# Ligands of Epidermal Growth Factor Receptor and the Insulin-Like Growth Factor Family as Serum Biomarkers for Response to Epidermal Growth Factor Receptor Inhibitors in Patients with Advanced Non-small Cell Lung Cancer

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**Introduction:** The selection of patients with non-small cell lung cancer (NSCLC) for epidermal growth factor receptor (EGFR) inhibitor (EGFR-tyrosine kinase inhibitors [TKIs]) therapy is suboptimal as tumor tissue is often unavailable. Ligands of EGFR, transforming growth factor-alpha (TGFa) and amphiregulin (ARG), and the insulin-like growth factor (IGF) family have been associated with resistance to EGFR-TKIs. The aim of our study was to explore whether concentrations of these factors measured in serum were predictive of response to EGFR-TKIs.

**Methods:** We assessed serum levels of marker candidates using enzyme-linked immunosorbent (TGFa and ARG) and chemiluminescent (IGF1 and IGF-binding protein-3) assays in 61 patients with advanced NSCLC treated with EGFR-TKIs and 63 matched advanced NSCLC control patients without EGFR-TKIs treatment. We dichotomized marker levels at the 20th, 50th, or 80th percentile and evaluated whether the effect of EGFR-TKIs treatment on disease-specific survival (DSS) differed by marker level based on multivariate proportional hazards regression with an interaction term.

**Results:** The effect of EGFR-TKIs treatment on DSS showed a significant difference by TGFa and ARG (interaction $p = 0.046$ and $p = 0.004$, respectively). Low concentrations of TGFa and high concentrations of ARG were associated with a better DSS in EGFR-TKIs patients compared with control patients. Patients with high concentrations of IGF-binding protein-3 had significantly longer DSS, independent of treatment (hazard ratio: 0.60 per 1 mg/liter, 95% confidence interval: 0.46–0.79).

**Conclusion:** Our results suggest that concentrations of TGFa and ARG measured in serum are predictive of EGFR-TKI response. The combination of these two biomarkers could be of value in the process of selecting patients for treatment with EGFR-TKIs.

**Key Words:** Non-small cell lung cancer, EGFR tyrosine kinase inhibitors, EGFR ligands, Insulin-like growth factor, Serum biomarkers.

(*J Thorac Oncol.* 2010;5: 1939–1948)

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality worldwide. Small molecule inhibitors competing with the adenosine triphosphate binding site of the epidermal growth factor receptor (EGFR) tyrosine kinase such as erlotinib and gefitinib (EGFR-tyrosine kinase inhibitors [TKIs]) have proven their efficacy in the treatment of NSCLC.[1–4] These orally administered EGFR-TKIs showed rapid tumor responses and improvements in quality of life in patients with advanced NSCLC who were irresponsive to platinum-based chemotherapy. Unfortunately, the response rates of erlotinib and gefitinib are low in unselected patients with NSCLC, and many studies tested EGFR for its predictive potential. EGFR mutations and amplification of EGFR were found to be predictive of response to EGFR-TKIs with response rates of up to 70%.[5–11] Nevertheless, there are several reasons why selection based on EGFR mutation or EGFR amplification status alone might not suffice. First, stabilization of disease has not been correlated with the presence of EGFR mutations or amplification, although this treatment effect is considered beneficial in the management of NSCLC.[6,12] Second, no mutations were identified in 10 to 20% of patients with a partial response to gefitinib.([5,6,13–16]) Finally, to select patients based on EGFR mutations or amplification, tumor tissue is required, which is often unavailable. Identifying new methods to select patients likely to respond to EGFR-TKIs, therefore, remains important.

Many studies have tested ligands of EGFR in relationship to EGFR-TKI response. Of the EGFR-specific ligands,

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Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/10/0512-1939

transforming growth factor-alpha (TGFa) and amphiregulin (ARG) have been studied intensively in vitro and in patients in relationship to EGFR-TKI treatment.[17–21] Gene expression microarray studies showed that both TGFa and ARG were overexpressed in tumors from patients with NSCLC not responding to gefitinib.[17] Subsequently, Ishikawa et al. and Masago et al.[18,21] found that serum measurements of TGFa and ARG were predictive of overall survival and tumor response in patients with advanced NSCLC treated with gefitinib. Nevertheless, because a control group was missing, differentiation between therapy benefit and survival benefit remains unclear.

In addition to ligands of EGFR, insulin-like growth factor (IGF) receptor type 1 (IGF1R) has been implicated in resistance to EGFR-TKI treatment.[22–25] Cross-talk between IGF1R and EGFR has been reported, and overexpression of IGF1R has been correlated with decreased efficacy of EGFR targeting in a glioblastoma model.[22] IGF1R is mainly activated by its ligand IGF1.[26–28] The bioavailability of IGF1 is regulated by a family of IGF-binding proteins (IGFBPs), particularly IGFBP3, a major serum binding protein for IGF1.[26–28] Consequently, the IGF1:IGFBP3 ratio is commonly used as readout for the bioactivity of IGF1.[26–31]

As IGF1R is activated by IGF1, whose bioavailability is regulated by IGFBP3, we hypothesize that serum concentrations of IGF1, IGFBP3, or the IGF1:IGFBP3 ratio might be predictive of response to EGFR-TKIs, in addition to levels of TGFa and ARG. The aim of this study, therefore, was to explore whether concentrations of TGFa, ARG, IGF1, or IGFBP3 measured in serum of patients with advanced NSCLC were predictive of response to EGFR-TKIs. We assessed serum levels of these candidate markers in patients treated with EGFR-TKI and a matched control group of untreated patients. We evaluated whether the effect of EGFR-TKI treatment on disease-specific survival (DSS) differed by marker level based on multivariate proportional hazards regression with an interaction term.

## PATIENTS AND METHODS

### Patient Selection

In this retrospective study, we studied patients with advanced NSCLC who had been enrolled between 2001 and 2005 in an Expanded Access Program. Patients had been treated on a compassionate use basis with gefitinib or erlotinib (EGFR-TKI) at the Netherlands Cancer Institute (NKI). Patients without brain metastases were eligible for this program in case of no response to conventional chemotherapy or unavailability of alternative treatment options.[11] Written informed consent was obtained from all patients. The inclusion criteria for this study consisted of EGFR-TKI treatment for more than 14 days and availability of serum collected within 100 days before start of treatment. To differentiate between prognostic and predictive value of the ligands, a control group of patients was identified, not treated with EGFR-TKIs and matched for gender, age, and histology to the EGFR-TKI treated patients. Controls were diagnosed between 1995 and 2006. In total, 124 patients were included in this study, which was designed following the REMARK guidelines.[32] This translational study was approved by the Institutional Review board of the NKI.

### Treatment

Patients receiving gefitinib were treated with a daily dose of 250 mg. Erlotinib was dosed at 150 mg daily, dose reductions to 50 mg occurred due to severe toxicity ($n = 2$). Treatment with EGFR-TKIs was continued until disease progression or the occurrence of a serious adverse event.

### Serum Analyses

Serum was stored at −30°C. TGFa concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA; ELISA-kit, DTGA00, R&D systems, MN) according to manufacturer's instruction. A standard curve was prepared for each plate using human recombinant TGFa diluted in assay diluent (provided by the ELISA-kit) for reference. The minimum detection limit of the assay was 3.0 ng/liter. For detection of ARG, an ELISA research kit (DY262, R&D systems) was used according to manufacturer's instructions (validation experiments Appendix). In short, 96-well clear flexible microtiter plates (DY990, R&D systems) were coated with 2 mg/liter capturing antibody overnight. After washing with phosphate-buffered saline (PBS) (pH, 7.4) containing 0.05% Tween20, wells were blocked with 300 $\mu$l reagent diluent (DY995, R&D systems) for 1 hour and washed again. Subsequently, wells were incubated for 2 hours with serum samples. After washing, the wells were incubated for 2 hours with 100 $\mu$g/liter biotinylated goat antihuman ARG (DY262, R&D Systems) followed by washing and a 20-minute reaction with streptavidin-conjugated horseradish-peroxidase. After washing, 100 $\mu$l substrate solution (DY 999, R&D systems) was added for 20 minutes. The color reaction was stopped by adding 50 $\mu$l 2 N sulfuric acid. Color intensity was determined at a wavelength of 450 nm with reference wavelength of 540 nm. The standard curve was drawn for each plate using recombinant ARG diluted in 10% fetal calf serum in PBS. Minimum detection limit of the assays for serum ARG was 3.0 ng/liter (validation experiments Appendix).

For the concentrations of IGF1 and IGFBP3, fully automated chemiluminescent immunometric technology was used (Immulite 2000, Siemens Medical Solutions Diagnostics). To calculate the IGF1:IGFBP3 ratio, IGFBP3 (mg/liter) was converted to molar concentrations with a conversion factor of 34.78. The normal range provided by the manufacturer for the age group 61 to 65 years was 9.75–27.56 nmol/liter for IGF1 and 3.2 to 6.6 mg/liter for IGFBP3.

### Statistical Analyses

DSS was defined as the time from start of treatment with the EGFR-TKI to death of disease or end of follow-up. Patients who were still alive at the end of follow-up, lost to follow-up, or who died due to non-NSCLC causes were censored at that time. For the control group, DSS was calculated from the start of first treatment (radiotherapy or chemotherapy) in the NKI or date of first visit if no treatment was started within the NKI.

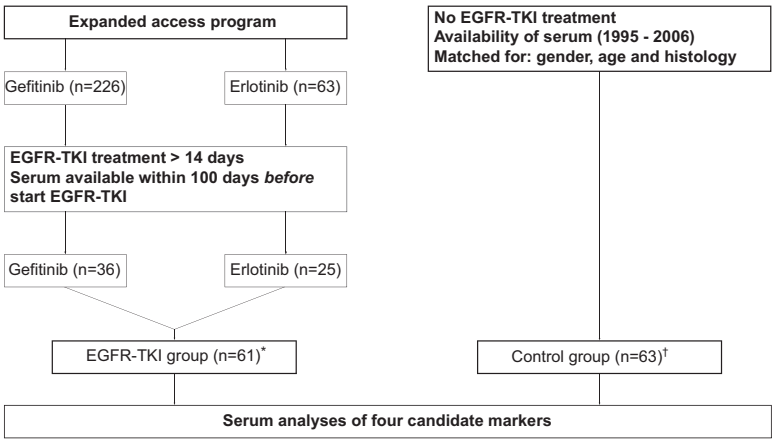


**FIGURE 1.** Flow chart. Flow chart of patient selection. *Included in epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) group: EGFR-TKIs treatment 12 days ($n = 1$). †Included control group: 9 days of EGFR-TKIs treatment before death of disease ($n = 1$).

Differences in clinicopathological variables between the EGFR-TKI group and the control group were tested using Fisher's exact test tests, Kruskal-Wallis tests, exact Mann-Whitney *U* tests, and Student's *t* tests. Patients with missing values for a variable were excluded from analyses involving that variable. Correlation between levels of candidate markers and age, and serum storage time were calculated using Spearman's rank and Pearson correlation analyses. Associations between marker candidates and gender, stage (III, IV), smoking status (smoker, nonsmoker), performance status (World Health Organization 0–1, 2–3), and histology (adenocarcinoma, other) were investigated by means of Mann-Whitney *U* tests and Student's *t* tests.

We evaluated whether the effect of EGFR-TKI treatment on DSS, expressed as hazard ratio (HR), differed by candidate marker level based on multivariate proportional hazards regression with an interaction term, adjusting for potential confounders (gender, smoking, prior chemotherapy, stage, and histology). Instead of searching for an optimal cutoff, we a priori selected three alternative cutoffs (20th, 50th, or 80th percentile) to dichotomize marker levels. For all cutoffs, interaction terms were calculated using the multivariate model; the cutoff with the largest absolute interaction coefficient was evaluated for the proportional hazards assumption by adding interaction terms between the time-dependent natural logarithm of follow-up time plus one and the interaction between treatment and candidate markers ($p$ value between 0.33 and 0.92). No evidence for nonproportional hazards was found, and the cutoffs of these candidate markers were further evaluated. Direct adjusted survival curves based on a multivariate Cox regression model including prior chemotherapy, performance status, and smoking and stratified for treatment were calculated using a SAS macro by Zhang et al.[33] All other calculations were performed using the statistical package SPSS (version 15.0 for Windows).

## RESULTS

Sixty-one EGFR-TKI treated patients were eligible for this study (Figure 1). The control group consisted of 63 patients, matched for gender, age, and histology as presented together with other clinical and pathologic characteristics in Table 1. Patients treated with EGFR-TKIs were significantly more often never smokers ($p = 0.03$), had more stage IIIa/b disease ($p < 0.01$, Table 1), and presented more often with a poor performance status ($p = 0.05$). Furthermore, this group had significantly more often received prior chemotherapy ($p < 0.01$, Table 1). All four marker candidates could be tested in all 124 patients. IGF1, IGFBP3, and the IGF1:IGFBP3 ratio showed a Gaussian distribution, whereas TGFa and ARG were lognormal distributed. Factors were not correlated with serum storage time (Supplementary Figure 1, http://links.lww.com/JTO/A48), age, stage, histology (data not shown), or prior chemotherapy (Supplementary Figure 2, http://links.lww.com/JTO/A49). Women had significantly higher concentrations of TGFa (Mann Whitney *U*, $p = 0.03$), whereas patients with higher levels of IGFBP3 were significantly more often nonsmokers (Student's *t* test, $p = 0.03$) and had a better performance status (Student's *t* test, $p = 0.005$, Supplementary Figure 2).

Smoking, prior chemotherapy, and performance status were significantly associated with DSS (Supplementary Table 1, http://links.lww.com/JTO/A52) and were, therefore, included in multivariate analyses as potential confounders. The 20% cutoff yielded the largest interaction coefficient in the multivariate model for IGF1 and IGFBP3. Similarly, the median cutoff for ARG and the 80% cutoff for TGFa and IGF1:IGFBP3 ratio proved to be the largest (data not shown). These cutoffs were selected for further analyses.

The effect of EGFR-TKI treatment differed significantly between patients with high and low levels of TGFa (interaction $p = 0.05$). Among patients with low levels of TGFa, the risk of death was almost twofold significantly decreased after EGFR-TKI treatment compared with the control group (Figure 2*A*, HR: 0.55, 95% confidence interval [CI]: 0.32–0.96, $p = 0.04$, Table 2), whereas in patients with high levels of TGFa, this risk was nonsignificantly increased (Figure 2*A*, HR: 1.51, 95% CI: 0.58–3.91, $p = 0.40$, Table 2).

ARG interacted significantly with the effect of EGFR-TKI treatment on DSS (interaction $p < 0.01$). In patients with high levels of ARG, EGFR-TKI treatment significantly decreased the risk of death more than threefold compared with the control patients (Figure 2*B*, HR: 0.31, 95% CI: 0.15–0.63, $p < 0.01$, Table 2). No significant difference between both

 

**TABLE 1.** Patient Characteristics

| | Total, *n* (%) | Treatment: EGFR-TKI Treated Patients, *n* (%) | Treatment: Control Patients, *n* (%) | *p* |
|---|---|---|---|---|
| Total | 124 (100) | 61 (100) | 63 (100) | |
| Gender | | | | |
| Male | 68 (55) | 33 (54) | 35 (56) | 1.000[a] |
| Female | 56 (45) | 28 (46) | 28 (44) | |
| Age at treatment | | | | |
| <65 yr | 73 (59) | 34 (56) | 39 (62) | 0.584[a] |
| ≥65 yr | 51 (41) | 27 (44) | 24 (38) | |
| Smoking | | | | |
| Never smoked | 16 (13) | 12 (20) | 4 (6) | 0.029[a] |
| (Former) smoker | 102 (82) | 44 (72) | 58 (92) | |
| Unknown | 6 (5) | 5 (8) | 1 (2) | |
| Performance status | | | | |
| 0–1 | 86 (69) | 37 (61) | 49 (78) | 0.052[a] |
| 2–3 | 29 (23) | 19 (31) | 10 (16) | |
| Unknown | 9 (7) | 5 (8) | 4 (6) | |
| Histology | | | | |
| Large cell | 31 (25) | 13 (21) | 18 (29) | 0.673[b] |
| Squamous cell | 20 (16) | 9 (15) | 11 (17) | |
| Adenocarcinoma | 59 (48) | 32 (52) | 27 (43) | |
| Bronchoalveolar carcinoma | 6 (5) | 3 (5) | 3 (5) | |
| Unknown | 8 (6) | 4 (7) | 4 (6) | |
| Stage of disease | | | | |
| IIIa/IIIb | 20 (16) | 16 (26) | 4 (6) | 0.003[a] |
| IV | 104 (84) | 45 (74) | 59 (94) | |
| TNM, T status | | | | |
| T1–T2 | 46 (37) | 16 (26) | 30 (48) | 0.356[a] |
| T3–T4 | 34 (27) | 16 (26) | 18 (29) | |
| Tx and unknown | 44 (35) | 29 (48) | 15 (24) | |
| TNM, N status | | | | |
| N0–N1 | 11 (9) | 6 (10) | 5 (8) | 0.310[a] |
| N2–N3 | 61 (49) | 21 (34) | 40 (63) | |
| Nx and unknown | 52 (42) | 34 (56) | 18 (29) | |
| Prior chemotherapy | | | | |
| No | 86 (69) | 29 (48) | 57 (90) | <0.001[a] |
| Yes | 38 (31) | 32 (52) | 6 (10) | |
| Disease-specific survival | | | | |
| Range (d) | 12–1638 | 16–1638 | 12–865 | |
| Median (d) | 151 | 115 | 171 | 0.156[c] |
| TGFa | | | | |
| Range (ng/liter) | 0–135.0 | 0–135.0 | 0–73.8 | |
| Median (ng/liter) | 10.5 | 11.1 | 9.6 | 0.383[c] |
| ARG | | | | |
| Range (ng/liter) | 0–2034.0 | 0–2034.0 | 0–1143.2 | |
| Median (ng/liter) | 9.5 | 8.7 | 9.8 | 0.476[c] |
| IGF1 | | | | |
| Range (nmol/liter) | 2.74–43.07 | 4.61–39.51 | 2.74–43.07 | |
| Median (nmol/liter) | 17.55 | 17.35 | 17.60 | 0.815[d] |
| IGFBP3 | | | | |
| Range (mg/liter) | 1.46–6.24 | 1.46–5.81 | 1.59–6.24 | |
| Median (mg/liter) | 3.92 | 3.93 | 3.91 | 0.800[d] |

[a] *p* values calculated using Fisher's exact test.
[b] *p* values calculated using Exact Kruskal-Wallis test.
[c] *p* values calculated using Exact Mann-Whitney *U* test.
[d] *p* values calculated using Student's *t* test.
TGFa, transforming growth factor-alpha; ARG, amphiregulin; IGF1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor binding protein-3; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; TNM, tumor, node, metastasis.

groups was found in patients with low ARG levels (Figure 2*B*, HR: 1.14, 95% CI: 0.59–2.17, $p = 0.70$).

We observed a borderline significant benefit from EGFR-TKI treatment (interaction $p = 0.09$) among patients, whose IGF1 levels exceeded the 20th percentile (Figure 2*C*, HR: 0.48, 95% CI: 0.27–0.83, $p < 0.01$, Table 2) but not among others (Figure 2*C*, HR: 1.27, 95% CI: 0.45–3.53, $p = 0.65$, Table 2).

EGFR-TKI treatment effects differed less strongly by levels of IGF1:IGFBP3 ratio or IGFBP3, and homogeneity was not rejected (Supplementary Table 2, http://links.lww.com/JTO/A53). Because IGFBP3 was significantly associated with performance status, a strong prognostic factor, we studied whether IGFBP3 was independently associated with DSS, regardless of treatment. We evaluated this factor as a continuous variable while adjusting for treatment. We observed a 40% decrease in risk per 1 mg/liter IGFBP3 increase (HR: 0.60, 95% CI: 0.46–0.79, $p < 0.001$, Table 3).

As the EGFR-TKI treated patients and the control patients were imbalanced for two important prognostic factors (i.e., prior chemotherapy and performance status), we subsequently repeated the analyses of TGFa, ARG, and IGF1 in patients who had not received prior chemotherapy only (number of EGFR-TKI treated patients and controls reduced from 61 and 63 to 29 and 57, respectively) and in patients with a good performance status only (number of EGFR-TKI treated patients and controls reduced to 37 and 49, respectively). We observed very similar patterns of treatment-related HRs by serum marker levels (Supplementary Table 3, http://links.lww.com/JTO/A54).

## DISCUSSION

In this study, we determined whether concentrations of TGFa, ARG, IGF1, IGFBP3, or the IGF1:IGFBP3 ratio measured in serum were predictive of EGFR-TKI response by comparing the DSS in patients treated with EGFR-TKIs compared with matched EGFR-TKIs untreated patients. We observed that patients with low serum concentrations of TGFa or high serum concentrations of ARG significantly benefited from EGFR-TKI treatment, whereas there was no such evidence for patients with higher and lower respective

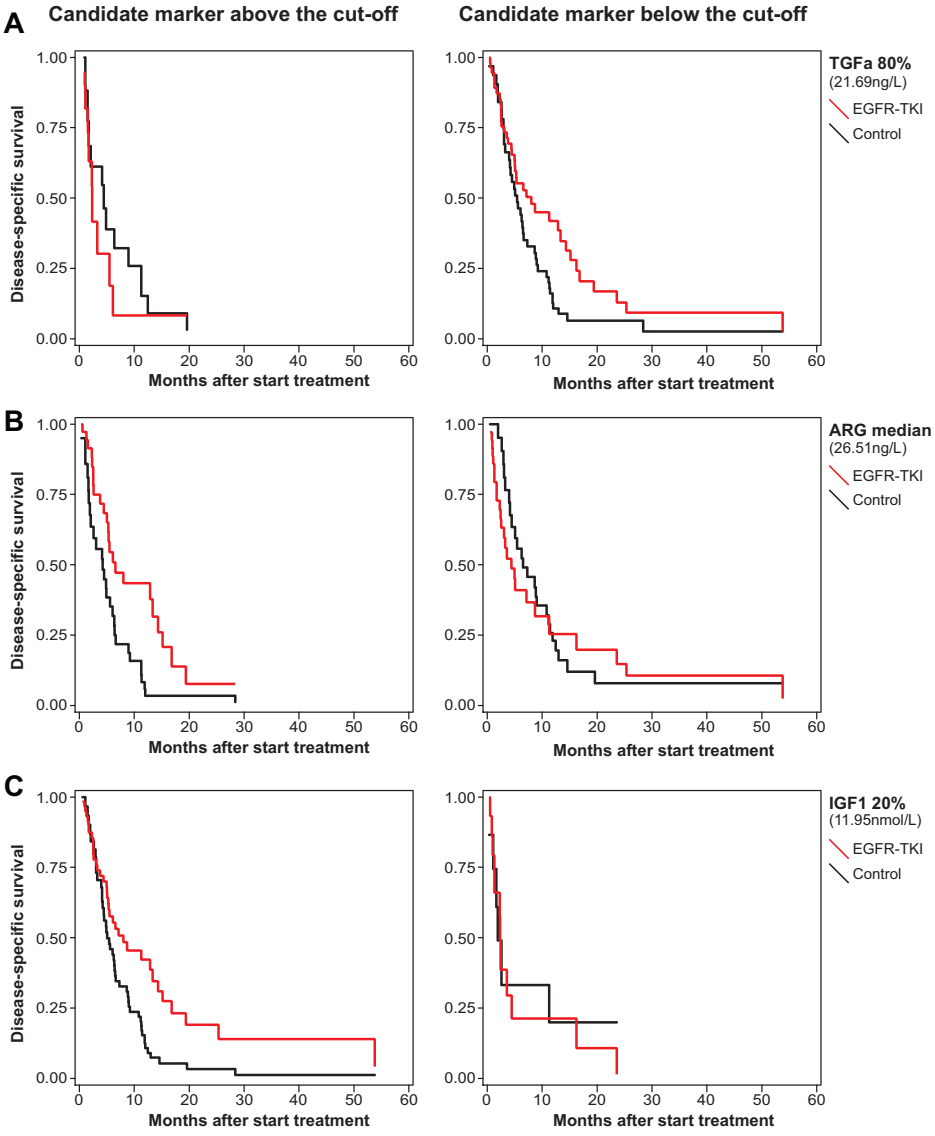


**FIGURE 2.** Relationship of candidate markers with outcome in epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) treated patients and their matched controls. Direct adjusted survival curves based on a multivariate Cox regression model including prior chemotherapy, performance status, and smoking and stratified for treatment. *A,* Disease-specific survival (DSS) according to transforming growth factor-alpha (TGFa) levels above and below 80th percentile among patients treated with EGFR-TKIs and their matched controls. *B,* DSS according to amphiregulin (ARG) levels above and below the median among patients treated with EGFR-TKIs and their matched controls. *C,* DSS according to insulin-like growth factor (IGF)1 above and below 20th percentile among patients treated with EGFR-TKIs and their matched controls.

values. Whether serum concentrations of IGF1 are predictive of response to EGFR-TKIs remains unclear, although our data suggest this may be the case.

Our study confirms previous findings in which patients with high levels of TGFa do not benefit from EGFR-TKI treatment. More specifically, two Japanese groups found high levels of both TGFa and ARG to be associated with progressive disease and a worse overall survival after gefitinib treatment in patients with NSCLC.[18,21] Because both studies lacked a control group, it was impossible to determine whether this difference in survival was due to therapy (prediction) or to tumor features (prognosis). Furthermore, both studies looked for an optimal threshold using internal data. Our results regarding ARG do not correspond with the findings mentioned earlier. Instead of a worse DSS, we observed a statistically significant benefit from EGFR-TKIs in patients with high-serum ARG levels compared with the control group but not among patients with low levels; this difference was statistically significant. This different direction of ARG has previously been observed. In vitro, primarily gefitinib-sensitive head and neck cancer cell lines were shown to secrete ARG.[34] Yonesaka et al.[35] showed that high-ARG expression as assessed by immunohistochemistry was associated with stable disease in patients with NSCLC treated with erlotinib or gefitinib, whereas low expression was associated with progressive disease. Furthermore, in patients with colorectal cancer treated with cetuximab (monoclonal antibody against EGFR), high-ARG expression by microarray analyses was associated with disease control (response and stable disease) and longer progression-free survival.[36,37] The discrepancy between our findings of ARG and both Japanese serum studies could be explained by the different ethnicity of the study populations. Patients with NSCLC from Asian origin are known to harbor EGFR mutations more frequently.[38,39] Subsequently, it is thought that these tumors may have a distinct pathogenesis in which

**TABLE 2.** Multivariate Cox Proportional-Hazard Regression Analysis of the Risk of Death (Disease-Specific Survival) per Factor of Interest

| | TGFa | | | | ARG[a] | | | | IGF1[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variables | No. of Events/No. of Patients | Hazard Ratio | 95% CI | *p* | No. of Events/No. of Patients | Hazard Ratio | 95% CI | *p* | No. of Events/No. of Patients | Hazard Ratio | 95% CI | *p* |
| Smoking | | | | | | | | | | | | |
| (Former) smoker | 95/102 | 1.00 | | | 95/102 | 1.00 | | | 95/102 | 1.00 | | |
| Never smoked | 13/16 | 0.76 | 0.38–1.52 | 0.440 | 13/16 | 0.59 | 0.29–1.16 | 0.127 | 13/16 | 0.59 | 0.30–1.16 | 0.123 |
| Prior chemotherapy | | | | | | | | | | | | |
| No | 79/86 | 1.00 | | | 79/86 | 1.00 | | | 79/86 | 1.00 | | |
| Yes | 35/38 | 2.97 | 1.68–5.23 | <0.001 | 35/38 | 3.47 | 1.98–6.09 | <0.001 | 35/38 | 3.78 | 2.17–6.61 | <0.001 |
| Performance status | | | | | | | | | | | | |
| 0–1 | 78/86 | 1.00 | | | 78/86 | 1.00 | | | 78/86 | 1.00 | | |
| 2–3 | 27/29 | 2.51 | 1.53–4.10 | <0.001 | 27/29 | 2.97 | 1.76–5.00 | <0.001 | 27/29 | 2.43 | 1.50–3.94 | <0.001 |
| Ligand concentration | | | | | | | | | | | | |
| Low | 90/99 | 1.00 | | | 56/62 | 1.00 | | | 22/25 | 1.00 | | |
| High | 24/25 | 1.41 | 0.74–2.68 | 0.296 | 58/62 | 1.88 | 1.07–3.30 | 0.029 | 92/99 | 0.73 | 0.31–175 | 0.481 |
| Ligand concentration high | | | | | | | | | | | | |
| Control | 13/14 | 1.00 | | | 31/33 | 1.00 | | | 49/53 | 1.00 | | |
| EGFR-TKI treatment | 11/11 | 1.51[c] | 0.58–3.91 | 0.399 | 27/29 | 0.31[a] | 0.15–0.63 | 0.001 | 43/46 | 0.48[b] | 0.27–0.83 | 0.009 |
| Ligand concentration low | | | | | | | | | | | | |
| Control | 44/49 | 1.00 | | | 26/30 | 1.00 | | | 8/10 | 1.00 | | |
| EGFR-TKI treatment | 46/50 | 0.55[c] | 0.32–0.96 | 0.035 | 30/32 | 1.14[a] | 0.59–2.17 | 0.702 | 14/15 | 1.27[b] | 0.45–3.53 | 0.654 |

[a] Homogeneity of both hazard ratios was rejected based on an interaction term with $p = 0.004$ (ARG: cutoff at median [ligand low ≤9.49 ng/liter; ligand high >9.49 ng/liter]).
[b] Homogeneity of both hazard ratios was not rejected based on an interaction term with $p = 0.087$ (IGF1: cutoff at 20% [ligand low ≤11.95 nmol/liter; ligand high >11.95 nmol/liter]).
[c] Homogeneity of both hazard ratios was rejected based on an interaction term with $p = 0.046$ (TGFa: cutoff at 80% [ligand low ≤21.69 ng/liter; ligand high >21.69 ng/liter]).
TGFa, transforming growth factor-alpha; ARG, amphiregulin; IGF1, insulin-like growth factor-1; CI, confidence interval; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor.

**TABLE 3.** Multivariate Cox Proportional-Hazard Regression Analysis of Death (Disease-Specific Survival) According to IGFBP3, Independent of Treatment

| Variables | No. of Events | Hazard Ratio | 95% CI | *p* |
|---|---|---|---|---|
| Smoking | | | | |
| (Former) smoker | 95 | 1.00 | | |
| Never smoked | 13 | 0.88 | 0.43–1.79 | 0.726 |
| Prior chemotherapy | | | | |
| No | 79 | 1.00 | | |
| Yes | 35 | 3.66 | 2.12–6.31 | <0.001 |
| Performance status | | | | |
| 0–1 | 78 | 1.00 | | |
| 2–3 | 27 | 2.04 | 1.26–3.31 | 0.004 |
| Ligand concentration | | | | |
| IGFBP3 per 1 mg/liter | 114 | 0.60 | 0.46–0.79 | <0.001 |
| Treatment | | | | |
| Control | 57 | 1.00 | | |
| EGFR-TKI treatment | 57 | 0.56 | 0.34–0.94 | 0.027 |

IGFBP3, insulin-like growth factor binding protein-3; CI, confidence interval; IGFBP3, insulin-like growth factor binding protein-3; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor.

tumors become completely dependent on the EGFR-pathway through mutations.[38,39] We speculate that high-ARG levels might represent a different pathogenic pathway in patients with NSCLC of white origin that leads to similar dependence on this pathway, albeit less complete. As a consequence, treatment of patients with high-ARG levels with EGFR-TKIs might lead to cell cycle arrest (i.e., stable disease) but not, as with EGFR mutations, to apoptosis (i.e., partial response and complete response) as has been described in cell line studies.[35,40] Furthermore, this could explain the rapid progression of disease ("tumor flare") after discontinuation of EGFR-TKIs[41] as has also been documented in gastrointestinal stromal tumors after withdrawal of the BCR-ABL TKI imatinib.[42]

In addition to ligands of EGFR, we studied components of the IGF1R-pathway. To the best of our knowledge, this is the first study evaluating the relationship between benefit from EGFR-TKIs and IGF1, IGFBP3, and the IGF1:IGFBP3 ratio. IGF1 is known to stimulate cell proliferation and to inhibit apoptosis by binding to IGF1R, a receptor tyrosine kinase.[27,43] Activation of IGF1R leads to signaling of the proliferative Ras-Raf-MAPK pathway and of the prosurvival phosphoinositol-3 kinase (PI3K)-Akt pathway.[27,43] Failure to down-regulate Akt characterizes insensitivity to EGFR-TKIs and has been shown to be mediated through PI3K signaling by IGF1R.[22–25,44,45] Furthermore, the combined use of anti-IGF1R and EGFR-TKIs has been shown to be more effective in vitro and in vivo than a single-agent approach.[25,46] In our study, patients with especially low levels of IGF1 did not seem to benefit from EGFR-TKIs. We speculate that truly low levels of IGF1 are the result of a negative-feedback loop

as the consequence of a constitutively active IGF1R-pathway in these patients. This negative regulation has not been documented but the opposite has in centennials, in whom a mutation in IGF1R was associated with reduced activity of IGF1R and high serum levels of IGF1.[47] Consequently, tumors of patients with low IGF1 may be able to sustain the prosurvival signals through Akt activation by depending on the IGF1R pathway for PI3K signaling instead of the EGFR pathway. The only study reporting on the relationship between the IGF1R pathway, in the form of IGF1R expression assessed by immunohistochemistry, and response to gefitinib showed no association with gefitinib resistance.[48] These results cannot directly be compared with our results, as we studied different components of the IGF1R pathway using different techniques.

Although studying prognostic factors was not an objective of our study, we found that IGFBP3 serum levels predicted DSS in patients with advanced NSCLC regardless of treatment. The protective effect of high-IGFBP3 levels could be explained not only by its ability to decrease the mitogenic action and inhibit the antiapoptotic effect of IGF1 but also by its IGF-independent inhibitory effect on cell growth.[26,28,49–51] Our results confirm findings of a previous study in which high-IGFBP3 plasma levels were associated with a significantly longer overall survival in patients with advanced NSCLC treated with irinotecan and cisplatin.[52] Furthermore, reduced IGFBP3 expression assessed by immunohistochemistry was associated with shorter DSS in patients with stage I NSCLC.[53] Use of serum IGFBP3 as a prognostic marker in stage I/II NSCLC would be appealing and warrants further study.

Our study was relatively small, and therefore, our evaluation could not attain the rigor of a training/validation study or a large randomized controlled trial. Furthermore, the EGFR-TKI group was not entirely homogeneous, as patients treated with gefitinib or erlotinib were included. Nevertheless, these agents act by similar mechanisms, and sensitivity analyses by drug, although based on small numbers, were largely consistent with the combined results. Therefore, as this is an exploratory study of candidate markers, we believe that we have assembled a sufficiently large data set to discover potentially predictive markers and have limited the number of cutoffs to avoid substantial overfitting. The retrospective nature of our study did not allow for a standardized measure for progression-free survival, which is considered to be more directly linked to response. This was due to the fact that imaging was not performed at set intervals. Nevertheless, using data of documented radiologic progression (or if unavailable, data of clinical progression) for progression-free survival resulted in very similar patterns of HRs for TGFa, ARG, or IGF1 (data not shown). Furthermore, until now, known markers predicting for response and progression-free survival, such as EGFR mutations, have not been shown to predict for an overall or DSS benefit after EGFR-TKIs in randomized controlled trials,[9,15,54] whereas this remains the ultimate goal in patient treatment. We, therefore, consider the presented data adequate to inform us whether and for which markers further studies are indicated. Although EGFR-TKIs response depends partly on presence of EGFR mutations or EGFR amplifications, the two markers we identified could greatly improve the prediction of EGFR-TKIs response in two ways. First, not all patients who respond to EGFR-TKIs are identified by EGFR mutations or amplifications, resulting in withholding patients with advanced NSCLC potential survival benefit from EGFR-TKIs (undertreatment). In this mostly palliative setting with limited treatment options, undertreatment would seem worse than overtreatment, which can be justified to some extent. Consequently, limiting undertreatment by identification of a subgroup of patients resistant to EGFR-TKIs seems just as or even more important. Second, serum measurements do not require tumor tissue, which is often unavailable, therefore greatly facilitating the prediction.

In conclusion, by using a matched control group, we were able to evaluate EGFR-TKI treatment benefit by marker level. Our results suggest that concentrations of TGFa and ARG measured in serum are predictive of EGFR-TKI response. This is the first study in patients with NSCLC of white origin in which this effect was evaluated and observed. The combination of these two biomarkers could be of value in the process of selecting patients for treatment with EGFR-TKI. The optimal cutoff for TGFa and ARG, the use of the combination of these two biomarkers as a predictive marker, and its additive value to known clinical predictors for EGFR-TKI resistance warrants further investigation and validation, preferably in a prospective randomized controlled trial.

## ACKNOWLEDGMENTS

*Supported by a cancer patient's family.*

*The authors thank Dorothe Linders and Bastiaan Evers for their technical assistance, and Christiaan Klijn, Karin Beelen, and Chiel de Bruin for their critical appraisal of the manuscript.*

## APPENDIX: VALIDATION AMPHIREGULIN DY262, R&D SYSTEMS

### Results

All samples were analyzed in duplicate on the same plate. Minimum detection limit of the assays for serum ARG was 3.0 ng/liter. The interassay variability of this ELISA was 20% (concentrations in the low range) and 8.5% (concentrations in the high range). The results shown earlier are a representation of all the validation tests we have run. Using reagents diluent of R&D, the recovery linearity of the assay ranged between 49% and 121%, with an average of 105% of the expected concentration. Nevertheless, when samples were diluted using 10% FCS/PBS, no signal intensity of endogenous ARG could be measured for 15 of 18 samples, whereas in three samples, a signal was present (data not shown). The recovery was determined by spiking in 200 ng/liter of recombinant ARG into serum samples. The range was between 76% and 102%, with an average of 93.6% of the expected concentration when reagents diluent of R&D was used.

### Conclusion

Although the recovery linearity of reagents diluent had a wide range, this was partly due to the samples with high

 

**TABLE A1.** Recovery Linearity of Amphiregulin: Dilution in Reagents Diluent of R&D

| Samples | Dilution | Measured Concentration (ng/Liter)[a] | Calculated Concentration (ng/Liter) | Recovery Linearity (%) |
|---|---|---|---|---|
| 32423 | 0× | >1000 | >1000 | NA |
| 32423 | 3× | 562.6 | 1687.7 | NA |
| 32423 | 5× | 330.4 | 1652.0 | NA |
| 9184 | 0× | 220.9 | 220.9 | 100.0 |
| 9184 | 5× | 25.6 | 127.9 | 57.9 |
| 9184 | 10× | 10.8 | 107.7 | 48.8 |
| 28489 | 0× | 117.3 | 117.3 | 100.0 |
| 28489 | 3× | 43.3 | 130.0 | 110.8 |
| 28489 | 5× | 25.9 | 129.7 | 110.6 |
| 24227 | 0× | 69.9 | 69.9 | 100.0 |
| 24227 | 2× | 39.8 | 79.7 | 114.0 |
| 24227 | 4× | 18.1 | 72.3 | 103.4 |
| 24227 | 5× | 11.7 | 58.4 | 83.5 |
| 24227 | 8× | 10.1 | 80.9 | 115.7 |
| 24227 | 10× | 8.5 | 84.9 | 121.4 |
| 24227 | 16× | 4.0 | 64.2 | 91.8 |
| 24227 | 20× | 3.7 | 73.5 | 105.2 |
| 4529 | 0× | 23.3 | 23.3 | 100.0 |
| 4529 | 5× | <3.0 | Min | NA |
| 4529 | 10× | <3.0 | Min | NA |
| 21701 | 0× | 12.0 | 12.0 | 100.0 |
| 21701 | 3× | 4.22 | 12.7 | 106.0 |
| 21701 | 5× | <3.0 | Min | NA |
| 28362 | 0× | 11.9 | 11.9 | 100.0 |
| 28362 | 2× | 4.4 | 8.7 | 73.2 |
| 28362 | 4× | <3.0 | Min | NA |
| 28362 | 8× | <3.0 | Min | NA |
| 37107 | 0× | <3.0 | Min | NA |
| 37107 | 2× | <3.0 | Min | NA |
| 37107 | 4× | <3.0 | Min | NA |
| 37107 | 8× | <3.0 | Min | NA |
| 37107 | 16× | <3.0 | Min | NA |

[a] Detection limit: 3.0 ng/liter.
Min, minimum under detection limit; NA, not applicable.

concentrations of ARG (>220 ng/liter) in which low-recovery linearity percentages arose. Nevertheless, the second dilution step of these higher concentrated samples did seem to be linear with the first dilution step. The results with fetal calf serum dilutions were unexpected. We suspect that the endogenous "free" ARG is bound by binding proteins in the FCS. We did not perform any additional experiments to prove this hypothesis, as this was beyond the scope of our research. Given the above findings, we proceeded the serum analysis for ARG as described in the methods section and as described many times in literature.[55–60]

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**TABLE A2.** Recovery by Spiking 200 ng/liter Recombinant Amphiregulin in Undiluted and Diluted Serum Samples

| Samples | Dilution | Unspiked Concentration (ng/Liter)[a] | Expected Concentration (ng/Liter) | Observed Spiked Concentration (ng/Liter) | Recovery % |
|---|---|---|---|---|---|
| 9184 | 0× | 220.9 | 420.9 | 429.0 | 101.9 |
| 9184 | 5× | 25.6 | 225.6 | 227.1 | 100.7 |
| 9184 | 10× | 10.8 | 210.8 | 216.2 | 102.6 |
| 4529 | 0× | 23.3 | 223.3 | 214.9 | 96.2 |
| 4529 | 5× | <3.0 | 201.0 | 181.5 | 90.3 |
| 4529 | 10× | <3.0 | 200.0 | 199.2 | 99.6 |
| 7571 | 0× | <3.0 | 201.8 | 153.5 | 76.1 |
| 7571 | 5× | <3.0 | 200.0 | 167.7 | 83.8 |
| 7571 | 10× | <3.0 | 200.0 | 181.9 | 90.9 |

[a] Detection limit: 3.0 ng/liter.

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