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Concomitant high gene copy number and protein overexpression of IGF1R and EGFR negatively affect disease-free survival of surgically resected non-small-cell-lung cancer patients

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Abstract

Background—Insulin-like growth factor 1 receptor (IGF1R) represents a novel molecular target in non-small-cell-lung cancer (NSCLC). IGF1R and epidermal growth factor receptor (EGFR) activation are essential to mediate tumor cell survival, proliferation, and invasion. This study investigates the prognostic role of IGF1R and EGFR in surgically resected NSCLC.

Materials and methods—*IGF1R and EGFR* copy number gain (CNG) were tested by fluorescence in situ hybridization (FISH) and protein expression by immunohistochemistry (IHC) in 125 stage I–II–IIIA NSCLC patients.

Results—Fourty-six tumors (40.3 %) were *IGF1R* FISH-positive (FISH+), and 76 (67.2 %) were *EGFR* FISH+. Tumors with concomitant *IGF1R*/*EGFR* FISH+ were observed in 34 cases (30.1 %). *IGF1R* and *EGFR* FISH+ were associated with SCC histology ($p = 0.01$ and $p = 0.04$, respectively). IGF1R and EGFR protein over-expression (IHC+) were detected in 45 (36.0 %) and 69 (55.2 %) cases, respectively. Tumors with concomitant IGF1R/EGFR IHC+ were detected in 31 (24.8 %) patients. *IGF1R*/*EGFR* FISH+ and IGF1R/EGFR IHC+ were significantly associated ($\chi^2 = 4.02$, $p = 0.04$). Patients with *IGF1R*/*EGFR* FISH+ and IGF1R/EGFR IHC+ were associated with shorter disease-free survival (DFS) ($p = 0.05$ and $p = 0.05$, respectively). Patients with concomitant IGF1R/EGFR FISH+/IHC+ had a worse DFS and overall survival ($p = 0.005$ and $p = 0.01$, respectively). The multivariate model confirmed that IGF1R/EGFR FISH+/IHC+ (hazard ratio (HR), 4.08; $p = 0.01$) and tumor stage (II–III vs I) (HR, 4.77; $p = 0.003$) were significantly associated with worse DFS.

Conclusions—*IGF1R/EGFR* FISH+ correlates with IGF1R/EGFR IHC+. IGF1R/EGFR FISH+/IHC+ is an independent negative prognostic factor for DFS in early NSCLC. These features may have important implications for future anti-IGF1R therapeutic approaches.

Keywords

IGF1R; EGFR; Non-small-cell-lung cancer; Prognosis

Introduction

Lung cancer remains the most frequent cause of cancer death worldwide [1], and the 5-year survival remains below 15 % [2]. Variable survival outcome among patients within the same clinical stage suggests the existence of unknown biological factors affecting prognosis.

Insulin-like growth factor receptor 1 (IGF1R) is a transmembrane heterotetrameric protein encoded by the IGF1R gene located on chromosome 15q25–q26, which promotes oncogenic transformation, growth, and survival of cancer cells [3–6]. The binding of IGF-I and IGF-II to the extracellular subunity domain of IGF1R activates the tyrosine kinase activity of IGF1R and triggers a cascade of reactions involving signal transduction pathways: the Ras, Raf, mitogen-activated protein kinase, and the phosphoinositol-3-kinase (PI3K)/AKT/BAD (Bcl-xL/Bc12-associated death promoter) [7]. Currently several IGF1R inhibitors are undergoing clinical evaluation, including blocking antibodies and tyrosine kinase blockers. Phase I/II studies of these compounds indicated favorable toxicity profiles and promising activity [8–11]. However, phase III trials of anti-IGF1R agent in NSCLC were recently discontinued owing to futility. These results stress the need to identify patient subpopulations that may preferentially benefit from anti-IGF1R therapy.

Several lines of evidence suggest an association between epidermal growth factor receptor (EGFR) and IGF1R pathways. Results of preclinical studies indicate that both receptors may heterodimerize [12], are capable of transphosphorylation [13], and share the same adaptor proteins and downstream signaling pathways [14]. Data on cell lines suggest that IGF1R mediates resistance to anti-EGFR therapy through continued activation of the PI3K-Akt pathway [15, 16]. Numerous ongoing clinical trials explore the efficacy of combination of EGFR and IGF1R inhibitors in patients with lung cancer.

The prognostic role of IGF1R has been previously investigated in human malignancies including NSCLC, leading to conflicting results. In breast cancer, IGF1R expression and activation have been linked to disease progression, increased resistance to radiotherapy, and poor prognosis [17, 18]. Fidler et al. [19], in gefitinib-treated patients, showed counter-intuitive improved outcomes with the co-expression of IGF1R and EGFR in exploratory analyses. Cappuzzo et al. [20] observed that IGF1R expression was significantly associated with better survival in metastatic NSCLC patients treated with tyrosine kinase inhibitors (TKIs). The same authors, in another study [21], showed that IGF1R expression did not represent a prognostic factor in resected NSCLC patients. Instead, Nakagawa et al. [22] observed that IGF1R expression was associated with reduced disease-free survival (DFS) in NSCLC. Recently, Dziadziuszko et al. [23] reported that IGF1R protein and gene expression did not associate with survival, whereas high *IGF1R* gene copy number harbored positive prognostic value. In a previous study, we showed that IGF1R and EGFR protein expression levels were associated, and high-level co-expression was a significant prognostic factor for worse DFS in early-stage NSCLC patients [24]. The conflicting available data and the growing interest on anti-IGF1R agents support the present study aimed to evaluate the relationship between IGF1R and EGFR gene copy number by fluorescence in situ hybridization (FISH) and protein expression by immunohistochemistry (IHC), and to investigate the prognostic significance of these receptors in a cohort of patients with NSCLC who underwent pulmonary resection.

Materials and methods

Patients

Patients with a histological diagnosis of stage I–III NSCLC who had complete surgical resection of the primary tumor and were followed on a regular basis in a specified program

were eligible for this study. Histological subtypes and grade of differentiation were determined according to the World Health Organization classification [25]. Patients were originally staged according to the 6th Edition of the IASLC TNM classification [26]. For this study, staging in all cases had been updated to reflect the 7th Edition [27]. Patients were classified as nonsmokers (never smoker), light smokers (<10 pack year and quit >10 years ago), and smokers. Neither chemotherapy nor radiotherapy had to be administered prior to surgery, and none of the patients had received EGFR target anticancer therapy during the follow-up period. Patients with disease recurrence or metastases were treated with platinum-based systemic chemotherapy. Follow-up including annual computed tomography (CT) scans of the chest and abdomen and chest X-rays in the 3-to 6-month intervals between the annual CT was planned for all patients in the first 3 years. Recurrences were detected by imaging techniques and when necessary confirmed by histological sampling.

The study was reviewed and approved by the institution's Ethics Committee, and the patients had to give their informed consent to surgical specimen analysis.

IGF1R and EGFR FISH analysis

Unstained 4 μm (+1 μm) sections of formalin-fixed paraffin-embedded (FFPE) tumor tissue were subjected to dual-color FISH assay using two probe sets: the laboratory-developed reagent IGF1R/CEP15 probe set combining the DNA insert encompassing IGF1R (BAC clone RP11 262P8) labeled with SpectrumRed d-UTPs and the SpectrumGreen CEP 15 probe and the commercial LSI EGFR Spectrum Orange/CEP7 Spectrum Green probe (Abbott Molecular). For both probe sets, assays were performed according to a previously described protocol [28]. Signals were enumerated in at least 100 tumor nuclei for each sample, using a fluorescence microscope (Zeiss AxioImager M1) equipped with single interference filters sets for green (FITC), red (Texas red), and blue (DAPI) as well as dual (red/green) and triple (blue, red, green) band pass filters. The copy number gain was evaluated utilizing a semi-quantitative grading system considering the increasing number of copies of the genes (disomy, low and high trisomy, low and high polysomy, and gene amplification) [29]. For documentation, images from representative fields were acquired by a charge-coupled device camera (Cool-Snap, Photometrics) and merged using dedicated software (CytoVision, Genetix, San Jose CA).

IHC staining of IGF1R and EGFR

The IHC staining of IGF1R and EGFR was performed according to a previously described protocol [24]. IGF1R and EGFR protein expression were evaluated using mouse antibodies (clones 24–31, 1:50 diluted, Lab Vision Neomarkers, and clone 3147, 1:100 diluted, Zymed, Laboratories Inc., respectively). There are at present no validated scoring systems for interpreting IHC staining for IGF1R and EGFR, thus we applied previous description for EGFR [30]. A cutoff value of 10 % positive cells was used in order to avoid inclusion of scattered positivity of the same intensity found in the normal bronchial tissue. A specimen was considered IHC-positive (IHC+) only when a distinct cell membrane staining was evident (Fig. 1e, f).

Statistical analysis

The χ^2-square or Fisher's exact test, where appropriate, was used to assess the association between clinical features and FISH analysis or the protein expression of biological factors. In a univariate exploratory analysis, maximum log-likelihood function was used to select the most discriminative cutoff values of IGF1R and EGFR FISH-positive (FISH+) for survival difference [31]. We found that the best discrimination was observed with a cutoff of ≥4.0 gene copies per cell displayed in 10 % of tumor cells to classify FISH+positive (FISH+) and FISH-negative (FISH-) patients. Overall survival (OS), disease-free survival (DFS), and the

95 % confidence intervals were evaluated by the Kaplan–Meier method comparing the different groups by log-rank test [32]. The Cox proportional hazards model was used to evaluate the prognostic role of each single studied parameter on OS and DFS, in univariate and multivariate analyses. Univariate survival analysis was conducted by exploring the interactions between the predictors, and the significant corrections were considered in the multivariate model (Cox, [33]). OS was defined as the time from diagnosis to the date of death from any cause; patients who were not reported as having died at the time of the analysis were censored at the date they were last known to be alive. DFS was defined as the time from diagnosis to first local, regional or distant recurrence, second primary malignancy or death from any cause, whichever came first. Patients who were alive and did not experience recurrence at the time of the analysis were censored at the last disease assessment date. Unless otherwise specified, all tests are with one degree of freedom (*df*). A probability value of <0.05 was considered as statistically significant. Statistical analysis was performed using Matlab software (The MathWorks ver. 7.2.0.232).

Results

Patient characteristics

From April 2002 to February 2006, we collected tissue samples of 125 consecutive early stage radically resected NSCLC patients, who were referred to the Thoracic Surgery Unit, Perugia University Hospital, Italy. Table 1 summarizes the demographic and clinical data. There were 96 males (84.8 %) and 19 females (15.2 %), with a median age of 66 years, ranging from 40 to 84. One hundred and sixteen patients (92.8 %) were current or former smokers. The histological types included 63 (50.4 %) squamous-cell carcinoma (SCC), 39 (31.2 %) adenocarcinoma, 3 (2.4 %) bronchioloalveolar, 12 (9.6 %) large cell and 8 (6.4 %) mixed histology. The majority of patients had poorly differentiated histology (57/125; 45.3 %) and surgery consisted of 102 lobectomies (81.6 %), 16 pneumonectomies (12.8 %), and 7 wedge resections (5.6 %), with hilar and mediastinal lymph node dissection. TNM staging consisted of 78 (62.4 %) Stage I, 20 (16.0 %) Stage II, 27 (21.6 %) Stage III. Following surgery, 3 patients (2.3 %) were treated with chemotherapy, 9 (6.9 %) with radiotherapy, 4 (3.1 %) with chemo-radiotherapy, and the remaining 109 (87.7 %) received no adjuvant treatment.

At a median follow-up time of 48.9 months, 66 patients (52.8 %) had died: 64 (95.6 %) deaths were due to disease recurrence and two (4.4 %) to unrelated causes. Nine (15.2 %) of the 59 patients still on follow-up experienced recurrence. The median survival time was 61.4 months.

IGF1R and *EGFR* gene copy number

FISH analyses were successful in 114 patients for *IGF1R* and in 113 patients for *EGFR* due to yielded insufficient tumor tissue. Mean gene copy number was 2.48 copies per cell (range 1.47–7.2) for *IGF1R* and 2.80 copies per cell (range 1.56–15) for EGFR. Using a cutoff of ≥4.0 gene copies per cell displayed in 10 % of tumor cells, FISH+ was detected in 46 tumors (40 %) for IGF1R and 76 tumors (77 %) for *EGFR*. Both *IGF1R* and *EGFR* showed true gene amplification in 5 patients each (4.4 %). *IGF1R* and *EGFR* concurrent by FISH+ was observed in 34 cases (30 %). Examples of microscope fields from *IGF1R* and *EGFR* FISH- and *IGF1R* and *EGFR* FISH+ tumors are shown in Fig. 1a–d.

IGF1R and EGFR immunohistochemistry

IHC of IGF1R and EGFR was evaluated in 125 tumors. With a cutoff value of >10 % positive cells (2+, 3+), IGF1R protein overexpression was detected in 45 patients (36.0 %) and was associated with larger tumor size ($p = 0.04$), but not with other clinical or biological

characteristics. IGF1R was more expressed in SCC histology than in non-SCC (57.8 vs 42.2 %, $p = 0.59$), although this difference was not statistically significance.

EGFR protein overexpression was observed in 69 patients (55.2 %), also more frequently in SCC than non-SCC (63.7 vs 36.3 %, $p = 0.001$). IGF1R protein expression was associated with EGFR protein expression ($\chi^2 = 4.49$, $p = 0.03$). Co-expression of both receptors was observed in 31 cases (24.8 %).

Correlation between *IGF1R* and *EGFR* FISH patterns and protein expression with clinical or demographic characteristics

Frequencies of *IGF1R* and *EGFR FISH* status, *FISH* status of both *IGF1R* and *EGFR* (indicated as *IGF1R*/*EGFR*), IHC status of IGF1R/EGFR and concomitant FISH and IHC status (indicated as FISH/IHC) of IGF1R/EGFR are shown in Table 2.

IGF1R FISH+ was associated with *EGFR* FISH+ ($p = 0.03$). *IGF1R* and *EGFR* FISH+ were associated with SCC histology ($p = 0.01$ and $p = 0.04$, respectively) as well as IHC+ of IGF1R/EGFR ($p = 0.008$) and concomitant FISH +/IHC+ of IGF1R/EGFR ($p = 0.04$). IHC+ of IGF1R/EGFR and concomitant FISH +/IHC+ of IGF1R/EGFR were associated with advanced stage ($p = 0.04$ and $p = 0.01$, respectively). No other clinical or demographic association was detected with these biological markers.

IGF1R/*EGFR* FISH+ and IGF1R/EGFR IHC+ were significantly associated ($\chi^2 = 4.02$, $p = 0.04$). Thirteen patients (11.5 %) showed concomitant FISH+/IHC+ of both receptors.

Prognostic implication of IGF1R and EGFR gene copy number and protein expression

Table 3 presents the results of the univariate analysis for DFS and OS. No statistically significant difference in DFS and OS was observed between patients with negative or positive FISH pattern for IGF1R (HR 1.48; 95% CI 0.77–2.84, $p = 0.10$, and HR 1.55; 95 % CI 0.93–2.59, $p = 0.08$, respectively) or EGFR (HR 1.48; 95 % CI 0.77–2.84, $p = 0.23$, and HR 0.98; 95 % CI 0.56–1.71, $p = 0.95$, respectively). Similar results between patients with negative or positive IHC patterns for IGF1R (HR 1.18; 95 % CI 0.68–2.03, $p = 0.53$, and HR 1.42; 95 % CI 0.87–2.33, $p = 0.15$, respectively) or EGFR (HR 1.61; 95 % CI 0.93–2.77, $p = 0.08$, and HR 1.39; 95 % CI 0.85–2.27, $p = 0.15$, respectively) were found for DFS and OS (data not shown).

The different associations of *IGF1R* and *EGFR* gene copy number by FISH and protein expression by IHC were also compared. Patients were classified into three groups: group I with FISH+ pattern for both IGF1R and EGFR, group II with IHC+ pattern for both IGF1R and EGFR, and group III with FISH +/IHC+ patterns for both IGF1R and EGFR (Fig. 2). A statistically significant difference in DFS was observed between patients with FISH+ of both genes compared to other group (25 months vs not reached; HR 1.76; 95 % CI 0.99–3.13, $p = 0.05$, Fig. 2a). The subset of patients with IGF1R/EGFR IHC+ protein expression showed a worse DFS (25 months vs 90 months; HR 1.71, 95 % CI 0.97–3.01, $p = 0.05$, Fig. 2b) when compared with other group. Also, a very significant shorter DFS was observed in the group III (FISH +/IHC+ of both IGF1R and EGFR) (10 months vs 85 months; HR 2.84; 95 % CI 1.38–5.84, $p = 0.005$, Fig. 2c). The same group III were, also, significantly associated with a poorer OS (33 months vs 87 months; HR 2.32, 95 % CI 1.20–4.44, $p = 0.01$, Fig. 2f). The groups I and II were not associated with OS ($p = 0.15$, $p = 0.09$, respectively, Fig. 2d, e).

Among clinical variables, stage (III vs I–II) was significantly associated with worse DFS (HR 2.86, 95 % CI 1.64–4.97, $p < 0.0001$) and OS (HR 2.47, 95 % CI 1.48–4.12, $p = 0.001$), whereas age (as continuous variable) was significantly associated with worse OS (HR 1.03, 95 % CI 1.00–1.06, $p = 0.04$). The multivariate model confirmed that FISH +/IHC+ patterns

for both IGF1R and EGFR (HR 4.08, 95 % CI 1.34–12.39, $p = 0.01$) and tumor stage (II–III vs I) (HR 4.77, 95 % CI 1.72–13.21, $p = 0.003$) were significantly associated with worse DFS (Table 4). Only stage (II–III vs I) was an independent prognostic factor for OS (HR 2.57, 95 % CI 1.38–4.79, $p = 0.003$) (data not shown).

Discussion

In this study, we evaluated the prognostic role of two of the most relevant biomarkers in NSCLC, and to our knowledge, report for the first time that FISH +/IHC+ of both IGF1R/EGFR receptors represents a negative prognostic factor in radically resected NSCLC. There is an increasing interest on IGF1R in NSCLC. Identification of biomarkers for selecting patients most likely to derive clinical benefit from IGF1R inhibitors is needed. *In vitro* studies showed that expression of IGF1R correlates with sensitivity to IGF1R inhibitors, such as BMS-536924 [34]. Preclinical models showed that IGF1R expression could be implicated in acquired resistance to anti-EGFR strategies [13, 15]. In breast and prostate cancer cells, Jones et al. [13] showed that increased signaling via the IGF1R pathway leads to acquired resistance to the EGFR tyrosine kinase inhibitor gefitinib. Chakravarti et al. [15] demonstrated that IGF1R can compensate for loss of EGFR function in primary glioma cell lines. Several new drugs, including mAbs and tyrosine kinase inhibitors, are currently under evaluation in patients with advanced NSCLC.

Gualberto et al. [35] reported prolonged clinical benefit to figitumumab (F) in tumors with intense membrane localization of IGF1R. They also saw that high IGF1R in the chemotherapy alone group was associated with rapid disease progression. However, these results should be considered preliminary due to small cohort of patients ($N = 45$) investigated.

A phase II study randomly assigned 150 chemonaive NSCLC patients to the standard combination of carboplatin (C) plus paclitaxel (P) versus the same regimen plus CP-751,871, an anti-IGF1R mAb [11]. The study showed a significant improvement in response rate favoring the experimental arm, with an impressive 78 % response rate in patients with squamous histology.

The higher expression of the IGF1R protein in patients with squamous-cell carcinoma compared with non-squamous histology observed in the Karp's study, as well as in our study, provides the biological background for the highest sensitivity to anti-IGF1R agents observed in clinical trials in this patient population. A recent phase III trial of F administered in combination with CP failed to demonstrate survival benefit in advanced NSCLC patients [36]. The study showed that the use of F with CP would be unlikely to improve overall survival compared to CP alone, mainly due to toxicity occurring in patients who randomly received F. However, in the subset of patients with high IGF serum levels, the addition of F appeared to offer benefit over CP. A second phase III trial investigating figitumumab with erlotinib versus erlotinib alone in the same population in the refractory setting (ADVIGO 1018; figitumumab plus erlotinib *versus* erlotinib in refractory advanced non-adenocarcinoma NSCLC) was also closed after an interim analysis in March 2010 because of the potential futility of the combination regimen. These results underscore the need to identify the patients most likely to benefit from anti–IGF1R therapy. The negative results in currently reported trials in unselected patients are disappointing, but they do not exclude the possibility that inhibition of the IGF-1R pathway could provide benefit in patients with specific molecular signatures. The preliminary results of presented trials suggest that high circulating levels of IGF-1 may be a potential, easy to test biomarker that may identify patients who may benefit from this class of agents.

A recent study has shown that the IGFIR and other molecules associated with the IGFIR pathway (e.g., IGF2R, IRS-1, -2) were overexpressed in NSCLC tumors undergoing epithelial-to-mesenchymal transition (EMT) [37]. The authors hypothesized that high circulating levels of free IGF-1(fIGF-1) could be associated with high IGF bioactivity in the tumor microenvironment, and this could favor EMT. Higher pre-treatment fIGF-1 levels were found to be predictive of the clinical benefit derived from the addition of Figitumumab to chemotherapy in NSCLC patients. In principle, the utilization of circulating factors as predictive biomarkers would appear to be more convenient than measurements requiring fresh or frozen neoplastic tissue. However, it should be noted that current assay methodologies, particularly those measuring IGF-1 bioactivity, are controversial, and it is necessary that assay methods be standardized [38]. In our study, *IGF1R* FISH+ pattern was detected in 40 % of NSCLC cases and *EGFR* FISH+ in 77 %, and there was a significant association between *IGF1R* and *EGFR* FISH+ and histologic subtypes, with the highest gene copy number in squamous-cell carcinoma. These data are consistent with a recent report [23].

The pattern of expression and prognostic value of IGF1R expression in NSCLC remains controversial. Prior studies have shown that IGF1R expression is associated with longer survival in patients treated with gefitinib [19, 20], poorer survival and higher expression in surgically treated lung adenocarcinomas versus squamous-cell carcinomas [39] or no association with survival in a similar population [40] and shorter DFS when co-expressed with EGFR in resected NSCLC [24].

Our study, conducted in early NSCLC, using FISH and IHC methods to understand the relationship between the gene and its protein levels, showed that tumors with IGF1R/EGFR FISH+/IHC+ were associated with a poorer DFS. The group of patients who had FISH+ patterns for both genes presents a DFS comparable to the group of patients with IHC+ patterns for both proteins ($p = 0.05$, $p = 0.05$, respectively, Fig. 2a, b). Finally, the group of patients who had FISH+/IHC+ of both genes had a significantly shorter DFS as shown in Fig. 2c, confirmed also in multivariate analysis. The FISH and IHC double positivity phenotype (IGF1R+/EGFR+) were found in about 10 % of the patients studied, and most of these patients were squamous-cell carcinoma. We could speculate that the cases FISH/IHC double positivity with this histological type (although the number is small) should be used to select patients for IGF1R or IGF1R + EGFR targeted therapy trials.

Furthermore, our results seem to suggest a correlation between high gene copy number and receptor activity, and they confirm the mathematical predictions of model developed by our group [41].

Interestingly, many of the genes regulated by IGF1R affect ribosome biogenesis and protein translation [3–5]. Thus, FISH+ of *IGF1R* and *EGFR* may cooperate in enhanced protein translation, either in a general way or for a specific subset of mRNAs. This finding generates the hypothesis that high gene copy number and overexpression of IGF1R with EGFR cooperatively shifts the repertoire of actively translated mRNAs to a set of genes that drive proliferation and growth, or inhibit apoptosis. Moreover, investigators have suggested that expression levels of IGF1R and EGFR are linked and that the IGF1R/EGFR ratio may serve as a more sensitive prognostic indicator of tumor phenotype and therapeutic response than expression of any single receptor [42, 43].

Given the possibility of cooperation and shared signaling pathways between the two systems, simultaneously targeting of the EGF and IGF systems may be more effective than targeting either system alone. Small molecule inhibitors and monoclonal antibodies that selectively inhibit either EGFR or IGF1R have to date predominated as effective treatments

in co-targeting strategies. The EGFR inhibition may sensitize tumor cells to anti-IGF-IR treatment, particularly in tumors that are strongly driven by EGFR. Prior evidence has indicated that acquired resistance to EGFR inhibition in NSCLC may be associated with enhanced dependency on IGF-IR signaling [44].

Thus, co-targeting EGFR and IGF-IR could be expected to have additive or synergistic antitumor effects.

In conclusion, in the present study, we have shown that IGF1R and EGFR are frequently FISH+ and IHC+ in early NSCLC and more frequently present in squamous-cell carcinoma. The patients group with IGF1R/EGFR FISH +/IHC+ might represent a subpopulation that is able to develop a more aggressive behavior. Furthermore, our finding could be used as new biomarkers because tumors with IGF1R/EGFR FISH +/IHC+ showed a worse DFS; however, due to limited percentage of this subpopulation and the short follow-up of our study, these results must be interpreted cautiously and need to be confirmed in large prospective trials.

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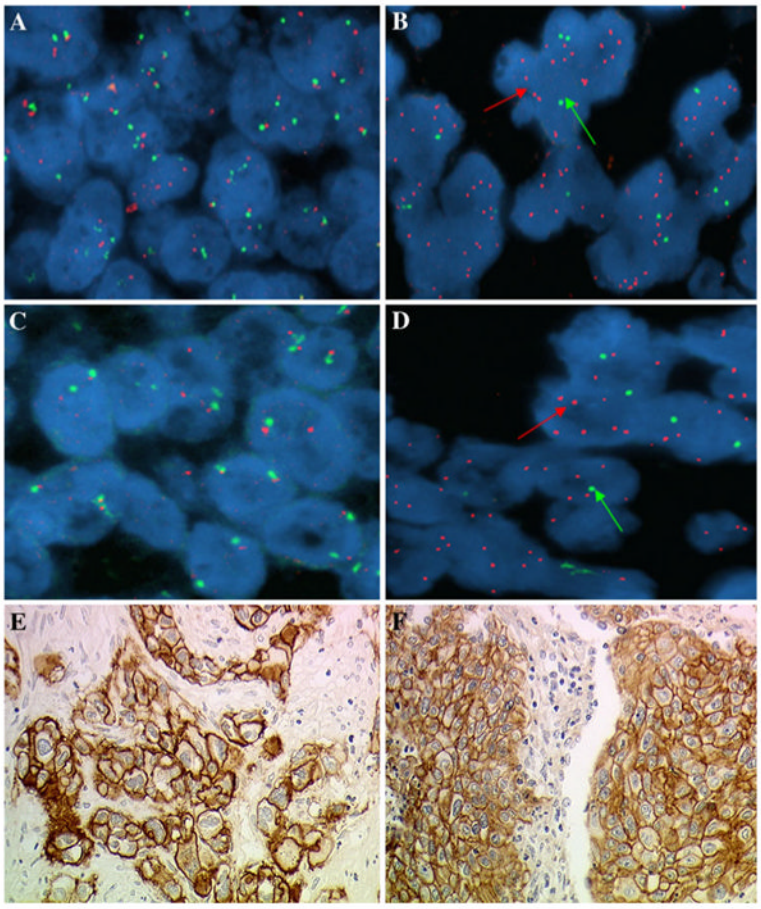

Fig. 1.
Dual color fluorescent in situ hybridization assays with probes for Insulin-like growth factor receptor 1(*IGF1R; red signal* indicated by the *arrow*) and chromosome 15 (CEP15; *green signal* indicated by the *arrow*). **a** *IGF1R* FISH−, **b** *IGF1R* FISH+. Dual color fluorescent in situ hybridization assays with probes for epidermal growth factor (*EGFR; red signal* indicated by the *arrow*) and chromosome 7 (CEP7; *green signal* indicated by the *arrow*). **c** *EGFR* FISH-, **d** *EGFR* FISH+. Examples of squamous-cell carcinoma with immunohistochemistry (IHC) positive for IGF1R (**e**) and for EGFR (**f**) (original magnification ×400)

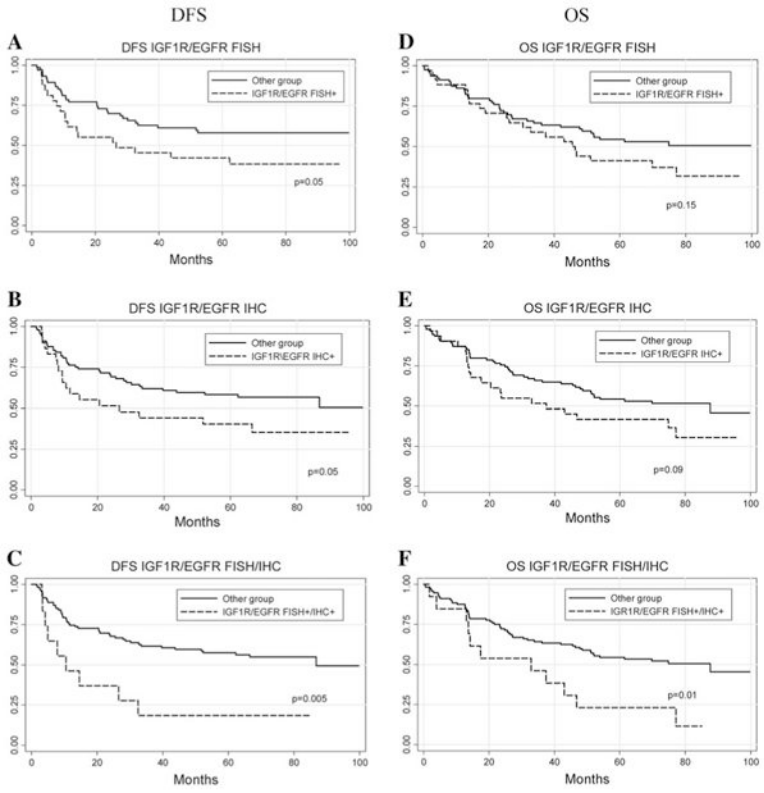

Fig. 2.

Kaplan-Meier curves of disease-free survival (DFS) and overall survival (OS) according to FISH status of *IGF1R*/*EGFR* (**a**, **d**), IHC status of IGF1R/EGFR (**b**, **e**), and FISH/IHC status of IGF1R/EGFR (**c**, **f**) in patients with non-small cell-lung cancer (NSCLC)

Table 1

Patient characteristics

Variable	Total patients	
	No.	(%)
Age *(years)*		
Median (range)	66 (40–84)	
Gender		
Female	19	15.2
Male	96	84.8
Smoking history		
Former/current smoker	116	92.8
Never smoker	9	7.2
Pathologic stage		
I	78	62.4
II	20	16.0
III	27	21.6
Histology		
Squamous-cell carcinoma	63	50.4
Adenocarcinoma	39	31.2
Bronchioloalveolar	3	2.4
Large cell carcinoma	12	9.6
Mixed histology	8	6.4
Grading		
Well	14	11.5
Moderately	54	43.2
Poorly	57	45.3
Resection		
Lobectomy	102	81.6
Pneumonectomy	16	12.8
Wedge	7	5.6

Table 2

Clinicopathologic characteristics of patients according to *IGF1R*, *EGFR* FISH status, *IGF1R*/*EGFR* FISH status, IGF1R/EGFR IHC status, and concomitant IGF1R/EGFR FISH/IHC status

	IGF1R FISH[a]		EGFR FISH[b]		IGF1R/EGFR FISH		IGF1R/EGFR IHC		IGF1R/EGFR FISH-IHC	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Both positive (%)	Other (%)	Both positive (%)	Other (%)	Both positive (%)	Other (%)
No. of Pts	46 (40.3)	68 (59.7)	76 (67.2)	37 (32.7)	34 (30.1)	79 (69.9)	31 (24.8)	94 (75.2)	13 (10.4)	112 (89.6)
Gender										
Female	6 (13.0)	12 (17.6)	14 (18.4)	4 (10.8)	4 (11.8)	14 (17.7)	3 (9.7)	16 (17.1)	1 (7.7)	18 (16.1)
Male	40 (87.0)	56 (82.3)	62 (81.6)	33 (89.2)	30 (88.2)	65 (82.3)	28 (90.3)	78 (82.9)	13 (92.3)	94 (83.9)
P	0.56		0.30		0.42		0.32		0.42	
Smoking history										
Smoker	44 (94.6)	61 (89.7)	68 (89.5)	36 (97.3)	33 (97.0)	71 (89.9)	29 (93.5)	87 (92.5)	12 (7.7)	104 (92.9)
Nonsmoker	3 (3.4)	7 (10.3)	8 (10.5)	1 (2.7)	1 (3.0)	8 (10.1)	2 (6.5)	7 (7.5)	1 (92.3)	8 (7.1)
P	0.24		0.14		0.19		0.85		0.94	
Histology										
Squamous	30 (65.2)	29 (42.6)	34 (44.8)	24 (64.9)	21 (61.3)	37 (46.8)	22 (71.0)	41 (43.6)	10 (76.9)	53 (52.7)
Other	16 (34.8)	39 (57.4)	42 (55.2)	13 (35.1)	13 (38.2)	42 (53.2)	9 (29.0)	53 (56.4)	3 (23.1)	59 (47.3)
P	0.01		0.04		0.14		0.008		0.04	
pTNM stage										
I	28 (60.9)	43 (63.2)	48 (63.2)	22 (59.4)	21 (61.8)	49 (62.0)	18 (58.1)	60 (63.8)	5 (38.6)	73 (65.2)
II	7 (15.2)	11 (16.2)	10 (13.2)	8 (21.6)	4 (11.7)	14 (17.7)	2 (6.5)	18 (19.2)	1 (7.7)	19 (16.7)
III	11 (23.9)	14 (20.6)	18 (23.6)	7 (19.0)	9 (26.5)	16 (20.3)	11 (35.5)	16 (17.0)	7 (53.8)	20 (17.9)
P	0.17		0.48		0.62		0.04		0.01	
Grading										
Well	5 (10.8)	7 (10.3)	9 (11.8)	3 (8.11)	5 (14.7)	7 (8.9)	4 (12.9)	10 (10.6)	2 (15.4)	12 (10.7)
Moderately	17 (36.9)	35 (51.47)	33 (43.4)	18 (48.6)	11 (32.3)	40 (50.6)	15 (48.4)	39 (41.5)	3 (23.1)	51 (43.5)
Poorly	24 (52.1)	26 (38.2)	34 (44.8)	16 (43.2)	18 (52.9)	32 (40.5)	12 (38.7)	45 (47.9)	8 (61.5)	49 (43.8)
P	0.28		0.78		0.18		0.67		0.25	

EGFR epidermal growth factor receptor, *IGF1R* insulin-like growth factor receptor 1, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry

[a] *IGF1R* FISH-positive = copy number gain (CNG), ≥ 4 gene copies in ≥ 10 % of tumor cells

[b] *EGFR* FISH-positive = copy number gain (CNG), ≥ 4 gene copies in ≥ 10 % of tumor cells

Table 3

Univariate analysis for variables considered (cox proportional hazard regression model)

Feature	Disease-free survival			Overall survival		
	HR	95 % CI	*P*	HR	95 % CI	*P*
Gender						
Female versus male	0.82	0.38–1.73	0.60	0.77	0.38–1.57	0.48
Age (years)						
Continuous	1.01	0.98–1.04	0.25	1.03	1.00–1.06	0.04
Smoking history						
Former/current smoker versus never smoker	0.86	0.31–2.38	0.77	0.70	0.30–1.64	0.42
Histology						
Squamous versus other	1.27	0.76–2.12	0.35	1.04	0.65–1.68	0.84
Grading						
G3 versus G1–G2	1.52	0.91–2.55	0.10	1.49	0.93–2.40	0.09
TNM stage						
II–III versus I	2.86	1.64–4.97	<0.001	2.47	1.48–4.12	0.001
IGF1R FISH[a]						
Positive versus negative	1.59	0.91–2.77	0.10	1.55	0.93–2.59	0.08
EGFR FISH[b]						
Positive versus negative	1.48	0.77–2.84	0.23	0.98	0.56–1.71	0.95
IGF1R/EGFR FISH						
Both positive versus other	1.76	0.99–3.13	0.05	1.46	0.83–1.47	0.15
IGF1R/EGFR IHC						
Both positive versus other	1.71	0.97–3.01	0.05	1.56	0.92–2.64	0.09
IGF1R/EGFR FISH/IHC						
Both positive versus other	2.84	1.38–5.84	0.005	2.32	1.20–4.44	0.01

HR hazard ratio, *CI* confidence interval, *EGFR* epidermal growth factor receptor, *IGF1R* insulin-like growth factor receptor 1, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry

[a] *IGF1R* FISH-positive = copy number gain (CNG), ≥4 gene copies in ≥10 % of tumor cells

[b] *EGFR* FISH-positive = copy number gain (CNG), ≥4 gene copies in ≥10 % of tumor cells

Table 4
Prognostic effect of studied parameters on DFS—multivariate analysis

Variable	Disease-free survival		
	HR	**95 % CI**	***P* value**
IGF1R/EGFR FISH			
Both positive versus other groups	1.44	0.61–3.40	0.40
IGF1R/EGFR IHC			
Both positive versus other groups	1.36	0.49–3.75	0.54
IGF1R/EGFR FISH/IHC			
Both positive versus other	4.08	1.34–12.39	0.01
TNM stage			
II–III versus I	4.77	1.72–13.21	0.003

HR hazard ratio, *CI* confidence interval, *EGFR* epidermal growth factor receptor, *IGF1R* insulin-like growth factor receptor 1, *FISH* fluorescence in situ hybridization, *IHC* immunohistochemistry