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Erythropoietin-mediated activation of JAK-STAT signaling contributes to cellular invasion in head and neck squamous cell carcinoma

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Originally characterized as a growth factor for erythrocytes, erythropoietin (EPO) is used to treat anemia and fatigue in cancer patients receiving radiation therapy and chemotherapy. EPO and the EPO receptor (EPOR) are expressed in nonhematopoietic cells and cancers. However, the role of EPO and EPOR within nonhematopoietic cancer cells remains incompletely understood. Although a recent clinical trial demonstrated worse tumor control and survival in head and neck cancer patients treated with EPO, the role of EPO and EPOR in head and neck squamous cell carcinoma (HNSCC) has not been examined. In the present study, we demonstrate the previously unrecognized EPO-mediated invasion by HNSCC cells through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway. Furthermore, we confirmed the expression of EPO and EPOR in a panel of human HNSCC cell lines and tissue specimens. Pharmacological doses of EPO also had a limited proliferation effect in these cell lines. These results define a novel role for EPO in mediating tumor cell invasion. Increased levels of EPO and EPOR in lymph node metastases as compared to primary tumors from HNSCC patients further support the role of EPO/EPOR in HNSCC disease progression and metastasis.
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Introduction

Cancer patients receiving radiation therapy (XRT) and chemotherapy have often been treated with recombinant human erythropoietin (EPO) to combat anemia and fatigue, since EPO promotes erythrocyte survival and differentiation (Jelkmann, 1994; Thatcher, 1998; Lacombe and Mayeux, 1999). A previous study suggested that EPO treatment in anemic patients receiving XRT and chemotherapy might improve survival (Littlewood *et al*., 2001). More recent evidence, however, demonstrates the expression and function of EPO and the EPO receptor (EPOR) in nonhematopoietic cells and cancers, suggesting that treatment with EPO could have unintended physiological consequences (Acs *et al*., 2001; Henke *et al*., 2003). EPO/EPOR expressing cancers include breast cancer, cervical carcinoma, melanoma, neuroblastomas and glioblastomas (Acs *et al*., 2001, 2002, 2003; Arcasoy *et al*., 2002; Yasuda *et al*., 2003). Although prior studies focused primarily upon the expression of EPO and EPOR in selected tumor types, some reports suggested a role for EPO in the proliferation and survival of cancer cells (Acs *et al*., 2001; Arcasoy *et al*., 2002; Yasuda *et al*., 2003; Pajonk *et al*., 2004).

Homodimerization of EPOR subunits stimulates numerous signaling pathways including activation of Lyn kinase, phosphatidylinositol 3-kinase and phospholipase C-γ (Wojchowski *et al*., 1999). The Janus kinase 2 (JAK2) is also recruited to the receptor complex and activates the signal transducer and activator of transcription (STAT) factors, STAT-5A and STAT-5B (Ihle *et al*., 1995; Liu *et al*., 1998). The activation of the STAT-5A and STAT-5B isoforms leads to distinct transcriptional regulation events (Zhang *et al*., 2000; Leong *et al*., 2002; Guo *et al*., 2004; Hwa *et al*., 2004). Recent studies have only begun to explore signaling pathways activated by EPO within nonhematopoietic cancer cells (Acs *et al*., 2001; Yasuda *et al*., 2003).

In addition to EPO/EPOR expression studies, two recent clinical trials raise serious concerns regarding the use of EPO in cancer patients. The Breast Cancer Erythropoietin Trial (BEST) in nonanemic patients was terminated early due to an increased mortality rate in patients given EPO, compared to those receiving a placebo; the increase was mainly due to early disease progression (BEST, 2003). Additionally, head and neck

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squamous (HNSCC) patients treated with XRT demonstrated increased locoregional recurrence and decreased survival when treated concomitantly with EPO (Henke *et al*., 2003). These results emphasize the need to evaluate carefully the use of EPO in cancer patients and to understand the role of EPO/EPOR signaling in cancer cells.

The presence of EPO and EPOR in HNSCC cells has only been shown recently, but their potential function(s) in this cancer has not been explored (Arcasoy *et al*., 2005). HNSCC represents about 5% of all human cancers and approximately 40 000 new cancer cases in the US annually (Jemal *et al*., 2002). The present study delineates a previously unrecognized functional role for EPO-mediated signaling in HNSCC cells. These findings may provide a potential molecular mechanism for the increased tumor recurrence and decreased survival in head and neck cancer patients treated with EPO.

Results

EPO mediates invasion in HNSCC cell lines

HNSCC morbidity and mortality are often the result of local tumor invasion into vital structures such as the carotid artery and the airway. Although several studies suggest that EPO mediates tumor proliferation, such an effect alone is unlikely to explain worse patient outcome in the recent EPO clinical trial in head and neck cancer patients (BEST, 2003; Henke *et al*., 2003). We explored the possibility that EPO might promote HNSCC tumor cell invasion. HNSCC cell invasion was measured in the Matrigel-GFR (growth factor reduced) invasion assay. Cells were plated into the upper chamber with no growth factor, and EPO (1 U/ml) or EGF (10 ng/ml) as a positive control. This laboratory has previously demonstrated EGF-stimulated invasion of HNSCC cells in the Matrigel-GFR invasion assay (Thomas *et al*., 2003). EPO- and EGF-stimulated 1483 cells demonstrated a significant increase in cellular invasion through the Matrigel-GFR matrix as compared to untreated cells (Figure 1a). Similarly, PCI-15B, UM-22A and UM-22B cells exhibited increased invasion with EPO stimulation (Figure 1). A dose–response curve experiment (0–100 U/ml EPO) in UM-22A and UM-22B cells demonstrated maximal invasion through the Matrigel-GFR matrix at 1 U/ml EPO (Supplementary data). In the untreated wells, HNSCC cell lines derived from metastatic disease sites (e.g. UM-22B and PCI-15B) demonstrated increased basal invasive activity as compared to cell lines derived from primary tumors (e.g. UM-22A). In all of the tested HNSCC cell lines, the difference between untreated and EPO-stimulated cell invasion was statistically significant ($P = 0.014$). Although prior studies suggested a role for EPO in tumor cell proliferation, these results demonstrated for the first time a role for EPO in mediating tumor cell invasion, specifically in HNSCC cells (Acs *et al*., 2002; Arcasoy *et al*., 2002; Yasuda *et al*., 2003).

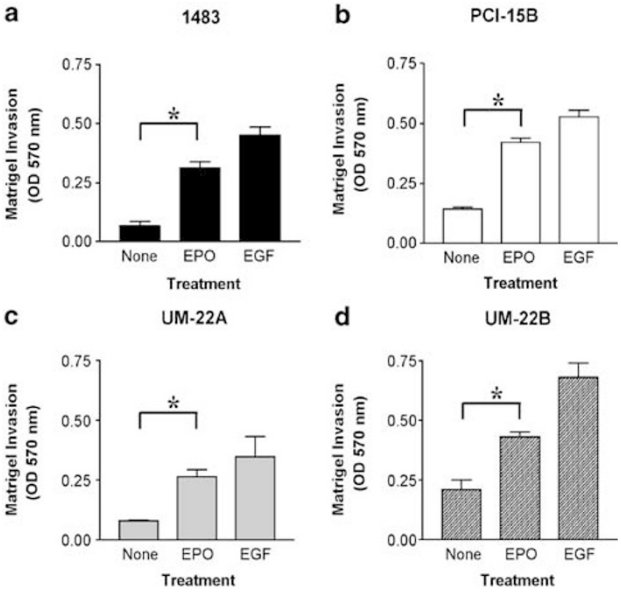

Figure 1 EPO stimulates invasion by HNSCC cells in the Matrigel invasion assay. The 1483 (**a**), PCI-15B (**b**), UM-22A (**c**) and UM-22B (**d**) cell invasion was examined in the Matrigel invasion assay. The difference in cellular invasion between untreated cells and EPO-stimulated cells is statistically significant (*$P = 0.014$)

JAK inhibitor (AG490) blocks HNSCC cell invasion

Ligand engagement of EPOR results in the activation of multiple signal transduction cascades including the JAK-STAT pathway (Wojchowski *et al*., 1999). To assess the possible role of JAK-STAT signaling in EPO-mediated invasion by HNSCC cells, we employed the JAK inhibitor, AG490 (Meydan *et al*., 1996). EPO stimulation of 1483 cells pretreated with AG490 (1 μM) demonstrated decreased invasion in the Matrigel-GFR assay as compared to EPO-stimulated control cells (Figure 2a). A total of 1483 cells stimulated with EGF were unaffected by AG490 and demonstrated invasiveness similar to EGF-treated control cells. Similarly, EPO-mediated cellular invasion in the Matrigel-GFR assay was inhibited by AG490 treatment in the HNSCC cell lines PCI-15B, UM-22A and UM-22B (Figure 2b–d). The baseline invasive activity in HNSCC cell lines derived from metastatic disease (UM-22B and PCI-15B) also declined with AG490 treatment. In all of the tested HNSCC cell lines, the reduction in EPO-stimulated cell invasion by AG490 was statistically significant ($P = 0.014$). The results from the AG490 inhibitor studies demonstrated a role for JAK-STAT signaling in EPO-mediated invasion by HNSCC cells.

A STAT-5A dominant-negative (DN) mutant inhibits HNSCC cell invasion

Since the AG490 inhibitor studies implicated JAK-STAT signaling in mediating EPO-stimulated HNSCC cell invasion, we sought to clarify further the role of this pathway by examining STAT factor activation. Our laboratory previously demonstrated a differential

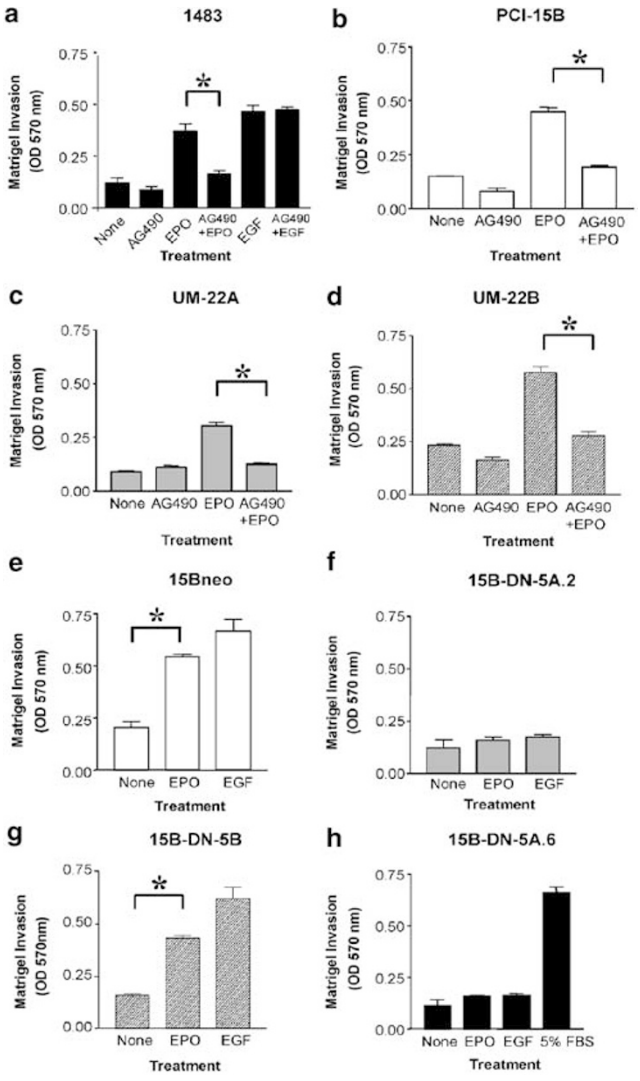

Figure 2 EPO-stimulated invasion by HNSCC cells is inhibited by a JAK2 inhibitor (AG490) or a DN mutant of STAT-5A. HNSCC cells (**a–d**) were pretreated with AG490 (1 μM) prior to growth factor stimulation in the Matrigel invasion assay. The HNSCC cell lines demonstrated diminished cellular invasion in response to EPO (1 U/ml) following AG490 treatment. Invasion due to EGF (10 ng/ml) stimulation in the 1483 cells was unaffected by the AG490 inhibitor. The difference in cellular invasion between EPO-stimulated cells in the presence and absence of the AG490 inhibitor is statistically significant (*$P = 0.014$). PCI-15B (**e**), PCI-15B-DN-5A (**f** and **h**) and PCI-15B-DN-5B (**g**) cells were treated with no growth factor, EPO (1 U/ml) or EGF (10 ng/ml). PCI-15B and PCI-15B-DN-5B cells were able to invade in response to EPO and EGF stimulation. The difference in cellular invasion between untreated cells and EPO-stimulated cells is statistically significant (*$P = 0.014$). Two clones of the PCI-15B-DN-5A cells expressing a DN form of STAT-5A did not invade in response to EPO or EGF. Cells of both PCI-15B-DN-5A clones were able to invade in response to 5% FBS (panel h and data not shown)

function for STAT-5 isoforms in HNSCC growth (Leong *et al*., 2002; Xi *et al*., 2003). Specifically, STAT-5B appeared to be essential for HNSCC growth but not STAT-5A. These studies employed DN mutants of STAT-5A and STAT-5B stably expressed in PCI-15B cells. We utilized these cells to investigate the role of

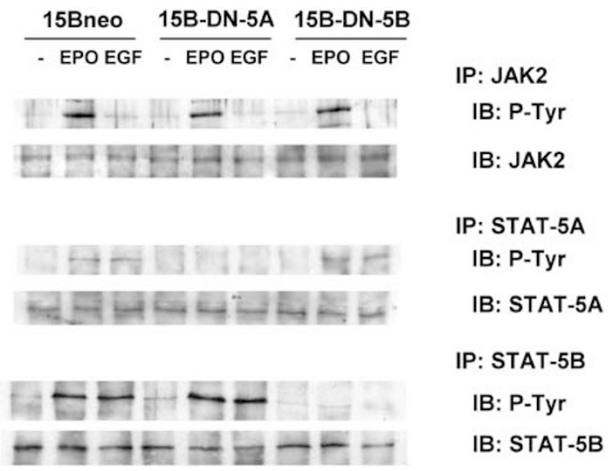

Figure 3 Activation of the JAK-STAT pathway is inhibited by the STAT-5A and STAT-5B DN mutants. Activation of the JAK-STAT pathway with the PCI-15B, PCI-15B-DN-5A and PCI-15B-DN-5B cell lines was assessed by immunoprecipitation of JAK2, STAT-5A or STAT-5B and immunoblotting with an antiphosphotyrosine antibody (4G10)

STAT-5A and STAT-5B in EPO-mediated cell invasion. The 15Bneo, 15B-DN-5A and 15B-DN-5B cells were subjected to the Matrigel-GFR invasion assay (Figure 2e–g). The 15Bneo and 15B-DN-5B cells demonstrated increased cellular invasion when stimulated with EPO and EGF. Interestingly, 15B-DN-5A cells did not demonstrate any increase in cellular invasion when stimulated with EPO or EGF. Cellular invasion was restored, however, when the 15B-DN-5A cells were treated with 5% FBS and these results were verified in an independent 15B-DN-5A clone (Figure 2h and data not shown).

To test the integrity of the JAK-STAT pathway in these cells, cell lysates from untreated, EPO- and EGF-stimulated cells were subjected to immunoprecipitation with the JAK2, STAT-5A or STAT-5B antibodies and immunoblotting with an anti-phosphotyrosine antibody (4G10). The membranes were stripped and reprobed with the immunoprecipitation antibody to ensure equivalent protein loading. EPO stimulation led to increased phosphorylation of JAK2 in 15Bneo, 15B-DN-5A and 15B-DN-5B cells (Figure 3). EGF stimulation did not result in the activation of JAK2 in these cell lines. Increased phosphorylation of STAT-5A was clearly demonstrated in both the 15Bneo and 15B-DN-5B cell lines with EPO stimulation. Additionally, EGF stimulation resulted in the activation of STAT-5A, possibly through the action of other JAK kinases or the intrinsic EGFR tyrosine kinase (David *et al*., 1996). No significant increase in STAT-5A phosphorylation was detected in the 15B-DN-5A cell line. Similarly, increased phosphorylation of STAT-5B was seen in the 15Bneo and 15B-DN-5A cell lines following EPO and EGF stimulation. However, no significant increase in STAT-5B phosphorylation was detected in the 15B-DN-5B cells. EPO-mediated activation of JAK-STAT signaling within HNSCC cells strongly suggests EPOR expression at the cell surface. Together with the AG490 inhibitor

studies, these results demonstrated the roles of JAK2 and STAT-5A, but not STAT-5B, in mediating HNSCC cell invasion following EPO stimulation.

EPO mediates a limited proliferative effect in HNSCC cell lines

A number of reports have suggested a proliferative effect by EPO on nonhematopoietic cancer cells (Acs *et al*., 2001; Arcasoy *et al*., 2002; Yasuda *et al*., 2003). Conversely, a recent study did not detect any proliferative effect by EPO on leukemia, renal carcinoma and colorectal adenocarcinoma cell lines (Liu *et al*., 2004). We serum starved HNSCC cells and treated them with no growth factor, increasing doses of EPO or EGF (10 ng/ml) as a positive control and assessed proliferation by total cell counts via vital dye exclusion. HNSCC cells demonstrated limited proliferation with EPO stimulation (Figure 4). Proliferation correlated with EPO dose, but was maximal at 10 U/ml and never as robust as EGF-stimulated proliferation in the HNSCC cell lines. PCI-37A, PCI-37B and 1483 cells exhibited similar responses to EPO and EGF stimulation (data not shown). Proliferation in cells treated with 1 U/ml or less of EPO was not statistically significant when compared to control cells treated with no growth factor ($P > 0.05$). These results demonstrate that EPO stimulation has a limited proliferative effect in HNSCC cells that is less than stimulation by EGF or serum (Figure 4 and data not shown).

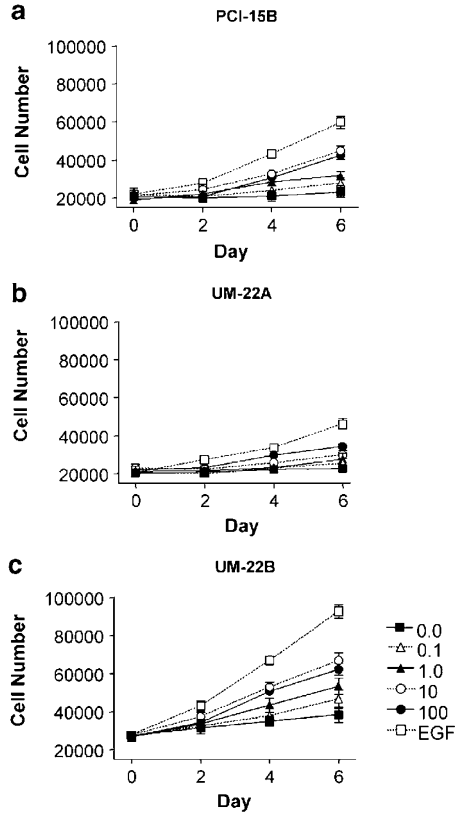

Figure 4 EPO mediates a modest proliferation response in HNSCC cells. Following treatment with no growth factor, EPO (0.1–100 U/ml) or EGF (□, 10 ng/ml), proliferation was assessed by total cell counts. Proliferation was not statistically significant in cells treated with 1 U/ml or less of EPO as compared to control cells that were not treated with growth factor ($P > 0.05$)

Expression of EPOR and EPO in HNSCC

EPO-stimulated activation of the JAK-STAT pathway and cell invasion provide evidence for the functional expression of EPOR in HNSCC cells. A recent study demonstrated the expression of EPO and EPOR by immunohistochemistry in human HNSCC specimens, but did not define any EPO-mediated function within these cells (Arcasoy *et al*., 2005). We assessed the expression of EPOR in a panel of well-characterized HNSCC cell lines, derived from various head and neck anatomic sites. Four of the six cell lines were derived from two patients with matched primary tumor (UM-22A/PCI-37A) and metastatic disease (UM-22B/PCI-37B) within cervical lymph nodes. In all six HNSCC cell lines, EPOR mRNA was detected by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis (Figure 5a). Controls included an RT-negative and water (H_2O)-only sample for each cell line (Figure 5a and data not shown). Additionally, RT–PCR products were isolated and confirmed by DNA sequencing (data not shown). Immunoblotting with an EPOR antibody demonstrated the expression of EPOR in all six representative HNSCC cell lines (Figure 5b). HEK293 cells transfected with EPOR or an empty vector served as the positive and negative controls, respectively. We also detected EPO mRNA by RT–PCR in the panel of HNSCC cell lines (Figure 5c). These RT–PCR products were also isolated and confirmed by DNA sequencing (data not shown). Combined with the functional data,

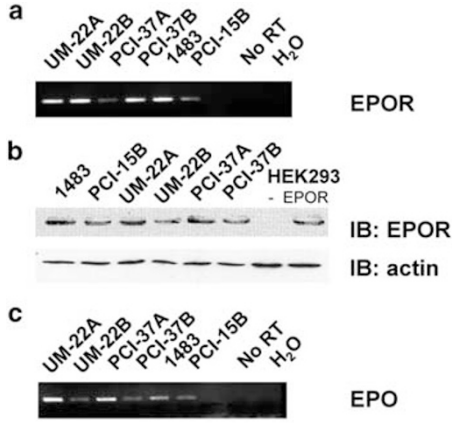

Figure 5 Expression of EPO and EPOR in HNSCC cell lines and tissue samples. (**a**) RT–PCR demonstrated the expression of EPOR mRNA in HNSCC cell lines. Controls included a RT-negative and water (H_2O)-only sample for each cell line. (**b**) Immunoblot analysis of HNSCC cell lines with the EPOR antibody demonstrated the expression of EPOR protein. HEK293 cells transfected with empty vector (−) or the pCDNA-EPOR expression vector (EPOR) served as controls. The membrane was stripped and reprobed with β-actin to verify equivalent protein loading. (**c**) RT–PCR demonstrated the expression of EPO mRNA in HNSCC cell lines. Controls included a reaction with no RT and an H_2O-only sample for each cell line

Table 1 Primary tumor and lymph node metastasis from HNSCC patients

Sample	*Age (years)*	*Sex*	*Primary site*	*Stage*	*Grade*
1	70	Male	Pyriform sinus	T4N2bM0	Mod
2	63	Male	Tongue	T4N2cM0	Mod
3	78	Male	BOT	T2N1M0	Mod
4	88	Male	FOM	T4aN1M0	Well
5	68	Female	BOT	T2N1M0	Poor
6	74	Male	Larynx	T3N2aM0	Mod
7	46	Male	Tongue	T4aN2bM0	Poor
8	73	Female	Oropharynx	T4N1M0	Mod
9	51	Male	FOM	T4N1M0	Mod
10	72	Male	Larynx	T3N2aM0	Mod
11	67	Male	Larynx	T3N2aM0	Mod
12	55	Female	Buccal	T4N2bM0	Poor

Tissue specimens were obtained from 12 HNSCC patients through the University of Pittsburgh Head and Neck Tissue Bank for quantitative real-time PCR analysis. Clinical staging was determined by American Joint Committee on Cancer (AJCC) classification (Greene *et al*., 2002). BOT, base of tongue; FOM, floor of mouth

these findings demonstrated that HNSCC cells express both EPOR and EPO, confirming recently reported immunohistochemistry studies (Arcasoy *et al*., 2005).

We also examined EPO and EPOR expression in tissue specimens obtained from the Head and Neck Cancer Tissue Bank at the University of Pittsburgh. In all, 12 patients had specimens available from both the primary tumor and cervical lymph node metastasis (Table 1). We assessed the levels of EPO and EPOR in these specimens by quantitative real-time RT–PCR. Levels of EPO and EPOR were normalized with the endogenous control β-glucuronidase (β-GUS) and compared by a relative standard curve method with cDNA from UM-22A cells. EPO was detected in 75% (9/12) of the primary tumors and 91.2% (11/12) of the lymph node metastases (EPO/β-GUS median: 2.215; range: 0.753–36.4). EPO could not be detected in the primary tumor and lymph node metastasis of one patient. EPOR was detected in 100% (12/12) of the primary tumors and lymph node metastases (EPOR/β-GUS median: 1.597; range: 0.635–219.1). EPO levels were increased in the lymph node metastasis as compared to the primary tumor in 83.3% (10/12) of patients (Figure 6; $P=0.0096$). EPOR levels were increased in the lymph node metastasis as compared to the primary tumor in 83.3% (10/12) of patients (Figure 6; $P=0.016$). These findings in human HNSCC specimens suggest the differential expression of EPO and EPOR *in vivo* that may contribute to HNSCC invasion and metastasis.

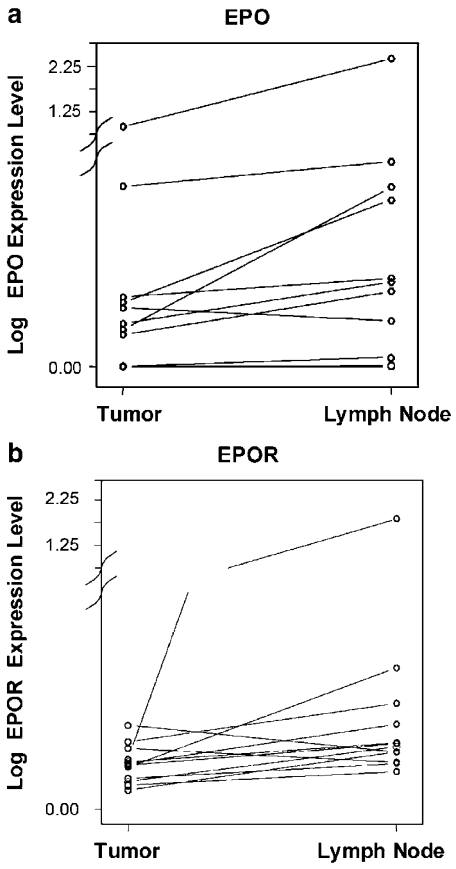

Figure 6 EPO and EPOR expression is increased in HNSCC lymph node metastases as compared to primary tumors in human tissue specimens. EPO and EPOR expression was measured in human tissue specimens by real-time quantitative PCR and normalized to β-GUS expression. (**a**) The expression levels of EPO in the primary tumor and matched lymph node metastasis specimens. (**b**) The expression levels of EPOR in the primary tumor and matched lymph node metastasis specimens. Lymph node metastases demonstrated increased the expression of EPO ($P=0.0096$) and EPOR ($P=0.016$) as compared to the matched primary tumors

Discussion

In the present study, EPO induced not only limited proliferation but also more robustly invasion in HNSCC cells. Our data delineate a molecular mechanism for EPO-stimulated HNSCC cell invasion, involving selective activation of JAK2 and STAT-5A. These findings are further supported by the expression of EPO and EPOR in HNSCC cell lines and patient tissue specimens, which confirm recent studies (Arcasoy *et al*., 2005). Collectively, these data support a role for EPO/EPOR in HNSCC cell invasion and disease progression. Combined with the decreased locoregional control and survival of patients treated with EPO in the BEST and head and neck cancer

clinical trials, these findings should lead clinicians to re-evaluate the therapeutic use of EPO in cancer patients (BEST, 2003; Henke *et al*., 2003).

Although EPO has been implicated in the regulation of tumor growth, the contribution of EPO signaling to tumor cell proliferation has not been well defined. A study implicating EPO in breast cancer cell growth employed inhibitors and soluble EPOR to measure inhibition of tumor growth (Arcasoy *et al*., 2002). Other studies measured proliferation by indirect methods such as 5-iodo-2′-deoxyuridine uptake (IdU) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Acs *et al*., 2001; Yasuda *et al*., 2003). Positive findings using these assays may also have resulted from an antiapoptotic effect. In addition, relatively high doses of EPO (10–250 U/ml) were required to detect proliferation effects. A more recent study found no proliferation effect due to EPO or GM-CSF in leukemic, renal cell carcinoma and colorectal adenocarcinoma cell lines (Liu *et al*., 2004). The analysis was performed using lower doses of EPO (0–20 U/ml) and it suggested that the proliferative effects of EPO may be cell type specific. We measured cell proliferation by cell count using vital dye exclusion and found that EPO has a limited effect on HNSCC proliferation at the 1 U/ml dose. Higher pharmacological doses (10–100 U/ml) were required to achieve a measurable proliferation response. In contrast, EPO induced cell invasion at a lower dose of 1 U/ml, supporting the hypothesis that the protumor effects of EPO may be related to invasion and metastasis instead of proliferation. Although the actual EPO concentration within the tumor microenvironment is undefined, EPO levels would likely have to be much higher than the levels found normally in the serum (5–20 mU/ml) to influence tumor proliferation (Tabbara, 1993).

While EPO may contribute to tumor growth, this effect alone is unlikely to explain the deleterious effects of EPO treatment upon patient survival and locoregional control in a recent head and neck cancer clinical trial (Henke *et al*., 2003). Increased tumor size may result in the compression of adjacent structures, but head and neck cancers, like many other solid tumors, cause patient morbidity and mortality through local invasion and extension into vital structures such as the trachea and carotid artery. The increase in HNSCC cell invasion following EPO stimulation has not been previously reported and provides a molecular mechanism for the potential negative effects of EPO therapy identified in the recent BEST and head and neck clinical trials (BEST, 2003; Henke *et al*., 2003). These poor outcomes may be explained, in part, by the modest increase in EPO and EPOR expression in the metastatic disease found in cervical lymph nodes. Although our present analysis was based upon a relatively small number of available tissue specimens, changes in EPO and EPOR expression between the primary HNSCC lesion and the metastatic deposits in the cervical lymph nodes were consistent and statistically significant. The upregulation of the EPO/EPOR signaling pathway is likely to be one of many factors contributing to disease progression and metastasis from the primary tumor site. Additional post-transcriptional regulatory mechanisms may also influence EPO and EPOR expression within metastatic HNSCC. Clinical administration of EPO in HNSCC patients receiving chemotherapy and XRT may further promote tumor invasion and metastasis, accounting for decreased patient survival and increased locoregional recurrence.

The present study also defines a molecular mechanism for EPO-stimulated HNSCC cell invasion. The involvement of JAK-STAT signaling in HNSCC cell invasion has not been previously reported, although a requirement for JAK activity in lymphoma invasion and metastasis was recently published (Opdam *et al*., 2004). HNSCC cell invasion requires STAT-5A, but not STAT-5B, as demonstrated by studies with DN mutants of the STAT-5 isoforms. Inhibition of EPO-mediated cell invasion, but not EGF-mediated invasion, by the JAK inhibitor AG490 provides further evidence that STAT-5A activity, specifically, is crucial for HNSCC invasion. The activation of STAT-5A by EGF does not require JAK2, but results from the intrinsic tyrosine kinase activity of EGFR (David *et al*., 1996). Conversely, HNSCC proliferation *in vitro* and *in vivo* requires STAT-5B but not STAT-5A (Leong *et al*., 2002; Xi *et al*., 2003). Although human STAT-5A and STAT-5B demonstrate 94% homology at the amino-acid level, they can be discretely activated and exhibit distinct DNA-binding specificities (Meinke *et al*., 1996; Boucheron *et al*., 1998). Thus, the different roles of STAT-5A and STAT-5B in tumor growth and invasion parallel the differences seen in mice with targeted disruption of the STAT-5A or STAT-5B gene (Liu *et al*., 1997; Udy *et al*., 1997).

Recognizing the role of EPO/EPOR in tumor progression is likely to influence significantly the development of other therapeutic interventions. EPO signaling influences uterine angiogenesis and likely contributes to angiogenesis in certain cancer types (Yasuda *et al*., 1998, 2003). To be effective, antiangiogenic therapies will need to focus on EPO as well as current molecular targets such as vascular endothelial growth factor. Perhaps, this combination approach will be more effective in not only inhibiting angiogenesis but also other cellular functions crucial for tumor progression.

EPO has been an important pharmacological agent in the treatment of patients with a variety of nonhematopoietic cancers. Anemia is a negative prognostic factor for cancer patients treated with XRT and chemotherapy (Shasha, 2001). Additionally, studies have suggested that the use of EPO may decrease the need for blood transfusions in head and neck cancer patients undergoing surgical treatment (Gall and Kerr, 2000; Glaser *et al*., 2001). The recent clinical trials and the accumulating data regarding previously unappreciated EPO-mediated functions in cancer cells demonstrate the need to increase our understanding of the role of EPO in tumor progression. However, these findings should not absolutely preclude the use of EPO in cancer patients, since the available data suggest that EPO may affect only certain types of cancer (Liu *et al*., 2004).

Therapeutic interventions targeting EPO-mediated signaling pathways specifically in cancer cells may permit the continued use of EPO for anemia in all cancer patients.

Materials and methods

Cell lines and materials

UM-22A and UM-22B (Virolainen *et al*., 1983); 1483 (Sacks *et al*., 1988); PCI-37A, PCI-37B and PCI-15B (Heo *et al*., 1989; Leong *et al*., 2002); and 15B-5A-DN, 15B-5B-DN and 15Bneo were maintained in DMEM + 10% FBS at 37°C in 5% CO_2. Cell lines derived from the same patient were designated 'A' for primary tumor and 'B' for metastatic tumor deposits. Recombinant human EPO (Epoetin-alfa; Amgen) was purchased through the University of Pittsburgh Pharmacy. EGF was obtained from Oncogene Research Products. EPOR antibody and the EPOR expression vector (pcDNA-EPORwt) were gifts from Dr Sarah A Gaffen (Yoshimura *et al*., 1990). Transfection of HEK293 cells was performed with a standard calcium phosphate precipitation method. Cell proliferation was assessed as described previously (Grandis *et al*., 1998).

RT–PCR for EPO and EPOR mRNA in HNSCC cell lines

Total RNA was isolated using Trizol reagent (Invitrogen) and RNA (2 μg) was treated with DNase I (Roche) in a 25 μl reaction. RNA (8 μl) was converted to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). EPO and EPOR were amplified as described previously (Yokomizo *et al*., 2002). RT–PCR products were separated on a 1.5% TBE/agarose gel, isolated and confirmed by DNA sequencing (University of Pittsburgh DNA Sequencing Core Facilities).

Real-time quantitative RT–PCR for EPO and EPOR in human HNSCC primary and lymph node tumor tissues

Tissue samples were obtained from the University of Pittsburgh Institutional Review Board (IRB)-approved Head and Neck Cancer Tissue Bank from patients treated between 2002 and 2004 with primary tumor and metastatic lymph node disease available. RNA was extracted and converted to cDNA. Separate quantitative PCR was performed with 1 μl of the cDNA reaction and 250 nM of each probe in an ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems). Triplicates of each 50 μl reaction were run using the following conditions: 50°C for 2 min, 95°C for 10 min and then 40 cycles of 95°C for 15 s, 60°C for 60 s. β-GUS primers and probes were as described previously (Godfrey *et al*., 2000). EPO and EPOR primers were used at 900 nM each. EPO and EPOR probes had the following sequences: 5′-FAM-TCCAGTGCCAGCAAT GACATCTCAGG-TAMRA-3′ and 5′-FAM-CCAGCTAT GTGGCTTGCTCTTAGGACA-TAMRA-3′, respectively. Quantitation was performed using the Relative Standard Curve method (ABI User Bulletin 'Relative Quantitation of Gene Expression' – updated 10/01). Standard curves were generated from UM-22A cDNA. EPO and EPOR ratios with the mean β-GUS (endogenous control) level were calculated for all three reactions of each sample. Averaged values were used to calculate lymph node to tumor ratios and group differences were tested with the Wilcoxon's signed rank sum test.

Matrigel™-GFR invasion assay

In all, 24-well plates with precoated growth factor reduced Matrigel (Matrigel-GFR) chambers (BD Biosciences) were prepared according to the manufacturer's instructions (Albini *et al*., 1987; Chu *et al*., 1993). The lower well of the chamber was filled with DMEM + 0.1% BSA. Cell suspensions (1×10^5 cells) were added to the upper chamber with no growth factor, EPO (1 U/ml) or EGF (10 ng/ml). AG490 (1 μM; LC Laboratories) inhibitor or DMSO was added to the cells within the upper chamber for 2 h prior to the addition of the appropriate growth factor. Dilutions of the cell samples placed in a 96-well plate served as loading controls. Following incubation for 24 h at 37°C, noninvasive cells were removed from the filter top and the filter was fixed in 1% formaldehyde in phosphate-buffered saline (PBS). Invading cells and loading controls were stained with 1% crystal violet. Filters were washed in PBS, were solubilized in 2% SDS overnight and the optical density determined at 570 nm. Mean ± s.d. values were calculated from three repeated observations in each of four separate experiments. Group differences were tested with the exact Wilcoxon's test.

Immunoprecipitation and Western blot analysis

Cells (1.5×10^6) were plated on 100 mm dishes. Following serum starvation for 48 h, cells were treated with no growth factor, EPO (1 U/ml) or EGF (10 ng/ml) for 15 min. Subconfluent cells were washed twice in ice-cold PBS and harvested in lysis buffer (Leong *et al*., 2002). The lysate samples were sonicated and centrifuged at 13 200 *g* for 5 min at 4°C. Protein lysates (800 μg) were subjected to immunoprecipitation with either the anti-JAK2, anti-STAT-5A or anti-STAT-5B antibodies (Upstate Biotechnology). Immunoblotting was performed with an antiphosphotyrosine antibody (4G10; Upstate Biotechnology). Secondary horseradish peroxidase-linked rabbit anti-mouse antibody was used and detected with the Western Blot Luminol reagent (Santa Cruz Biotechnology). Membranes were stripped and reprobed with the antibody used for immunoprecipitation to verify equivalent target protein levels.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)