Hypoxia-inducible Factors HIF-1α and HIF-2α in Head and Neck Cancer: Relationship to Tumor Biology and Treatment Outcome in Surgically Resected Patients

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Abstract

Hypoxia within head and neck squamous cell carcinoma (HNSCC) predicts a poor response to radiotherapy and poor prognosis. Hypoxia-inducible factor (HIF)-1 and HIF-2 are nuclear transcription factors that regulate the cellular response to hypoxia and are important for solid tumor growth and survival. Overexpression of HIF-1α and HIF-2α was demonstrated in three HNSCC cell lines under hypoxia and tumor tissue versus normal tissue (n = 20, HIF-1α, P = 0.023; HIF-2α, P = 0.013). On immunostaining, HIF-1α and HIF-2α expression were localized to tumor nuclei; HIF-2α expression was also seen in tumor-associated macrophages. Expression of HIF-1α in surgically treated patients with HNSCC (n = 79) was associated with improved disease-free survival (P = 0.016) and overall survival (P = 0.027).

Introduction

A low intratumoral pO2 in HNSCC predicts a poor response to radiation and low overall survival (1). This is partly because of lack of fixation by oxygen of radiation-induced damage but may also be related to overexpression of hypoxia-inducible genes that promote cell survival (2). Two of the most important transcription factors mediating the cellular response to hypoxia are HIF-1α and HIF-2α. These transactivate many target genes, including glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (3). Induction of these genes is part of the cellular response to an adverse environment and may give cells a survival advantage by promoting glucose transport, anaerobic metabolism, and angiogenesis (2, 4). HIF-1α and HIF-2α are critical for solid tumor growth, survival, and promotion of aggressiveness (5, 6) with overexpression seen in many human tumors (7, 8). Overexpression of HIF-1α has been linked to poor outcome in cervical and oropharyngeal carcinoma in patients treated with radiotherapy (9, 10). We sought to investigate the role of HIFs in the biology of HNSCC treated by a modality not dependent on hypoxia, i.e., surgery to determine whether the effect of HIF expression may be different in this context.

Materials and Methods

Cell Lines. Human head and neck squamous carcinoma cell lines UM-SCC22A, UM-SCCC22B (courtesy of Dr. T. Carey, University of Michigan), SCC-25, and HeLa cells (American Type Culture Collection) were maintained in DMEM with 10% heat-inactivated FCS and 2 mM fresh glutamine. Cells were exposed to normoxia or hypoxia (94.9% N2, 5% CO2, 0.1% O2) for 16 h. Cells were harvested on ice and homogenized in lysis buffer [8 mM urea, 10% glycerol, 10 mM Tris-HCl (pH 6.8), 1% SDS, 5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin].

Immunoblotting. Cell and tissue extracts were protein quantified using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) to ensure even distribution of total protein between lanes. Hypoxic and normoxic HeLa cell extracts were used as positive and negative controls, respectively (8). Proteins were resolved in SDS 6% polyacrylamide gels and transferred with a semidry blotter to Immobilon-P membrane (Millipore, Bedford, MA) overnight. For HIF-1α detection, MoAb 28Bb (11) was used at 4 μg/ml; for HIF-2α, MoAb 190B (11) was used at 2 μg/ml; Glut 1 polyclonal rabbit antibody GT11-A (Alpha Diagnostic, San Antonio, TX) was used at 2 μg/ml. Detection was performed with horseradish peroxidase-conjugated goat antimouse (HIF-1α and HIF-2α) and antirabbit (Glut 1) immunoglobulins (DAKO, Ely, United Kingdom) at 1:1000 and enhanced chemiluminescence (Amersham Corp., Little Chalfont, Buckinghamshire, United Kingdom). To additionally control for protein loading, blotted membranes were stained with Ponceau Red (Sigma Diagnostics, St. Louis, MO) to ensure even distribution of total protein between lanes. Quantitative analysis of HIF-1α and HIF-2α protein expression was performed on the immunoblotting gels by densitometry (Fluorochrom; Alpha Innotech Corp., San Leandro, CA).

LDH Assay. Tissue extracts were protein quantified using the Bio-Rad dendritic cell protein assay to ensure even protein loading and LDH levels were measured using a colorimetric assay (Sigma Diagnostics).

Patient Material. Previously untreated patients (79) with HNSCC (31 oral cavity, 23 oropharynx, 16 larynx, and 9 hypopharynx) were studied. Their median age was 62 years (17–92); there were 54 men and 25 women. A group of 29 tumors was early stage (T1/2), and 50 were late stage (T3/4); 38 were N0 at presentation, and 41 had metastatic spread to the neck nodes (N+). All had surgery with pathologically clear resection margins as their first line of management; 28 received postoperative radiotherapy because of their advanced stage. Tumor grade, margin of invasion, inflammatory infiltrate, and percentage tumor necrosis were assessed.

Immunostaining. Tissue sections were cleared of paraffin, rehydrated, and blocked in hydrogen peroxide. Then they were pressure cooked for 3 min in Tris/EDTA lysis buffer (pH 9.0) before incubation with 1:20 MoAb ESEE 122 (anti-HIF-1α; Ref. 8) or neat MoAb EP190b (anti-HIF-2α; Ref. 8) with 5% normal human serum for 60 min or incubated with 1:100 MoAb Qbend 10 (anti-CD34, Dako) or 1:10 MoAb PGM1 (anti-CD68) in Tris-buffered saline for 60 min. HIF-1 and HIF-2 were developed using the horseradish peroxidase Envision System (DAKO) and CD34 and CD68 using the alkaline phosphatase antialkaline phosphatase system. Slides were counterstained with Hematoxylin. Tumor sections were graded as showing no expression (negative) or expression (positive) of HIF-1α and HIF-2α in the tumor nuclei and tumor-associated macrophages (HIF-2α only). A case of renal carcinoma with known

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2 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; Glut 1, glucose transporter 1; HIF, hypoxia-inducible factor; LDH, lactate dehydrogenase; MoAb, monoclonal antibody; NS, not significant; MVD, microvessel density; TAM, tumor-associated macrophage.
overexpression of HIF-1α and HIF-2α protein and normal kidney were used as positive and negative controls, respectively. MVD was determined in tumor microvessel hotspots using a Chalkley point counting.

Statistics. The difference between expression of HIF-1α, HIF-2α, and LDH in paired tumor and normal tissue was determined using the Wilcoxon signed rank test. Correlation between the level of HIF-1α/HIF-2α and LDH levels in fresh tissue or MVD in the corresponding paraffin sections was examined using Spearman’s correlation. The difference in age, percentage of tumor necrosis, and MVD were compared for the two different categories of HIF-1α (nuclear) or HIF-2α (nuclear or TAM) expression using the Mann-Whitney U test. The association between the patient’s sex, tumor stage (two categories: T1/2 or T3/4), nodal state (N0 and N+), tumor grade, margin of invasion, and inflammatory infiltrate was compared for the negative and positive categories of HIF-1α or HIF-2α expression using Pearson’s χ² test.

Multiple logistic regression, using a forward variable selection technique (entry P < 0.1, removal P > 0.2), was used to identify which of the above variables were significant in predicting HIF-1α and HIF-2α expression. Disease-free and overall survival curves were estimated using the Kaplan-Meier method and compared using the Log-rank test. Cox proportional hazards model, using a forward variable selection technique (entry P < 0.1, removal P > 0.2), was used to identify which of the above variables (including postoperative radiation) were of independent statistical significance in predicting disease-free and overall survival. All statistics were done using SPSS software v9.0.

Results and Discussion

Expression of HIF-1α, HIF-2α, and Glut 1 in HNSCC Cell Lines. HIF-1α and HIF-2α were overexpressed in all three head and neck cell lines under hypoxia with little or no expression in normoxia. Glut 1 was overexpressed in two of the three cell lines under the same conditions (Fig. 1a). These findings confirm that HIF-1α and HIF-2α are not overexpressed constitutively in these HNSCC cell lines because of genetic factors, such as mutation of the von Hippel Lindau gene, and that overexpression occurs in response to hypoxia. Overexpression of Glut 1 confirms the activity of these HIFs on down-stream genes.

Expression of HIF-1α, HIF-2α, and LDH in HNSCC Tissue Samples. HIF-1α was expressed in all 20 HNSCC tumor samples with 15 of the 20 showing greater expression in tumor tissue compared with adjacent normal tissue (Fig. 1b). Using densitometry to measure the level of expression of HIF-1α on Western blotting, a significantly higher level of expression was observed in tumor tissue (n = 20, tumor median 1.4 units of density (50–219), normal median 69 units of density (19–163), P = 0.023, Wilcoxon’s rank-sum test; Fig. 1b). HIF-2α was expressed in 19 of the 20 HNSCC tumor samples with 14 of the 19 showing greater expression in tumor tissue compared with adjacent normal tissue (Fig. 1b). A significantly higher level of HIF-2α expression was observed in tumor tissue (n = 19, tumor median 54 units of density (27–267), normal median 32 units of density (17–97), P = 0.013, Wilcoxon’s rank-sum test; Fig. 1b). HIF-1α and HIF-2α expression were seen in some samples of adjacent normal tissue. Levels of LDH were significantly higher in the 16 of the 20 tumor samples when compared with adjacent normal tissue (n = 20, tumor median 161 units of density (41–450), normal median 76 units of density (20–211), P = 0.002, Wilcoxon’s rank-sum test; Fig. 1b). Overexpression of HIF-1α and HIF-2α in tumor samples demonstrates that these hypoxia-inducible transcription factors are induced in HNSCC. Overexpression of the downstream target gene LDH in tumor tissue suggests they are transcriptionally active. This overexpression is most likely because of changes in the local environment, particularly hypoxia (1). Overexpression of the HIFs in adjacent normal tissue fits with their demonstration in normal epithelium, overlying the tumor on immunohistochemistry in this study (data not shown) and others (7, 8, 12). This overexpression may be because of local hypoxia in the tissues secondary to the increased interstitial pressure exerted by the tumor, shunting, or the accumulation of metabolic products that affect oxygen delivery and consumption by tissues proximal to the tumor (12).

Correlation between HIF-1α/HIF-2α Expression and LDH/ MVD. There was no significant correlation between HIF-1α/HIF-2α expression and LDH expression in frozen tumor tissue or MVD in corresponding paraffin-embedded tissue. This lack of direct correlation between the HIFs and LDH or MVD is not surprising, given the diversity of other biological processes involved in the expression of LDH and promotion of angiogenesis. Other studies have similarly found a lack of direct correlation between hypoxia and angiogenesis (10, 13).

Localization of HIF-1α and HIF-2α Expression on Immuno- staining in HNSCC. HIF-1α expression was identified in 69 of 79 tumors on immunostaining; 10 cases were excluded (excessive background/poor staining). Expression was largely nuclear and was seen throughout the tumor area, including the perinecrotic region of the tumor and the tumor stromal interface (Fig. 2a). In positive areas, only a proportion of the nuclei stained positive. Nuclear localization of HIF-1α and the heterogeneous distribution of expression throughout the tumor area are consistent with other studies, where expression was often concentrated in the perinecrotic regions, and at the tumor/stroma interface (7, 8, 10, 12, 14). This heterogeneous expression pattern may be in part explained by the variable blood flow, high oxygen consumption, and longitudinal vascular oxygen gradients within the tu-
mors, resulting in zonal regional of hypoxia (15–17). Another factor may be the recently demonstrated improved survival of HIF-1α/HIF-2α cells distant from blood vessels in animal tumor models (18). HIF-1α is known to play a role in the promotion of apoptosis in tumor cells (19), and HIF-1α/HIF-2α cells may have a survival advantage being less dependent on vascular supply.

HIF-2α expression was identified in 70 of 79 tumors; 9 were excluded (excessive background/poor staining). Staining was identified throughout the tumor in some of the nuclei without any particular predilection for perinecrotic tissues or the tumor/stroma interface (Fig. 2b), but marked overexpression of HIF-2α was identified in tumor-associated macrophages either in close proximity to the tumor or infiltrating the tumor stroma. These were confirmed as macrophages by examining serial sections stained for HIF-2α and CD68, a cell surface antigen specific to macrophages (Fig. 2, c–f). CD 68-positive macrophages identified distant from the tumor in a sample of normal tonsil were negative for HIF-2α (Fig. 2, g and h). This may be because of macrophages hypoxia from high metabolic activity and low perfusion or other local stimuli.

**HIF-1α/HIF-2α Expression and Tumor Necrosis and Other Tumor Variables.** A higher percentage of tumor necrosis was the only variable associated with HIF-1α (nuclear) expression (n = 69, P = 0.035, Mann-Whitney) on univariate analysis; this significance was lost with the introduction of T stage into the multiple logistic regression model (Table 1). None of the variables were associated with HIF-2α (nuclear) expression, and tumor necrosis was the only variable associated with HIF-2α (TAM) expression (n = 70, P = 0.001, Mann-Whitney) on univariate analysis. In the multiple logistic regression model, necrosis remained significantly predictive of HIF-2α (TAM) expression [P = 0.001, odds ratio 9.61 (2.76–31.52)]. Nodal stage was the only other variable introduced, using the forward selection criteria described, but it was NS

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**Fig. 2.** Expression of HIF-1α and HIF-2α on immunostaining in HNSCC. a, nuclear expression of HIF-1α (×400). Arrows, positive nuclei. b, nuclear expression of HIF-2α (×400). Arrows, positive nuclei. c and e, expression of HIF-2α in TAMs (×250). d and f, serial section of c and e showing CD 68-positive macrophages (×250). g, lack of expression of HIF-2α in normal tonsil tissue (×250). h, serial section of g showing normal CD68 macrophages present in the tonsil tissue.
independently ($P = 0.09$). The association between HIF-1α (nuclear) and HIF-2α (TAM) expression with tumor necrosis is consistent with the underlying mechanism of induction of the hypoxia-induced transcription factors. The lack of correlation with other tumor and host factors reflects the wide range of complex biological processes that mediate HIF expression and the effect they have on downstream genes.

**HIF-1α/HIF-2α Expression and Disease-free and Overall Survival.** This study demonstrates a significantly better disease-free survival ($n = 69$, $P = 0.016$, Log-rank test) and overall survival ($n = 69$, $P = 0.027$, Log-rank test) in HNSCC patients with nuclear expression of HIF-1α (Fig. 3, $a$ and $b$). There was no significant difference in disease-free or overall survival with HIF-2α (nuclear or TAM) expression (Fig. 3, $c$–$f$). In the Cox proportional hazards model, HIF-1α expression was the only significant predictor of disease-free ($P = 0.028$, relative hazard 0.364 (0.148–0.898)) and overall ($P = 0.035$, relative hazard 0.292 (0.093–0.915)) survival. No other variables significantly changed the model. These findings contrast with recently published results suggesting that HIF-1α overexpression is associated with poor outcome in patients with cervical and oropharyngeal carcinoma treated with radiotherapy (9, 10). However, in this series of surgically treated patients, the significant interaction of tumor hypoxia with radiation (1) can be separated from that because of tumor biology, and a superior disease-free and overall survival have been demonstrated in HIF-1-expressing tumors. This may reflect the potentially aggressive nature of HIF-1−/− tumor cells, which lose their normal ability to undergo apoptosis at a distance from blood vessels reducing their dependence on vascular supply. It is known that tumor cells show reduced proliferation and increased apoptosis under hypoxia in HIF-1-competent cells (20), and it has been demonstrated recently that low, rather than high, expression of HIF-1α and HIF-2α is associated with better clonogenic survival in breast carcinoma cell lines (3). Thus, although HIF regulates many genes that may enhance tumor growth, the overall balance of activation effects may depend on the type of cancer and treatment modality.

There is hope that the HIFs may be suitable targets for future therapy, but caution should be exercised, as the specificity of interactions with this system, its specificity to areas of tumor rather than physiological hypoxia, and the overall effect of inhibiting the HIF system are unclear but could have significant implications for its potential clinical use (4).

**Table 1 Association between HIF-1α or HIF-2α expression in HNSCC on immunostaining and patient and tumor variables**

<table>
<thead>
<tr>
<th>A. Continuous variables</th>
<th>HIF-1α (nuclear)</th>
<th>HIF-2α (nuclear)</th>
<th>HIF-2α (TAM)</th>
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</thead>
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<tr>
<td>Age (years)</td>
<td>$n^a$</td>
<td>Negative Median (range)</td>
<td>Positive Median (range)</td>
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<tr>
<td>Necrosis (%)</td>
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<td>62 (17–79)</td>
<td>60 (27–79)</td>
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<td>MVD</td>
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<td>5.33 (2.67–10.33)</td>
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<table>
<thead>
<tr>
<th>B. Categorical variables</th>
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<th>HIF-2α (nuclear)</th>
<th>HIF-2α (TAM)</th>
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<td>32</td>
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<tr>
<td>T stage</td>
<td>F</td>
<td>9 12</td>
<td>14 23 18</td>
</tr>
<tr>
<td>N stage</td>
<td>N0</td>
<td>13 21</td>
<td>6 20 15 28</td>
</tr>
<tr>
<td>Tumor grade (differentiation)</td>
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<td>2</td>
<td>4 2</td>
</tr>
<tr>
<td>Tumor margin</td>
<td>Pushing</td>
<td>17 25</td>
<td>23 7 36</td>
</tr>
<tr>
<td>Inflammatory infiltrate</td>
<td>Patchy</td>
<td>15 21</td>
<td>18 8 27</td>
</tr>
<tr>
<td>Confluent</td>
<td>8</td>
<td>19</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$ number of patients.

$^b$ As a percentage of the total tumor area.
HYPOXIA-INDUCIBLE FACTORS IN HNSCC

References

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