Relation of Erythropoietin and Erythropoietin Receptor Expression to Hypoxia and Anemia in Head and Neck Squamous Cell Carcinoma

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Abstract

Purpose: The use of erythropoietin in head and neck squamous cell carcinoma (HNSCC) has been associated with poor survival. This study examines the protein and mRNA expression of erythropoietin and erythropoietin receptor in HNSCC and their relation to hypoxia, hemoglobin (Hb), and clinical outcome.

Experimental Design: The immunohistochemical expression of erythropoietin and erythropoietin receptor was assessed in 151 cases of HNSCC. Expression was compared with the hypoxia-dependent proteins hypoxia-inducible factor-1α (HIF-1α) and carbonic anhydrase-9 (CA-9) and correlated with clinical outcome. The mRNA expression of erythropoietin and erythropoietin receptor was measured in paired samples of HNSCC.

Results: Erythropoietin and erythropoietin receptor were expressed in 95% and 99% of tumors, respectively. Using a weighted expression score, there was a positive correlation between erythropoietin and erythropoietin receptor expression (r = 0.18, P = 0.03), HIF-1α (r = 0.38, P < 0.01) and CA-9 (r = 0.26, P = 0.002) correlated with erythropoietin expression, but there was no correlation with CA-9 and erythropoietin receptor. No correlation was found between Hb and erythropoietin (r = 0.07, P = 0.36) or erythropoietin receptor (r = –0.02, P = 0.8), and no survival difference between high and low erythropoietin or erythropoietin receptor expression (P = 0.59 and P = 0.98, respectively). The mRNA expression of erythropoietin (P = 0.03) but not erythropoietin receptor (P = 0.62) was significantly increased in 11 paired samples of HNSCC.

Conclusion: In vivo, the HIF pathway regulates erythropoietin at the mRNA level but not erythropoietin receptor expression in HNSCC. Anemia does not seem to influence the hypoxic microenvironment of tumors sufficiently to alter the expression of erythropoietin. The effects of exogenous erythropoietin may be acting via receptors expressed on tumor cells in vivo, or on vascular cells, which also express the pathway.

Head and neck cancer represents the fifth most common cancer in men and eighth most common in women worldwide, with about 600,000 new cases each year (1). More than 90% of the cancers are of squamous origin, arising from any part of the upper aerodigestive tract (2), and despite advances in treatment, the 5-year survival of advanced disease remains only around 50% (3).

Anemia, identified in a large number of patients with cancer, is recognized to have a strong relationship with disease-related fatigue and quality of life (4). Anemia is also recognized to be associated with poor overall and disease-free survival in HNSCC (5), although its definition varies widely from 11 to 14.5 g/dL (6). Recombinant human erythropoietin, developed to increase the hemoglobin (Hb) levels of patients with chronic renal failure, is also licensed to treat anemia in cancer patients (7). Although this has been shown to improve disease-related quality of life (8), concerns over its effects on patient survival have arisen following trials in HNSCC and breast cancer patients (9, 10). In a randomized, double-blind, placebo controlled trial of anemic HNSCC patients receiving recombinant human erythropoietin, it was reported that disease-free survival and overall survival was reduced compared with placebo (9). Furthermore, a study in non-anemic breast cancer patients treated with recombinant human erythropoietin was terminated prematurely, when interim survival results showed decreased survival in patients receiving recombinant human erythropoietin compared with placebo (10).

Endogenous erythropoietin is produced primarily in the fetal liver and adult kidney in response to hypoxia. It is a glycoprotein hormone that serves as the primary regulator of erythropoiesis by stimulating growth, preventing apoptosis, and inducing differentiation of RBC precursors (11, 12). In addition to hematopoietic cells, erythropoietin and the
Erythropoietin, Erythropoietin Receptor, and Hypoxia

eythropoietin receptor expression has been shown in a variety of non-tumor cells, including neural, cardiac, and muscle cells (13, 14), and has been shown to be important in angiogenesis (15). More recently, erythropoietin and erythropoietin receptor have also been shown to be expressed in a number of tumors, including HNSCC (16), breast (17), lung (18), prostate (19), and melanoma (20).

Studies into the hypoxic signaling of erythropoietin led to the discovery of the transcription factor hypoxia-inducible factor (HIF) pathway (11). Although hypoxia results in cell death if it is severe or prolonged, it can also induce transcriptional changes, mediated by the HIF pathway. The HIF signaling pathway is critically important in tumor biology, regulating several important biological pathways in addition to erythropoiesis, including cell proliferation, glucose and energy metabolism, apoptosis, and pH regulation (21).

HNSCC, in common with many solid tumors, is characterized by heterogeneous regions of hypoxia (22, 23). The degree of hypoxia can be quantified by measuring the expression of hypoxia-regulated proteins, including HIF-1α, a key transcription factor and carbonic anhydrase-9 (CA-9), a downstream gene regulated by the HIF pathway (23). High levels of these hypoxic proteins are associated with decreased survival in HNSCC (24–26).

The finding that both erythropoietin and erythropoietin receptor are expressed in non-hemopoietic tissues coupled with the clinical evidence for a detrimental role of recombinant human erythropoietin in tumor behavior has raised the possibility that erythropoietin can act on the erythropoietin receptor in the tumor to enhance its malignant properties. We therefore did this study to examine the expression of erythropoietin and erythropoietin receptor in HNSCC and to investigate the mechanism of their regulation and their relationship with hypoxia and clinical outcome. The study is also the first to consider whether anemia, a physiologic stimulus to endogenous erythropoietin production, is important in regulating tumor expression of erythropoietin.

Materials and Methods

Patients and tissue microarray. The work was carried out with the approval of the Oxford Ethics Committee. One hundred fifty-one consecutive cases of HNSCC from the Ear Nose and Throat Department, Radcliffe Infirmary, Oxford were analyzed. All patients were treated with curative intent with primary surgery. Patients who had previously been treated for HNSCC or who were to receive radiotherapy before surgery were excluded. Clinical data, including date of birth, sex, tumor subsite, tumor stage, preoperative Hb, disease recurrence, and survival, were recorded.

The tissue microarray was constructed using formalin-fixed, paraffin-embedded specimens derived by surgical resection from the Pathology Department, John Radcliffe Hospital, Oxford. At least two representative areas of viable tumor and when present, tumor adjacent to necrotic regions were identified for each case. One-millimeter cores from the paraffin-embedded blocks were taken, and 4-μm sections of cores were adhered to Micro Slides in an 8 × 15 grid arrangement and provided with a unique tissue microarray identifier.

Immunohistochemistry. For erythropoietin and erythropoietin receptor, after dewaxing and rehydration, samples were pressure cooked in sodium citrate buffer (0.01 mol/L, pH 6.0) for 6 minutes then an endogenous peroxidase block (DAKO Envision, Carpinteria, CA) applied for 5 minutes. Slides were washed in TBS and incubated overnight at 4°C, with either the erythropoietin antibody (H-162, rabbit polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA; 1:200 dilution in 10% human serum) or the erythropoietin receptor antibody (C-20, rabbit polyclonal antibody, Santa Cruz Biotechnology; 1:400 dilution in 10% human serum). Slides were washed in TBS containing Tween 20 (pH 7.6; Sigma, St. Louis, MO) for 5 minutes. Then the secondary polymer from the Envision kit (DAKO) was applied for 30 minutes at room temperature. Visualization of the immunostaining was by diaminobenzidine substrate. After rinsing in water, slides were lightly counterstained with hematoxylin, dehydrated, and mounted.

For erythropoietin and erythropoietin receptor, slides of fetal liver and placenta were used as positive controls, and substitution of primary antibody with TBS was used as a negative control.

We have previously examined the immunohistochemical expression of HIF-1α (antibody ESEE122, 1:30), and CA-9 (antibody M75, 1:50) for this series, using previously described immunohistochemical techniques (24).

Assessment of protein expression. All tissue microarray immunostained slides were reviewed by light microscopy by two observers (K.A.S. and S.W.), blindly and scored independently, and then reviewed at a conference microscope and a consensus determined. Fields were assessed at low (×100) and intermediate (×200) magnification. A score was only obtained if >10% of surface area of the sample contained tumor cells. The intensity and percentage of cancer cells staining with cytoplasmic erythropoietin and erythropoietin receptor expression were separately assessed in all optical fields. Staining was scored according to intensity 0 to 3 (0, absent; 1, weak; 2, moderate; and 3, strong), and percentage of cells involved 0 to 4 (0, 0%; 1, 1-10%; 2, 11-50%; 3, 51-80%; 4, >80%). With up to four tissue cores per case, the highest score in each category was used as the final result. The final weighed expression score for each case was obtained by multiplying the intensity score with the percentage score (0-12).

The weighed expression score for erythropoietin and erythropoietin receptor was used as a continuous variable for all correlation assessments. To assess survival the erythropoietin and erythropoietin receptor, weighed expression scores were divided into high and low scores based on the mean expression score for the whole group.

The tissue microarray had previously been scored for HIF-1α and CA-9 expression as part of an ongoing study. HIF-1α expression was assessed using the same weighed expression score as above and a score of 0 to 12 derived for each tumor. For CA-9, the membrane percentage of tumor cells was assessed using the categories described above and a score of 0 to 4 obtained.

RNA extraction, reverse transcription, quantitative real-time PCR. Samples were collected and transported in RNA later (Ambion, Austin, TX), stored at 4°C for 24 hours, before being transferred to liquid nitrogen until RNA extraction. The RNA extraction was performed with half transferred to formalin; paraffin embedded; and used to confirm the presence of tumor and half used for total RNA extraction using Tri-reagent (Sigma) and RNeasy spin columns (Qiagen, Chatsworth, CA), according to the manufacturer’s instructions. RNA concentration and purity was assessed using the NanoDrop ND-1000 spectrophotometer and the Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE).

cDNA production was carried out using the high-capacity cDNA achieve kit (Applied Biosystems, Foster City, CA), in a total volume of 50 μL: 25 μL of master mix (5 μL 10× reverse transcriptase buffer; 2 μL, 25× deoxynucleotide triphosphate mixture; 5 μL, 10× random primer; 2.5 μL, MultiScribe reverse transcriptase; 10.5 μL DEPC water) and 25 μL of total RNA. The reaction was allowed to proceed for 10 minutes at 25°C then 2 hours at 37°C then 4°C. cDNA was stored at −20°C until further use.

Quantitative real-time PCR reactions were done using the Human ProbeLibrary (Exiqon, Copenhagen, Denmark), and intron spanning primer-probe combinations designed using the ProbeFinder software (http://www.probefinder.com): erythropoietin, forward TCCAGACACCGAATAATTCTGA and reverse CGGCTGAAGCTCTACGGC primers (probe 58): erythropoietin receptor, forward CCTGGCCTTTGGCGACAC and reverse CCGGGGTCTTAGGAGCCTACTA primers.
paired differences between quartile distributions of protein expression. Analyzed using the Kaplan-Meier method, and prognostic factors were using GraphPad Prism (v3.0). Locoregional control and survival was the mean value of PCR reactions done in triplicate. Expression of all the normal samples, and all calculations were based on the comparator and the sample. The mean comparator was the pooled efficiency of the reference gene, and DCt = the cycle difference between (probe 75); Ubiquitin C, forward, CCTGACCACAGGTGTGAT and reverse TCTTGAGTACGACAGGTTGATGA primers (probe 11).

Quantitative real-time PCR reactions were done in a Rotor Gene, RG-3000 (Corbett Research, Sydney, Australia). All reactions were done in triplicate, in a final volume of 25 μL containing 10 μL cDNA (1 ng/μL), 12.5 μL Absolute QPCR Mix (Abgene, Rochester, NY), 1 μL forward primer (10 μmol/L), 1 μL reverse primer (10 μmol/L), 0.25 μL water, and 0.25 μL Exiqon probe. After an initial step of 10 minutes at 95°C, 45 amplification cycles were done: 95°C for 15 seconds and 60°C for 60 seconds.

The reaction efficiency for each gene was calculated after obtaining standard curves for each PCR reaction by making 2-fold serial dilutions covering the range equivalent to 20 to 0.625 ng of RNA. Relative quantification of gene expression was done using the method described by Pfaffl (27) and Ubiquitin C as an internal reference gene. In brief, comparisons were made between the number of cycles required for the fluorescence of a sample to reach a predetermined threshold that lay within the exponential phase and above nonspecific background. The relative ratio of gene expression was calculated as follows:

\[
\text{Relative Ratio} = \frac{(E_{\text{target}} \cdot \text{DCt TARGET})}{(E_{\text{ref}} \cdot \text{DCt REF})} \text{ (mean comparator – mean sample)}
\]

where \( E_{\text{target}} \) = reaction efficiency of the gene of interest, \( E_{\text{ref}} \) = reaction efficiency of the reference gene, and DCt = the cycle difference between the comparator and the sample. The mean comparator was the pooled expression of all the normal samples, and all calculations were based on the mean value of PCR reactions done in triplicate.

**Statistical analysis.** Statistical analysis and graphs were generated using GraphPad Prism (v3.0). Locoregional control and survival was analyzed using the Kaplan-Meier method, and prognostic factors were assessed by log-rank analysis. Correlations between continuous variables were obtained by linear regression analysis and Spearman’s rank correlation. The one-way ANOVA test was used to examine differences between quartile distributions of protein expression. Paired t tests were used to analyze matched cDNA expression. Two-tailed Ps are given and considered statistically significant when \( P < 0.05 \).

### Results

The clinicopathologic characteristics of the cases reviewed are detailed in Table 1. The mean follow-up of this cohort was 33 months (range, 0-108 months); 71 patients are alive and disease-free, four are alive with disease, and 76 are dead.

**Protein expression of erythropoietin and erythropoietin receptor.** Assessment of immunohistochemical expression using the tissue microarray was obtainable in 146 (97%) cases for erythropoietin and 139 (92%) cases for erythropoietin receptor. Failure to obtain a result from some patients was due to inadequate tumor sample (<10% of the surface area contained tumor cells) or loss of tumor sample from the array slide and compares favorably with other reported studies (28, 29). This is shown as “unavailable” in Table 1.

Within tumor cells, expression of erythropoietin and erythropoietin receptor was cytoplasmic. Expression of erythropoietin receptor was stronger than that of erythropoietin (Fig. 1). The pattern of expression ranged from negative through to strong cytoplasmic staining in up to 100% of cells. Erythropoietin and erythropoietin receptor expression was seen in vascular endothelial cells and in smooth and striated muscle cells adjacent to tumor cells.

Ninety-five percent of tumors expressed some erythropoietin immunoreactivity, and 99% expressed erythropoietin receptor. Using the weighed expression score described, there was a positive correlation between erythropoietin and erythropoietin receptor expression (\( r = 0.18, P = 0.03 \)).

**Correlation among protein expression of erythropoietin, erythropoietin receptor, hypoxia-inducible factor-1α, and carbonic anhydrase-9.** In this cohort, 30% of cases were assessed as having high HIF-1α and 62% as having high CA-9 expression. Using linear regression analysis and the immunohistochemical expression score as a continuous variable, erythropoietin expression correlated with HIF-1α (\( r = 0.38, P < 0.01 \)) and CA-9 (\( r = 0.26, P = 0.002 \); Fig. 2). There was no correlation among erythropoietin receptor expression, HIF-1α (\( r = 0.14, P = 0.11 \)), or CA-9 (\( r = 0.08, P = 0.37 \)).

**Protein expression of erythropoietin and erythropoietin receptor compared with anemia and clinical outcome.** Pretreatment Hb values were available in 146 of 151 (97%) cases. The weighed expression score for erythropoietin and erythropoietin

<table>
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<tr>
<th>Table 1. Patient and tumor characteristics for the whole group</th>
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<tr>
<td><strong>All patients</strong></td>
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<td><strong>Sex, (%)</strong></td>
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<tr>
<td>Male</td>
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<td><strong>Erythropoietin, (%)</strong></td>
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receptor was assessed with pretreatment Hb as a continuous variable. No correlation between Hb and erythropoietin (r = 0.07, P = 0.36) or erythropoietin receptor (r = −0.02, P = 0.8) was found.

In a previous study, we have shown that Hb ≤ 11 g/d was associated with worse overall survival and disease-free survival in this cohort. Using the Mann-Whitney test, we found that there was no significant difference between the erythropoietin or erythropoietin receptor weighed expression score in anemic (Hb ≤ 11 g/d) and nonanemic (Hb > 11 g/d) patients (P = 0.44 and P = 0.86, respectively).

Using the criteria described in Materials and Methods, we considered 89 and 59 cases (59% and 39%) to have high erythropoietin and erythropoietin receptor reactivity, respectively. Using Kaplan-Meier analyses and log-rank test, no correlation was found between erythropoietin or erythropoietin receptor expression and survival (P = 0.59 and P = 0.98, respectively) or disease-free survival (P = 0.88 and P = 0.66, respectively). At 5 years, the survival for high erythropoietin expression compared with low expression was 45 and 40 months, respectively, and disease-free survival was 49 and 44 months, respectively. The high erythropoietin receptor expression compared with low expression survival at 5 years was 35 and 49 months, respectively, and disease-free survival was 43 and 51 months, respectively.

**mRNA expression of erythropoietin and erythropoietin receptor.**

Using RNA from 11 paired samples of HNSCC and normal tissue, the expression of erythropoietin and erythropoietin receptor was examined using quantitative real-time PCR. Overall, there was a significant increase in mRNA transcript expression of erythropoietin between normal and tumor tissue (P = 0.03; Fig. 3), but no significant difference between the mRNA expression of the erythropoietin receptor transcript (P = 0.62; Fig. 4). Furthermore, there was no correlation between erythropoietin and erythropoietin receptor mRNA expression (r = 0.08, P = 0.7).

Formalin-fixed, paraffin-embedded sections of representative tumor sections corresponding to the cases in which RNA had been extracted were assessed by immunohistochemistry for erythropoietin and erythropoietin receptor protein expression. Using the same weighed expression score described, no correlation between mRNA and protein expression for erythropoietin (r = 0.13, P = 0.74) or erythropoietin receptor (r = 0.6, P = 0.09) was found.

**Discussion**

In this study, both erythropoietin and erythropoietin receptor were expressed in the cytoplasm, a pattern of expression that has been previously reported in HNSCC (16). Similar expression has been reported in breast (30), non–small cell lung (18), cervix (31), endometrial (32), and prostate (19) cancers. We did not observe membrane expression of erythropoietin receptor as reported in some studies (19, 31).

The high level of erythropoietin and erythropoietin receptor expression we report and the positive correlation between the weighted expression scores are similar to a previous study of 21 patients with HNSCC (16). In other tumor types, a high level of erythropoietin and erythropoietin receptor expression has also
been reported, and a positive correlation was found in prostate tumors, using a weighed intensity score and non–small cell lung cancers, using contiguous sections (18, 19). This study represents the largest number of HNSCC patients reviewed and supports the evidence that there is widespread coexpression of erythropoietin and erythropoietin receptor in HNSCC. Further to this, we have investigated the regulating stimuli for both erythropoietin and erythropoietin receptor in vivo and examined their functional significance.

Physiologically, hypoxia and the HIF pathway regulate endogenous erythropoietin production (11). In HNSCC, it has been reported that the expression of erythropoietin/erythropoietin receptor increases around necrotic regions assumed to be hypoxic (33). There is further supporting evidence that hypoxia regulates erythropoietin expression. In HNSCC, erythropoietin but not erythropoietin receptor expression correlated with the hypoxic marker pimonidazole (16), and in cervical and endometrial cancers, erythropoietin and HIF-1α expression colocalized (31, 32). In this study, the finding that erythropoietin, but not erythropoietin receptor, colocalized with HIF-1α and CA-9 provides further evidence that hypoxia is the main stimulus for tumor expression of erythropoietin. Furthermore, this is the first study to examine the correlation between erythropoietin and CA-9, strengthening the association between erythropoietin and the HIF pathway.

It has been postulated that anemia, common in HNSCC and associated with a poor outcome, may contribute to tumor hypoxia by reducing the O2 carrying capacity of the blood (34, 35). This study is the first to examine the relationship between Hb and tumor erythropoietin/erythropoietin receptor expression. The lack of correlation between the factors may be because anemia is not contributing significantly to tumor hypoxia. This would have implications for the therapeutic approach of correcting anemia to alter tumor oxygenation and suggest that altering Hb levels would not modify the molecular response to hypoxia. Alternatively, anemia may be interacting with other pathways that obscure its independent assessment.

We also considered whether erythropoietin/erythropoietin receptor expression could be correlated with survival or disease-free survival. No correlation was found, in contrast to one previous study in endometrial cancer that found that a weighed intensity score for erythropoietin was associated with decreased survival (32). This finding is interesting, because markers of hypoxia have been used as predictors of outcome in HNSCC. It may suggest that due to the relatively long half-life of erythropoietin (hours) compared with HIF-1α (minutes; refs. 36, 37), erythropoietin offers a poor discriminatory score for the level of hypoxia. It may also be that there are differences in tumor types, but further studies in other tumors are required to examine this association.

Having shown high levels of erythropoietin and erythropoietin receptor protein expression in HNSCC, we then considered the mRNA expression in paired samples. A recent in vivo study in non–small cell lung cancer found, using reverse transcription-PCR, that although erythropoietin and erythropoietin receptor transcripts could be detected, their distribution was heterogeneous and no correlation could be found between them (18). Using paired samples, although we found a range of tumor expression, in 9 of the 11 cases, the mRNA expression of erythropoietin was increased in the tumor compared with the corresponding normal sample and that this was significant when considering the cohort as a whole. However, there was no corresponding increase in erythropoietin receptor mRNA expression between the two groups. In addition, there was no correlation...
found between erythropoietin and erythropoietin receptor mRNA or between mRNA and protein expression for either erythropoietin or erythropoietin receptor. The latter observation shows the complexity of in vivo pathways, and other post-translational mechanisms may further regulate the protein expression.

This finding shows that although the erythropoietin mRNA expression in tumors is heterogeneous, using paired samples, erythropoietin but not erythropoietin receptor expression is up-regulated in HNSCC. Because the samples are paired, we can be confident that this is a tumor-specific response. It may therefore be that erythropoietin is involved in a local autocrine feedback loop, stimulated by hypoxia, and act as an effector molecule for the erythropoietin receptor to stabilize it post-translationally.

The finding that the mRNA and protein expression of erythropoietin is increased in HNSCC in association with hypoxia raises the question of the biological significance. Clinical trials using recombinant human erythropoietin have indirectly suggested a functional role for erythropoietin and erythropoietin receptor in HNSCC by adversely influencing survival but have not explained the mechanism underlying this (9). Statistical imbalances in the trial have been postulated, but preclinical studies support a functional role for erythropoietin/erythropoietin receptor in vitro.

In vitro, the expression of both erythropoietin and erythropoietin receptor was increased by hypoxia in breast cancer cells, whereas in HNSCC, only erythropoietin receptor expression was up-regulated (17, 33). The latter finding is in contrast to our clinical data that suggests erythropoietin is regulated by hypoxia in HNSCC. Recent studies have also shown that treating HNSCC cells with recombinant human erythropoietin increases Janus kinase 2 phosphorylation and causes enhanced cell invasion (33, 38). Other studies have shown that recombinant human erythropoietin increases cell proliferation in breast cancer cells (17) and inhibits the cytotoxic effects of cisplatin in cervical cancer cells (31). Furthermore, erythropoietin stimulated growth and survival of tumor cells in xenografts (39), indicating that erythropoietin can be important in tumor biology. Although we have investigated the role of erythropoietin/erythropoietin receptor in tumor epithelial cells, we also found vascular endothelial expression of erythropoietin/erythropoietin receptor. The signaling of erythropoietin and erythropoietin receptor is recognized to be important in angiogenesis (15). Therefore, it is possible that the tumor expression of erythropoietin/erythropoietin receptor while reflecting hypoxia may be exerting an angiogenic effect on the tumors.

This study supports the increasingly recognized phenomenon of erythropoietin and erythropoietin receptor expression in tumors and provides strong evidence for the hypoxic regulation of erythropoietin and a local autocrine signaling pathway for erythropoietin/erythropoietin receptor. This is the first study to examine the relationship between erythropoietin/erythropoietin receptor and anemia. It would be of interest to investigate directly the effects of transfusion on this pathway by serial biopsies to elucidate the interactions while on therapy. In addition, if erythropoietin is targeting vessels, the combined use of erythropoietin with an antiangiogenic agent may address some of the clinical concerns.

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References

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