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HIF-1α mRNA gene expression levels in improved diagnosis of early stages of prostate cancer

CHRISTODOULOS P. PIPINIKAS[1], NICHOLAS D. CARTER[1], CATHERINE M. CORBISHLEY[2], & CHRISTIANE D. FENSKE[1]

[1]*Division of Clinical Developmental Sciences, Medical Genetics and* [2]*Cellular Pathology, St George's University of London, London, UK*

Abstract

Objectives: Several clinical studies have indicated that the rates of invasive growth and metastatic disease in cancer depend on the degree of hypoxia, which is mediated by hypoxia-inducible factor 1 α (HIF-1α). To determine its potential role as a marker for prostate cancer (CaP) diagnosis, *HIF-1α* mRNA levels were measured in blood samples of patients diagnosed with different stages of prostatic disease. *Methods: HIF-1α* mRNA levels were measured by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) and correlated with accurate clinicopathological data. Quantitative data were compared with serum prostate-specific antigen (PSA) measurements to determine variations in the accuracy of the CaP diagnosis. *Results: HIF-1α* mRNA levels were significantly upregulated in patients with localized CaP (LocCaP; $n=63$; $p<0.0001$), compared with patients with no evidence of malignancy (NEOM) and benign prostatic hyperplasia (BPH) ($n=35$ for both patient groups combined). Receiver operator characteristic (ROC) curve analysis demonstrated that *HIF-1α* specificity for the NEOM/BPH diagnosis was 88.6%. Sensitivity for LocCaP was 74.6% with an overall diagnostic efficiency of 79.6%. Specificity of the NEOM diagnosis at PSA levels of 4.0 ng ml^{-1} was 28.6% and sensitivity of the LocCap diagnosis was 65.1%, demonstrating a reduced overall diagnostic efficiency, compared with that given by *HIF-1α* measurements, of 52.0%. Levels of *HIF-1α* in patients with metastatic CaP (MetCaP; $n=27$)) were similar to those in the NEOM/BPH group. *Conclusions: HIF-1α* is upregulated early in CaP development with subsequent downregulation at later metastatic stages. This study demonstrates increased accuracy of early-stage disease diagnosis using *HIF-1α* qRT-PCR compared with serum PSA measurements. *HIF-1α* may therefore be a useful adjunct, together with other diagnostic markers used in relative qRT-PCR and current diagnostic techniques (including serum PSA and PSA velocity) to minimize unnecessary biopsies indicated by elevated serum PSA levels alone.

Keywords: *HIF-1α,* diagnostic marker, prostate cancer, prostate-specific antigen, quantitative RT-PCR, ROC curve analysis

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Introduction

Currently, prostate cancer (CaP) is diagnosed in essence on the basis of biopsy following raised serum prostate-specific antigen (PSA) levels (Pinthus et al. 2007).

Correspondence: Christodoulos P. Pipinikas, Division of Clinical Developmental Sciences, Medical Genetics, St George's University of London, Jenner Wing, Cranmer Terrace, London SW17 0RE, UK. Tel: +44 (0) 2087250192. Fax: +44 (0) 2087251039. E-mail: cpipinik@sgul.ac.uk

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However, while PSA levels >10 ng ml^{-1} usually indicate CaP (Mikolajczyk et al. 2004), patients with both localized and benign disease may exhibit levels in the range 4–10 ng ml^{-1}. Raised serum PSA levels in 60–70% of patients who have a negative biopsy are found to be due to benign disease or prostatitis (Bunting 1995). Conversely, 20% patients with levels below 4 ng ml^{-1} (Thompson et al. 2004) deemed to mitigate the need for biopsy, have CaP. In addition, due to the limitations of biopsy sampling, a number of patients with raised PSA and a negative biopsy may have cancer that is undetected. These data highlight the limitations of serum PSA in CaP diagnosis and the need for markers that may replace or be used in conjunction with current diagnostic techniques to improve diagnostic specificity.

Hypoxia, a disruption of oxygen homeostasis, has been associated with the most common causes of mortality worldwide, including cancer (Greenlee et al. 2000, Semenza 2000a). It is well established that the median partial O_2 pressure (PO_2) is much lower in tumours compared with their tissue of origin and a further reduction in O_2 supply in tumours after disease relapse is observed compared with their primary tumours (Höpfl et al. 2004). O_2 sensing, which allows cells to respond to hypoxic conditions (Figure 1A, B) (Semenza 2000b) is associated with the activation of genes involved in cell survival and proliferation and is mediated by the transcription factor, hypoxia-inducible factor-1 (HIF-1) (Figure 1C–H).

HIF-1 provides tumour cells with a survival advantage as it allows them to benefit from these normal cellular responses (Semenza 2002). HIF-1 is a heterodimer consisting of two subunits, HIF-1α and HIF-1β (Wang & Semenza 1995). The HIF-1α subunit is overexpressed in tumours as a result of physiological signals (hypoxia) and alterations in gene expression (Kiriakidis et al. 2007). Several clinical studies have indicated that the degree of hypoxia defines the rates of invasive growth and formation of metastatic disease (Zhong et al. 1999). For example, human colon cancer cells transfected with a HIF-1α-carrying vector exhibit increased growth rates compared with untransfected cells (Hanahan & Folkman 1996); reduced growth rates and vascularization have been observed in mouse hepatoma cell xenografts lacking an active *HIF-1α* gene (Maxwell et al. 1997).

Due to its role in early tumour formation, *HIF-1α* mRNA levels were evaluated as a potential candidate marker for early-stage CaP diagnosis. Gene expression levels were correlated with patient diagnosis and *HIF-1α* diagnostic accuracy compared with that obtained for serum PSA measurements to determine any improved outcome.

Materials and methods

Patient groups

Following fully informed consent, blood samples were taken from 125 patients attending the Prostate Clinic at St George's Hospital (London, UK). Patients, investigated for benign and malignant prostate disease by a single urology team, had undergone at least one transrectal ultrasound (TRUS)-guided octant prostate biopsy on the basis of serum PSA and/or clinical findings (undertaken by one of three specialist uroradiologists) following the national standard optimized protocol. This study was approved by the Wandsworth Local Research Ethics Committee and carried out in accordance with the declaration of Helsinki (2000) of the World Medical Association.

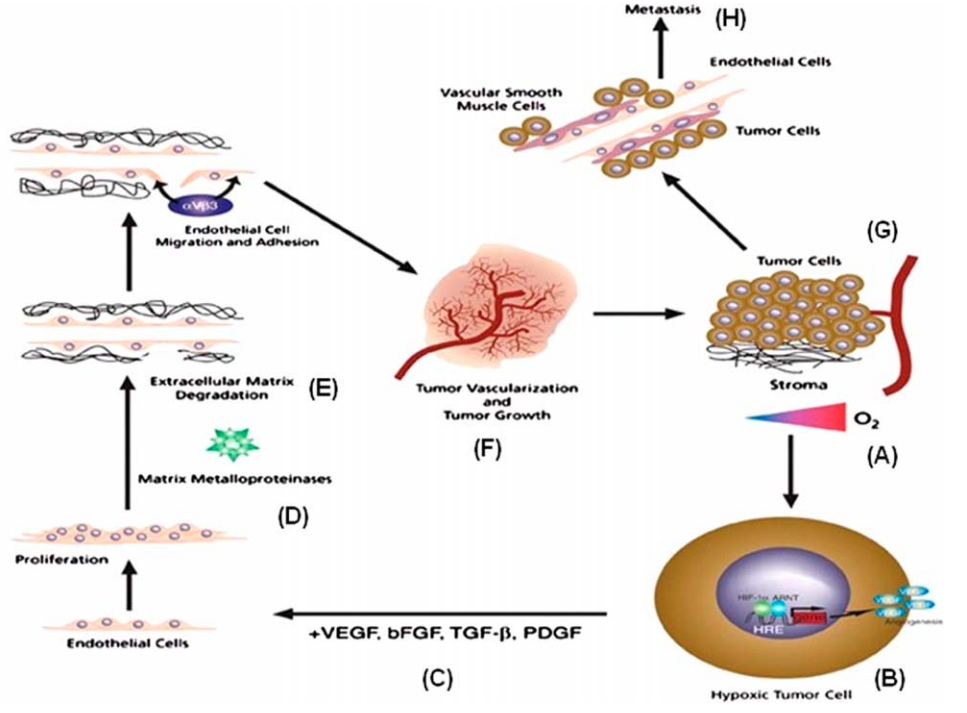

Figure 1. Events leading to angiogenesis and metastasis. Early tumours begin as small masses no larger than about 1 mm^3 in diameter. In the absence of angiogenesis, the blood supply is suspended and the cell becomes hypoxic. In response to the hypoxic environment (A), hypoxia-inducible factor-1α (HIF-1α) is activated (B) and promotes transcription of many downstream target genes, including vascular endothelial growth factor (VEGF) (C). This results in the formation of a neovasculature necessary for tumour growth. Interactions between tumour cells, stromal cells and endothelial cells trigger the secretion and activation of matrix metalloproteinases that degrade the extracellular matrix (D, E) and permit the budding of new blood vessels from existing vessels (F). Blood vessels that are established in the tumour tissue (G) permit the invasion of tumour cells into the bloodstream and the establishment of secondary metastatic sites (H). bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor β; PDGF, platelet-derived growth factor; HRE, hypoxic response elements; ARNT, aryl hydrocarbon receptor nuclear translocator. (Reproduced with permission from Sigma-Aldrich).

Patient data, including age, date of histology and blood sampling, PSA at time of initial histological diagnosis and blood sampling, follow-up PSA values, histology (including Gleason scores) and bone scan/computed tomography/magnetic resonance imaging results, ensured accurate correlation of research results with clinical data.

Histopathological and radiological information were used to classify patients into groups. The NEOM group (no evidence of malignancy, $n = 35$) included patients whose biopsy results were negative for cancer and patients diagnosed with benign prostatic hyperplasia (BPH) for which they consequently underwent channel transurethral resection of the prostate (TURP). The LocCaP group ($n = 63$) included patients with biopsy-proven prostate adenocarcinoma but no clinical and/or radiological evidence of metastatic disease. At the time of blood sampling, none of these patients had undergone radical prostatectomy. The MetCaP group ($n = 27$) included patients with evidence of widespread disease (mainly bone metastasis). Histological examination was available from the time of initial patient diagnosis. Patient serum

PSA levels were subsequently monitored – significant changes in clinical symptoms or increased serum PSA levels dictated further histological examination. Clinicopathological characteristics are given in Table I.

Specificity of *HIF-1α* for CaP diagnosis was evaluated using blood samples of randomly selected healthy controls (males, $n=7$ and females, $n=7$). A further control group included patients with inflammatory disease ($n=13$) as demonstrated by high levels of serum C-reactive protein (CRP). Levels of *HIF-1α* expression were measured in this control group to ensure that any variation was specific to CaP development, and not due to an inflammatory response.

Blood collection and RNA extraction

Blood samples (2×5 ml in EDTA tubes, BD Vacutainer) were stored and transported on ice to minimize the risk of RNA degradation. Total RNA was extracted in duplicate within 1 h of blood sampling using the VERSAGENE blood RNA kit (Flowgen Bioscience, Nottingham, UK). On-column DNAse treatment was carried out using

Table I. Clinicopathological characteristics of the 125 patients with different stages of CaP included in the study.

	Patient groups		
	NEOM $n=35$	LocCaP $n=63$	MetCaP $n=27$
Age (years)			
Range	52–80	56–94	39–84
Median	67	74	72
Gleason score (GS)			
2–4		8	—
5–7	No	46	9
8–10	GS	8	15
Serum PSA (ng ml^{-1})			
Histological diagnosis			
≤4	7	4	2
4.1–10	16	18	3
≥10.1	12	37	22
Median PSA	7.2	14.6	180.0
PSA range	0.5–103.0	2.1–150.0	0.2–5000.0
Blood collection			
≤4	10	22	4
4.1–10	17	19	5
≥10.1	8	22	18
Median PSA	6.2	7.9	32.8
PSA range	0.5–28.9	0.1–139.0	0.2–1570.0
Median interval (days) between histological diagnosis and blood sampling (range)	141 (12–1944)	196 (12–2559)	246 (16–2100)

NEOM, no evidence of malignancy; LoCaP, localized prostate cancer, MetCaP, metastatic CaP; PSA, prostate-specific antigen.

NEOM patient group no GS due to negative biopsy results; GS not available for 1 LocCaP and 3 MetCaP patients. Serum PSA measurements at time of histological diagnosis not available for 4 LocCaP patients. Age range and median age refers to that at blood sampling.

the RNeasy Micro kit (Qiagen, Crawley, UK) according to the manufacturer's protocols. RNA samples were stored at −70°C.

Complementary DNA (cDNA) synthesis and relative quantitative reverse transcriptase–polymerase chain reaction

RNA was reversed transcribed into complementary DNA (cDNA) using SuperScript™ II and oligo(dT)$_{12–18}$ primer according to the manufacturer's protocol (Invitrogen, Paisley, UK). cDNA samples were stored at −20°C prior to PCR amplification. No template and no enzyme controls were used as described previously (Pipinikas et al. 2007).

Quantitative RT-PCR (qRT-PCR) was carried out (LightCycler 2.0) using FastStart™ SYBR Green I DNA master mix (both Roche Diagnostics, Mannheim, Germany) and *HIF-1α* primers: sense 5′-CGCATCTTGATAAGGCCTCT-3′ and antisense 5′-TACCTTCCATGTTGCAGACT-3′ (Sigma-Genosys, Suffolk, UK). The reaction comprised 5.0 μl cDNA, 2.0 μl of SYBR Green I master mix, 4 mmol L^{-1} $MgCl_2$ and 0.3 μmol l^{-1} of each primer in a final volume of 20 μl. The reaction was preheated at 95°C for 10 min followed by 40 amplification cycles of denaturation at 95°C for 5 s, annealing at 60°C for 5 s and extension at 72°C for 16 s. The temperature transition rate (slope) was 20°C per s. A standard curve was prepared using PC-3 cell line cDNA to calculate the efficiency of the reaction and to quantitate levels of *HIF-1α* expression. Amplification efficiency was 1.995. Melting curve analysis showed a single product with a distinct narrow peak at 82°C with no primer dimers or products of non-specific binding, indicating a highly specific reaction. *HIF-1α* data were related to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *GAPDH* qRT-PCR primers, reaction and amplification conditions were as described previously (Pipinikas et al. 2007).

Assay validation

The accuracy of the assay was determined by investigating the intra-assay (test precision) variation using triplicates of the standard PC-3 dilution samples ($10E^{-03}$ to $10E^{-09}$) included in a single run. The reproducibility of the assay was investigated by calculating the interassay (test variability) variation using the crossing points (Cp) of the three standard dilution samples ($1.00E^{-03}$, $1.00E^{-05}$ and $1.00E^{-07}$) that were included in a total number of 15 individual experiments run separately. The mean intra-assay and interassay variations, 1.57% and 6.39%, respectively, were within the expected range (Pfaffl 2004).

These standard dilutions were included in each patient sample run allowing calculation of amplification efficiency within individual experiments and accounting for any matrix effect (day-to-day or sample-to-sample variation). The optimized standard curve was imported at the end of each experiment using the LightCycler software. In this way, all quantitative runs had the same amplification efficiency allowing for accurate *HIF-1α* quantification.

Statistical analysis

Statistical analysis was carried out using relative *HIF-1α* quantitative data (*HIF-1α*/*GAPDH*). Data were processed with Analyse-it™ General and Clinical laboratory

statistics version 1.73 (Analyse-it Software Ltd, Leeds, UK) and SYSTAT version 11 (Systat Software Inc, London, UK). Levels of *HIF-1α* expression in the patient groups were determined using descriptive statistics. Analysis of variance (*ANOVA*) was carried out to determine levels of significance with $p < 0.05$ considered statistically significant. Receiver operator characteristic (ROC) curve analysis was used to calculate the area under the curve (AUC) as a factor of discrimination between the different pair-wise comparisons and to determine cut-off values of *HIF-1α* expression for evaluation of the diagnostic specificity and sensitivity and positive and negative predictive values (PPV and NPV). A direct comparison with PSA ROC curve analysis data was carried out to investigate any additional value of *HIF-1α* for CaP diagnosis.

Results

HIF-1α mRNA levels

All patients included in the study were positive for *GAPDH* qRT-PCR demonstrating nondegraded mRNA. All LocCaP patients ($n = 63$) showed high levels of *HIF-1α* expression. Six NEOM (of 35) and one MetCaP (of 27) patients demonstrated *HIF-1α* gene expression levels below the limit of detection of the qRT-PCR assay. Four (of seven) healthy male controls showed very low levels of expression of *HIF-1α*, with the remaining three showing no expression at all. Similarly, CRP patients and healthy female controls showed extremely low *HIF-1α* expression levels (Table II).

HIF-1α was significantly upregulated ($p < 0.0001$) by a mean 15-fold increase between NEOM and LocCaP. A significant mean 14-fold decrease ($p < 0.0001$) was observed in *HIF-1α* levels between LocCaP and metastatic disease, with levels in the MetCaP group similar to those in the NEOM group ($p = 0.4740$; Figure 2).

Levels of *HIF-1α* in the CRP controls were significantly lower compared with those in the NEOM, LocCaP and MetCaP groups (Table III). No significant differences were found between the CRP and the healthy controls, indicating that *HIF-1α* upregulation in the prostate patient groups (NEOM, LocCaP and MetCaP) was independent of the inflammatory response.

Table II. Descriptive statistics for blood *HIF-1α* mRNA expression levels in patient diagnostic and control groups.

Patient group	Mean[a,b]	95% CI of mean	Median	95% CI of median
NEOM	1.77	0.73–2.81	0.62	0.32–1.90
LocCaP	26.2	12.7–39.6	7.61	5.23–14.5
MetCaP	1.91	0.85–2.98	0.7	0.54–1.89
CRP	0.35	0.2–0.49	0.31	0.13–0.49
Healthy males	0.15	0.1–0.2	0.142	–
Healthy females	0.1	0.07–0.13	0.104	0.051–0.146

[a]The values refer to *HIF-1α* data normalised to *GAPDH* and are unitless ratios. [b]Data have been multiplied by a 10^4 factor.

NEOM, no evidence of malignancy; LoCaP, localized prostate cancer, MetCaP, metastatic CaP; CRP, C-reactive protein.

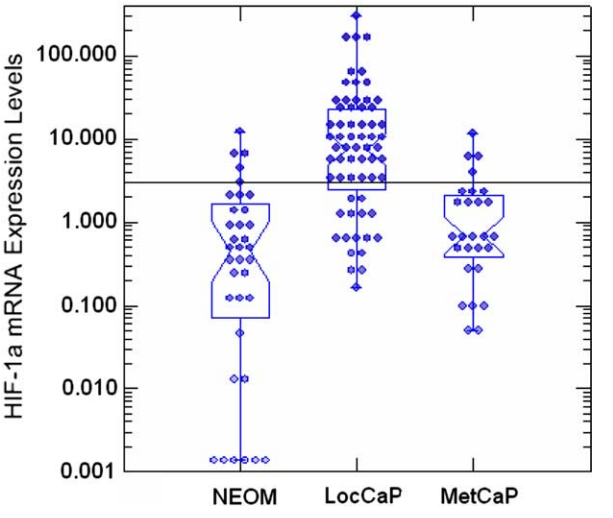

Figure 2. A combined box plot and symmetrical dot density plot showing distribution of relative *HIF-1α* mRNA levels (*HIF-1α*/*GAPDH*) in the three different patient diagnostic groups. Boxes are notched (narrowed) at the median and return to full width at the lower and upper 95% confidence interval values. The straight line parallel to the *x*-axis represents the chosen cut-off point of 3.00. Note that the patients with levels of *HIF-1α* below the limits of detection have also been included in the classification box plots. To enable their inclusion in statistical analysis, values equivalent to a 100-fold reduction of the lowest normalized level of *HIF-1α* (*HIF-1α*/*GAPDH*) in the respective patient group was taken.

Diagnostic validity of HIF-1α assay

ROC curve analysis was carried out to determine the diagnostic specificity and sensitivity of the assay in discriminating between the different prostate patient groups (Table IV).

Using a single cut-off point of 3.00 the sensitivity of the *HIF-1α* assay for LocCaP diagnosis was 74.6% with a specificity of 85.2% for MetCaP and 88.6% for NEOM diagnosis. Positive and negative predictive values were equally high with an overall efficiency for correct diagnosis close to 80.0% (Table V).

ROC curve analysis demonstrated limited discrimination between NEOM and MetCaP disease.

Table III. Calculated *p*-values between the different pair-wise comparisons.

	Healthy males	Healthy females	CRP	NEOM	LocCaP	MetCaP
Healthy males	–	0.6557	0.4542	0.0332	<0.0001	0.0136
Healthy females		–	0.1325	0.0009	<0.0001	0.0002
CRP			–	0.0332	<0.0001	0.0081
NEOM				–	<0.0001	0.4740
LocCaP					–	<0.0001
MetCaP						–

CRP, C-reactive protein; NEOM, no evidence of malignancy; LoCaP, localized prostate cancer, MetCaP, metastatic CaP.

Table IV. ROC curve analysis showing area under the curve (AUC) results as a function of the level of discrimination between the different comparisons.

Comparison	AUC[a]	*p*-Value	95% CI of AUC
LocCaP/NEOM	0.883	<0.0001	0.808–0.958
LocCaP/MetCaP	0.87	<0.0001	0.789–0.950
LocCaP/CRP	0.957	<0.0001	0.915–1.000
LocCaP/healthy[b]	0.999	<0.0001	0.994–1.000
MetCaP/NEOM	0.558	0.229	0.405–0.712
MetCaP/CRP	0.781	<0.0001	0.633–0.929
MetCaP/healthy	0.888	<0.0001	0.778–0.998
NEOM/CRP	0.695	0.0072	0.539–0.851
NEOM/healthy	0.859	<0.0001	0.741–0.977
CRP/healthy	0.888	<0.0001	0.757–1.000

[a]Higher AUCs indicate higher levels of discrimination. [b]Because of the limited number of healthy controls (males and females) and due to similar gene expression levels, the two groups were combined in one (Healthy).
LoCaP, localized prostate cancer, NEOM, no evidence of malignancy; MetCaP, metastatic CaP; CRP, C-reactive protein.

Blood HIF-1α mRNA and serum PSA levels

All diagnoses were based on clinical and radiological investigations carried out using the nationally recognized standard optimized protocols at the time of the study. At histological diagnosis, seven NEOM patients (7/35) (i.e. biopsy showing no evidence of malignancy) had serum PSA levels ≤ 4 ng ml^{-1}. However, 16 NEOM patients had PSA levels between 4.1 and 10 ng ml^{-1} and 12 had levels >10.1 ng ml^{-1}. At blood sampling, patient serum PSA levels had decreased slightly: 10 NEOM patients with levels ≤ 4 ng ml^{-1} and 25 with levels between 4.1 and 10 ng ml^{-1} or above (Table I).

In the LocCaP group, 18 and 19 patients had serum PSA levels between 4.1 and 10 ng ml^{-1} at time of histological diagnosis and blood sampling, respectively. These figures reiterate the extent of overlap using serum PSA for CaP diagnosis, especially in the range of 4.1–10 ng ml^{-1}. As serum PSA/PSA velocity are the main drivers for prostate biopsy, their combination with additional marker(s) of enhanced diagnostic

Table V. Diagnostic criteria of *HIF-1α* utility for the discrimination of the different group comparisons.

Comparison	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)	Cut-off point[a]
LocCaP/NEOM[b]	74.6 (47/63)	88.6 (31/35)	92.2 (47/51)	66.0 (31/47)	79.6 (78/98)	≥3.00
LocCaP/MetCaP	74.6 (47/63)	85.2 (23/27)	92.2 (47/51)	59.0 (23/39)	77.7 (70/90)	≥3.00
MetCaP/NEOM	No discrimination due to *HIF-1α* downregulation at advanced stages of disease					

[a]The cut-off value (3.00) for *HIF-1α* is normalized to *GAPDH* and is a unitless ratio. [b]Patients who were negative for *HIF-1α* were also included in the analysis to allow accurate determination of the diagnostic criteria.
LocCaP patient group is considered as positive for the pair-wise comparison (i.e. having higher *HIF-1α* mRNA levels). For specificity and sensitivity, numbers in parentheses refer to cases correctly classified over total number of patients in each group. For PPV and NPV numbers in parentheses refer to positive and negative predictions correct, respectively. PPV, positive predictive value; NPV, negative predictive value; LoCaP, localized prostate cancer, NEOM, no evidence of malignancy; MetCaP, metastatic CaP; CRP, C-reactive protein.

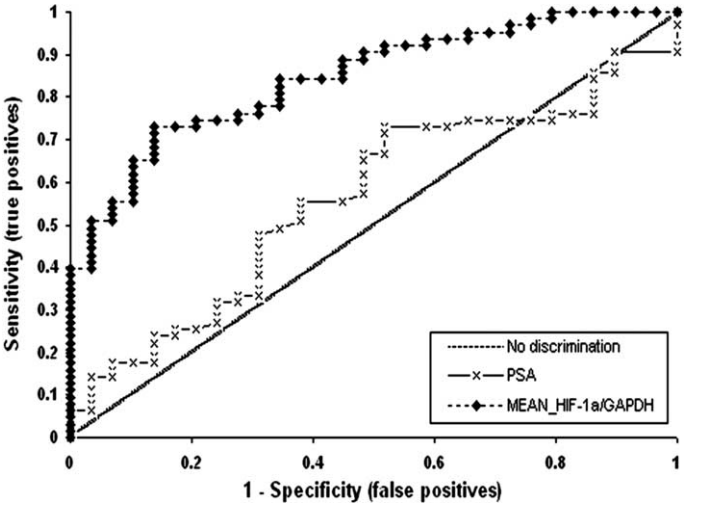

Figure 3. Serum prostate-specific antigen (PSA) vs *HIF-1α* qRT-PCR performance in early disease diagnosis. Comparison of PSA and *HIF-1α* ROC curves to discriminate between LocCaP and NEOM.

accuracy such as *HIF-1α*, would arguably improve diagnosis, and consequently avoid unnecessary biopsies.

The diagnostic capacity of serum PSA was investigated at different cut-off points: 4.0 ng ml^{-1} (the level currently used in the UK) and 2.6 ng ml^{-1}. The latter has been recommended as an alternative cut-off serum PSA level which results in more frequent detection of small, organ-confined tumours without overdetecting possibly clinically insignificant ones (Krumholtz et al. 2002). *HIF-1α* qRT-PCR demonstrated improved discrimination between LocCaP and NEOM (AUC 0.883) compared with serum PSA levels (AUC 0.562) (Figure 3).

PSA ROC curve analysis demonstrated that lowering the cut-off point increases sensitivity but reduces specificity (Table VI). This agrees with previous studies (Punglia et al. 2003, Schrader & Kranse 2003, Carter 2004). Sensitivity and specificity of *HIF-1α* qRT-PCR at the proposed cut-off point (unitless ratio of 3.00) was higher than that

Table VI. Serum prostate-specific antigen (PSA) levels versus blood *HIF-1α* mRNA levels in differentiating between LocCaP and NEOM.

	Cut-off[a]	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Efficiency (%)	TP	TN	FP	FN
PSA (ng ml^{-1})	2.6	68.3	14.3	58.9	20.0	48.9	43	5	30	20
	4.0	65.1	28.6	62.1	31.3	52.0	41	10	25	22
	6.0	61.9	48.6	68.4	41.5	57.1	39	17	18	24
	8.0	49.2	74.3	77.5	44.8	58.1	31	26	9	32
	10.0	34.9	77.1	73.3	39.7	50.0	22	27	8	41
HIF-1α	3.00	74.6	88.6	92.2	66.0	79.6	47	31	4	16

[a]Cut-off points: PSA cut-off points taken as ng ml^{-1}; the cut-off value (3.00) for *HIF-1α* is normalised to *GAPDH* and is a unitless ratio.

LocCaP above (>) cut-off point; NEOM less or equal (≤) to cut-off point.

PPV, positive predictive value; NPV, negative predictive value; TP, true positives; TN, true negatives; FP, false positives; FN, false negatives.

of serum PSA for all five cut-off points investigated (Table VI), indicating improved differential diagnosis of NEOM and LocCap, i.e. early stages of CaP development.

HIF-1α also improved discrimination between LocCaP and MetCaP compared with serum PSA (*HIF-1α* AUC 0.87, PSA AUC 0.696; data not shown). As *HIF-1α* is downregulated at advanced stages of disease development, little difference in measured levels was observed between those of MetCaP and NEOM patient groups. PSA, however, demonstrated improved discrimination between these groups (PSA AUC = 0.744, $p = 0.0003$, 95% CI 0.605–0.883; *HIF-1α* AUC 0.558, $p = 0.229$, 95% CI 0.605–0.883).

Discussion

Using relative qRT-PCR, expression levels of *HIF-1α* were measured using blood samples from patients with benign (NEOM/BPH) and malignant (LocCaP and MetCaP) prostatic disease. To our knowledge, this is the first study describing the potential of *HIF-1α* qRT-PCR measurements in early-stage CaP diagnosis.

Consistent with the known function of *HIF-1α* in tumour biology (Maxwell 2005), our study confirms that *HIF-1α* upregulation in LocCaP is an early event in cancer development associated with the hypoxic conditions of the tumour and the transcriptional activation of downstream target genes (e.g. vascular endothelial growth factor, VEGF) necessary for the survival and further development of the tumour. This is further supported by previous immunohistochemical studies (Zhong et al. 2004). Downregulation of *HIF-1α* in MetCaP patients indicates that once new blood vessels have been established, hypoxia is not characteristic of the tissue environment (Figure 1). Alternative mechanisms may be necessary for the invasion of tumour cells into the bloodstream and the formation of secondary metastatic sites, e.g. activation of matrix metalloproteinases (MMPs), which play a central role in the process of metastasis including destruction of the extracellular matrix and cell invasion (Johnsen et al. 1998, Yoon et al. 2003); or suppression of RECK (reversion-inducing-cysteine rich protein with Kazal motifs), which normally suppresses invasion and angiogenesis by downregulating MMP activity and limiting VEGF bioavailability (Takeuchi et al. 2004).

Using relative qRT-PCR and ROC curve analysis, *HIF-1α* was assessed as a potential marker for the diagnosis of prostate cancer and diagnosis of stages of disease development. Results were compared with the specificity and sensitivity of serum PSA measurements. These showed improved specificity, sensitivity, PPV, NPV and overall diagnostic efficiency for *HIF-1α* in differential diagnosis between NEOM and LocCaP (Table VI), and LocCaP versus MetCaP compared with serum PSA measurements. With this increased diagnostic accuracy of early disease stage, this marker has the potential to significantly reduce the number of unnecessary biopsies, as demonstrated in Tables V and VI: these tables include NEOM patients diagnosed on the basis of prostate biopsy which, in turn, was driven by elevated serum PSA levels.

The current study correlates patient qRT-PCR results with existing clinical data, obtained from current diagnostic techniques, with recognized and well-documented limitations. Patients with raised serum PSA levels may have LocCaP undetected by biopsy or radiological techniques, and therefore misdiagnosed as NEOM. Similarly, patients diagnosed with LocCaP may have micrometastases or circulating cells, undetected by current techniques (bone scans) used to diagnose MetCaP.

Our assay identified 16 LocCaP patients with *HIF-1α* levels similar to those seen in NEOM, or (due to *HIF-1a* downregulation at advance disease stages) in MetCaP patients. Positive biopsy results in the LocCaP patients eliminate the possibility of NEOM diagnosis; therefore these patients may well have advanced disease. This may explain the range of serum PSA levels in our LocCaP patient group (Table I), with some levels higher than would be expected for LocCaP diagnosis, and indicative of more advanced disease. Repeat bone scans would be recommended in these cases.

In addition to its diagnostic potential, due to its tumour-specific upregulation, the HIF pathway may provide a potential target for CaP therapy, enhancing selectivity of action and subsequently potentially decreasing potential side-effects. The oncotic pressure is elevated in solid tumours due to increased VEGF transcriptional activity, and therefore minimizes efficient drug delivery (Stohrer et al. 2000). Furthermore, the aggressive tumour phenotype relies on different events, e.g. neovascularization and glycolysis, which are HIF-1α-dependent. Therefore, it may be possible, by targeting HIF-1α, to improve the success of chemotherapeutic treatment and impact tumour aggressiveness. Several different approaches targeting the HIF pathway have been described (Semenza 2003).

In conclusion, our findings demonstrate that *HIF-1α* may be used to differentially diagnose NEOM vs LocCaP and LocCaP vs MetCaP with high accuracy, thus facilitating CaP diagnosis at early stages of disease development and monitoring disease progression. While recognizing the small patient sample size of this study, there is clear demonstration that *HIF-1α* may prove to be a useful adjunct to other diagnostic techniques including serum PSA, in particular identifying patients with low serum PSA levels, but with localized cancer undetected by biopsy, and those diagnosed with clinically localized disease, but who may have early metastatic disease. Further studies, involving increased patient samples, are envisaged to strengthen statistical analysis and study conclusions. As well as enhanced diagnostic potential, *HIF-1α* also presents opportunities for improved therapeutic approaches by gene-specific inhibition, e.g. by inhibiting angiogenesis and tumour growth.

Declaration of interest: The information in this paper forms part of the patent application filed by St George's Hospital Medical School (SGHMS) trading as St George's University of London (SGUL). SGHMS has entered into an agreement with a commercial company as a result of the research reported in this article, with the objective research commercialisation. This commercial company has not funded any of the research reported in this article.

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