

The Androgen Receptor Is Significantly Associated with Vascular Endothelial Growth Factor and Hypoxia Sensing via Hypoxia-Inducible Factors HIF-1a, HIF-2a, and the Prolyl Hydroxylases in Human Prostate Cancer

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Abstract Purpose: Hypoxia regulates key biological processes including angiogenesis via the transcription factor, hypoxia-inducible factor (HIF). In prostate cancer, angiogenesis is also influenced by androgens, and recent cell line studies suggest that this effect is partly mediated by HIF. The study aimed to assess whether a relationship exists in human prostate cancer between expression of the androgen receptor, HIFs, and the key angiogenesis factor, vascular endothelial growth factor (VEGF).

Experimental Design: A tissue microarray comprised of 149 radical prostatectomy specimens was constructed. Semiquantitative immunohistochemical analysis was used to assess the expression of the androgen receptor, VEGF and HIF-1a and 2a, and their regulatory prolyl hydroxylase enzymes (PHD1, PHD2, and PHD3). Statistical analysis compared these factors with each other and with prostate-specific antigen relapse.

Results: There was a significant correlation between HIF-1a and HIF-2a expression ($P = 0.02$), and with androgen receptor ($P = 0.04$ and $P < 0.001$, respectively) and VEGF expression ($P = 0.05$ and $P < 0.001$, respectively). VEGF was also significantly related to the androgen receptor ($P = 0.05$), whereas PHD2 was inversely related to HIF-2a expression. No significant association was shown between HIF-1a or HIF-2a and time to prostate-specific antigen recurrence ($P = 0.20$ and $P = 0.94$, respectively).

Conclusions: These findings confirm the relationship between hypoxia and the androgen receptor in prostate cancer, and show for the first time, the role of HIF-2a in this disease process. They provide clinical evidence to support the recent cell line findings that androgens may regulate VEGF levels through the activation of HIF in androgen-sensitive tumors. Inhibition of both the HIF pathways may provide new therapeutic options in the management of this disease.

One of the key pathways involved in the response to hypoxia is the HIF-1 pathway. HIF-1 is an oxygen-dependent transcriptional activator which is composed of two basic helix-loop-helix PAS proteins, HIF-1a and HIF-1b (1). HIF-1b (aryl hydrocarbon nuclear translocator) is constitutively expressed and largely unaffected by oxygen tension in contrast to HIF-1a, which is the responsive HIF-1 element

(2). Although three α subunits (HIF-1a, HIF-2a, and HIF-3a) have been identified, most of the studies to date have concentrated on the role of HIF-1a, with those of HIF-2a and HIF-3a remaining less clear (3).

The level and activity of the HIF-1a subunit is tightly regulated through a number of posttranslational modifications. In the presence of oxygen, the prolyl hydroxylase enzymes (PHD1, PHD2, and PHD3) cause site-specific hydroxylation of two proline residues, P402 and P564, within the oxygen-dependent degradation domain of HIF-1a. This hydroxylation allows for the recognition of HIF-1a by the tumor suppressor von Hippel-Lindau protein, an E3 ubiquitin ligase complex, which targets HIF-1a for degradation (4). Hydroxylation of a conserved asparagine residue (Asp⁸⁰³) within the caspase-activated DNase domain of HIF-1a, by factor-inhibiting HIF, controls the activity of HIF-1a by preventing it from binding to the coactivators, p300/CBP, and hence modifying the transcriptional activity of key regulatory genes (5). However, under hypoxic conditions, molecular oxygen is unavailable and these enzymes are inactive, resulting in both an increase in the level of nuclear HIF-1a and its activity.

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Immunohistochemical studies have confirmed the up-regulation of HIF-1 α in areas of human prostate cancer compared with normal prostate and benign prostatic hyperplasia (BPH; ref. 6). They have also indicated that this is likely to be an early event in the development of prostate cancer as levels are higher in areas of high-grade prostatic intraepithelial neoplasia, a presumed precursor lesion, relative to normal prostate and benign disease (7). This pattern of up-regulated expression in areas of prostate cancer and high-grade prostatic intraepithelial neoplasia has also been reported for vascular endothelial growth factor (VEGF), one of the key genes regulated by HIF (8, 9).

In the prostate gland, the expression of VEGF has also been correlated with the presence of androgens. Lissbrant et al. showed in castrated adult male mice that testosterone indirectly stimulates vascular growth in the ventral prostate lobe by increasing epithelial VEGF synthesis and that this is a necessary component in testosterone-stimulated prostate growth (10). This control over VEGF levels is also seen in prostate cancer where androgen deprivation has been shown to result in a down-regulation of VEGF levels, whereas expression is increased in androgen-independent tumors (11). Indeed, it is the down-regulation of VEGF and the resultant vascular degeneration that is believed to underlie the therapeutic effect of anti-androgens in this disease process (12).

Mabjeesh et al. recently postulated that androgens exert their control over VEGF levels by activating HIF, in a similar manner to hypoxia (13). They found that androgens increase HIF-1 α nuclear expression in LNCaP cells via an autocrine loop of epidermal growth factor induction by androgens and stimulation of HIF-1 α production by epidermal growth factors. They also reported that the effect of HIF-1 α could be blocked by anti-androgens. Because the data suggesting the link between HIF, VEGF, and the androgen receptor is based on a single cell line report, the aim of the current study was to analyze the coexpression of the members of the HIF pathway with VEGF together with their regulatory components and the androgen receptors in human prostate cancer specimens, to establish evidence for their *in vivo* role in human prostate cancer. The potential clinical utility was also investigated using time to prostate-specific antigen (PSA) recurrence as a surrogate for relapse-free survival.

Patients and Methods

With approval from the local ethics committee, archival radical prostatectomy specimens were collected from the Royal Berkshire Hospital, Reading, United Kingdom. One hundred and forty-nine samples were available for tissue microarray construction. The age of the patient at the time of surgery and their preoperative PSA levels were recorded together with the presence of capsular invasion and positive surgical margins (circumferential and urethral). Postoperative Gleason score (2–10, 14) was also documented, as was disease recurrence, defined as a consistent increase in the PSA of >0.2 ng/mL or three or more consecutive increases. In the absence of PSA recurrence, the time to the last PSA test was calculated.

Each radical prostatectomy specimen had two cores of prostate cancer removed (1 mm diameter) for tissue microarray construction. Tissue microarray sections were cut from each block at 4- μ m-thick intervals. All slides underwent initial H&E staining to verify the presence of cancer. In those specimens where carcinoma could not be identified in either of the two cores, an attempt was made to replace them.

Immunohistochemistry. After dewaxing, all slides underwent antigen retrieval. For HIF-1 α , HIF-2 α , and VEGF, this involved steaming under pressure at 15 psi for 3 minutes in a buffer of Tris EDTA (pH 9.0; Sigma, St. Louis, MO). Androgen receptor retrieval was carried out in citrate buffer (pH 6.0) while steaming for 10 minutes. The slides undergoing PHD1, PHD2, and PHD3 staining did not require antigen retrieval. Endogenous peroxidases were then blocked in all specimens using 0.3% hydrogen peroxidase in 0.1% sodium azide for 10 minutes.

The antibodies for HIF-1 α (Esee 122), HIF-2 α (EP 190b), VEGF (VG1), PHD1 (PHD112), PHD2 (366G/76), and PHD3 (EG188e) were generated in the Nuffield Department of Clinical Laboratory Science, John Radcliffe Hospital (Oxford, United Kingdom), and their validation on transfected cell lines with positive and negative controls have previously been published (15–17). The androgen receptor antibody was purchased from Novocastra (Newcastle-Upon-Tyne, United Kingdom; Clone 2F12). Antibodies were used at the following concentrations: 1:30 for 60 minutes (HIF-1 α), neat supernatant for 60 minutes (HIF-2 α), 1:2 for 30 minutes (VEGF), neat supernatant for 60 minutes (PHD1, PHD2, and PHD3), and 1:25 overnight at 4°C (androgen receptor). After antibody incubation, slides were treated with a secondary antibody, an anti-rabbit anti-mouse antibody complex (Envision HRP, Dako-Cytomation, Cambridgeshire, United Kingdom), following which 3,3'-diaminobenzidine was applied to complete development (1:5 of substrate buffer and 3,3'-diaminobenzidine and chromogen; Dako-Cytomation). Finally, slides were counterstained with hematoxylin.

Scoring criteria. A single pathologist using a semiquantitative method did the scoring for the tissue microarrays. As reported by

Table 1. The results of the statistical analysis investigating the relationships between clinical factors

	Time to PSA recurrence (mo)	Positive margin (\pm)	Capsular invasion (\pm)	Gleason score (2-5, 6, 7-10)	Preoperative PSA (ng/mL)
Age (y)	0.66 (Cox reg.)	0.20 (Mann-Whitney)	0.36 (Mann-Whitney)	0.27 (Kruskal-Wallis)	0.15 (Spearman)
Preoperative PSA (ng/mL)	0.38 (Cox reg.)	0.08 (Mann-Whitney)	0.24 (Mann-Whitney)	0.42 (Kruskal-Wallis)	
Gleason score (2-5, 6, 7-10)	0.43 (log-rank)	0.26 χ^2	0.81 χ^2		
Capsular invasion (\pm)	0.03 –ve doing better (log-rank)	0.001 (+ve) χ^2			
Positive margin (\pm)	0.03 –ve doing better (log-rank)				

NOTE: The nature of the relationship, positive (+ve) versus negative (–ve), is presented with the statistically significant relationships presented in boldface. The statistical test used for each relationship is presented in parentheses.

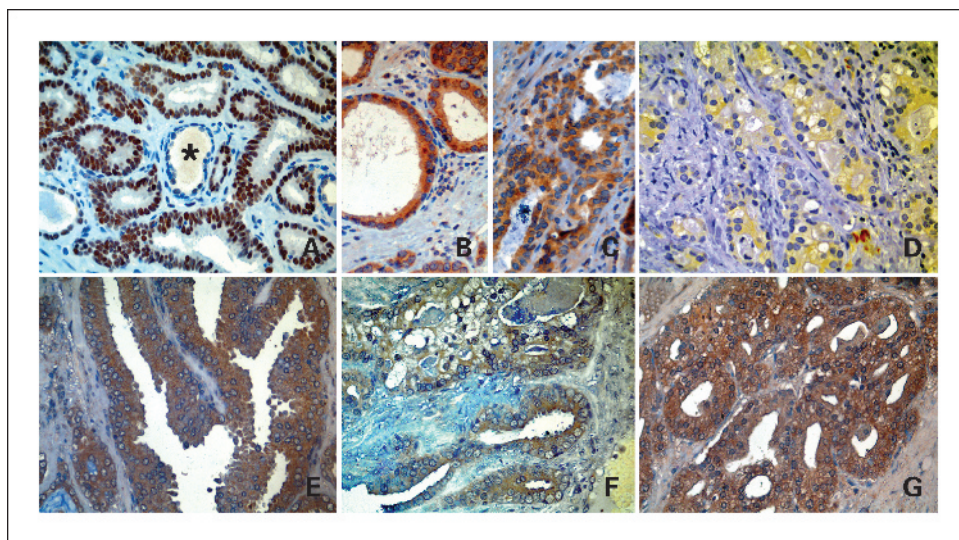


Fig. 1. A-E, immunohistochemistry showing examples of staining in prostate cancer for the hypoxia pathway and androgen receptor. A, strong nuclear staining for the androgen receptor in the tumor cells of an invasive prostate carcinoma; a single entrapped normal acinus is only weakly stained (*). Strong immunoreactivity for HIF-1a (B) and HIF-2a (C) together with moderate staining for VEGF-A (D) in the neoplastic cells of an invasive prostatic carcinoma. Strong, moderate, and weak staining for PHD1 (E), PHD2 (F), and PHD3 (G), respectively, in the neoplastic cells of prostate cancer.

others, HIF-1a and HIF-2a cores were scored according to the presence of nuclear staining (18). Because the level of HIF that is required to initiate transcription is currently unknown, and because the range of staining intensity was narrow, the HIF cores were scored only according to the presence (1+) or absence (0) of nuclear expression. For PHDs and VEGF expression, the intensity of the staining provided a sufficient range of intensities to enable scoring of the specimens as 0, negative; 1, weak; 2, moderate; and 3, strong staining. Tumors were considered positive for the PHDs with 2+ or 3+ staining and positive for VEGF with any staining (1+, 2+ and 3+). These data split the patients into approximately equal groups. As previously reported, the androgen receptor was assessed using both the intensity of the immunohistochemical stain (0, 1, 2, 3, as described above) and the percentage of neoplastic nuclei stained (0-100%; ref. 19). This was then used to calculate the sum value for each core (intensity \times percentage) with a score of >200 considered positive for the analysis. For all markers, the highest score from the two cores was used in the data analysis.

Statistical analysis. Given the categorical nature of the staining factor data, contingency tables were analyzed using Pearson's χ^2 test, a test suitable for the analysis of this type of data. When comparing the staining factor data to the presence of clinicopathologic factors, Mann-Whitney and Kruskal-Wallis tests were used for those analyses where continuous data was involved. The association with time to PSA increase was assessed by log-rank test. All statistical analyses were done using the Stata package release 8.0 (Stata Corporation, College Station, TX).

Results

Relationships between clinical characteristics. The mean age of patients was 61 years (range, 44-76), the median PSA was 8.2 ng/mL (range, 1.5-25). Median Gleason score on surgical excision was 6 (range, 2-9), 86 of 149 (57.7%) had capsular invasion, and 51 of 149 (34.2%) had positive surgical margins. PSA recurrence was significantly associated with the presence of capsular invasion ($P = 0.03$) and positive surgical margins ($P = 0.03$). There was also a significant relationship between the presence of capsular invasion and positive surgical margins ($P = 0.001$; Table 1).

Relationships between members of the hypoxia pathway. HIF-1a, HIF-2a, and all PHDs showed a nuclear and cytoplasmic neoplastic cell expression (Fig. 1B, C and E, F, G, respectively)

as previously reported (20). Similarly, VEGF and androgen receptor exhibited tumor cell cytoplasmic and nuclear expression (Fig. 1A and D, respectively). The number of tumors staining positive and negative for each factor according to their expression of HIF-1a is presented in Table 2.

There was a significant correlation between HIF-1a and HIF-2a expression ($P = 0.02$). Both HIF-1a and HIF-2a expression were significantly related to VEGF ($P = 0.05$ and $P < 0.001$, respectively). Likewise, the presence of androgen receptor was also significantly related with HIF-1a ($P = 0.004$), HIF-2a ($P < 0.001$), and VEGF expression ($P = 0.05$; Table 3). Expression of PHD1, PHD2, and PHD3 were significantly related with each other ($P < 0.001$). PHD2 expression was also

Table 2. The number of tumors staining positive and negative for each factor according to their expression of HIF-1a (χ^2 analysis)

	HIF-1a negative	HIF-1a positive	P
Number of patients	20	119	
HIF-2a			
Negative	10	29	0.02
Positive	10	89	
VEGF			
Negative	8	24	0.05
Positive	12	95	
Androgen receptor			
Negative	13	39	0.004
Positive	6	78	
PHD1			
Negative	12	91	0.37
Positive	6	28	
PHD2			1.00
Negative	15	91	Fisher exact
Positive	4	27	
PHD3			
Negative	11	73	0.99
Positive	7	46	

Table 3. The results of the statistical analysis between hypoxia factors, presented in terms of the *P* value

	HIF-1a	HIF-2a	VEGF	AR	PHD1	PHD2
PHD3	0.99 (-ve)	0.67 (+ve)	0.18 (+ve)	0.92 (+ve)	<0.001 (+ve)	<0.001 (+ve)
PHD2	0.86 (-ve)	0.02 (-ve)	0.71 (-ve)	0.03 (+ve)	<0.001 (+ve)	
PHD1	0.37 (-ve)	0.06 (-ve)	0.96 (-ve)	0.09 (-ve)		
AR	0.004 (+ve)	<0.001 (+ve)	0.05 (+ve)			
VEGF	0.05 (+ve)	<0.001 (+ve)				
HIF-2a	0.02 (+ve)					

NOTE: The nature of the relationship, positive (+ve) versus negative (-ve), is presented with the statistically significant relationships presented in boldface (χ^2 analysis).

significantly but negatively correlated with HIF-2a ($P = 0.02$). PHD2 was negatively correlated with the androgen receptor ($P = 0.03$).

The relationship between clinical and hypoxia pathways. HIF-1a expression was significantly negatively related to the preoperative PSA level ($P = 0.03$). VEGF expression was significantly but negatively related to the postoperative Gleason score ($P = 0.05$), and the androgen receptor expression was significantly but negatively related to the preoperative PSA levels ($P = 0.05$). Androgen receptor expression was also significantly related with time to PSA recurrence ($P = 0.03$); those with more androgen receptor expression having a longer time before PSA recurrence (Table 4).

Discussion

This study aimed to assess the relationship between expression of the androgen receptor and a key angiogenesis factor, VEGF, in clinical samples, as it has been reported in *in vitro* cell line studies of human prostate cancer, and investigate the relationship with hypoxia signaling pathways. Analysis of the clinical factors used in this study showed that the cohort is similar to other published series (21, 22), e.g., PSA recurrence significantly related with the presence of capsular invasion and positive surgical margins in addition to the Gleason score (Table 1). These results are expected as the presence of both capsular invasion and positive margins indicate the existence of local spread and Gleason score identifies those patients with more aggressive tumors. Thus, these findings support the use of this tissue microarray as a template with which to study prostate cancer specimens.

Du et al. have previously confirmed the up-regulation of HIF-1a expression in areas of prostate cancer as compared with those of BPH and normal prostate (6). Using an immunohistochemistry technique on formalin-fixed and paraffin-embedded specimens obtained from 13 cases of normal prostate, 28 cases of BPH, and 34 cases of prostate cancer, they found that normal prostate manifested no immunoreactivity, whereas both cancer and BPH specimens

showed significantly increased HIF-1a protein expression, which was highest in cancer specimens. Zhong et al. (7) went on to show that HIF-1a expression was also up-regulated in the proposed precursor lesion, high-grade prostatic intraepithelial neoplasia. They reported that HIF-1a levels were up-regulated in 11 of 14 high-grade prostatic intraepithelial neoplasia lesions relative to the respective normal epithelium, stromal cells, and BPH, but was lower than in areas of adjacent prostate cancer. The current study confirms the expression of HIF-1a in prostate cancer and is the first to show the presence of HIF-2a expression. Furthermore, expression of both HIF-1a and HIF-2a were significantly correlated in neoplastic tissues, suggesting that both isoforms play a functional role in the tumor response to hypoxia and therefore play an important role in the development and progression of this disease.

The identification of mutations within the oxygen-dependent domain of HIF-1a was recently shown in a small number of prostate cancers (23, 24). The presence of mutations implies that the activation of HIF-1a can be selected for enabling the expression of this protein under inappropriate conditions. This could lead to an increase in aggressive biological behavior or contribute to the development of therapeutic resistance by the cancer cells.

A key factor regulated by HIF is VEGF. Stefanou et al. (8), using an immunohistochemical technique in 60 prostate adenocarcinomas and 64 BPH samples, reported that VEGF levels were higher than those in BPH. In addition, akin to HIF-1a, VEGF levels have also been shown to be higher in some high-grade prostatic intraepithelial neoplasia lesions than in normal epithelium, suggesting that the increase in VEGF expression is an early event in the development of prostate cancer (9). The current study not only confirms the presence of VEGF expression in prostate cancer, but additionally shows, for the first time, a positive relationship between VEGF expression and both HIF-1a and HIF-2a expression.

However, the principal aim of this study was to investigate the hypothesis that androgens use the hypoxia pathway, at least in part, to exert their control over VEGF levels in

Table 4. The results of the statistical analysis between clinical data and hypoxia factors, presented in terms of the *P* value

	Preoperative PSA (ng/mL), Mann-Whitney	Gleason score (2-5, 6, 7-10), χ^2	Capsular invasion (\pm), χ^2	Positive margin (\pm), χ^2	Time to PSA recurrence (mo), log-rank
HIF-1a (0 versus 1)	0.03 (-ve)	0.90	0.06	0.73	0.20
HIF-2a (0 versus 1)	0.53	0.13	0.54	0.30	0.94
PHD1 (0, 1 versus 2, 3)	0.79	0.96	0.42	0.77	0.51
PHD2 (0, 1 versus 2, 3)	0.28	0.56	0.94	0.47	0.61
PHD3 (0, 1 versus 2, 3)	0.64	0.13	0.28	0.52	0.36
VEGF (0 versus 1, 2, 3)	0.52	0.05 (-ve)	0.87	0.57	0.60
Androgen receptor (<200 versus >200)	0.05 (-ve)	0.80	0.49	0.16	0.03, >200 doing better

NOTE: The nature of the relationship, positive (+ve) versus negative (-ve), is presented with the statistically significant relationships presented in boldface. The statistical test used for each relationship is presented in the column heading.

human prostate cancer. The current study shows that the expression of both HIF-1a and HIF-2a were highly significantly correlated with the androgen receptor ($P = 0.004$ and $P < 0.001$, respectively; Table 3), supporting the recent *in vitro* cell line findings by Mabjeesh et al. (13) that androgens activate HIF-1a, driving VEGF expression in androgen-sensitive human prostate cancer. Furthermore, the significant association between VEGF and androgen receptor and both HIF-1a and HIF-2a strongly suggest that the HIF activation is functional.

In addition to confirming the important relationship between the hypoxia pathway and androgen receptor, this study has made several other novel observations. In particular, we found that the expression of all three PHD enzymes were significantly correlated with each other in prostate cancer, suggesting that all three are coregulated by a common mechanism in this disease. Although the expression of HIF-1a was unrelated to that of the PHD1, PHD2, and PHD3 enzymes, HIF-2a was significantly inversely associated with PHD2 ($P = 0.02$). The significance of this observation is difficult to determine as these pathways are still poorly defined but it may indicate that although both HIF-1a and HIF-2a are present within prostate cancer, it is HIF-2a, which is the more active isoform, with PHD2 down-regulation allowing higher expression because of lack of degradation. Knowles et al. (25) have previously shown that the availability of critical factors such as oxygen, ascorbate, and iron have major effects on HIF, indicating that their presence may control which isoform is active, even when more than one is expressed.

The potential clinical utility of the factors investigated was analyzed by measuring their relationship to PSA recurrence, a surrogate marker for disease-free survival (Table 4). With the exception of the androgen receptor ($P = 0.03$), none of the factors showed a significant relationship with this marker, but the majority of patients have not relapsed; therefore, longer follow-ups will be necessary. The higher expression of androgen receptor was, however, associated with a lower frequency of PSA recurrence.

The implications of androgen receptor regulating HIF in normoxia are that a major pathway known to regulate the

hypoxia response, transcriptome, that promotes aggressive tumor growth, can be activated in normoxic conditions. Other growth factors are also known to activate this pathway e.g., epidermal growth factor, insulin-like growth factor, and HER2. Hence, this represents a subgroup of androgen receptor-regulated pathways, and we would suspect that with longer follow-up, they will be more likely to relapse. Furthermore, bypass of androgen receptor blockade by production of peptide growth factors could also lead to an increase in the expression of the HIF pathway, which would be interesting to analyze in relapsed patients. Hypoxia has been shown in prostate cancer and would activate this pathway independently of androgen receptor, therefore, this may also contribute to growth in the absence of androgen receptor. Finally, the combination of hypoxia induction of HIF with androgen receptor induction in normoxic areas may be particularly detrimental. All this suggests that HIF antagonists should be investigated in hormone-resistant prostate cancer. Indeed, preliminary results from our laboratory indicate that RNAi treatment to down-regulate HIF-1 in the androgen receptor-positive prostate cancer cell line could inhibit androgen-induced growth.

In summary, this study, using an immunohistochemistry technique, is the first to investigate the relationship between the hypoxia pathway, angiogenesis, and androgen receptor. The result suggest that although both HIF-1a and HIF-2a are present, HIF-2a could be the dominant HIF isoform in prostate cancer. In addition to providing important new data regarding the expression of some of these novel factors and how they relate to each other, it also supports the *in vitro* work that androgens regulate VEGF levels through the activation of HIF-1a in androgen-sensitive tumors. This finding has important clinical implications, as alternative strategies of inhibiting HIF-1a may be of therapeutic value in androgen-resistant tumors.

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