Abstract. Background: Adverse outcomes in breast cancer patients treated with recombinant human erythropoietin (rhEpo) have been linked to the expression of Epo-receptors (EpoR) in cancer cells, although limited data on the clinical significance of these observations are available. Patients and Methods: Tissue samples from 107 patients with breast cancer who did not receive rhEpo and from 12 patients with benign lesions were retrospectively analysed for EpoR expression by RT-PCR and Western blot, and the results were correlated to clinical and demographic data. Results: While EpoR levels were not linked to anaemia or inflammation, they were positively associated with progesterone and oestrogen receptor status. Patients with increased EpoR-mRNA expression had a higher local cancer recurrence rate (p=0.021), however, no significant difference in overall survival was observed. Conclusion: Since EpoR expression is associated with hormone receptor positivity and decreased locoregional disease control, this parameter characterises a specific cancer phenotype rather than being a negative predictor itself.

Anaemia is a frequent complication in patients suffering from cancer. The incidence of anaemia increases with the progression of the disease and the age of patients. Thus, up to 40% of cancer patients are anaemic at diagnosis, and the prevalence increases to almost 80% during radio- and/or chemotherapy (1-3).

The pathogenesis of anaemia in cancer is multifactorial and includes classical features of the inflammatory anaemia of chronic disease, such as iron restriction within the reticuloendothelial system, an impaired biological activity of erythropoietin and a diminished proliferation of erythroid progenitor cells, all of which are the reflection of a cancer driven activated immune system (2). In addition, co-existing factors such as vitamin deficiency, renal insufficiency, tumour infiltration into the bone marrow and local cytokine production by malignant cells within this compartment further contribute to cancer-related anaemia. While anaemia and the associated restriction of iron in the circulation is hypothesised to result from a defence strategy of the body to limit the availability of this essential nutrient for rapid proliferating tissues (2, 4), the associated development of tumour hypoxia is considered to contribute to tumour resistance towards chemo- and radiotherapy (5). Thus, it has been hypothesised that an increase of oxygen delivery to tumour tissues upon correction of anaemia may improve response rates to radio- and chemotherapy (6). Indeed, cancer-associated anaemia is negatively associated with survivals regardless of tumour type (7), however, a more advanced anaemia is probably a reflection of an advanced tumour stage and an enhanced immune activation (2, 8).

Erythropoietin (Epo) is a 30.4 kDa glycoprotein that regulates erythropoesis by stimulating growth, preventing apoptosis and inducing differentiation of red blood cell precursors. Because the Epo response to cancer anaemia appears to be limited, human recombinant erythropoetin or so called erythropoiesis stimulating agents (ESAs) have been introduced for the treatment of patients with cancer-related or chemotherapy-induced anaemia (9). ESAs have been proven to be effective in varying numbers of cancer patients by correcting anaemia and improving anaemia related symptoms such as fatigue (9, 10). Recently, clinical trials involving anaemic and non-anaemic cancer patients have raised questions concerning the safety of ESAs with respect to survival (11, 12) and promotion of thrombembolic events (13-15). In addition, several cancer cells were found to express EpoR on their surface whose function is a matter of discussion (16-18). However, to date, very little information
is available on the potential role of EpoR for the course of the disease in patients suffering from breast cancer not receiving ESAs. The present study retrospectively examined the EpoR expression in 107 mammary cancer patients and analysed their association with tumour biology and the course of the disease.

Patients and Methods

Patients. The clinical data of 107 breast cancer patients and 12 patients with benign lesions of the breast were retrospectively analysed. EpoR expression was investigated in frozen mammary biopsy samples tissues which were obtained during surgery performed at the Department of Obstetrics and Gynecology, Innsbruck Medical University, Austria between December 1994 and May 1998. Breast cancer specimens were obtained immediately after resection of the breast or lumpectomy. Specimens were brought to the pathologist for patho-histological examination and stored in liquid nitrogen at –70°C. Demographic data, treatment history and data on the course of the disease were available for all patients, as well as detailed information on tumour pathology and laboratory values from blood samples drawn at the time of admission during a routine blood draw. Patient baseline characteristics and treatment modalities are shown in Table I.

Quantitative determination of EpoR mRNA expression by real-time PCR. Total RNA was extracted from tumour specimen and control tissues, the latter were obtained from age matched patients with non-malignant or normal biopsy results, using a guanidinium–isothiocyanate–phenol–chloroform-based procedure as previously described (19).

Reverse transcription was performed with 1 μg of total RNA, random hexamer primers (5 μM), dNTPs (62.5 μM; Roche, Mannheim, Germany), and 200 U M-MLV reverse transcriptase (GIBCO, Gaithersburg, MD, USA) in 1× reverse transcription buffer for 1.5 hours at 37°C. TaqMan (Applied Biosystems, Vienna, Austria) reverse transcriptase–polymerase chain reaction (RT-PCR) primers and probes were designed, and quantification of target genes by RT-PCR was performed as previously described (19). The following primers and TaqMan probes were used: Hum EpoR primer sense 5’-ACCGCCGGGCTCTGAA-3’; antisense 5’-TTCAAACTCGCTCTCTGGGC-3’; probe 5’-AGAAGATCTGGCCTGGCATCCCG-3’. For quantification of the human housekeeping gene β-actin, the Applied Biosystems) predeveloped assay kit was used.

Western blotting. Protein extracts were prepared from tumour tissues as previously described (20) and run on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Proteins were transferred onto a nylon membrane (Hybond-P; Amersham-Pharmacia, Vienna, Austria) and, after blocking, incubated with a rabbit polyclonal antibody raised against the C-terminal cytoplasmic domain of human EpoR (1:400, C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA). K-562 whole cell lysate sc-2203 (Santa Cruz Biotechnology) was used as a positive control.

Data analysis. Statistical analysis was performed using statistical analysis software package (SPSS version 15.0; SPSS Inc, Chicago, IL, USA). Calculations for statistical differences between the various groups were performed by non-parametric tests, namely Kruskal-Wallis and Mann-Whitney tests. Associations among the various parameters in the different groups were calculated using the Spearman’s rank correlation technique. Survival was analysed by Kaplan-Meier analysis (differences between groups were calculated by Wilcoxon-Gehan-test), and univariate binary logistic regression analysis was employed to identify prognostic factors.

Results

EpoR expression in malignant and non-malignant mammary tissues. The expression of EpoR was quantified in biopsies of 107 patients with breast cancer and 12 controls with non-malignant lesions of the breast. There was no significant difference in the mean expression of EpoR mRNA between malignant and benign lesions (Figure 1). Importantly, the EpoR mRNA expression was paralleled by corresponding alterations of EpoR protein levels as quantified by Western blots (Figure 2).

The association of EpoR mRNA expression with the course of the disease was then analysed. No correlation was
observed between the patient overall survival and the expression of EpoR mRNA in cancer biopsies. However, there was a significant association between EpoR mRNA expression and decreased localised disease free survival ($rs=–0.221, p=0.022$; $rs$: correlation coefficient). Increased EpoR expression at presentation was significantly associated with local recurrence of cancer ($rs=0.215, p=0.029$). Higher EpoR mRNA expression was observed in patients with local recurrence of cancer ($p=0.021$, Figure 3).

EpoR mRNA expression was not associated with menopausal status, age, tumour stage or differentiation (Table II). Interestingly, a significant positive correlation was found between expression of EpoR and progesterone receptor expression ($rs=0.201, p=0.038$); also, the association with oestrogen receptor status approached significance ($rs=0.177, p=0.069$). Moreover, there were no significant differences in serum C-reactive protein, urinary neopterin and haemoglobin concentrations between controls and cancer patients, and accordingly, EpoR mRNA expression was neither correlated to haemoglobin levels nor to the immune activation markers neopterin and C-reactive protein at presentation (Table II).

Table II. Univariate correlations between EpoR mRNA expression in breast cancer tissue and clinical, pathological and laboratory parameters in 107 patients with breast cancer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$rs$</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.151</td>
<td>0.121</td>
</tr>
<tr>
<td>Stage</td>
<td>0.042</td>
<td>0.669</td>
</tr>
<tr>
<td>Lymph node-positive</td>
<td>–0.021</td>
<td>0.831</td>
</tr>
<tr>
<td>Grading</td>
<td>–0.021</td>
<td>0.825</td>
</tr>
<tr>
<td>Overall survival</td>
<td>–0.002</td>
<td>0.983</td>
</tr>
<tr>
<td>Disease-free survival generalised</td>
<td>–0.162</td>
<td>0.096</td>
</tr>
<tr>
<td>Disease-free survival localised</td>
<td>–0.221</td>
<td>0.022</td>
</tr>
<tr>
<td>Relapse overall</td>
<td>–0.097</td>
<td>0.323</td>
</tr>
<tr>
<td>Relapse localised</td>
<td>0.215</td>
<td>0.029</td>
</tr>
<tr>
<td>Oestrogen receptor-positive</td>
<td>0.177</td>
<td>0.069</td>
</tr>
<tr>
<td>Progesterone receptor-positive</td>
<td>0.201</td>
<td>0.038</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>0.069</td>
<td>0.428</td>
</tr>
<tr>
<td>C-Reactive protein</td>
<td>0.300</td>
<td>0.212</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>0.039</td>
<td>0.709</td>
</tr>
<tr>
<td>Neopterin</td>
<td>0.176</td>
<td>0.247</td>
</tr>
</tbody>
</table>

$rs$, Correlation coefficient; only $p$-values below 0.05 were considered as statistically significant.

Discussion

Based on concerns related to the expression of EpoR on mammary carcinoma cells and on several studies reporting a negative association between administration of rhEpo and the clinical course in patients with advanced cancer (12-14), the present study examined the putative effects of EpoR expression on the clinical course in breast cancer patients not receiving rhEpo.

By means of PCR, EpoR expression was detected in both, malignant and normal breast tissues, which is in contrast to the study by Acs et al. where EpoR was detected exclusively in breast cancer cells while adjacent normal cells had no such receptors (18). Importantly, differences in EpoR mRNA expression between individual patients were paralleled by corresponding alterations of EpoR protein expression. However, although the mean EpoR mRNA expression was slightly higher in cancer patients than in controls with non-malignant lesions, there was no significant difference. While a correlation between disease progression and EpoR expression in patients with head and neck cancer was previously reported (12), the results of the present study did not show an effect of EpoR expression on survival of breast cancer patients. However, in vitro results indicated that different cell lines and tissue types may respond differently to rhEpo (21). This indicates that rhEpo does not consistently provide a proliferative stimulus for cancer cells and/or that EpoR on certain tissues may not be functional.

In the present study, higher EpoR levels were associated with a significantly increased likelihood of locoregional recurrence.
recurrence of cancer and were also significantly correlated to the expression of oestrogen and progesterone receptors. Thus, one may suggest that the expression of EpoR on breast cancer cells is a reflection of a specific differentiation type of the tumour rather than being a poor prognostic marker per se.

Moreover, since the retrospective analysis of the present study was performed in 107 breast cancer patients diagnosed with breast cancer between 1994 and 1998, of whom only 5 received rhEpo at least once, it was not possible to draw any conclusions on the effects of rhEpo therapy towards the course of the disease in these individuals nor to demonstrate whether there was an association with the expression of EpoR on cancer tissue.

While in the present study an increased expression of EpoR mRNA was associated with a significantly reduced local disease-free survival, there was positive correlation of EpoR mRNA expression with oestrogen and progesterone receptor status, respectively. This is surprising since a positive oestrogen and progesterone receptor status is known to be an indicator for a better prognosis, because such patients can be treated with hormone therapy (22). The observation of a correlation between EpoR and hormone receptor expression on the one hand, and increased EpoR mRNA and reduced locoregional disease control on the other hand is in agreement with the recent assumption that EpoR and membrane steroid receptors are not independent prognostic markers, but may represent possible new therapeutic targets (23). An increased EpoR expression on tumour cells may, however, also be a reflection of reduced tumour perfusion since both Epo and EpoR are induced by hypoxia signals (24). Accordingly, the injection of a monoclonal antibody against Epo as well as the administration of EpoR signalling blockers into certain

Figure 2. EpoR protein expression in biopsy specimens of cancer patients and controls. Protein was subjected to Western blotting with anti-EpoR specific antibody (size 78 kDa) and a β-actin antibody as a loading control. Representative blots are shown for breast cancer tissues in which high or low EpoR mRNA expression was detected by quantitative RT-PCR and for controls.

Figure 3. Association between EpoR mRNA expression and the clinical course of disease. Quantitative EpoR mRNA expression in tumour tissue biopsies was compared between breast cancer patients still alive after 10 years and those who has died, as well as those patients with or without local relapse. Data are depicted as lower quartile, median and upper quartile (boxes) and minimum/maximum ranges (whiskers). Outliers are shown. The significance levels for differences are shown as determined by the non-parametric Wilcoxon test.
tumour tissues dramatically reduced capillary density and resulted in tumour cell destruction (16, 25). One alternative explanation for the differences in EpoR expression on breast cancer tissues may relate to the differences in haemoglobin levels or iron status. At least in iron deficiency anaemia, EpoR expression on erythroid progenitors is inversely correlated to haemoglobin levels (25, 26), and Epo has been shown to specifically increase transferrin mediated iron uptake and to stimulate haeme biosynthesis in erythroid progenitor cells (27-29). However, there was no association between haemoglobin levels and EpoR expression, while data on iron status were not available. Secondly, one may assume that EpoR expression may be linked to immune activation. This hypothesis was also tested in the present study, but no correlation between C-reactive protein levels and urinary neopterin concentrations with EpoR mRNA expression was found.

In summary, this study provided evidence that EpoR mRNA expression may have ambivalent biological functions in tumour biology since it was associated with a decreased locoregional disease control, while it had a positive association with oestrogen and progesterone receptor expression. An increased EpoR mRNA expression in breast cancer biopsies was not associated with an increased mortality, thus EpoR on cancer cells may rather be indicative for a specific, likewise more aggressive, breast cancer phenotype than being a negative predictor per se.

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References


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