Erythropoietin and Erythropoietin Receptor Coexpression Is Associated with Poor Survival in Stage I Non–Small Cell Lung Cancer

Pierre Saintigny,1,4 Benjamin Besse,8 Patrice Callard,5 Anne-Claire Vergnaud,2 Sébastien Czernichow,2,3 Magali Colombat,5 Philippe Girard,6 Pierre Validire,7 Jean-Luc Breau,1 Jean-François Bernaudin,4 and Jean-Charles Soria8

Abstract

Purpose: This study was designed to evaluate the prognostic effect of erythropoietin (EPO) and EPO receptor (EPO-R) expression in stage I non–small cell lung cancer (NSCLC) patients.

Experimental Design: EPO and EPO-R expression in 158 tumor samples from resected stage I NSCLC was evaluated using immunohistochemistry and tissue array technology.

Results: EPO-R and EPO were highly expressed in 20.9% and 35.4% of tumors, respectively. High EPO-R expression compared with negative or low-level expression was associated with a poor 5-year disease-specific survival (80.6% versus 80.8%; P = 0.01, log-rank test). High EPO expression compared with negative and low-level expression was associated with a trend toward a poor 5-year disease-specific survival (69.6% versus 80.4%; P = 0.13, log-rank test). A high level of EPO-R and EPO coexpression was associated with a poor 5-year disease-specific survival compared with other groups of patients (50.0% versus 80.0% survival at the end of follow-up; P = 0.005, log-rank test). In multivariate analysis for disease-specific survival, high-level EPO-R and EPO coexpression was an independent prognostic factor for disease-specific survival (hazard ratio, 2.214; 95% confidence interval, 1.012–4.848; P = 0.046).

Conclusion: These results establish the pejorative prognostic value of EPO and EPO-R expression in early-stage resected NSCLC and suggest a potential paracrine and/or autocrine role of endogenous EPO in NSCLC aggressiveness.

Erythropoietin (EPO) was originally shown to be the major cytokine regulating erythropoiesis by binding to the EPO receptor (EPO-R), member of the cytokine receptor superfamily (1). For many years, EPO was considered to act only on erythroid cells and its synthesis was thought to be limited to the fetal liver and adult kidney (2). Over the last decade, there has been growing evidence from both cell lines and human tissues that nonhematopoietic tissues also express EPO and/or EPO-R, suggesting an autocrine or paracrine role for EPO/EPO-R signaling, involved in mitosis, inhibition of apoptosis, and angiogenesis (3–10). Furthermore, coexpression of EPO and EPO-R has been shown in various pediatric and adult solid tumors, and in vitro studies have shown a significant role of EPO/EPO-R signaling in cancer cell proliferation, migration, invasiveness, and inhibition of apoptosis (2). The significance of this potential EPO/EPO-R signaling in tumors is still under debate, although it has been recently shown that recombinant EPO (rEPO) could have adverse effects on head and neck cancers expressing EPO-R (11). On the other hand, the role of endogenous EPO synthesis in tumor tissues is still hypothetical and controversial (2).

EPO/EPO-R coexpression has been recently shown in a limited series of tissue samples from non–small cell lung cancer (NSCLC) at both the mRNA and protein levels (12). A potential role of EPO/EPO-R autocrine and/or paracrine signaling in NSCLC has therefore been hypothesized. Based on these premises, we decided to test whether EPO/EPO-R expression was associated with prognosis in 158 patients undergoing complete surgery for stage I NSCLC.

Materials and Methods

Patients and survival data. All patients of this study consecutively underwent lobectomy or pneumonectomy and mediastinal lymph
node dissection for NSCLC in the Thoracic Surgery Department of
Institut Montsouris (Paris, France) from April 1994 to September 2002.
In a first step, patients with stage I tumors according to the international
tumor-node-metastasis system (International Union Against Cancer,
2005) were selected when paraffin-embedded tumor blocks were
available (n = 232). Survival data were available for 190 patients of
this series with a median follow-up of 48.78 months for disease-specific
survival (range, 0.43-110.89). Survival status was verified and updated
as of June 13, 2006. The study finally included 158 patients based on
the availability of both EPO and EPO-R expression data on tissue
microarray (TMA). None of these patients received neoadjuvant
chemotherapy, and only 13 received platinum-based adjuvant chemo-
therapy and 3 received postoperative mediastinal radiotherapy.
No patient of this series received rEPO. This study was conducted according
to the French legislation on biomedical studies.

Tissue samples and immunohistochemical detection of EPO-R and
EPO. Three different TMA blocks were constructed from formalin-
fixed, paraffin-embedded tissue samples as described previously (13).
Representative areas of the tumor were carefully selected on H&E-
stained sections, and to be representative of the tumor, three tissue
cores (0.6 mm in diameter) were sampled, two in the periphery of the
tumor and one in the center avoiding necrotic areas.

After construction of the TMA block, serial 4-μm sections were placed
on charged polylysine-coated slides. H&E-stained microarray sections
were reviewed by one pathologist (P.C.) to confirm that the sample was
representative of the original tumor. Following deparaffinization with
xylol, alcohol, and rehydration, slides were steamed in 0.01 mol/L
sodium citrate buffer (pH 6.0) for 15 min in a microwave oven. Slides
were thereafter incubated overnight at 4°C with either the anti-human
EPO rabbit polyclonal antibody (clone H-162, 1:100 dilution
corresponding to 0.5 μg/mL; Santa Cruz Biotechnology, Inc.) or the
anti-human EPO-R rabbit polyclonal antibody (clone C-20, 1:400
dilution corresponding to 2 μg/mL; Santa Cruz Biotechnology) as
previously reported (12). Sections were washed with TBS containing
0.1% Tween 20 (pH 7.0), loaded onto the Ventana IHC Instrument
using the Ventana Medical System iVIEW 3,3¢-diaminobenzidine
detection kit (Ventana Medical Systems, Inc.), and counterstained with
hematoxylin.

Human placenta sections and fetal liver were used as positive controls
for EPO-R and EPO immunolocalization, respectively (data not shown).
Omission of the primary antibody and incubation with normal rabbit
IgG (1:200 dilution, 2 μg/mL concentration; Santa Cruz Biotechnology)
instead of the primary antibody were used as negative controls.

Two independent reviewers (P.S. and P.C.) unaware of all clinical
data did TMA analysis and scoring of the immunohistochemical results.
Differences between the two investigators were resolved by consensus.
At the first evaluation, the two independent observers both evaluated
the staining intensity and the percentage of tumor cells expressing EPO-
R and EPO. As more than 85% of core sections showed 100% of cells
expressing EPO-R or EPO when positive staining was observed, we
decided to express the results only in terms of staining intensity.

For EPO-R and EPO, cytoplasmic and/or membranous labeling were
considered to be positive. A three-point scale (from 0 to 2) was applied
to semiquantitatively evaluate EPO-R and EPO expression. Absence of
labeling, very faint, or equivocal immunoreactions were scored as 0.
Score 1 indicated weak or moderate cellular staining, and score 2
corresponded to intense cellular staining.

Statistical analysis. Results are expressed as mean ± SD for continuous variables or % for dichotomous variables. Population
characteristics according to EPO-R and EPO expression were compared
by Student’s t tests or χ² tests when appropriate. Associations of EPO-R
and EPO expression with 5-year disease-specific survival were studied
using Cox models with adjustment for tobacco consumption, histology,
age, and T stage. Survival curves were plotted using the Kaplan-Meier
method, and the significance of differences was determined by the log-
rank test. A P value of <0.05 was considered to be statistically
significant. All survival curves were calculated from the date of surgery.

Disease-specific survival time was calculated from the date of surgery
to death from cancer-related causes.

All statistical analyses were done using Statistical Analysis System
version 8.2 software (SAS).

Results

**Patient characteristics.** Assessment of EPO-R and EPO
expression using TMA and survival data was obtainable in

| Table 1. Patient characteristics according to EPO and EPO-R expression levels in tumor cells (high level of
expression versus other groups) |
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<td><strong>EPO (%)</strong></td>
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<td>T stage</td>
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NOTE: EPO negative or low = 0 or 1 values of EPO; EPO high = 2 values of EPO; EPO-R negative or low = 0 or 1 values of EPO-R; EPO-R high = 2
values of EPO-R.
Erythropoietin and Its Receptor in Lung Cancer

As previously reported, EPO-R expression was more intense and EPO expression was more frequent in NSCLC samples. At the first analysis, complete concordance between the two independent observers was obtained for 87% of TMA tissue spots for EPO-R expression and 79% for EPO expression. A consensus was easily reached for the other TMA tissue spots after joint analysis. When analyzing the three tissue cores from the same tumor, a similar staining intensity was observed for 83% of cores for EPO-R and 76% of cores for EPO. When a different staining intensity was observed between various tissue cores, for both EPO-R and EPO, the core with the highest expression level was considered for the final result. Assessment of the slides by different examiners can therefore be considered to be fairly easy and reproducible.

As usual, nonspecific staining for EPO-R and EPO was observed over plasma cells. Positive staining was occasionally observed over fibroblasts and rare endothelial cells. EPO and EPO-R immunostaining was cytoplasmic in cancer cells. In rare cases, EPO-R staining was membranous.

As previously reported, EPO-R expression was more intense than EPO expression (12). EPO-R expression was observed in 94.3% (149 of 158) of tumors, with a high level of expression (score 2) in 20.9% (33 of 158), whereas EPO expression was observed in 79.1% (125 of 158) of tumors, with a high level (score 2) of expression in 35.4% (56 of 158) of tumors. All controls were negative for EPO-R and EPO staining. Representative areas of immunohistochemical staining are presented in Fig. 1. EPO-R and EPO expression was concomitantly observed in 76.5% (120 of 158) of tumors. A concurrent high level of EPO-R (score 2) and EPO (score 2) expression was observed in 11.4% (18 of 158) of tumors. Correlation of EPO-R and EPO expression with clinicopathologic data is presented in Table 1.

EPO-R and EPO expression and patient characteristics and survival. As very few tumors (n = 9) were considered to be negative for EPO-R expression, subsequent analysis of this very small group of patients was not feasible; we therefore decided to add this group to the low EPO-R expression group (n = 116) for survival analysis and to compare it to the high EPO-R expression group (n = 33). For EPO expression, the disease-specific survival curve of the EPO-negative group (n = 33) was almost identical to the disease-specific survival curve of the low EPO group (n = 69; data not shown). We therefore decided to add the EPO-negative group to the low EPO group for survival analysis and to compare it to the high EPO expression group (n = 56). Thus, for further analyses of EPO-R and EPO expression, scores 0 and 1 were considered to reflect negative or a low level of expression, whereas score 2 was considered to reflect a high level of expression.

No difference in EPO-R and EPO expression was observed according to age, gender, race, and T stage. A high level of EPO-R expression was more frequently observed in squamous cell carcinomas compared with other histologic subtypes, whereas a high level of EPO expression was more frequently observed in smokers compared with nonsmokers.

We subsequently analyzed the relationship between EPO-R and EPO expression and survival time. A high level of EPO-R expression (score 2) versus negative or low expression (scores 0 and 1) was associated with a statistically significant shorter 5-year disease-specific survival (60.6% versus 80.8%; P = 0.01). A high level of EPO expression (score 2) versus negative or low expression (scores 0 and 1) was associated with a trend toward a poor 5-year disease-specific survival (69.6% versus 80.4%; P = 0.13). Moreover, concomitant high levels of EPO-R and EPO expression were associated with a shorter 5-year disease-specific survival (50.0% versus 80.0%; P = 0.005).

Kaplan-Meier survival curves showed a statistically significant shorter disease-specific survival in patients with tumors with either a high level of EPO-R expression compared with patients with negative or low EPO-R (Fig. 2A) expression or a high level of EPO expression compared with patients with negative or low EPO (Fig. 2C) and a high level of concomitant EPO-R/EPO coexpression compared with other groups of patients (Fig. 2E). Similarly, a statistically significant shorter overall survival was observed in patients with a high level of EPO-R expression compared with patients with negative or low EPO-R expression (Fig. 2B) and in patients with a high level of concomitant EPO-R/EPO coexpression compared with other groups of patients (Fig. 2F).

Univariate and multivariate Cox proportional hazard models were used to evaluate the association between EPO-R, EPO expression, and EPO-R/EPO coexpression, clinicopathologic variables (age, gender, race, smoking history, histologic subtype, and tumor-node-metastasis), and disease-specific survival (Table 2). In univariate analysis, a high level of EPO-R expression as well as a high level of concomitant EPO-R/ EPO coexpression were associated with a poor disease-specific survival. In multivariate analysis, a high level of EPO expression was nearly significant [hazard ratio, 1.958; 95% confidence interval (95% CI), 0.999-3.837; P = 0.0504] and a high level of concomitant EPO-R/EPO coexpression was associated with poor disease-specific survival (hazard ratio, 2.214; 95% CI, 1.012-4.848; P = 0.0467).
Fig. 2. Disease-specific survival and overall survival curves according to the level of expression of EPO-R (A and B), EPO (C and D), and concomitant EPO-R/EPO expression (E and F); EPO negative or low = 0 or 1 values of EPO; EPO high = 2 values of EPO; EPO-R negative or low = 0 or 1 values of EPO-R; EPO-R high = 2 values of EPO-R.
of EPO signaling with local soluble EPO-R or anti-EPO x xenograft models of ovarian and uterine carcinomas, blockade Akt-dependent signaling pathway (27, 28). In preclinical was recently detected in human melanoma cells activating an tumor-promoting functions in various cancer models. For cells may contribute to disease progression via a wide variety of studies show that functional EPO-R/EPO signaling in cancer currently a subject of debate (2, 26). A growing number of findings impairs outcome in patients positive for EPO-R expression. EPO-R and EPO high versus negative or low 1.894 (0.984-3.645) 0.0559 1.958 (0.999-3.837) 0.0504 EPO-R and EPO high versus other groups 3.113 (1.466-6.611) 0.0031 2.214 (1.012-4.848) 0.0467 NOTE: EPO negative or low = 0 or 1 values of EPO; EPO high = 2 values of EPO; EPO-R negative or low = 0 or 1 values of EPO-R; EPO-R high = 2 values of EPO-R. Abbreviation: HR, hazard ratio.

Discussion

Several recent studies have reported EPO-R and EPO expression in tumor cell lines and primary cancers (14–21). In a previous study, we reported that EPO-R and EPO coexpression at both the mRNA and protein levels is a common finding in NSCLC (12). The present study, based on a larger series of patients with NSCLC, confirms that EPO-R and EPO are frequently coexpressed in tumor cells. Additionally, we show that high EPO-R expression and high EPO expression in tumor cells in this series of resected stage I patients were associated with poor disease-specific survival. Moreover, EPO-R/EPO coexpression seemed to be an independent prognostic factor for disease-specific survival in multivariate analysis. As reported by Henke et al. (11), the specificity of the C-20 antibody for EPO-R detection used in the present study as in other studies (12, 15, 17–22) has been recently questioned by Elliot et al. (23). However, this antibody has been shown to reliably detect EPO-R by immunohistochemistry (11, 12, 15, 17–22) and by Western blot (20, 24). It should also be emphasized that a pioneer work (25) showed the presence of EPO-R in lung carcinomas using binding of biotinylated EPO. Furthermore, in our previous study, we not only detected EPO-R by immunohistochemistry but also detected EPO-R mRNA by reverse transcription-PCR (12). Finally, using the same C-20 antibody for evaluation of EPO-R expression in head and neck cancer, Henke et al. (11) showed that rEPO only impairs outcome in patients positive for EPO-R expression. The role of EPO and EPO-R signaling in carcinomas is currently a subject of debate (2, 26). A growing number of studies show that functional EPO-R/EPO signaling in cancer cells may contribute to disease progression via a wide variety of tumor-promoting functions in various cancer models. For example, a functional EPO autocrine signaling mechanism was recently detected in human melanoma cells activating an Akt-dependent signaling pathway (27, 28). In preclinical xenograft models of ovarian and uterine carcinomas, blockade of EPO signaling with local soluble EPO-R or anti-EPO antibody was associated with an increase in apoptotic death of tumor cells (29). I.p. injections of an EPO-R antagonist have also been shown to block signal transducers and activators of transcription 5 phosphorylation and inhibit melanoma and stomach choriocarcinoma tumor cell survival and angiogenesis (30). In a rat mammary adenocarcinoma model, EPO-R/EPO inhibitors induced tumor growth delay (16).

Our series of resected stage I NSCLC patients showed homogeneous results, suggesting that not only EPO but also EPO-R expression is an indicator of poor prognosis. Furthermore, and in line with a potential biological role underlying these findings, our work confirmed that EPO-R and EPO coexpression by tumors is associated with a shorter disease-specific survival and represents an independent prognostic factor in multivariate analysis. This suggests that locally secreted EPO may play a significant role in the aggressiveness of NSCLC via autocrine or paracrine activation mechanisms. In at least two recent studies, EPO-R gene overexpression appeared as a significant player of NSCLC behavior, thereby confirming its biological relevance. Using the lung metagene model, Potti et al. (31) defined a group at low risk of recurrence and a group at high risk of recurrence in stage IA NSCLC. EPO-R gene expression was found to be a discriminating gene predicting recurrence in stage IA resected NSCLC. In another study, the authors analyzed the expression profile of 1,289 genes in 92 NSCLC cancer tissues divided into two groups according to lymph node metastasis (32). Using the optimal set of genes, it was possible to stratify the patients for lymph node metastasis at 100% (23 genes) for squamous cell carcinomas and 100% (43 genes) for adenocarcinomas. EPO-R overexpression was one of the related genes associated with lymph node metastasis in squamous cell carcinomas along with hypoxia-induced factor-1α. Local secretion of EPO may be related to local intratumoral hypoxia via hypoxia-induced factor-1α regulation (2). As in other primary tumors, hypoxia-induced factor-1α and β expression in tumor cells is associated with poor prognosis in NSCLC (33). Hypoxia has also been shown to induce EPO-R

Table 2. Univariate and multivariate Cox proportional hazard model on disease-specific survival

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<th>Multivariate</th>
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<tr>
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<td>HR (95% CI)</td>
<td>P</td>
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<tr>
<td>Age</td>
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<tr>
<td>&lt;60</td>
<td>1</td>
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<tr>
<td>60-70</td>
<td>2.496 (0.963-6.471)</td>
<td>0.0597</td>
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<tr>
<td>≥70</td>
<td>3.164 (1.236-8.098)</td>
<td>0.0163</td>
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<td>Gender (female vs male)</td>
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<tr>
<td>Female</td>
<td>1.237 (0.644-2.376)</td>
<td>0.5234</td>
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<tr>
<td>Male</td>
<td>0.794 (0.243-2.601)</td>
<td>0.7037</td>
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<td>Ethnic origin (other vs Caucasian)</td>
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<tr>
<td>Other</td>
<td>1.329 (0.511-3.458)</td>
<td>0.5603</td>
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<tr>
<td>Caucasian</td>
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<td>Smoker (yes vs no)</td>
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<tr>
<td>Yes</td>
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<tr>
<td>No</td>
<td>2.810 (1.121-7.047)</td>
<td>0.0276</td>
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<td>T stage (T2 vs T1)</td>
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<td>T1</td>
<td>1.819 (0.925-3.578)</td>
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<td>T2</td>
<td>1.894 (0.984-3.645)</td>
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<td>EPO-R high versus negative or low</td>
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<td>3.113 (1.466-6.611)</td>
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expression in cancer cell lines (14, 22) and functional EPO-R may present impaired down-regulation after EPO stimulation (34), suggesting the absence of down-regulation of this potential autocrine/paracrine EPO/EPO-R loop when activated.

Multivariate analysis showed that squamous cell carcinoma histology is an independent prognostic factor. This finding is at variance with previous reports (35), although the prognosis after resection of the various histologic subtypes is still a subject of debate (36, 37). In the present series, a positive correlation was observed between squamous cell carcinoma histology and EPO-R expression, which can be compared with a previously described association between this histology and hypoxia-induced factor-1α expression (38). The effect of hypoxia and anemia on EPO-R/EPO expression in tumor cells has not been fully evaluated. The observed relationship between high levels of EPO expression in tumor cells and tobacco consumption needs to be confirmed in further studies. In accordance with the potential unfavorable role of endogenous EPO, it has recently been reported that survival was significantly lower in patients with high preoperative plasma levels of endogenous EPO (39).

The present results raise questions about the use of rEPO in patients with NSCLC, particularly in a curative setting in patients with completely resected tumors. It is well known that rEPO can act in certain conditions as a tissue-protective protein in nonhematopoietic tissue, such as the central nervous and cardiovascular systems (40). Effects of rEPO in cancer cells have been evaluated in both preclinical and clinical studies. An increased proliferation of breast (22), renal (41), and prostate cancer cell lines (42) and inhibition of apoptosis of human breast cancer cell lines have been reported (43). Furthermore, reports have shown stimulation of breast cancer cell migration under hypoxic conditions by EPO (44) and stimulation of invasion by head and neck squamous carcinoma cells via the Janus-activated kinase-signal transducers and activators of transcription signaling pathways (45, 46). A significant correlation was observed between disease progression and EPO-R/EPO coexpression in a series of 32 patients (46). Pretreatment with rEPO has also been reported to protect certain cancer cell lines from the cytotoxic effects of the chemotherapeutic agent cisplatin, particularly via an antiapoptotic effect (16, 47). Three recent randomized clinical trials reported an adverse outcome associated with rEPO therapy in metastatic breast cancer (48), head and neck carcinoma (49), and locally advanced cervical carcinoma (50) patients undergoing radiation and concurrent chemotherapy. Henke et al. subsequently showed that locoregional progression-free survival was poorer when rEPO was administered to patients positive for EPO-R expression compared with placebo (adjusted relative risk, 2.07; 95% CI, 1.27-3.36; P = 0.01). In contrast, rEPO did not impair outcome in receptor-negative patients (adjusted relative risk, 0.94; 95% CI, 0.47-1.90; P = 0.86; ref. 11).

Altogether, these results suggest that further studies are required to investigate the effects of rEPO therapy on disease progression and survival at least for patients with resected NSCLC.

References
33. Giannopoulos A, Koukourakis MI, Sivridis E, et al. Relation of hypoxia inducible factor 1α and 2α in


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