

## Contrasted Outcomes to Gefitinib on Tumoral IGF1R Expression in Head and Neck Cancer Patients Receiving Postoperative Chemoradiation (GORTEC Trial 2004-02)

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### Abstract

**Purpose:** Intermediate/high-risk operated patients with head and neck cancer may benefit from the addition of EGF receptor (EGFR) inhibitor gefitinib to chemoradiation. This study was designed to assess improved outcomes and identify predictive biomarkers.

**Experimental Design:** Patients provided informed consent for tumor biomarker analyses and, when eligible, were further enrolled in the therapeutic CARISSA multicenter randomized phase II trial of postoperative irradiation with cisplatin + gefitinib (GORTEC 2004-02-NCT00169221).

**Results:** Seventy-nine patients were included in the biomarker study, whereas 27 did not meet prerequisites for randomization between gefitinib and placebo. Two-year disease-free survival (DFS) rate was 65.0% and did not differ between randomized patients treated with gefitinib or placebo ( $P = 0.85$ ). The similarity of DFS curves between nonrandomized patients ( $n = 27$ ), randomized patients without gefitinib ( $n = 27$ ), and randomized patients receiving gefitinib ( $n = 25$ ), and similar histoclinical parameter distributions for all groups, allowed us to conduct statistical analyses on the entire population. On multivariate analysis, elevated expression of PAK1 by Western blotting, CD31 and membranous insulin-like growth factor 1 receptor (IGF1R) both by immunohistochemistry was significantly associated with shorter DFS. There was a significant interaction between IGF1R and gefitinib. Gefitinib abolished the prognostic discriminative power of high IGF1R expression; patients with elevated IGF1R expression benefited from gefitinib whereas those with low IGF1R fared worse.

**Conclusion:** Gefitinib treatment affords no significant clinical benefit on DFS in an unselected population of patients with head and neck cancer. Our results point to the potential advantage of personalizing treatment for gefitinib based on tumoral IGF1R expression. This should foster confirmatory analyses in trials involving EGFR-targeting agents. *Clin Cancer Res*; 18(18); 5123–33. ©2012 AACR.

### Introduction

Surgery and radiotherapy are the mainstay of treatment in patients with head and neck cancer (HNSCC) with

locally advanced resectable disease. Cisplatin-based chemoradiation combinations have been administered since 2004 on the basis of level I evidence from the 2 large-scale

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### Translational Relevance

Intermediate/high-risk operated patients with head and neck cancer may benefit from the addition of EGF receptor (EGFR) inhibitors to chemoradiation. In a group of 79 patients, including 25 patients treated with gefitinib (GORTEC 2004-02-NCT00169221), the prognostic value of 28 tumor markers was examined. Biomarkers covered EGFR signaling, cross-talk, and tumor-stroma interactions. Elevated expression of PAK1 by Western blotting, CD31 and IGF1R, both by immunohistochemistry, was significantly associated with worse outcomes. A significant interaction was observed between insulin-like growth factor 1 receptor (IGF1R) and gefitinib; patients with elevated IGF1R expression benefited from gefitinib whereas those with low IGF1R fared worse. These findings indicate that EGFR tyrosine kinase inhibitors may not afford clinical benefit in unselected populations of patients with head and neck cancer and they point to the potential advantage of personalizing treatment for gefitinib based on tumoral IGF1R expression.

EORTC22931-RTOG9501 trials showing improved outcomes compared with postoperative radiation therapy alone (1) in high-risk HNSCCs. Despite that, 5-year relapse-free survival rates remain poor, and many patients die from locoregional disease progression. Increasing knowledge of molecular radiation biology spurred the development of rational strategies for combining radiotherapy with molecular therapeutics. Preclinical investigations on EGF receptor (EGFR; refs. 2, 3) revealed its role in carcinogenesis (4) and tumor progression (5). Collectively, preclinical research on EGFR (6) and correlative data of the RTOG 9003 study (5) validated the concept of selective radiation modulation by targeting EGFR and established a new combined strategy for the treatment of HNSCCs (7, 8). In line with preclinical data from our laboratory and others' on the sensitivity of HNSCC cell lines to gefitinib, a small-molecule inhibitor of EGFR tyrosine kinase inhibitors (TKI; refs. 9, 10), and the synergy between irradiation and gefitinib *in vitro* (9–11), there was a strong rationale for the addition of gefitinib to chemoradiation in HNSCCs (12). On the basis of several feasibility reports on its addition to chemoradiation, the combination was used in the CAR-SSA-GORTEC 2004-02-NCT00169221 trial to increase disease-free survival (DFS). Given that EGFR expression does not predict the response to EGFR antagonists and cannot help to select patients who should receive gefitinib, other molecular biomarkers were studied. The current study included a stepwise approach, namely a clinical preselection of patients agreeing to have their operative specimen used for biomarker analyses, followed by randomization for gefitinib or placebo in combination with cisplatin-based chemoradiation.

### Materials and Methods

#### Patient treatment and tumor specimens

Patients with histologically proven HNSCCs and planned surgery for locally advanced disease provided informed consent for tumor biomarker analyses. Patients with intermediate/poor prognosis [extracapsular spread (microscopic extension over nodal capsule), >3 N+ ± lymphatic or vascular emboli ± perineural invasion ± microscopically positive margins ± pT4 on operative specimen] were further enrolled into the French multicenter blinded Institutional Review Board–approved randomized phase II trial of postoperative irradiation with cisplatin ± gefitinib versus placebo. Gefitinib (250 mg twice-daily, AstraZeneca Pharmaceuticals) was administered orally for 9.5 weeks (1 week prior, 6.5 weeks during and 2 weeks after chemoradiation).

To meet inclusion criteria, tumor samples had to contain ≥50% tumor cells, determined by hematoxylin eosin saffron staining of an apposition smear on a portion of fresh tumor (≥0.5 cm<sup>3</sup>) excised from the operative specimen. Biologic data were reported using quality recommendations for reporting on tumor markers in prognostic studies (13). Half of the tumor fragment (mean = 190 mg) was frozen in liquid nitrogen within 15 minutes after surgery and subsequently processed (14, 15). The remaining mirrored tumor fragment was used for histologic control and fixed in formalin for immunohistochemical analyses. A 25% waste rate of biologic samples for the abovementioned reasons was planned in the initial trial design.

#### Biomarker and statistical analyses

**Biomarker analyses.** Antibodies used for biomarker detection are listed in Tables 1 and 2. For Western blot analyses, frozen tumor samples were homogenized in presence of protease and phosphatase inhibitors. Homogenates and membranes were prepared as previously described (14, 15). Control and tumor samples were subjected to SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore). Before analysis of patient tumor samples, Western blotting conditions were optimized (quantity of starting material, antibody, selection of appropriate controls) using the CAL33 human HNSCC cell line (10), HEK293 cells, and frozen tumor samples. Immune complexes were detected by enhanced chemiluminescence (Pierce). Signal intensity was visualized by autoradiography. Chemiluminescence was quantified using the GeneGnome Bio Imaging System (Syngene), allowing a wide linear response range (gray scale from 1 to 65,500). Biomarker interassay variability calculated on control values from independent runs ranged around 25%. Quantitative data were normalized to control values within each series of 6 to 8 patient samples. Actin staining was conducted to evaluate the integrity and cellularity of the tumor samples. Biomarker results are provided in Supplementary Data.

Primary antibodies used to detect immune complexes, the percentage of acrylamide gels, and the quantity of tumor samples deposited for Western blot analyses are indicated in Table 2. Secondary antibodies coupled to horseradish peroxidase were from Jackson ImmunoResearch Laboratories.

**Table 1.** Immunohistochemical analyses: antibodies used, procedures, and staining patterns

Antibody	Clone	Supplier (address)	Pretreatment	Dilution	Incubation, min	Detection kit	Staining
EGFR	31G7	Ventana Medical System (Meylan, France)	Protease 8 min	1	32	Ultraview-DAB	Membranous
HER2/neu	4B5	Ventana Medical System (Meylan, France)	CC1 30 min	1	20	Ultraview-DAB	Membranous
IGF1R	G11	Ventana Medical System (Meylan, France)	CC1 60 min	1	16	Ultraview-DAB	Membranous and cytoplasmic
CD31	JC70A	Dako (Trappes, France)	CC1 60 min	1:50	32	Ultraview-DAB	Cytoplasmic
Cleaved caspase-3	Asp175	CST (Ozyme, Saint Quentin, France)	CC1 60 min	1:100	22	Ultraview-DAB	Nuclear and cytoplasmic
E-Cadherin	NCH-38	Dako (Trappes, France)	CC1 30 min	1:50	24	Ultraview-DAB	Membranous
Vimentin	V9	Dako (Trappes, France)	CC1 30 min	1:600	32	Ultraview-DAB	Cytoplasmic
Cortactin	4F11	Millipore (Temecula, CA)	CC1 8 min	1:500	32	Ultraview-DAB	Cytoplasmic and Membranous
Ki67	MIB-1	Dako (Trappes, France)	CC1 30 min	1:100	32	Ultraview-DAB	nuclear
p16	16P07	CINtec (Velizy, France)	CC1 30 min	1	12	Ultraview-DAB	Nuclear and cytoplasmic
p21	SX118	Dako (Trappes, France)	CC1 60 min	1:25	36	Ultraview-DAB	Nuclear
Akt	C67E7	CST (Ozyme, Saint Quentin, France)	CC1 60 min	1:100	32	Ultraview-DAB	Nuclear and cytoplasmic
pAkt	D9E (Ser473)	CST (Ozyme, Saint Quentin, France)	CC1 60 min	1:50	120	Ultraview-DAB	Nuclear and cytoplasmic
Cyclin D1	SP4	Dako (Trappes, France)	CC1 60 min	1:50	32	Ultraview-DAB	Nuclear
ERCC1	8F1	Thermo Scientific (Courtaboeuf, France)	CC1 30 min	1:50	32	Ultraview-DAB	Nuclear

NOTE: Results of biomarker expression levels are provided in the Supplementary Material.

Controls corresponded to homogenates or membranes prepared from exponentially growing cells treated with EGF, insulin (INS), or 10% fetal calf serum (FCS) for 5 minutes. The control for active caspase-3 corresponded to a cytosolic extract of HEK293 cells treated for with cyto-

chrome *c* for 5 minutes. Cell membranes were prepared by hypotonic lysis and high-speed centrifugation.

Representative carcinoma areas were selected on hematoxylin-phloxin saffron (HPS)-stained sections. Three tissue cores (0.6 mm in diameter) from patient paraffin blocks

**Table 2.** Antibody protocols in brief for Western blotting staining

Antibody	Clone	Supplier (address)	Source	Gel	Protein, $\mu$ g	Control
EGFR	Rabbit polyclonal	Cell Signalling (Danvers, MA)	Rabbit	7%	60	CAL33 + EGF
pEGFR (Y1068)	Rabbit polyclonal	Cell Signalling (Danvers, MA)	Rabbit	7%	60	CAL33 + EGF
ERK1	C-16	Santa Cruz Biotech. (Santa Cruz, CA)	Mouse	9%	40	HEK293 + INS
pERK	MAPK-YT	Sigma (St. Louis, MO)	Mouse	9%	40	HEK293 + FCS
PTEN	A2B1	BD Biosciences (San Diego, CA)	Mouse	9%	60	HEK293 cytosol + cyt c
$\beta$ -Actin	AC-15	Sigma (St. Louis, MO)	Mouse	9%	10	
Fibronectin	10/fibronectin	BD Biosciences (San Diego, CA)	Mouse	7%	40	CAL33 + EGF
FADD	1F7	MBL (Nagoya, Japan)	Mouse	12%	40	HEK293 cytosol + cyt c
PAK1	Rabbit polyclonal	Cell Signalling (Danvers, MA)	Rabbit	9%	40	HEK293 + FCS
Src	36D10	Cell Signalling (Danvers, MA)	Rabbit	9%	60	CAL33 + EGF
pSrc (Y416)	Rabbit polyclonal	Cell Signalling (Danvers, MA)	Rabbit	9%	60	CAL33 + EGF
ILK	3/ILK	BD Biosciences (San Diego, CA)	Mouse	9%	40	CAL33 + EGF

were transferred into recipient blocks using a tissue microarray (TMA) Beecher Instrument Inc. workstation. Two blocks were built, one with 189 tumoral cores (63 patients) and the other with 48 tumoral cores (16 patients). Immunohistochemistry (IHC) was conducted on 3- $\mu$ m formalin-fixed, paraffin-embedded sections. Biomarker expression was assessed on triplicate TMA spots and ultraviolet DAB detection kits in a Benchmark XT (Ventana) autostainer. Samples were deemed unsuitable for analysis when cores were missing or when no tumor cells were found. For each biomarker, 6 normal tissue cores per TMA were included as technical controls; a positive control was tested in the same staining run as TMA slides and omission of the primary antibody served as negative control. Immunohistochemical slides were scanned and evaluated by 2 pathologists blinded to each other's scoring. Biomarker stainings were attributed Quick Score (QS) values, defined as the percentage of stained tumor cells multiplied by the staining intensity (0, 1, 2, or 3). Immunohistochemical values ranged from 0 to 300 for those assessed as quick scores and from 0% to 100% for those assessed as percentages. Analyses were conducted on mean QS obtained from triplicate TMA cores. For assessment of interobserver reproducibility, the data were compared and slides were reviewed by both pathologists in case of major discrepancies (>10%).

**Statistics.** Patients' characteristics corresponding to continuous variables (number of involved nodes, nodal density (number of metastatic nodes over number of resected nodes), irradiation dose, cisplatin dose intensity), and tumor markers (Western blot data, IHC data including Quick scores) did not fit a Gaussian distribution and could not be normalized. These data were thus analyzed as continuous variables (using nonparametric tests), and as binary variables according to their median values ( $\leq$  vs.  $>$  median). Relationships between continuous variables were analyzed by means of Spearman rank correlations. The impact of categorical variables on continuous variables was tested by means of Mann-Whitney test for 2-group comparisons or Kruskal-Wallis tests for comparisons of more than 2 groups ( $P_{\text{exact}}$  values computed according to the Monte Carlo method). Links between categorical variables were assessed by means of  $\chi^2$  tests or Fisher exact tests.

DFS and overall survival (OS) were computed from the date of diagnosis according to the Kaplan-Meier method. Median follow-up was computed according to the inverse Kaplan-Meier method. Primary endpoint was DFS calculated until date of first relapse (local, regional, or distant). Comparison of DFS between groups was tested by the log-rank test, with and without adjustment on nodal status. Univariate and multivariate Cox proportional hazard regression models were also applied for testing categorical or Gaussian continuous variables; relative risks (RR) along with 95% confidence intervals (CI95) were estimated. For stepwise multivariate analyses, both forward and backward analyses were conducted with  $P = 0.05$  for entry and  $P = 0.10$  for removal. The search for a possible influence of gefitinib as a function of biomarker expression was assessed

using a Cox model including an additional specific interaction term between gefitinib and the biomarker of interest. All tests were 2-sided. A  $P < 0.01$  was considered as significant. Statistics were drawn up on SPSS software v 15.0.

## Results

### Patient characteristics and treatment

Seventy-nine HNSCC samples fitting quality criteria for biologic collection, obtained from patients eligible on histoclinical characteristics, were included in biomarker analyses. Of these, 84% were male and median age was 58 years (range, 42–76; Table 3). Tumors were well, moderately, poorly, or undifferentiated in 41%, 42%, 16%, and 1%, respectively. Tumor stage was I–II and III–IV in 23% and 77%, respectively. Nodal stage was N0–1 in 46%, N2 in 14%, and N3 in 41%, respectively, 58% of cases displayed nodes with extracapsular spread. Nodal density, extracapsular spread, and nodal stage were significantly associated ( $P < 0.001$ ). Poorly differentiated tumors had significantly greater nodal density ( $P = 0.029$ ).

Among these 79 patients, 27 did not meet prerequisites for inclusion in the therapeutic study (time-to-freezing tumor samples and/or chemotherapy contra-indication and/or gefitinib refusal). Thus, 52 patients were further included in the therapeutic trial (Fig. 1). Among the 52 randomized patients, 27 did not receive gefitinib and 25 received gefitinib. The 27 nonrandomized patients did not receive gefitinib.

Considering the entire cohort, 87% of them received more than 60 Gy. Among randomized patients, compliance with chemoradiation, as well as toxicities, were similar in standard versus gefitinib arms (16). Accounting for the specific study design (Fig. 1), we checked whether patient characteristics differed between the 25 patients receiving gefitinib and the 54 who did not. Histoclinical parameters, radiotherapy as well as cisplatin dose intensity, were similar between patients  $\pm$  gefitinib (Table 3). We thus merged the 27 nonrandomized with the 27 randomized patients who did not receive gefitinib in further analyses.

### Patient outcomes

At time of analysis for the 79 patients, median follow-up was 38 months, 3 patients were lost to follow-up, 20 patients developed progression (local failure, 11; nodal failure, 4; distant metastasis, 12) and 28 patients were deceased (14 cancer-specific deaths, 2 toxic deaths, 4 other cancer-related deaths, 8 other/unknown causes).

Two-year DFS rate (primary endpoint) was 65.0% and DFS did not differ between randomized patients treated with gefitinib or placebo ( $P = 0.85$ ). Figure 2, left, illustrates the similarity of DFS curves between nonrandomized patients, randomized patients without gefitinib, and randomized patients receiving gefitinib in the whole population ( $P = 0.97$ ). Comparison of DFS between the 25 patients receiving gefitinib and the 54 who did not confirmed that gefitinib did not influence DFS ( $P = 0.85$ ). Two-year locoregional control (LRC) was 80.1% and did not differ

**Table 3.** Patient characteristics with respect to gefitinib treatment

	No gefitinib (N = 54) n (%)	Gefitinib (N = 25) n (%)	P
Gender: male/female	46/54 (84.6)	20/25 (81.5)	0.57
Tumor site			
Oropharynx	30/48 (60.9)	14/25 (59.3)	0.590
Oral cavity	18/49 (36.2)	10/25 (40.7)	0.784
Hypopharynx	10/48 (21.7)	7/25 (25.9)	0.492
Larynx	5/48 (10.9)	3/25 (11.1)	0.837
Differentiation			
Well	15 (34.1)	13 (51.9)	
Moderately	22 (53.7)	7 (25.9)	
Poorly	6 (12.2)	5 (22.2)	0.171
T stage			
I	1 (2.1)	0 (0)	
II	9 (19.1)	7 (25.9)	
III	19 (40.4)	7 (25.9)	
IV	20 (38.3)	11 (48.1)	0.692
0-II vs. III-IV	10 (21.3) vs. 37 (78.7)	7 (25.9) vs. 20 (74.1)	
N stage			
0-I	22 (46.8)	12 (44.4)	
IIa	6 (12.8)	4 (14.8)	
IIb-III	21 (40.4)	9 (40.7)	0.838
Number of involved nodes (mean)	4.2	2.4	0.576
Nodal density (involved/dissected, mean)	13.2	6.7	0.770
Extracapsular spread	26/43 (58.5)	13/24 (57.7)	0.616
Positive margins	2/23 (9.1)	1/24 (4.0)	0.525
Treatment			
Dose (mean in Gy)	60.6	61.2	0.46
Dose intensity (% planned 300 mg cisplatin, mean)	73.1	72.2	0.544

between patients treated  $\pm$  gefitinib ( $P = 0.92$ ). Two-year OS rate was 56.0% and did not differ among patients  $\pm$  gefitinib ( $P = 0.56$ ). The causes of death did not vary between patients receiving or not gefitinib ( $P = 0.81$ ). One toxic death was observed in the gefitinib group and one in the group without gefitinib. Further analyses were thus conducted for the whole group.

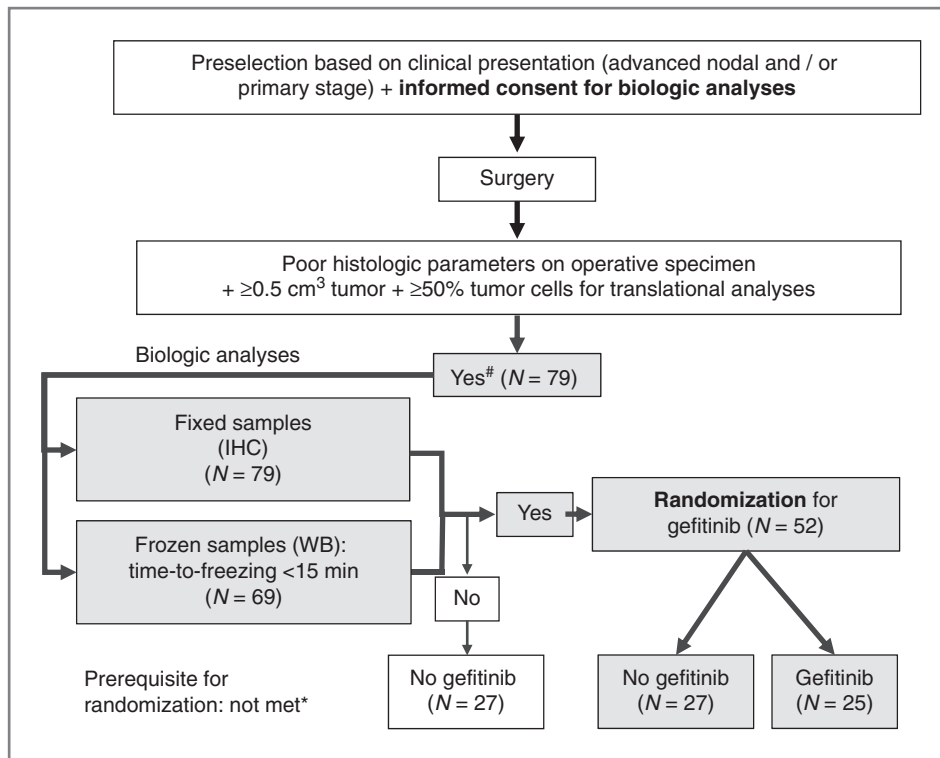
Univariate and multivariate analyses of histoclinical parameters showed that nodal status (N0-1 vs. N2-3) was the only prognostic factor for DFS, with patients having N0-1 disease displaying longer DFS ( $P = 0.050$ ; RR, 0.40; 95% CI, 0.16-1.00). Other histoclinical parameters were not correlated with DFS in this population selected on poor histoclinical prognostic factors. The nonsignificant impact of gefitinib on DFS was confirmed after adjustment for nodal status ( $P = 0.91$ ). No significant interaction between histoclinical parameters and gefitinib benefit was identified. OS was mostly affected by local failure ( $P = 0.0001$ ) and to a lesser extent by metastasis ( $P = 0.02$ ; Fig. 2, right).

#### Impact of tumor markers on DFS

Immunohistochemical analyses were available for 79 patients. Because of technical deviation (time-to-freezing

$\geq 15$  minutes), 10 frozen samples were excluded, and Western blot analyses were thus conducted on tumor homogenates from 69 patients (Fig. 1). We looked for correlations between each protein marker, histoclinical data, and outcomes. Among all tumor markers (Fig. 3), IGF1R, CD31, and cyclin D1 expression determined by IHC was significantly greater in N2-3 tumors than in N0-1 tumors ( $P = 0.010$ , 0.013, and 0.001, respectively). p16 expression by IHC ( $P = 0.010$ ) was significantly greater in T0-2 tumors than in T3-4 tumors. Lower cyclin D1 expression was observed in poorly differentiated tumors than in well- and moderately differentiated tumors ( $P = 0.001$ ). No other significant relationship was observed between tumor markers and histoclinical parameters.

Univariate analyses of tumor markers revealed that elevated expression of PAK1 by Western blotting, CD31 by IHC, and membranous IGF1R by IHC were significantly associated with shorter DFS (Table 4, Fig. 4, left). A multivariate analysis showed that PAK1, CD31, and IGF1R remained independent prognostic factors, even after adjustment on nodal status. Among patients with low IGF1R levels, the 6 patients who exhibited high PAK1 expression relapsed, illustrating the high independent prognostic value of PAK1 and IGF1R. Univariate and multivariate analyses

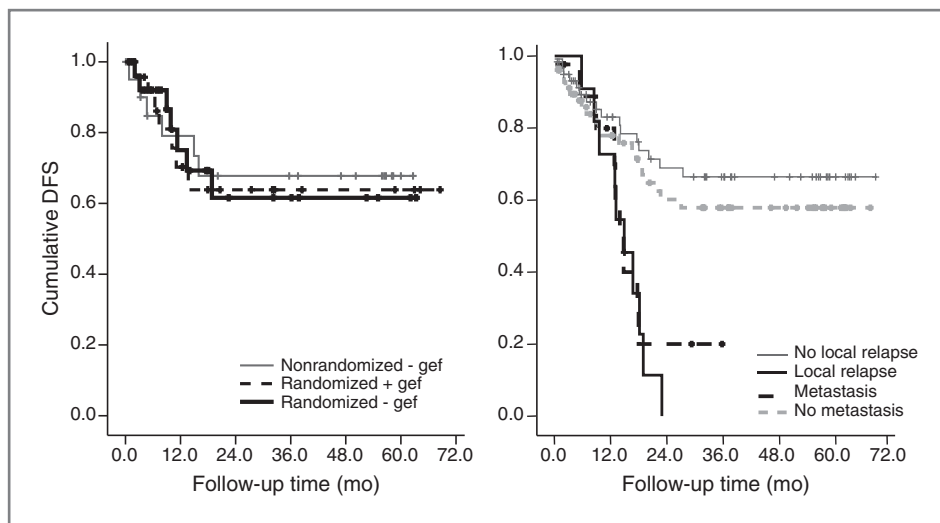


**Figure 1.** CONSORT diagram. Multicenter blinded Institutional Review Board–approved randomized phase II trial of postoperative irradiation with cisplatin ± gefitinib CARISSA GORTEC 2004-02-NCT00169221. Poor histologic prognostic factors confirmed on operative specimen (N+R+ or >3 N+ R– ± lymphatic or vascular emboli ± perineural invasion ± microscopically positive margins ± pT4). \*, Freezing ≥ 15 minutes and/or refusal to sign informed consent to gefitinib treatment and/or contra-indication to systemic treatment. #, Patients who did not fit these conditions were excluded from the study.

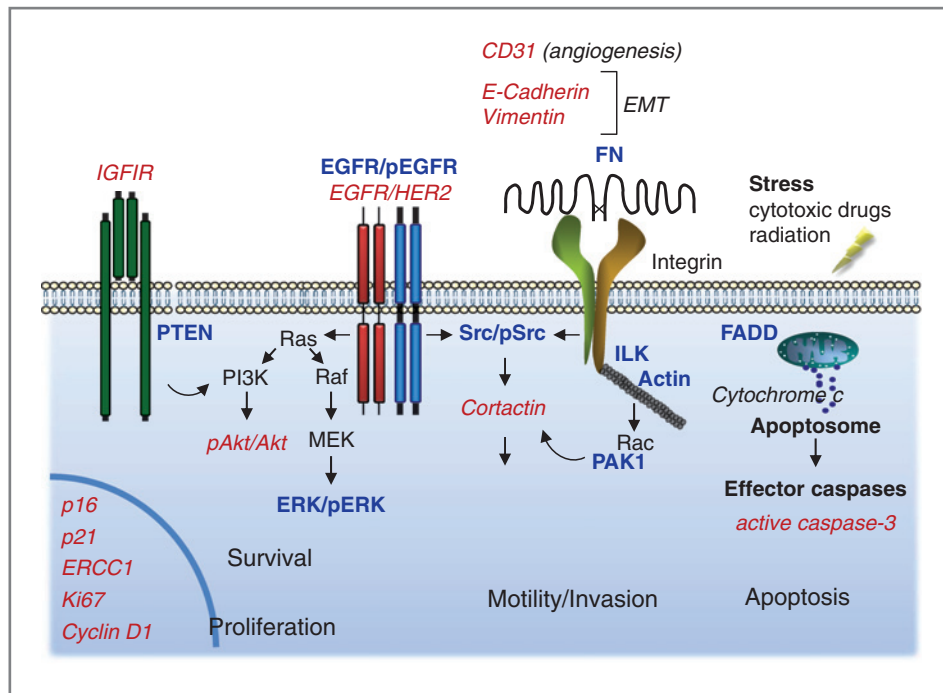
revealed that elevated PAK1 by Western blotting, CD31 by IHC, and membranous IGF1R by IHC were significantly associated with worse locoregional failure rates. Univariate analyses revealed that elevated expression of the same tumor markers was also significantly associated with shorter OS. However, only CD31 remained significant in multivariate analysis of OS.

We then looked at biomarker distributions between groups receiving gefitinib or not. Distributions of tumoral IGF1R expression (Fig. 5), CD31, and PAK1 were not similar between patients ± gefitinib. Analyses of a possible inter-

action between each of the above prognostic tumor markers and gefitinib efficacy was thus tested. No interaction was observed between PAK1 or CD31 and gefitinib. In contrast, there was a significant interaction between IGF1R expression and gefitinib. As illustrated in Fig. 4, right, gefitinib improved DFS in patients with elevated membranous IGF1R staining whereas it proved to be deleterious in patients with low IGF1R expression (Cox analysis:  $P = 0.052$  for gefitinib,  $P = 0.006$  for IGF1R,  $P = 0.028$  for the gefitinib × IGF1R interaction). Results were similar after adjustment for nodal status.



**Figure 2.** DFS (left) according to randomization and gefitinib (Gef) treatment ( $P = 0.97$ ) and OS (right) by local control (solid lines) and metastasis (dashed lines)



**Figure 3.** Signaling pathways studied in the CARISSA ancillary study. In addition to EGFR expression levels, assessed by 3 different methods (15), the activation status of EGFR signaling pathways (namely Ras/Raf/ERK and PI3K/Akt) and apoptosis markers were analyzed. HER2 and IGF1R expression was also examined, as these receptors are involved in cross-talk with EGFR. The activation status of Raf/ERK and PI3K/Akt pathways was determined by monitoring pERK/ERK and pAkt/Akt levels, respectively. Extended biomarker analyses included proteins involved in apoptosis (active caspase-3, FADD); markers of invasion, tumor–stroma interactions, and angiogenesis [cortactin, PAK1, integrin linked kinase (ILK), fibronectin (FN), pSrc/Src, vimentin, E-cadherin, CD31]; markers of human papilloma virus–mediated carcinogenesis, cell cycle, and DNA repair (p16, p21 nuclear, Ki67, cyclin D1, ERCC1). Several markers were analyzed by both IHC and quantitative Western blotting (WB), including ERK/pERK, Akt/pAkt, and active caspase-3. For duplicate WB/IHC biomarkers, results were chosen for statistical analyses on the basis of their association with DFS (lower *P* value), yielding a total of 28 biomarkers. WB biomarkers are indicated in blue and IHC biomarkers are in red. Other markers involved in the presented pathways are represented in black.

**Correlations between biomarkers**

To identify biomarker sets associated with IGF1R expression, we further analyzed the correlations between membranous IGF1R expression by IHC and each of the other markers analyzed as continuous variables. Membranous IGF1R expression by IHC positively correlated with CD31 ( $r = 0.58, P < 0.001$ ), cyclin D1 ( $r = 0.49, P < 0.001$ ), vimentin ( $r = 0.42, P < 0.001$ ), all measured by IHC. Membranous IGF1R also correlated positively with cytoplasmic IGF1R staining ( $r = 0.46; P < 0.001$ ) and negatively with fibronectin expression levels ( $r = -0.37, P < 0.002$ ). IGF1R expression was not correlated with phosphorylated

Akt, phosphorylated extracellular signal–regulated kinase (ERK), or any other marker tested.

**Discussion**

We here report the results of a series of intermediate/poor prognosis patients enrolled in the GORTEC 2004-02 trial to undergo biomarker analyses and receive gefitinib with postoperative cisplatin-based chemoradiation. We determined whether gefitinib improved DFS in patient subsets based on their biomarker profiles (17). Two-year DFS rate was 65% and was independent, in the overall population, of gefitinib. Despite extensive preclinical rationale (9–11) for

**Table 4.** Prognostic factors for DFS

	UVA		MVA	
	RR (95% CI)	P	RR (95% CI)	P
PAK1 (median value, by WB)	3.1 (1.02–9.47)	0.046	5.0 (1.47–14.60)	0.009
IGF1R (Quick score, membranous staining by IHC)	4.0 (1.51–10.41)	0.005	2.4 (1.06–8.01)	0.039
CD31 (percent cells positively stained by IHC)	3.0 (1.21–7.41)	0.018	4.8 (1.64–13.84)	0.005

Abbreviations: MVA, multivariate analysis; UVA, univariate analysis; WB, Western blotting.

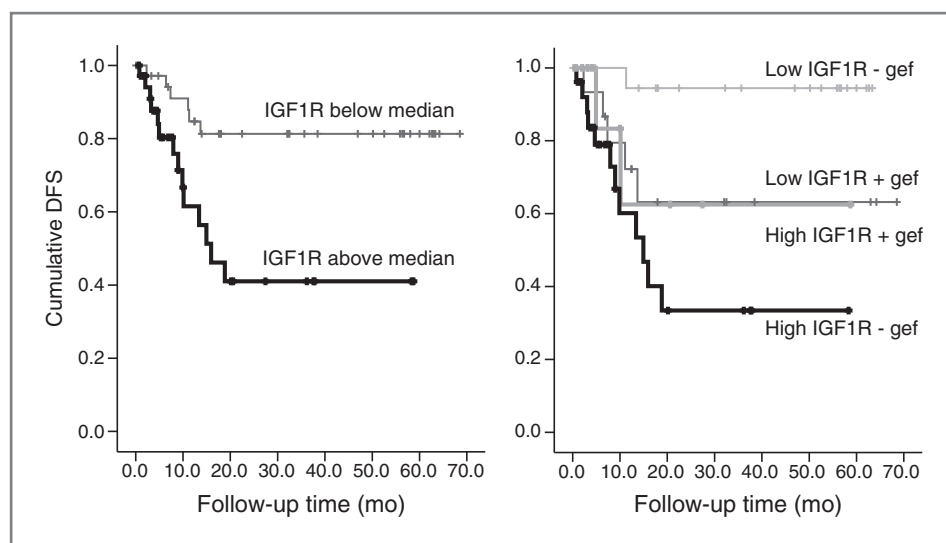


Figure 4. DFS by IGF1R ( $P = 0.003$ ; log-ranks) expression levels (left) and DFS by IGF1R expression levels in patients treated with gefitinib (+gef) or not (-gef).  $P = 0.003$  (right).

the use of EGFR TKI, only marginal clinical benefits have been observed in broad HNSCC patient populations treated with gefitinib (18, 19) with most clinical trials being underpowered (20, 21) despite acceptable toxicity profiles and initial promising response rates (22). The nonrandomized phase II study by Cohen and colleagues, using induction, concurrent hyperfractionated split-course irradiation with gefitinib and maintenance in advanced stages, was the sole trial suggesting a benefit of gefitinib, as compared with historical series (23, 24). Hainsworth and colleagues showed no superiority of adding gefitinib to chemoradiation (25). In a large but complex randomized phase II design, the addition of gefitinib with concomitant chemoradiotherapy  $\pm$  maintenance did not improve 2-year LRC

either (26). In the current study, we observed good compliance rates to overall treatment with no excess of toxicity (16) but no benefit of gefitinib in the overall population. This finding concurs with the notion that gefitinib does not benefit unselected HNSCC populations (20, 21, 27–29).

Probably only a fraction of patients with HNSCCs benefit from gefitinib, yet this gain may be much greater in populations enriched for specific molecular traits whereas lack of patient selection may mask activity. Even though preclinical studies have shown that PTEN loss, ERK or Akt activation may explain resistance to anti-EGFR therapies, there has been no unequivocal robust predictive marker of the response to EGFR inhibitors in HNSCCs in the clinics to date (7, 20–21, 27–29). Consistent with previous studies

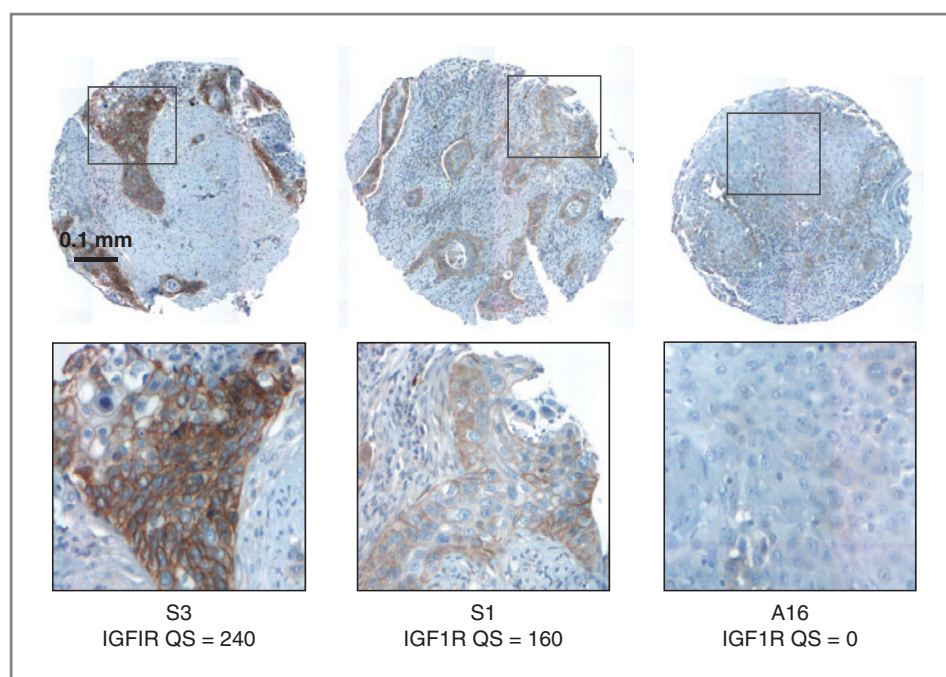


Figure 5. Immunohistochemical staining of TMA tumor cores from patients with high, intermediate, and low IGF1R levels (median QS, 39; range, 0–300).



(21), we showed herein that basal EGFR expression level was not predictive of DFS in patients receiving gefitinib. Similar data were obtained using monoclonal antibodies such as cetuximab, although published results on predictive markers are somewhat inconsistent (7). We thus tested the prognostic value of 28 baseline tumor biomarkers in the whole population, as well as their impact on gefitinib efficacy.

It is not surprising that p16 was not a prognostic factor, as most patients in this series were heavy smokers and consumers of alcohol. It is known that the relatively improved prognosis that p16 confers to those patients is overridden by a smoking consumption of more than 10 package-years (30).

Regarding the investigated biomarkers, we found that elevated tumoral expression of CD31, PAK1, and IGF1R were significantly and independently associated with a poor DFS. Association of elevated CD31 with poor prognosis is in line with the well-established enabling role of angiogenesis in sustaining and expanding neoplastic growths. PAK1 belongs to a serine/threonine protein kinase family known to regulate several signaling pathways through kinase-dependent and -independent mechanisms (31). In addition to their role in cytoskeletal remodeling, cell motility, and invasion, PAKs have been shown to regulate cell survival, mitosis, and gene transcription. Located on 1 of the 2 11q13-q14 amplicons, the *PAK1* gene, is overexpressed in oral carcinomas (31–33). This locus also harbors genes encoding the *cyclin D1* gene. In light of the poorer outcomes associated with high PAK1 expression in the current study and consistent with recent clinical studies (33), further investigations are clearly warranted in HNSCCs. IGF1R is a heterotetrameric protein with 2 identical  $\alpha$ -subunits containing an IGF-binding site and 2 transmembrane  $\beta$ -subunits that possess intrinsic tyrosine kinase activity. Major downstream signaling pathways of IGF1R include Ras/Raf/mitogen-activated protein kinase and PI3K/Akt signaling cascades. IGF1R signaling can stimulate a wide variety of responses in cells, including proliferation, differentiation, adhesion and motility, angiogenesis, and survival (34), and has also been associated with poorer prognosis in patients with oral carcinoma (35).

The present study revealed a positive correlation between tumoral IGF1R and vimentin, cyclin D1, and CD31 expression by IHC. The activation of PI3K/Akt pathway, downstream of IGF1R, increases cyclin D1 accumulation and subsequent cell-cycle progression (36). Vimentin expression increases during epithelial–mesenchymal transition (EMT) and is associated with poorer prognosis and resistance to gefitinib in cultured HNSCCs (37). Interestingly, a significant negative correlation was observed between IGF1R expression by tumor cells and levels of fibronectin measured by Western blotting in tumor homogenates. Fibronectin is an extracellular matrix protein secreted by fibroblasts, which stimulates tumor cell invasion and adhesion through activation of transmembrane receptors called integrins. This warrants further investigations on the role of fibronectin and IGF1R interactions in HNSCCs.

Given that distributions of the above prognostic markers were similar between patient subgroups with respect to gefitinib, we explored the possible impact of their expression on gefitinib efficacy in the whole population. Indeed, a significant interaction was observed between IGF1R and gefitinib efficacy, with a beneficial effect in the subpopulation of patients with a high expression of membranous IGF1R measured by IHC (Fig. 4). No interaction was observed between gefitinib and any of the other biomarkers. Strikingly, gefitinib treatment abolished the prognostic discriminative power of high IGF1R expression. Screening patients for their tumoral IGF1R expression is thus relevant for gefitinib treatment. Such a result could be explained by off-target, either or indirect, drug effects (38), affecting IGF1R (39), despite the absence of published data. In line with prior reports, Huang and colleagues showed that high IGF1R expression has prognostic value in patients with *Kras* wild-type colorectal cancers treated with cetuximab (40).

Another potentially relevant point here is that patients with low IGF1R levels fare worse under gefitinib than patients without gefitinib. At the experimental level, breast and prostate cancer cells resistant to gefitinib have low basal IGF1R expression, with high levels of basal phosphorylated IGF1R (41), thus strengthening our findings that the poor prognosis under gefitinib can be linked to low IGF1R expression levels. Interestingly, attenuated therapeutic response, following relief of negative feedback mechanisms, has already been described in response to anti-cancer treatments (42, 43). Specific Akt inhibition *in vitro* induces IGF1R expression and phosphorylations with levels that proportionally exceed induction of expression (43). Thus, in the current study, EGFR blockade possibly induced IGF1R upregulation in tumors with low baseline IGF1R expression. It probably also increased IGF1R phosphorylation and activation of pathways downstream of IGF1R or other receptors, that is, acquired IGF1R-induced resistance, following gefitinib inhibition (41, 43). Noteworthy, signaling through IGF1R recruits adaptor molecules including insulin receptor substrate (IRS)-1. Such complex feedback loops and compensatory mechanisms have been reported for other EGFR inhibitors. Lapatinib, a dual HER2-EGFR inhibitor, increases IGF1R transcription in breast cancer cell types and induces alternative receptor pathways such as HER3 (44). Corroborating recent data of the literature, our results suggest that unselected EGFR inhibition in combination with chemoradiation not only leads to lack of benefit but also to inferior outcome. Observations of a deleterious effect of anti-cancer drugs have been reported in melanomas where vemurafenib, a RAF inhibitor, induces paradoxical activation of RAF/ERK signaling in subsets of patients with wild-type B-RAF (45). In cells expressing high levels of RAF dimers, binding of vemurafenib to one member of RAF dimer transactivates non–drug-bound ATP-bound RAF, leading to ERK activation, cell proliferation, and cutaneous squamous cell carcinomas (45). Deleterious effects of EGFR inhibitor cetuximab were previously reported in colorectal studies as a result of *KRAS* mutations (40, 46–49). Interestingly, Winder and colleagues showed that wild-type

KRAS patients with germline polymorphisms in the IGF1 pathway treated with cetuximab had a significant increase in their risk for tumor progression (50). On the whole, initially puzzling and counterintuitive findings may indeed result in the identification of compensatory feedback loops (42, 47, 51), that may be at least partially governed by driver mutations in key components downstream of receptors.

Hierarchical clustering was applied separately to IHC and Western blotting data. This approach revealed significant intertumor heterogeneity among tumor biomarker profiles. Whereas no significant tumor cluster was identified for the immunohistochemical results using pvclust, 2 significant patient tumor clusters were revealed with Western blotting data. However, these clusters did not predict DFS, and IGF1R expression levels were not different among these groups (results not shown).

Overall, the present data show a strong prognostic value of IGF1R in patients with HNSCCs, and there appears to be little future for gefitinib in unselected HNSCC populations where gefitinib activity may be masked. Albeit a small population size, our striking observations on outcomes suggest that determination of IGF1R expression may guide patient selection for gefitinib treatment. This should foster confirmatory studies in the numerous ongoing studies involving cetuximab, afatinib, and lapatinib, as these molecules are currently used in many HNSCC clinical trials.

#### Disclosure of Potential Conflicts of Interest

The authors have received gefitinib for research and clinical trials from AstraZeneca Inc., under a cooperative research development and clinical trials agreement with the National Cancer Institute. G. Milano is a consultant for Roche and has received honoraria from GSK and Saladax.

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