerebral biopsy</td>
<td>1 (2)</td>
<td>1</td>
</tr>
<tr>
<td>Bone biopsy</td>
<td>1 (2)</td>
<td>1</td>
</tr>
<tr>
<td>Spinal biopsy</td>
<td>1 (2)</td>
<td>1</td>
</tr>
<tr>
<td>AFPE cytologic specimens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>2 (5)</td>
<td>2</td>
</tr>
<tr>
<td>Bronchial washing</td>
<td>1 (2)</td>
<td>1</td>
</tr>
<tr>
<td>Lung FNA</td>
<td>3 (7)</td>
<td>3</td>
</tr>
</tbody>
</table>

Abbreviations: FFPE, formalin fixed, paraffin embedded; AFPE, alcohol fixed, paraffin embedded; FNA, fine-needle aspiration.

The objectives of the present study were as follows: (a) to confirm the efficiency of FISH/chromogenic in situ hybridization (CISH) analysis in assessing EGFR and HER2 in histologic biopsies and cytologic specimens of NSCLC and (b) to define in small-sized specimens the optimal scoring method of FISH or CISH for identifying NSCLC patients eligible for TKI treatment.

Materials and Methods

Patient and Tissue Samples

We obtained material from 42 NSCLC patients treated with gefitinib: 23 were biopsies and 6 were cytologic specimens (Table 1) obtained from our pathology department archives. For the remaining 13 tumors, we had paraffin blocks of surgical samples, and to reproduce the study procedure on small specimens, we prepared a tissue array (Advanced Tissue Arrayer model ATA-100, Chemicon International, Temecula, CA) taking two cores of 1 mm in diameter from one representative block. Eleven additional surgical samples were used as control for in situ hybridization procedures. Human tissue samples were used according to the guidelines of the local Ethics Committee.

All 42 patients had received compassionate therapy with gefitinib at the Oncology Division of San Giovanni Battista Hospital, Molinette (Turin, Italy; Table 2). Patients received 250 mg/d p.o. gefitinib for an indefinite period until disease progression or unacceptable toxicity. Objective tumor response was assessed using the Response Evaluation Criteria in Solid Tumors (17). Duration of overall response was defined as the time from the date of the first objective assessment of a complete/partial response until the date of objectively documented progressive disease. Computed tomography of the chest and upper abdomen was done every 8 weeks. Survival and progression-free survival were defined as the period from the start of gefitinib treatment to the date of death or disease progression, respectively, or last follow-up.

DNA Extraction and PCR

Genomic DNA was extracted from four 10-μm-thick sections of formalin-fixed, paraffin-embedded blocks or alcohol-fixed, paraffin-embedded blocks. After deparaffinizing with xylene-ethanol, specimens were incubated overnight at 55°C in lysis buffer containing proteinase K (20 mg/mL) followed by DNA isolation after phenol-isopropanol extraction. DNA concentration was measured with a spectrophotometer (BioPhotomer, Eppendorf AG, Hamburg, Germany). The quality of DNA extracted from formalin-fixed, paraffin-embedded blocks or alcohol-fixed, paraffin-embedded blocks was tested by doing amplification of a 300-bp fragment of the human MHC class II DRβ gene with the following primers: 5′-CCGGTC-GACTGTCCCCCAGACGTTC-3′ (DRBF) and 5′-GAATTCTCGCCGTACGTGAACGTTTC-3′ (DRBR). PCR amplification of EGFR (exons 19 and 21) was done using the following primers: 5′-CAATATCAGCCTATTAGGGTGGCGGCTC-3′ (EGFR19F), 5′-CATAGAAAGTAACATTAGGATGTG-3′ (EGFR19R), 5′-CTAACGTTCGCCACCATTAAGGTTCGCCAGC-3′ (EGFR21F), and 5′-GGCTGGGACCTCAACAAAATCTTGG-3′ (EGFR21R). The primers were obtained from ref. 9.

Table 2. Characteristics of the 42 NSCLC patients

<table>
<thead>
<tr>
<th></th>
<th>Responder</th>
<th>Nonresponder</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, n (%)</td>
<td>12 (28.5)</td>
<td>30 (71.5)</td>
<td></td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>60 (37–77)</td>
<td>63 (37–77)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Female</td>
<td>9 (21.5)</td>
<td>5 (12)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Male</td>
<td>3 (7)</td>
<td>25 (59.5)</td>
<td></td>
</tr>
<tr>
<td>Age (y), n (%)</td>
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</tr>
<tr>
<td>&lt;50</td>
<td>3 (7)</td>
<td>1 (2.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>&gt;50</td>
<td>9 (21.5)</td>
<td>29 (69)</td>
<td></td>
</tr>
<tr>
<td>Stage, n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>2 (5)</td>
<td>0.3</td>
</tr>
<tr>
<td>IV</td>
<td>12 (28.5)</td>
<td>28 (66.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>Previous radiotherapy lung</td>
<td>3</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Previous chemotherapy</td>
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<td>28</td>
<td></td>
</tr>
<tr>
<td>Previous surgery lung</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Site of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>9</td>
<td>18</td>
<td></td>
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Table 3. Correlation of EGFR and HER2 copy number to EGFR mutation and response to gefitinib

<table>
<thead>
<tr>
<th>EGFR disomy</th>
<th>EGFR trisomy</th>
<th>EGFR polysomy</th>
<th>EGFR disomy</th>
<th>EGFR trisomy</th>
<th>EGFR polysomy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2 disomy, %</td>
<td>HER2 disomy, %</td>
<td>HER2 disomy, %</td>
<td>HER2 disomy, %</td>
<td>HER2 disomy, %</td>
<td>HER2 disomy, %</td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>EGFR mutated</td>
<td>0</td>
<td>0</td>
<td>1 (14)</td>
<td>0</td>
<td>1 (14)</td>
</tr>
<tr>
<td>EGFR unmutated</td>
<td>2 (40)</td>
<td>0</td>
<td>0</td>
<td>3 (10)</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Not responder</td>
<td>EGFR mutated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EGFR unmutated</td>
<td>24 (80)</td>
<td>2 (7)</td>
<td>0</td>
<td>3 (7)</td>
<td>1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>26 (62)</td>
<td>2 (5)</td>
<td>1 (2.5)</td>
<td>3 (7)</td>
<td>7 (16)</td>
<td>42</td>
</tr>
</tbody>
</table>

PCR was done in a total volume of 50 μL containing 1× PCR buffer (20 mmol/L Tris-HCl, 50 mmol/L KCl), 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, 0.4 μmol/L each primer, 0.2 unit Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 500 ng of genomic DNA. Thermal cycling conditions were as follows: 5 min at 94°C followed by 40 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 7 min.

DNA Sequencing

PCR products were separated on a 2% agarose gel, purified using the PCR Cleanup Gel Extraction kit (Macherey-Nagel, Düren, Germany), and sequenced in both directions by dye terminator sequencing with the BigDye Terminator v1.1 Sequencing kit (Applied Biosystems, Foster City, CA). Sequencing fragments were detected by capillary electrophoresis on an ABI Prism 310 DNA analyzer (Applied Biosystems). In all cases, samples harboring mutations were subjected to a second round of amplification and sequencing. EGFR mutation analysis was done by laboratory personnel blinded to clinical response.

Fluorescence In situ Hybridization

Probes for EGFR (Vysis, Inc., Downers Grove, IL) and HER2 (Vysis) were used for FISH according to the manufacturer’s instructions. Briefly, sections were baked overnight at 56°C, deparaffinized in xylene, dehydrated in 100% ethanol, and air dried. Slides were pretreated in sodium thiocyanate for 30 min at 80°C and then with proteases for 7 to 10 min at 37°C; they were then washed in 2× SSC, air dried, covered with ~10 μL of probe (LSI EGFR/CEP7 dual-color probe or LSI HER2/CEP17 dual-color probe, Vysis), codenatured in HYBrite System (Vysis) for 5 min at 72°C, and hybridized overnight at 37°C with the probes. Finally, slides were washed with posthybridization buffer at 72°C and counterstained with 4’,6-diamidino-2-phenylindole. Tumor sections were first scanned at low power with a 4’,6-diamidino-2-phenylindole filter to identify areas of optimal tissue digestion and nonoverlapping nuclei. Analysis was done independently by two observers (A.S. and L.M.) blinded to the clinical response of the patients.

Chromogenic In situ Hybridization

Digoxigenin-labeled EGFR and HER2 DNA probes (Zymed, South San Francisco, CA) and biotin-labeled centromeric probes for chromosome 17 (Chr17; Spot-Light Chr17 centromeric probe, Zymed) and Chr7 (Spot-Light Chr7 centromeric probe, Zymed) were used following the manufacturer’s instructions. Sections were covered with coverslips and denatured on a hot plate at 94°C. Hybridization was done overnight at 37°C. Signals were detected using a CISH detection kit (Zymed). For EGFR and HER2 tests, a peroxidase quenching solution was applied to the sections followed by nonspecific blocking solution for 10 min at room temperature, FITC-conjugated sheep antidigoxigenin for 30 min at room temperature, horseradish peroxidase-goat anti-FITC for 30 min at room temperature, and 3,3’-diaminobenzidine chromogen for 30 min at room temperature. For biotin-labeled probes, horseradish peroxidase-streptavidin (30 min) followed by 3,3’-diaminobenzidine chromogen was used to reveal the reactions. Slides were counterstained with Mayer’s hemalum. Genes and chromosomes, visualized as dark brown dots, were counted in parallel sections.

In situ Hybridization Categories and Statistical Analysis

FISH and CISH results for EGFR and HER2 genes were assessed using the categories proposed by Hirsch et al. (18): balanced disomy (1.6–2.0 genes and chromosomes in all cells), balanced trisomy (2.2–3.0 genes and chromosomes in at least 10 cells), balanced polysomy (3.1–4.4 genes and chromosomes in at least 10 cells), low amplification (gene/chromosomes 2.1–3.0), and high amplification (gene/chromosomes >3). We considered as positive cutoff the presence of at least 10 neoplastic cells showing gene gain. The results were compared with those obtained on the whole sections, considering 40% of cells showing gene increase as cutoff. Clinical response was evaluated blinded to molecular results.

FISH/CISH analysis and clinical response to gefitinib were compared using the two-tail Fisher’s exact test, and statistical significance was defined as P < 0.05. Statistical analyses were done using software freely available online. 4 The overall concordance between the different procedures was evaluated with K statistic. The Kaplan-Meier estimates of median time to disease progression (TTP) and overall survival (OS) of patients were analyzed.

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4 http://www.quantpsy.org


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Results
The median follow-up for the patients who were still alive at the last follow-up was 14.8 months. The Kaplan-Meier estimates of median TTP for the overall population were 2.8 months. The median OS was 6 months. The 2-year survival rate was 12.5%.

**EGFR Mutation Status**
Among the 42 patients, 6 had a deletion in exon 19 (3 E746-A750, 1 L747-A750, 1 L747-P751insP, and 1 E746-P750insS) and 1 patient had the L858R missense mutation in exon 21. These seven patients with a mutated EGFR responded to gefitinib (100%); of the 35 patients without EGFR mutation, 14% responded to gefitinib ($P < 0.0001$; Table 3; Fig. 1A, C, and E). In mutated and unmutated cases, neoplastic cells represented from 10% to 90% of cells on the slide sections.

**EGFR and HER2 Gene Copy Number**
FISH analysis was successful in 37 of 42 cases; in the other 5 cases, CISH for genes and chromosomes was done (Table 1). The results obtained by tissue array, biopsy, and cytologic specimen showed good agreement with the result of full histologic analysis of surgical samples. Two cases showed balanced polysomy of EGFR/HER2 in small specimens and high polysomy in full sections. The other cases showed balanced disomy for the two genes in both types of specimens.

None of the 42 cases analyzed by FISH (37 cases) or CISH (5 cases) showed amplification of either the EGFR or...
HER2 gene. However, 16 cases (38%) presented a balanced gain of chromosomes and genes (Table 3). Specifically, Chr7-EGFR balanced polysomy was observed in eight cases (Fig. 1B and F) and balanced trisomy (Fig. 1D) in five. Chr17-HER2 balanced trisomy was observed in six cases and balanced polysomy in seven. The in situ hybridization analysis subset was compared with mutation analysis and showed an overall concordance of 78.6% with a k value of 49% (P < 0.001; Table 3). In particular, Chr7-EGFR balanced polysomy was present in 6 of 7 (86%) mutated cases and in 2 of 35 (5.7%) unmutated cases. Chr7-EGFR balanced trisomy was present in 1 of 7 (14%) mutated cases and in 4 of 35 (11%) unmutated cases. Chr17-HER2 balanced polysomy was present in 5 of 7 (71.4%) mutated cases and in 2 of 35 (6%) unmutated cases, whereas Chr17-HER2 balanced trisomy was present in 14% of both mutated (1 of 7) and unmutated (5 of 35) cases (Table 3).

Seven cases (five mutated) with coupled HER2/EGFR balanced polysomy responded to gefitinib (mean TTP, 15.8 months; mean OS, 18.7 months) and one responder with Chr7-EGFR polysomy alone had a TTP and OS of 6.74 months. Two (one mutated) of three cases with EGFR/HER2 trisomy had a clinical response to gefitinib but showed a TTP and OS similar to other five cases with balanced trisomy per single gene (three for HER2 and two for EGFR) that did not respond (mean TTP, 2.4 months; mean OS, 8.38 months).

In conclusion, 10 of 13 (77%) patients with genomic gain of EGFR and 9 of 13 (69%) cases with genomic gain of HER2 responded to gefitinib compared with 2 of 29 (6.8%) and 3 of 29 (10%) balanced disomic cancers, respectively (P < 0.0001 and 0.0005; Table 3; Fig. 2). The score categories for FISH/CISH results in small specimens considered as positive, doubtful, or negative responders to gefitinib are reported in Fig. 3.

Figure 2. Kaplan-Meier curves for TTP and OS. Median TTP was 16.7 mo for EGFR ≥3/HER2 ≤3 and 2.3 mo for EGFR ≤2/HER2 ≤2 patients (P = 0.00014). Median OS was 16.4 mo for EGFR ≥3/HER2 ≥3 and 5.0 mo for EGFR ≤2/HER2 ≤2 patients (P < 0.00001). Statistical significance of differences between the two groups was evaluated with log-rank tests.

Figure 3. Diagnostic interpretation of EGFR and HER2 gene gain by FISH/CISH in small specimens of NSCLC.
Discussion

The principal findings of this study are the following: (a) the results of FISH/CISH analysis done on small-sized specimens represent a successful method for establishing the EGFR/HER2 gene content in NSCLC; (b) as reported in the literature, gain of EGFR and HER2 genes is more frequently a consequence of Chr7 and Chr17 polysomy, respectively, rather than gene amplification; and (c) concurrent polysomy and, less specifically, concurrent trisomy of EGFR and HER2 may be considered as positive markers for selecting NSCLC patients eligible for TKI treatment.

The first aim of our study was to validate the EGFR/HER2 gene study by FISH or CISH in very small tissue samples of lung cancers. In our series, in 68% of cases, the first diagnosis of NSCLC was obtained from small biopsies or cytologic samples. This limited the possibility of characterizing the histotype of tumors in 7% of cases. Several studies have reported successful use of FISH and CISH in alcohol-fixed ﬁne-needle aspiration cytology or tissue array (19–22). In this study, either FISH or CISH was successful in all specimens analyzed. The use of CISH to enumerate both genes and chromosomes was limited to samples that were difﬁcult to interpret by ﬂuorescence microscopy because of highly heterogeneous cell populations (pleural effusion or bronchial lavage) or high autofluorescent background due to hemosiderin or necrosis.

The second aim was to deﬁne, when dealing with small tissue specimens analyzed by FISH/CISH, the diagnostic criteria useful for selecting patients with NSCLC who are eligible for TKI treatment. To this end, a Medline search was done using as query term “EGFR and gene number and lung” (Supplementary Table S4), and we selected eight studies that used FISH to evaluate the status of the EGFR gene with or without correlation to mutations and clinical response (13, 14, 18, 23–27). First, the literature conﬁrms that gain of EGFR according to FISH correlates with gene mutation (13, 27). However, these results cannot be interpreted in a straightforward manner because FISH positivity or negativity is variably deﬁned (14, 26, 27).

Furthermore, EGFR ampliﬁcation by FISH has been reported in ~10% of NSCLC (13, 18, 24, 25), but balanced trisomy (three copies of the gene and Chr7) and polysomy (four or more copies of the gene and Chr7) are the most frequent events leading to EGFR gain. In large case series of NSCLC, trisomy or polysomy is further subdivided into “low” or “high” depending on the cutoff of 40% of cells showing three or four or more copies of EGFR and Chr7 (13). As recently discussed in the article by Dziadziuszko et al. (8), the use of this cutoff even in high polysomy does not exclude that there may be plenty of diploid cells. Thus, the risk of underestimating FISH results has to be taken into account when dealing with small, paucicellular specimens. Our data show that, in small samples, any gene gain of EGFR and HER2 in 10 neoplastic cells is comparable with the 40% cutoff in tumor surgical samples and correlates with the presence of speciﬁc mutations in the EGFR gene. In addition, although EGFR gene copy number detected by FISH is associated with improved response to gefitinib (13–15), conﬂicting results are reported on the role of HER2 gene gain (14, 16). As previously reported (16) we conﬁrmed that only concurrent gain of both HER2 and EGFR genes is associated with sensitivity to gefitinib.

In conclusion, because none of the clinical predictors, such as smoking status, race, patient sex, and histology, could guarantee or exclude clinical beneﬁt from gefitinib, molecular factors may be incorporated to deﬁne the probability of response to TKIs. However, to prevent incompatibility in techniques and interpretation, we suggest that, for endoscopic biopsies or cytologic specimens, FISH/CISH analysis may be used as the ﬁrst-choice laboratory test as an alternative to gene mutation analysis. The probability of response will be lower in cases without EGFR and HER2 gene gain.

References


Molecular Cancer Therapeutics

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