Overexpression and Nuclear Translocation of Hypoxia-Inducible Factor Prolyl Hydroxylase PHD2 in Head and Neck Squamous Cell Carcinoma Is Associated with Tumor Aggressiveness

Terhi Jokilehto,[1,2,3] Krista Rantanen,[1,2] Marjaana Luukkaa,[4] Pekka Heikkinen,[1,2] Reidar Grenman,[5,6] Heikki Minn,[4] Pauliina Kronqvist,[7] and Panu M. Jaakkola[1,2]

Abstract

Purpose: Hypoxia in tumors is associated with poor prognosis and resistance to treatment. The outcome of hypoxia is largely regulated by the hypoxia-inducible factors (HIF-1α and HIF-2α). HIFs in turn are negatively regulated by a family of prolyl hydroxylases (PHD1-3). The PHD2 isoform is the main down-regulator of HIFs in normoxia and mild hypoxia. This study was designed to analyze the correlation of the expression and subcellular localization of PHD2 with the pathologic features of human carcinomas and HIF-1α expression.

Experimental Design: The expression of PHD2 was studied from paraffin-embedded normal tissue (n = 21) and head and neck squamous cell carcinoma (HNSCC; n = 44) by immunohistochemistry. Further studies included PHD2 mRNA detection and HIF-1α immunohistochemistry from HNSCC specimens as well as PHD2 immunocytochemistry from HNSCC-derived cell lines.

Results: In noncancerous tissue, PHD2 is robustly expressed by endothelial cells. In epithelium, the basal proliferating layer also shows strong expression, whereas the more differentiated epithelium shows little or no PHD2 expression. In HNSCC, PHD2 shows strongly elevated expression both at the mRNA and protein level. Moreover, PHD2 expression increases in less differentiated phenotypes and partially relocalizes from the cytoplasm into the nucleus. Endogenously high nuclear PHD2 is seen in a subset of HNSCC-derived cell lines. Finally, although most of the tumor regions with high PHD2 expression show down-regulated HIF-1α, regions with simultaneous HIF-1α and PHD2 expression could be detected.

Conclusions: Our results show that increased levels and nuclear translocation of the cellular oxygen sensor, PHD2, are associated with less differentiated and strongly proliferating tumors. Furthermore, they imply that even the elevated PHD2 levels are not sufficient to down-regulate HIF-1α in some tumors.

All solid tumors contain a varying degree of hypoxic regions due to aberrant or limited amount of vasculature. In head and neck squamous cell carcinomas (HNSCC), hypoxia and reduced vascular density correlate to poor prognosis and cause resistance to radiation therapy and chemotherapy (1–3). The best characterized molecular responses to hypoxia are mediated through the hypoxia-inducible factor 1 and 2 (HIF-1 and HIF-2) transcription factor complex (1, 4). Under normoxic conditions, the regulatory α-subunit (HIF-1α) is posttranslationally hydroxylated at two proline residues (Pro^{402} and Pro^{564}; refs. 5–8). These are recognized by the von Hippel-Lindau tumor suppressor proteins that subsequently leads to ubiquitination and proteosomal destruction of HIF-1α (9–13). Three human prolyl hydroxylases that use oxygen as a cosubstrate have been characterized (14, 15) and termed prolyl hydroxylase domain proteins (PHD), HIF prolyl hydroxylases, or Egl-9 homologues. Under restricted oxygen availability, the PHD activity decreases and the degradation of HIF-1α is blocked, leading to the transcription of a wide range of genes. These have key functions in glucose homeostasis and angiogenesis (1, 16, 17). HIF-1α is expressed in most of the common human cancer types, including HNSCC and breast cancer (4, 18). The expression correlates, together with the downstream targets, with poor prognosis and resistance to treatment (19–21), apart from surgically removed HNSCC (22).

The expression of the three human PHD isoforms has been studied at mRNA level. PHD2 shows most abundant mRNA expression across tissues, whereas PHD1 and PHD3 show more restricted tissue distribution (23–25). The three isoforms have

Authors' Affiliations: [1]Turku Centre for Biotechnology, Turku University, [2]Åbo Akademi University, [3]Turku Graduate School of Biomedical Sciences, Departments of [4]Oncology and Radiotherapy, [5]Otorhinolaryngology-Head and Neck Surgery, [6]Medical Biochemistry, and [7]Pathology, Turku University and Turku University Hospital, Turku, Finland

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Requests for reprints: Panu M. Jaakkola, Turku Centre for Biotechnology, Tykistökatu 6B, FIN-20520, Turku, Finland. Phone: 358-2333-8030; Fax: 358-2333-8000; E-mail: panjaa@utu.fi.

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similar oxygen and cosubstrate requirements, but they show different preferences for HIF-1α and HIF-2α as well as for the two prolyl hydroxylation sites (24, 26, 27). The PHD2 isoform targets the degradation of HIF-1α mainly through hydroxylation of P564. In cell culture models, PHD2 has been shown to be the most important isoform for the down-regulation of HIF in normoxic as well as mild hypoxic conditions (26, 28). Several studies have shown that PHD2 is up-regulated transiently by hypoxia in a HIF-dependent manner by a number of cell types *in vitro*, implying that HIF may induce an autoinhibitory feedback on its own activity (26, 28–31). However, several questions as to how PHD2 is expressed and regulated at protein level in diverse cell types *in vivo* and how this correlates with HIF-1 expression, as well as how the expression of PHD2 would correlate with the clinicopathologic features of human cancers, remains unknown.

Here, we have studied the expression of PHD2 in normal human tissues and HNSCC specimens. We show that PHD2 is robustly expressed by endothelial cells and proliferating epithelial cells, but is less expressed by most other normal cell types. In cancerous epithelia, the PHD2 expression is markedly increased, and furthermore, is partially translocated from the cytoplasm into the nucleus following less differentiated tumor phenotype.

Materials and Methods

Patient samples. Following informed consent, tumor samples were collected from surgically removed oral and oropharyngeal HNSCC (n = 44) from both genders, between 1990 to 2002, in Turku University Central Hospital, Finland. All HNSCC samples were removed before any treatment, either radiation therapy or chemotherapy. The use of tumor samples was approved by the National Authority for Medicolegal Affairs. Normal tissue samples (n = 21) were collected from patients undergoing uvulo-palato-pharyngoplasty. Samples were from both genders and patient ages ranged from 29 to 87 years.

Immunohistochemistry. Five-micrometer-thick sections were stained for antibodies against PHD2 (NB100-137, Novus Biologicals, Littleton, CO; 1:500 dilution) and Hif-1α (BD Transduction Laboratories, San Jose, CA; 1:100 dilution). Visualization of primary antibodies was done with Vectastain ABC reagent and diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CO), which is based on an indirect streptavidin-biotin method. Slides were counterstained with hematoxylin. Antigen retrieval was carried out using a microwave oven. The staining for CD34 (BD Biosciences, San Jose, CA; dilution 1:100) was done using the TechMate 500 immunostainer and a peroxidase/diaminobenzidine multilink detection kit (DAKO, Carpinteria, CA).

In each HNSCC sample, the PHD2 staining intensity was assessed by two independent observers using a ×40 objective in three separate evaluation areas with a total of 300 squamous cell carcinoma cells. Staining intensity was assessed on a scale of 0 to 3, where 3 indicated an intense staining corresponding to the staining intensity of endothelial cells. HIF-1α staining intensity was assessed on a scale of 0 to 2. Nuclear PHD2 staining was evaluated in the whole tumor area with a ×20 objective. This gave an estimation of the percentage of cells showing nuclear staining in the whole sample. Thereafter, the most representative tumor area was identified and the previous estimation was verified by quantitative assessment of the percentage of cells showing nuclear staining with ×40 objective in three separate optical fields in a total of 300 hundred carcinoma cells. Each tumor was scored for the amount of cells with nuclear staining as a percentage of all cells. The results are displayed as mean values in Table 2. Finally, nuclear staining and staining intensity were combined into a histoscore by multiplying the percentage of nuclear positivity with the staining intensity.

RNA preparation and real-time quantitative PCR. RNA was extracted with the acid guanidium thiocyanate-phenol-chloroform method. First-strand cDNA synthesis was done with 200 units of Moloney murine leukemia virus RNase H^- reverse transcriptase (Promega, Madison, WI) using 0.5 μg poly$(dT)_{15}$ primers (Promega) and 1 μg of DNaseI treated RNA from each tumor sample.

Primers and fluorogenic probes for real-time PCR were designed with the Primer Express computer software (PE Biosystems, Foster City, CA) and obtained from MedProbe (Oslo, Norway). EF-1α (eukaryotic elongation factor-1α, UniGene no. Hs.439552) was used as a reference gene. The sequences of the primers and probes are: EF-1α, fwd 5′-CTGAACCATCCAGGCCAAAT-3′, rev 5′-GCCGTGTGGCAATCCAAT-3′; probe, 5′(FAM)-AGCGCCGGCTATGCCCCTG-(TAMRA)-3′; *PHD2*, fwd 5′-AGCAGCATGGACGACCTGAT-3′, rev 5′-TCGTCCGGCCATTGATTT-3′; probe 5′-(FAM)-CCCAGCTTCCCGTTACAGTGGCG-(TAMRA)-3′. Real-time PCR reactions were done using 2 μL of diluted cDNA, 5 μL of the TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA), 300 nmol/L oligonucleotide primers, and 200 nmol/L FAM-labeled probes. The amplification was detected with ABI Prism 7700 sequence detector as an increase in the FAM fluorescence. The C_T values were detected and the relative expressions of the analyzed genes were calculated using the formula: relative expression = $2^{-\Delta CT}$, where ΔC_T = C_T (target gene) − C_T (EF-1α) for each sample.

The nuclear localization signal of PHD2 was identified using PSORT database (http://psort.ims.u-tokyo.ac.jp/).

Cell culture, transfections, and cell imaging. HeLa and HaCaT cells were obtained from the American Type Culture Collection, Rockville, MD. Normal human skin fibroblasts were established from a healthy male volunteer donor. HUVEC cells were a kind gift from Dr. E. Iivanainen. The recently established cancer patient–derived HNSCC cell lines (UT-SCC2, UT-SCC8, and UT-SCC9) used in the study have been described previously (32). Cells were cultured in DMEM, supplemented with 10% FCS, penicillin-streptomycin and L-glutamine. SCC cells were also supplemented with nonessential amino acids (Sigma, St. Louis, MO). Cells were cultured in humidified air containing 5% CO_2 at 37°C. For hypoxia treatments, air was replaced by nitrogen to reach 1% oxygen in a hypoxia workstation (Invivo$_2$, Ruskinn Technology, Ltd., United Kingdom).

Laemmli buffer extracted proteins were detected by Western blotting and enhanced chemiluminescence with specific primary antibodies against PHD1 (BL525 Bethyl Laboratories Inc., Montgomery, TX; 1:2,000 dilution), PHD2 (NB100-137, Novus Biologicals; 1:2,500 dilution), PHD3 (NB100-139, 1:2,000 dilution), and β-actin (AC-40, Sigma).

For visualizing endogenous PHD2, cells were fixed with PTEMF [100 mmol/L PIPES (pH 6.8), 10 mmol/L EGTA, 1 mmol/L $MgCl_2$, 0.2% Triton X-100, and 4% formaldehyde] and stained with PHD2 antibody followed by Cy3-conjugated secondary antibody (Jackson Immunochemicals, West Grove, PA). The nuclei were stained with Hoechst 33342 (Sigma). For immunocytochemistry, cells were plated on coverslips, and for fluorescence recovery after photobleaching (FRAP), cells were placed on MatTek glass-bottomed culture dishes (MatTek Corporation, Ashland, MA). Transfections were done with Effectene transfection reagent (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Construction of the PHD2–enhanced green fluorescent protein fusion plasmid (PHD2-EGFP) has been described elsewhere (33).

For live cell microscopy, cells were grown to 60% confluence. The experimental conditions for FRAP have been described elsewhere (34). Shortly thereafter, images were acquired with Zeiss LSM 510 META confocal microscope and Physiology software (Carl Zeiss Corporation, Jena, Germany), GFP fluorescence was excited using the 488 nm laser and detected with LP505 filter. All experiments were done at 37°C and 5% CO_2 in a humidified cell culture chamber. Each FRAP experiment started with an image scan followed by a bleach pulse of 100 iterations and 100% laser power. A series of 60 images were collected at 5-second intervals. The mobile fraction was calculated by using the equation

$y = A\ (1 - \exp(kt))$ and custom-designed software (Dr. Rolf Sara, Turku Centre for Biotechnology). In this analysis, bleaching compensation is calculated from the declining tail of the bleached region of interest instead of using the reference cell region of interest.

Results

Expression of PHD2 in noncancerous tissues. Several studies have indicated that PHD2 is expressed abundantly over a wide range of diverse normal tissues at the mRNA level, but the immunohistochemical localization of PHD2 has not been described (24, 25). We used a commercially available antibody to study the expression of PHD2 in several normal cell types from the oral and pharyngeal region (Fig. 1). The specificity of the antibody was first validated by Western analysis, which showed hypoxic induction of endogenous PHD2 and no cross-reactivity with other PHD isoforms in transient expression (Fig. 1A). The normal pharyngeal tissue, which included neural tissue, fibroblasts, muscle cells, salivary glands, endothelial cells, and epithelial cells showed that endothelial cells have robust PHD2 expression regardless of the anatomic site (Fig. 1B-F). This was apparent in all sections studied and was further verified by CD34 endothelial cell marker from parallel sections (Fig. 1B, inset). Strong staining of endothelial cells was seen in the lining of large blood vessels as well as in capillaries. Specialized endothelial cells such as neuroendothelial cells (Fig. 1E), endothelial cells of muscle tissue (Fig. 1C), and salivary glands (Fig. 1D) as well as endothelial cells of the placenta (Fig. 1F) showed equally robust staining. Muscle cells and mucous acini of salivary glands showed clearly less staining and fibroblasts did not show detectable staining.

Normal epithelium showed a dual expression pattern. The basal proliferating layer of stratified squamous epithelium showed strong PHD2 expression, which gradually decreased towards the surface of epithelia. The expression was already weaker in the more cuboidal cells above the proliferating layer and was completely lost in the flattened superficial cells

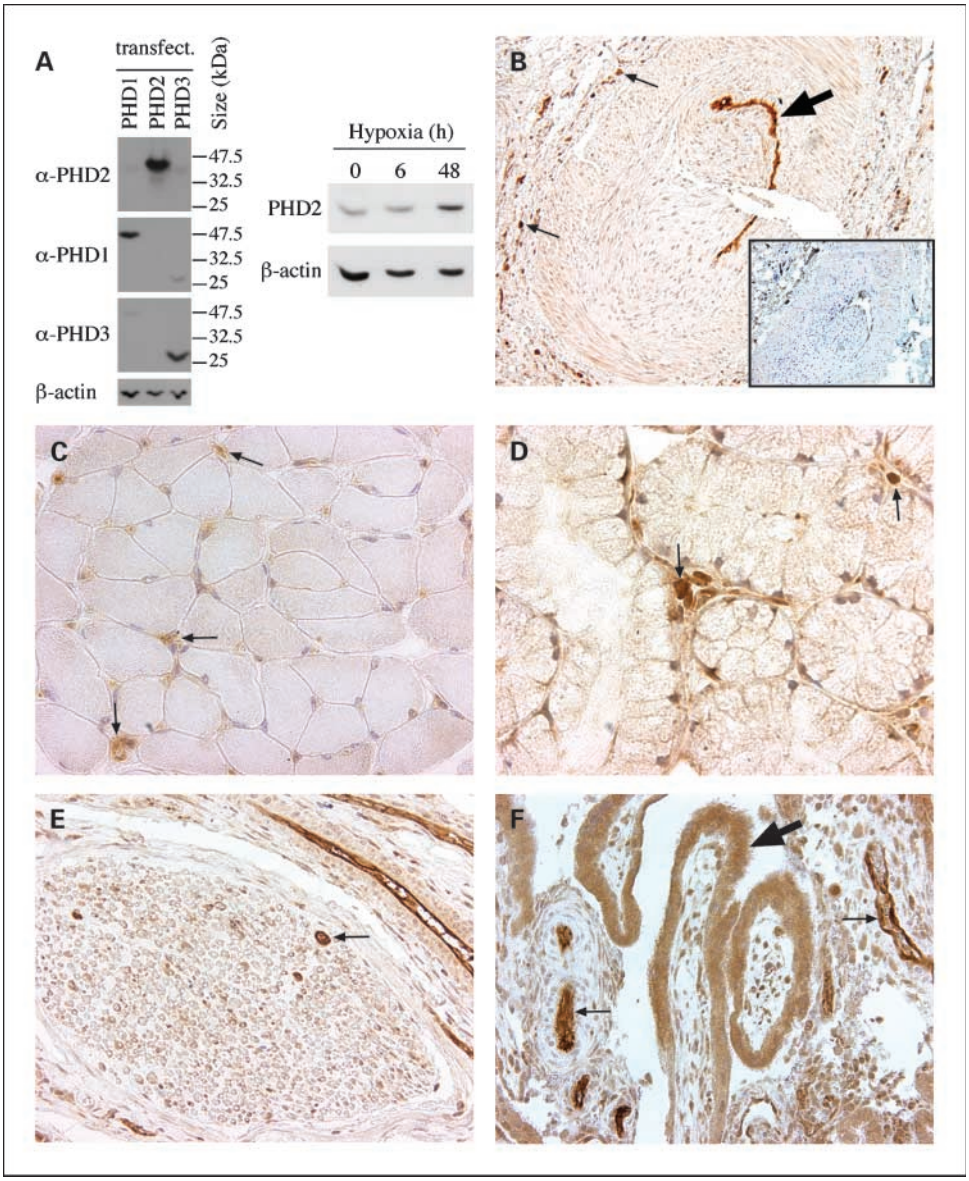

Fig. 1. Expression of PHD2 in various normal tissues. *A,* validation of the specificity of the PHD2 antibody. HeLa cells were transfected by PHD1, PHD2, or PHD3 expression constructs (*transfect.*) followed by Western blotting for each PHD isoform. HeLa cells were further grown in normoxia (0 hours) or exposed to 6 and 48 hours of hypoxia followed by Western blotting for PHD2. *B,* arterial endothelial cells (*thick arrow*) and small surrounding capillaries (*thin arrows*) show strong PHD2 staining (*brown*). Inset, CD34 staining of a parallel section. All magnifications are ×400 if not otherwise mentioned. *C,* muscle tissue. Capillary endothelial cells between the muscle cells (*arrows*). *D,* salivary gland. Capillary endothelial cells (*arrows*). *E,* peripheral nerve bundle. Endothelial cells of endoneurial blood vessels (*arrows*; magnification, ×200). *F,* placental tissue. Trophoblast cells (*thick arrow*) and endothelial cells (*thin arrows*).

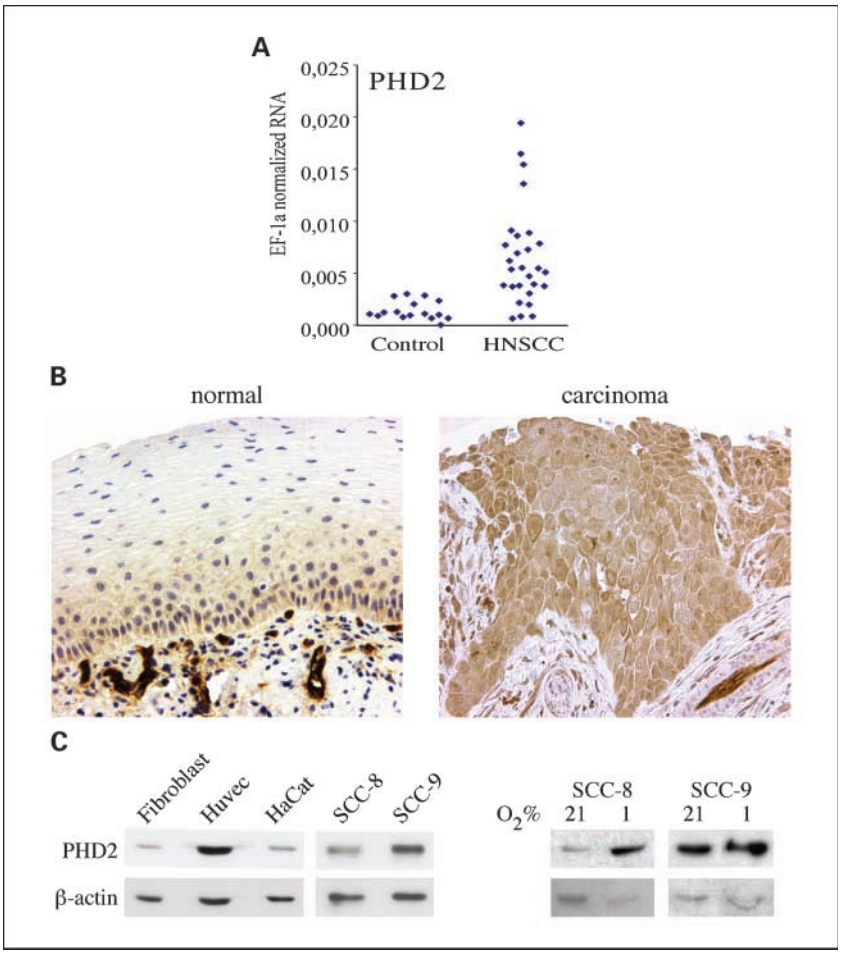

Fig. 2. PHD2 is overexpressed in HNSCC. *A,* real-time PCR analysis of PHD2 mRNA levels in normal and HNSCC patient samples. Dots, expression level of EF-1α normalized PHD2 in one sample. *B,* immunohistochemical staining of PHD2 in representative normal epithelial tissue (note expression restricted to the basal layer) and carcinoma tissue (magnification, ×200). *C,* Western blot analysis of PHD2 protein in human primary and transformed cells. Primary human endothelial cells (HUVECs), primary fibroblasts, immortalized epithelial cells (HaCaT), and two HNSCC-derived cell lines (UT-SCC8 and UT-SCC9). The SCC cells were further exposed to 21% or 1% oxygen for 48 hours and analyzed for PHD2 expression by Western blotting.

(Fig. 2B). The expression of PHD2 was restrained to the cytoplasm. Besides endothelial and proliferating epithelial cells, placental trophoblasts also showed strong PHD2 expression (Fig. 1F).

PHD2 is overexpressed in HNSCC. We next studied the expression of PHD2 in human HNSCC at the mRNA level by real-time PCR. Twenty-nine HNSCC tumor samples from oral and pharyngeal cancers were used. The expression was compared with 13 normal samples derived from surgically removed oropharyngeal tissue and the levels were normalized to the EF-1α housekeeping gene. The expression of PHD2 was clearly elevated in cancer specimens when compared with normal tissue (Fig. 2A). The fold change in the mean expression levels of the cancer group compared with the control group was 4.5. The highest values for PHD2 exceeded the mean expression level of the control group by 14-fold. The up-regulation was comparable to lactate dehydrogenase-A expression (data not shown).

We next studied the expression of PHD2 by immunohistochemistry in histologic sections derived from HNSCC patients (n = 44). Compared with the normal pharyngeal epithelia (n = 21), PHD2 expression was strongly up-regulated in HNSCC samples. The HNSCCs showed moderately (43% of tumors) to strongly elevated (57% of tumors) PHD2 expression (Fig. 2; Table 1). Similar to normal tissue, the tumor endothelial cells showed intense PHD2 expression, but the stromal fibroblasts were negative. It is noteworthy that the granulation tissue also showed strongly staining inflammatory cells (data not shown).

We further compared the PHD2 expression in primary human cells and cells derived from HNSCC by Western blotting. In accordance with immunohistochemistry, primary human fibroblasts showed little PHD2 but primary endothelial cells (HUVEC) had high basal PHD2 levels (Fig. 2C). The HNSCC cell lines showed varying levels of basal PHD2 expression ranging from low expression (UT-SCC8) comparable to fibroblasts, to robustly expressing cell lines (UT-SCC9) comparable to endothelial cells (Fig. 2C). Hypoxia is the only stimulus known to increase PHD2 expression. However, the increased expression in HNSCC did not superimpose with hypoxic areas. Robust PHD2 expression was detected away from any vessel, as well as proximal to vessels and without any visible necrosis. We therefore studied the expression of PHD2

Table 1. PHD2 intensities in normal and tumor tissue

	*n**	0	1	2	3
Noncancerous	21	3	18	0	0
HNSCC	44	0	19	21	4

*n, number of cases analyzed; (0-3), staining intensity.

in cancer cell lines in hypoxic conditions. Cell lines with low to moderate expression, such as UT-SCC8, showed clear hypoxic up-regulation of PHD2. However, the expression was not further elevated by hypoxia in cell lines that had high normoxic PHD2 (UT-SCC9). These cells, however, retained normal hypoxic HIF-1α induction (data not shown), suggesting that in cancer cells, PHD2 expression may also be up-regulated by means other than hypoxia.

Overexpressed PHD2 translocates into nuclei in poorly differentiated tumors. As PHD2 expression in normal epithelia was restricted to the proliferative layer, we next correlated the staining intensity to the histologic grade of the tumors. A clear trend of increasing PHD2 expression was seen with higher tumor grade (Fig. 3; Table 2). Grade 1 tumors showed low PHD2 expression (17 out of 19 tumors), whereas grade 2 (18/20) as well as the grade 3 tumors (5/5) showed clearly increased expression. The association of PHD2 expression with histologically aggressive tumors was further corroborated by Ki67 proliferation marker staining from adjacent sections (data not shown).

Early in the course of the study, we noticed an unexpected trend of nuclear localization of PHD2 in a subset of tumor cells. The percentage of cells that had PHD2-positive nuclei was counted within each specimen and correlated with the histologic grade of the tumors. We observed a strong association of nuclear staining with higher-grade tumors. The grade 1 tumors showed 0% to 5% of positive nuclei and the rest of the cells showed completely cytoplasmic staining similar to the normal epithelium. However, 50% to 90% of cells in grade 3 tumors showed simultaneous nuclear and cytoplasmic PHD2 expression (Fig. 3A; Table 2). To further investigate the correlation between PHD2 staining intensity and nuclear translocalization, we calculated histoscore values for each specimen (for details, see Materials and Methods; Table 2). The histoscore analysis revealed the stronger association of PHD2 expression with tumor grade compared with PHD2

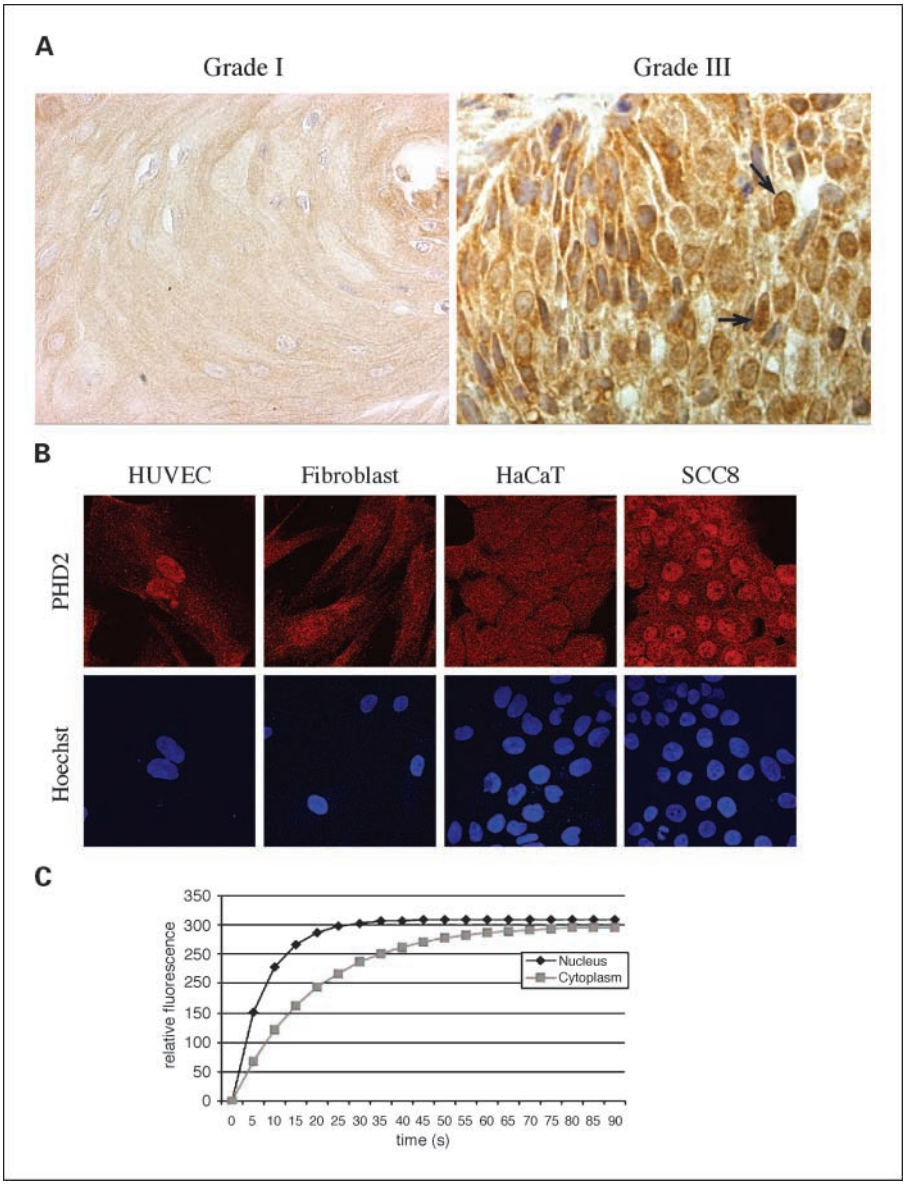

Fig. 3. Nuclear translocation of PHD2 correlates with histologic tumor grade. *A,* in poorly differentiated (grade 3) carcinoma, PHD2 expression can be seen both in the cytoplasm and nucleus in a subset of cells (*arrows*, cells with increased nuclear PHD2 expression), whereas in more differentiated carcinomas (grade 1), PHD2 expression is strictly cytoplasmic (magnification, ×200). *B,* confocal microscopy of cultured cells stained for PHD2 (*red*) and nuclei (*blue*). *C,* cultured UT-SCC2 cells transfected with PHD2-EGFP fusion protein (see Supplemental Figure) followed by FRAP analysis. The PHD2 mobility was studied in the cytoplasmic and nuclear fractions. Dots, restoration of EGFP fluorescence at given time points (5-90 seconds) after photobleaching (0 seconds).

Table 2. PHD2 intensities and nuclear staining in HNSCC

Histological grade	n^* (44)	1	2	3	Nuclear staining (mean, %)	Histoscore (mean)	Histoscore (median)
1	19	17	2	0	11	17	5
2	20	2	17	1	42	83	70
3	5	0	2	3	60 (<0.0001)†	164 (<0.0001)‡	140

*n, number of cases analyzed; (0-3), staining intensity; nuclear staining, percentage of cells with nuclear PHD2; histoscore, nuclear staining multiplied by staining intensity.
†P value of Spearman correlation for nuclear staining and tumor grade.
‡P value of Spearman correlation for histoscore and tumor grade.

intensity alone. Spearman correlation coefficient for tumor grade and nuclear staining was 0.80 ($P < 0.0001$) and the histoscore value was 0.83 ($P < 0.0001$). On the contrary, no correlation between PHD2 expression and tumor size, lymph node status, or metastasis was seen in HNSCC (P values for Spearman correlation of PHD2 intensity and tumor-node-metastasis were 0.91, 0.22, and 0.96, respectively).

Previous studies using overexpressed fusion proteins in cell culture have suggested that the localization of PHD2 is restricted to the cytoplasm (33). However, a putative bipartite nuclear localization signal (RRDNASGDAAKGKVKAK) in the NH_2 terminus was identified and has also been described in earlier studies (35). This prompted us to further investigate the subcellular distribution of PHD2. We examined the expression of endogenous PHD2 in normal and HNSCC-derived cell lines (Fig. 3B). All cultured cells showed some degree of nuclear PHD2 in addition to cytoplasmic expression. Primary fibroblasts and immortalized epithelial cells (HaCaT) showed little nuclear PHD2 expression compared with primary endothelial cells (HUVEC), which is in agreement with the staining pattern seen in tissue samples. However, some HNSCC-derived cell lines, such as UT-SCC8, showed strongly increased nuclear translocation of PHD2. We further studied the distribution of endogenous PHD2 after 24-hour hypoxic exposure, but could not detect any increase in nuclear localization.[8]

Finally, to study the possible differences in the dynamics of the cytoplasmic and nuclear PHD2, we used transient expression of PHD2-EGFP fusion protein followed by FRAP. PHD2-EGFP was ectopically expressed in HNSCC cell lines and the localization was compared with endogenous PHD2 (Supplementary Figure). The subcellular localization of the PHD2-EGFP was comparable to that of endogenous PHD2 in several HNSCC-derived cell lines. This allowed us to perform FRAP experiments both for the cytoplasmic and nuclear fractions from an HNSCC-derived cell line (UT-SCC2; Fig. 3C). Both fractions showed active movement of PHD2. Surprisingly, however, the kinetics of the nuclear fraction was particularly fast with a half-time of approximately 5 seconds compared with 2- to 3-fold longer half-time within the cytoplasm, implying faster movement of PHD2 in the nucleus and suggesting differential complex formation of PHD2 in the cytoplasm and nucleoplasm.

Expression of PHD2 partially correlates with down-regulation of HIF-1α. HIF-1α protein is not detectable in normal epithelia, but is up-regulated in most human carcinomas (18). The HIF-1α expression is mainly seen at the perinecrotic severely hypoxic areas. Cell culture models have indicated that PHD2 is the hydroxylase isoform mainly responsible for marking HIF-1α for destruction in normoxic and non–severe hypoxia (26, 28). We stained parallel HNSCC sections ($n = 14$) for HIF-1α and PHD2. First, we scored the tumors for overall HIF-1α and PHD2 expression. Most sections with negative or low HIF-1α expression had moderate or high PHD2 staining, but in a subset of tumors, both high HIF-1α and PHD2 expression was detected. However, neither HIF-1α nor PHD2 expression is uniform in any given section, and the overall staining might not reveal the causal relationship between the two proteins. Therefore, we identified 21 consecutive optical fields from the tumors and scored them for HIF-1α and PHD2 staining intensity. As expected, most regions that showed low or no HIF-1α expression stained strongly for PHD2 (73%). However, we could also identify areas of simultaneously high PHD2 and HIF-1α expression (27%). An example of two tumor regions is shown in Fig. 4. The other tumor region stains strongly and the other has no HIF-1α staining. Both of these regions, however, show strong PHD2 expression. Further details of the correlation of PHD2 and HIF-1α expression are presented in the Supplementary Table. This data implies that in a subset of cancer cells, PHD2 expression is not sufficient to down-regulate HIF-1α.

Discussion

PHD2 has been implicated as the main regulator of HIF-1α in cell culture conditions (14, 15), and therefore, to be the most important hydroxylase isoform in the oxygen-sensing pathway. Here, we have reported that PHD2 is strongly expressed by endothelial cells and the proliferating layer of epithelium in normal tissue, and rather surprisingly that PHD2 is overexpressed in epithelial cancer. PHD2 expression is increased in less differentiated phenotypes and is further translocated into the nuclei following dedifferentiation. This pattern of PHD2 expression provides a novel biomarker for the differentiation status of carcinomas.

The robust expression of PHD2 in endothelial cells and in a subset of epithelial cells, as well as the lower expression in some other cell types such as muscle cells or mucous acini suggests that PHD2 may account for keeping HIF-1 levels low in these cells. The PHD2 levels are undetectable in several other cell types including nonproliferating and more differentiated epithelial cells, fibroblasts, and neural cells. It may be that in

[8] T. Jokilehto, unpublished results.

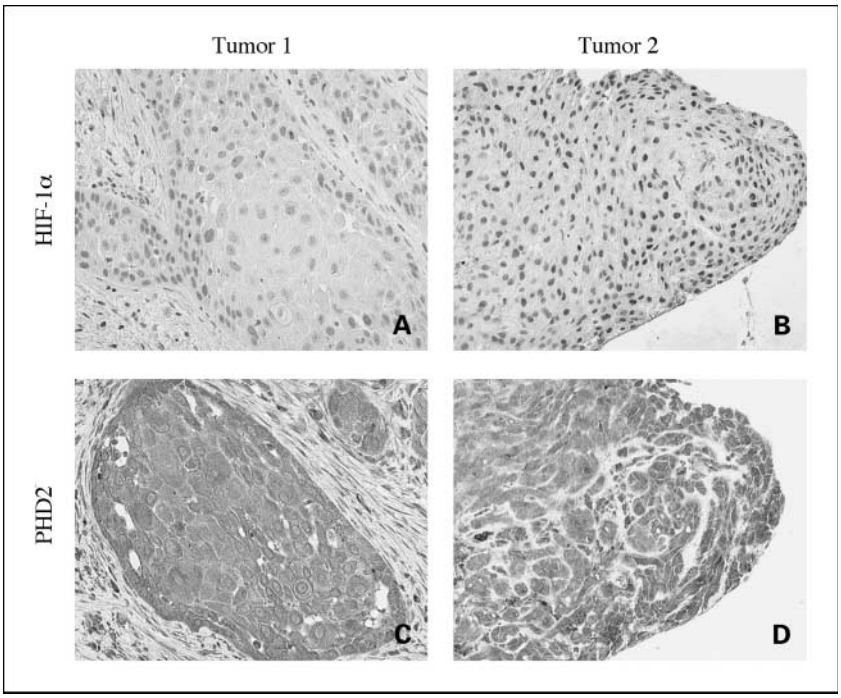

Fig. 4. The expression of PHD2 partially correlates with the down-regulation of HIF-1α. Parallel optical fields showing the coexpression of HIF-1α (*A* and *B*) and PHD2 (*C* and *D*) in two HNSCC sections. *A* and *C*, parallel optical fields from one tumor; *B* and *D*, parallel optical fields from another tumor (magnification, ×200).

these cells, the two other HIF hydroxylase isoforms, PHD1 and PHD3, account for the low HIF-1α expression or that very low PHD2 expression, undetectable by immunohistochemistry, still partially plays a role in HIF-1α regulation.

Previous studies have implicated hypoxia as a strong inducer of PHD2 expression. However, PHD2 was widely expressed throughout the tumor samples and on close contact with capillaries in nonnecrotic regions. Furthermore, PHD2 was inherently overexpressed in some of the studied HNSCC-derived cell lines, which retain normal hypoxic HIF induction. These data imply that hypoxia is not the only trigger of altered PHD2 expression, but other oncologic events may take part. In keeping with this, the down-regulation of PHD2 by Siah2 ubiquitin ligase has been described and its loss could up-regulate PHD2 (36).

The expression and nuclear translocalization of PHD2 are strongly increased in more aggressive, i.e., more proliferating and less differentiated carcinomas, suggesting that the altered expression may depend on growth-promoting events. However, in HNSCC, the histologic tumor grade does not correlate with the tumor-node-metastasis classification. In line with this, we did not detect any correlation between PHD2 expression and clinical variables (tumor-node-metastasis or tumor stage) in our HNSCC material. In contrast, we detected a strong correlation between nuclear PHD2 expression—and not only histologic grade—but also tumor size in a set of breast cancer specimens (n = 22).[8] Taken together, the data suggests that PHD2 may have a function in the regulation of cellular proliferation and differentiation. This is supported by studies showing that the expression of several cyclins and their regulators are regulated by hypoxia (37–39) and that the *Drosophila* homologue of PHD2, which acts upstream of the cell cycle regulators, is required for cell growth (40). Given the enhanced nuclear expression of PHD2 in less differentiated epithelial cells, it is also noteworthy that hypoxia induces dedifferentiated phenotypes of breast cancer (41), and that a nuclear tumor suppressor ING4 is associated with PHD2 (42). Each member of the PHD family may have a very different function in the control of tumor growth because in contrast to our proposed function of PHD2 in tumor biology, increased expression of PHD1 has been suggested to suppress tumor growth in mouse xenograft models (43).

In the prevailing model, the function of PHD2 was to keep HIF-1α at a low level in normoxic and mildly hypoxic conditions. On the other hand, HIF-1α is a marker of poor prognosis in several carcinomas, including HNSCC (19–21). Therefore, one might argue that elevated PHD2 expression is not in accordance with the known association of HIF in tumors. However, we have identified regions staining strongly for both PHD2 and HIF-1α, showing that in a subset of cancer cells, the elevated PHD2 is not sufficient to down-regulate HIF-1α. Further studies to investigate the consequence of increased PHD2 levels and subcellular localization on the expression of HIF-1α and HIF-2α, which is suggested to be the main isoform responsible for tumor promotion (44, 45), will be required. However, it is interesting to note that stabilization of HIF and loss of prolyl hydroxylation has been reported to occur during carcinogenesis (4, 46). Several possibilities for reduced hydroxylase activity—in addition to the lack of oxygen—exist. These include the fact that many factors such as insufficient amounts of ascorbate (47), elevated amounts of succinate (48), or 2-oxoacids (49) could suppress hydroxylase activity, but also that for full activity, PHD2 requires interacting partners (50). In this regard, it is also interesting that in some cancers, there may not be any correlation between oxygenation status and HIF-1α expression (51).

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