Analysis of hypoxia-associated gene expression in prostate cancer: lysyl oxidase and glucose transporter-1 expression correlate with Gleason score

GRANT D. STEWART[1,2], KELLY GRAY[3], CAROLINE J. PENNINGTON[3], DYLAN R. EDWARDS[3], ANTONY C.P. RIDDICK[1], JAMES A. ROSS[2] and FOUAD K. HABIB[1]

[1]Prostate Research Group, Edinburgh Cancer Research Centre, School of Molecular and Clinical Medicine, University of Edinburgh, 4th Floor, MRC Human Genetics Building, Western General Hospital, Crewe Road South, Edinburgh EH4 2XU; [2]Tissue Injury and Repair Group, Room FU501, 1st Floor, Chancellor's Building, Edinburgh University Medical School, 49 Little France Crescent, Edinburgh EH16 4SB; [3]School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK

Received June 25, 2008; Accepted August 18, 2008

DOI: 10.3892/or_00000180

Abstract. Prostate cancer cells exist under hypoxic conditions. Hypoxia has a detrimental effect on the efficacy of treatment and final outcome in patients with prostate cancer. There have been a large number of endogenous markers of hypoxia described previously across a range of cancer types, both *in vitro* and *in vivo*. The aim of this study was to evaluate the expression of a range of hypoxia-associated genes within benign prostatic hypertrophy (BPH) and prostate cancer tissue. Messenger RNA was extracted from primary prostate tissue obtained from 67 men with benign prostatic hypertrophy or prostate cancer (Gleason score 5 to 10). Real-time polymerase chain reaction was performed to quantify the expression levels of 12 hypoxia-associated genes in these tissues. Expression of lysyl oxidase (LOX) and glucose transporter-1 (GLUT-1) genes were significantly higher in prostate cancer compared with BPH tissue ($P<0.05$) and correlated with Gleason score (LOX: $R=0.297$, $P=0.015$; GLUT-1: $R=0.274$, $P=0.026$). HIF-2α had a negative correlation with Gleason score ($R=-0.309$, $P=0.012$). The remaining hypoxia-associated genes did not show any specific pattern of expression in prostate tissue. Numerous molecules have been proposed as endogenous markers of hypoxia. The findings of this study illustrate that not all hypoxia-associated molecules are relevant to prostate cancer *in vivo*. However, LOX and GLUT-1 are candidate markers of hypoxia in prostate cancer and may prove useful in identifying patients with hypoxic prostate cancer. Not all hypoxia-associated molecules are relevant in prostate cancer *in vivo*.

Correspondence to: Mr. Grant D. Stewart, Prostate Research Group, Edinburgh Cancer Research Centre, School of Molecular and Clinical Medicine, University of Edinburgh, 4th Floor, MRC Human Genetics Building, Western General Hospital, Crewe Road South, Edinburgh EH4 2XU, UK
E-mail: grant.stewart@ed.ac.uk

Key words: benign prostatic hypertrophy, glucose transporter-1, hypoxia, hypoxia inducible factor-2α, lysyl oxidase, prostate cancer, real-time PCR

Introduction

It is well established that solid tumours >1 cm^3, including prostate cancer, exist under fluctuating oxygen tensions and are exposed to both acute and chronic hypoxia (1,2). The presence of a hypoxic cancer microenvironment correlates with increased tumour invasiveness, metastases and resistance to radio- and chemotherapy (3-6). Furthermore, hypoxia is an independent prognostic indicator of poor clinical outcome for patients with prostate and other cancers (5,7). Numerous genes and proteins have been demonstrated to be upregulated under hypoxic conditions in a range of tumours. However, none of the various exogenous and endogenous markers of hypoxia described previously has emerged as the gold-standard method of identifying tumour hypoxia (1). A mechanism of reliably and easily identifying the oxygen status of a man's prostate cancer would be very useful in individualising treatment, especially with respect to the timing of radiotherapy.

Existing exogenous methods of measuring oxygen levels in a nidus of prostate cancer (e.g. Eppendorf probe, nitro-imidazole agents) give heterogeneous results and do not correlate with each other or clinical outcome (1). As such, the aim of this study was to study the expression by benign prostatic hypertrophy (BPH) and prostate cancer tissue of a range of biological markers purported to be produced by tumour cells in response to hypoxia. We aimed to determine how expression of hypoxia-associated genes correlated with pathology and identify if any of the genes showed promise for use within a panel of markers for the identification of hypoxic prostate cancer.

Materials and methods

Clinical samples. Samples of prostate cancer and BPH tissue used for quantitative real-time PCR were obtained from the Partners in Cancer Research Tissue Bank, held in the Department of Histopathology at the Norfolk and Norwich University Hospital. Details of the ethical approval, obtaining informed patient consent, tissue acquisition, and histopathological and molecular quality control and validation have already been described (8). Samples of prostate cancer tissue were collected from patients undergoing radical prostatectomy or channel transurethral resection of the prostate (TURP) and BPH samples were obtained from patients undergoing radical cystoprostatectomy for transitional cell carcinoma of the bladder or TURP for benign prostatic hyperplasia (BPH). Sixty-seven primary prostate specimens were obtained. The prostate samples were made up of 16 BPH and 51 prostate cancer specimens. Of the prostate cancer samples eight were Gleason score 5-6, thirty-one Gleason score 7 and twelve Gleason score 8-10.

RNA extraction. Total RNA from the prostate tissues was isolated by first homogenising tissues in RNAzol (Biogenesis, Poole, UK) and then by using the Promega SV Total RNA Isolation System (Promega Corporation, USA) to remove DNA and purify the RNA. RNA was re-suspended in nuclease free water and concentrations determined using a Nanodrop spectrophotometer (LabTech International, Ringmoor, UK).

Quantitative real-time PCR. cDNA preparation and real-time PCR were performed as previously described (9,10). Briefly, 1 μg of total RNA was reverse transcribed using 2 μg random hexamers (Amersham) and Superscript II reverse transcriptase (Life Technologies, Paisley, UK) according to the supplier's instructions. cDNA was stored at -20˚C until used in the PCR.

Relative expression levels were quantified for the following hypoxia-associated genes: vascular endothelial growth factor A (VEGF-A), prolyl hydroxylase 2 (PHD2), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), carbonic anhydrase IX (CAIX), lysyl oxidase (LOX), hypoxia inducible factor-1α (HIF-1α), hypoxia inducible factor-2α (HIF-2α), glucose transporter-1 (GLUT-1), erythropoietin (EPO), E-cadherin, angiopoietin-2 (Ang2). Gene-specific primers were designed to span Universal Probes using Roche Applied Science (Burgess Hill, UK) Universal Probe Library Assay Design Centre. Universal probe identities are listed in Table I. Primers were synthesised by Sigma Genosys (Gillingham, UK) sequences for all primers and universal probe identities are given in Table I. VEGF-A primers and probes were pre-existing and designed using Primer Express 1.0 software (Applied Biosystems, Warrington, UK) and synthesised by Applied Biosystems. All primers spanned exon boundaries. BLASTN searches were conducted on all primer/probe nucleotide sequences to ensure gene specificity. The 18S ribosomal RNA (18S rRNA) gene was used as an endogenous control to normalise for differences in the amount of total RNA in each sample, using previously validated procedures (9,11); 18S rRNA primers and probe were purchased from Applied Biosystems. PCR reactions were performed using the ABI Prism 7500 Fast Sequence Detection System (Applied

Table I. Primer sequences of the genes analysed by quantitative RT-PCR.[a]

Gene	Ref Seq accession no.	Forward	Reverse	Probe
Vascular endothelial growth factor A (VEGF-A)	NM_001025366	tggaattggattcgccattt	tatgtgggtgggtgtctacag	ctgctaaatcaccgagcccggaaga
Prolyl hydroxylase 2 (PHD2)	NM_022051	cgacctgatacgccactgta	ccattgcccggataacaa	44
Inducible nitric oxide synthase (iNOS)	NM_00625	tcggcagaatctacaaagtcc	tggccatcctcacaggag	31
Cyclooxygenase-2 (COX-2)	NM_000963	tcacgcatcagttttcaaga	tcaccgtaaatatgatttaagtccac	23
Carbonic anhydrase IX (CAIX)	NM_001216	cttggaagaaatcgctgagg	tggaagtagcggctgaagtc	51
Lysyl oxidase (LOX)	NM_002317	gaatggcacagttgtcatcaa	aaacttgctttgtggccttc	82
Hypoxia inducible factor-1α (HIF-1α)	NM_181054	ggttcacttttcaagcagtagg	tggtaatccacttttcatccattg	3
Hypoxia inducible factor-2α (HIF-2α)	NM_001430	aatcagcttcctgcgaacac	gcttcggactcgttttcaga	55
Glucose transporter-1 (GLUT-1)	NM_006516	ggttgtgccatactcatgacc	cagataggacatccagggtagc	67
Erythropoietin (EPO)	NM_000799	tcccagacaccaaagttaatttcta	ccctgccagacttctacgg	58
E-cadherin	NM_004360	cccgggacaacgtttattac	gctggctcaagtcaaagtcc	35
Angiopoietin-2 (Ang2)	NM_001147	tgcaaatgttcacaaatgctaa	aagttggaaggaccacatgc	75

[a]Sequences for the primers are shown in the 5' to 3' orientation.

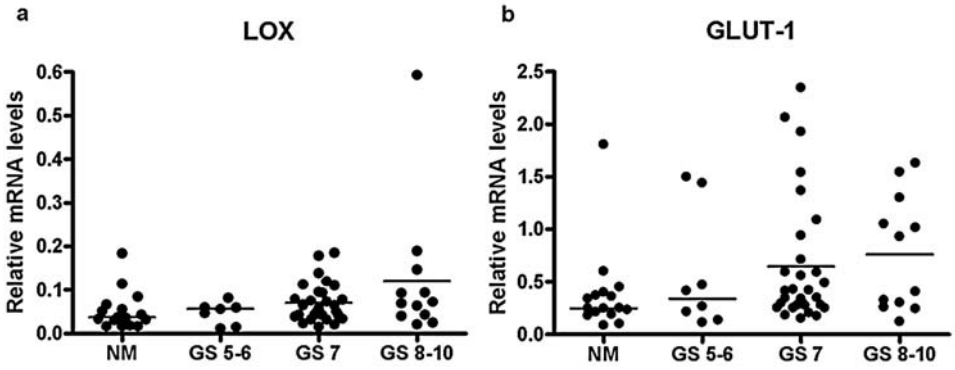

Figure 1. Quantitative RT-PCR analysis of (a) lysyl oxidase (LOX) and (b) glucose transporter-1 (GLUT-1) gene expression which was significantly higher in prostate cancer than BPH tissue. Prostate cancer specimens were sorted by Gleason Score (GS) and grouped as those with scores of 5-6, 7 or 8-10. The values of gene output are after normalisation to 18S rRNA. The bars represent the median value. For a summary of the statistics see Table II. NM, non-malignant.

Biosystems). Each reaction was performed in 25 μl and contained the equivalent of 5 ng of reverse transcribed RNA (1 ng RNA for 18S rRNA analyses), 33% Taq Man 2X PCR Master Mix (Applied Biosystems), 200 nM each of the forward and reverse primer, and 100 nM of probe. Conditions for the PCR reaction were 2 min at 50˚C, 10 min at 95˚C and then 40 cycles, each consisting of 15 sec at 95˚C and 1 min at 60˚C. Samples with cycle threshold (C_T) values varying by >1.5 C_T values from the mean were removed from the analysis. To determine the relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using the cDNA from one sample and making 2-fold serial dilutions covering the range equivalent to 20-0.625 ng of RNA (for 18S rRNA analyses, the range was 4-0.125 ng). Additionally, the median C_T values of the genes analysed were used to classify expression as: very high (C_T <25.5), high (≥25.5 C_T <30.5), moderate (≥30.5 C_T <35.5), low/absent (≥35.5 C_T <40), or not detected/below the limits of detection (C_T = 40) (9). Because of a drop off in sensitivity of the instrument, C_T values ≥35.5 are unreliable, in terms of exact levels of mRNA expression.

Statistical analysis. The data did not satisfy normality or equal variance, so nonparametric tests were used. The Mann-Whitney U test was carried out to compare prostate cancer and BPH samples. Further tests were carried out using the two-tailed Spearman rank correlation coefficient to determine whether there were associations with the Gleason sum score. The results were analysed using SPSS (version 13.0; SPSS Inc. Headquarters, Chicago, IL, USA), P<0.05 was regarded as significant.

Results

Comparison of hypoxia-associated gene expression by BPH and prostate cancer. Of the 12 hypoxia-associated genes quantified using real-time PCR two, LOX (Fig. 1a; P=0.047) and GLUT-1 (Fig. 1b; P=0.03), demonstrated a significant difference between prostate cancer and BPH mRNA samples (Table II). Furthermore, a correlation existed between increasing Gleason score and increasing expression levels of both LOX (R=0.297, P=0.015) and GLUT-1 (R=0.274, P=0.026) (Table II, Fig. 1).

None of the other 10 hypoxia-associated genes examined correlated with Gleason score, other than HIF-2α which demonstrated a negative correlation with Gleason score (R=-0.309, P=0.012; Table II and Fig. 2). However, there was not a significant difference in HIF-2α expression between BPH and prostate cancer mRNA samples.

Absolute expression levels of hypoxia-associated genes. Table II details the median C_T values of BPH and prostate cancer samples for each hypoxia-associated gene assessed. Using the arbitrary grouping of expression levels outlined in the methods section all hypoxia-associated genes had moderate, high or very high levels of expression. The C_T values for BPH and cancer samples for the following genes: COX-2, Ang2 and HIF-2α were in different 'level of expression' groups. However, as there was no significant difference between the BPH and prostate cancer mRNA expression levels for any of these genes this variation in categorisation does not represent a real difference between the samples. E-cadherin, VEGF-A and HIF-1α had very high levels of expression in both BPH and prostate cancer samples. EPO, CAIX and iNOS had moderate levels of mRNA expression in both BPH and prostate cancer. The remaining genes all had high levels of expression.

Correlations between individual hypoxia-associated genes. There were a variety of correlations between individual hypoxia-associated genes. However, as LOX and GLUT-1 were the only 2 genes to demonstrate a significant difference between BPH and prostate cancer samples correlation between other genes were not meaningful.

Discussion

It is well established that prostate cancer cells are found under hypoxic conditions *in vivo*. However, there has been no evaluation of the expression of hypoxia-associated genes in primary prostate tissue. This study assessed the mRNA expression of hypoxia-associated molecules in BPH and prostate cancer tissue. Of the hypoxia-associated genes assessed, expression of LOX and GLUT-1 were significantly higher in prostate cancer than BPH tissue and correlated with Gleason score. Although many endogenous markers have been

Table II. Summary of expression of hypoxia-associated genes by BPH and prostate cancer tissue and the relationship of hypoxia-associated gene expression with Gleason score.

Gene	BPH median C_T value	Prostate cancer median C_T value	BPH vs. prostate cancer	Correlation with Gleason score	Direction of change in malignancy
GLUT-1	26.6	26.1	P=0.030	P=0.026/R=0.274	↑
LOX	30.1	29.8	P=0.047	P=0.015/R=0.297	↑
COX-2	30.7	30.4	P=0.062	NS	
E-cadherin	24.0	23.9	P=0.166	NS	
HIF-1α	23.9	23.7	P=0.206	NS	
VEGF-A	24.2	23.2	P=0.217	NS	
Ang2	29.9	30.8	P=0.240	NS	
EPO	35.0	34.2	P=0.252	NS	
HIF-2α	25.4	25.8	P=0.298	P=0.012/R=-0.309	↓
iNOS	33.2	32.6	P=0.461	NS	
PHD2	30.4	29.4	P=0.575	NS	
CAIX	33.7	33.2	P=0.899	NS	

NS, not significant.

associated with the hypoxia response in cancers they are not all necessarily upregulated in primary prostate cancer tissue.

Strengths of this study were the use of RNA from primary prostate samples of 67 men with both BPH and prostate cancer and the representation of a range of Gleason scores by the specimens from which mRNA was extracted. However, we recognise the limitations of this study. Firstly, there were no matched protein samples from the same patients donating samples for RNA extraction. Matched patient samples would have strengthened the analysis by allowing validation of the genes upregulated at the RNA level and direct comparison of gene and protein expression. Secondly, there was incomplete data regarding endocrine therapy or radiotherapy given to patients in this study which may have altered the oxygen status and potentially the hypoxic proteome (12). Lastly, although several hundred hypoxia-associated molecules have been identified, only a few common molecules were chosen for analysis in this study.

Approximately 1-1.5% of the genome is transcriptionally responsive to hypoxia, although this varies between different cell types (13). All of the genes studied in this study have previously been demonstrated to be upregulated by hypoxia *in vitro* and/or *in vivo* tumour models, resulting in a more aggressive, treatment-resistant phenotype (14-17). Furthermore, a recent study from Vergis and co-workers demonstrated that two of the molecules assessed in the present study, HIF-1α and VEGF, identified patients with localised prostate cancer at high risk of biochemical failure (18). However, few of the hypoxia-associated genes analysed in this study revealed major differences between BPH and prostate cancer at the transcriptional level.

Two of the 12 genes assessed, LOX and GLUT-1, were significantly upregulated in prostate cancer tissue compared with benign prostate tissue. Furthermore, LOX and GLUT-1 expression correlated with Gleason score. Both LOX and GLUT-1 have previously been reported as hypoxia-associated genes involved in metastasis and glucose transport respectively (14,19). Hypoxic cancer cells overexpress GLUT-1 to accelerate glucose intake mainly for low effective, anaerobic respiration, preventing death due to oxygen deficiency (20). LOX is an extracellular matrix protein that is consistently overexpressed by hypoxic human tumour cells (13) and is also a useful marker of the hypoxia response *in vitro* (21). Further studies at the protein level are needed to confirm if LOX and GLUT-1 will form useful hypoxia markers in prostate cancer. However, in other unpublished studies from our laboratory we have failed to find a reliable antibody against LOX for immunoblotting or immunohistochemistry.

Expression of COX-2 and iNOS are known to increase with increasing grade/stage of prostate cancer (22,23). In the present study, neither COX-2 nor iNOS showed a significant upregulation of mRNA expression in prostate cancer vs. BPH samples. Although COX2 and iNOS have previously been associated with the hypoxia response of tumours (15-17), the varying known regulatory factors controlling expression of COX-2 and iNOS implies that it is unclear if hypoxia, or different factor(s), were regulating expression in prostate cancer.

HIF-1 is a heterodimeric transcription factor which is the prototypical hypoxia-associated molecule (24). HIF-1α is the master regulator of the hypoxia response, causing upregulation of effector genes by binding to the hypoxia response element within their promoter regions. HIF-1α protein staining has been shown to be greater in prostate cancer than BPH tissue and to identify patients at high risk of biochemical failure (18,25). As such, HIF-1α mRNA levels would intuitively be expected to be increased as grade of cancer increased. In the current study there was a trend for higher HIF-1α mRNA expression in prostate cancer vs. BPH samples but this was not statistically significant. However, this finding agrees with previous studies showing that HIF-1α is regulated at the post-translational rather than transcriptional level (26).

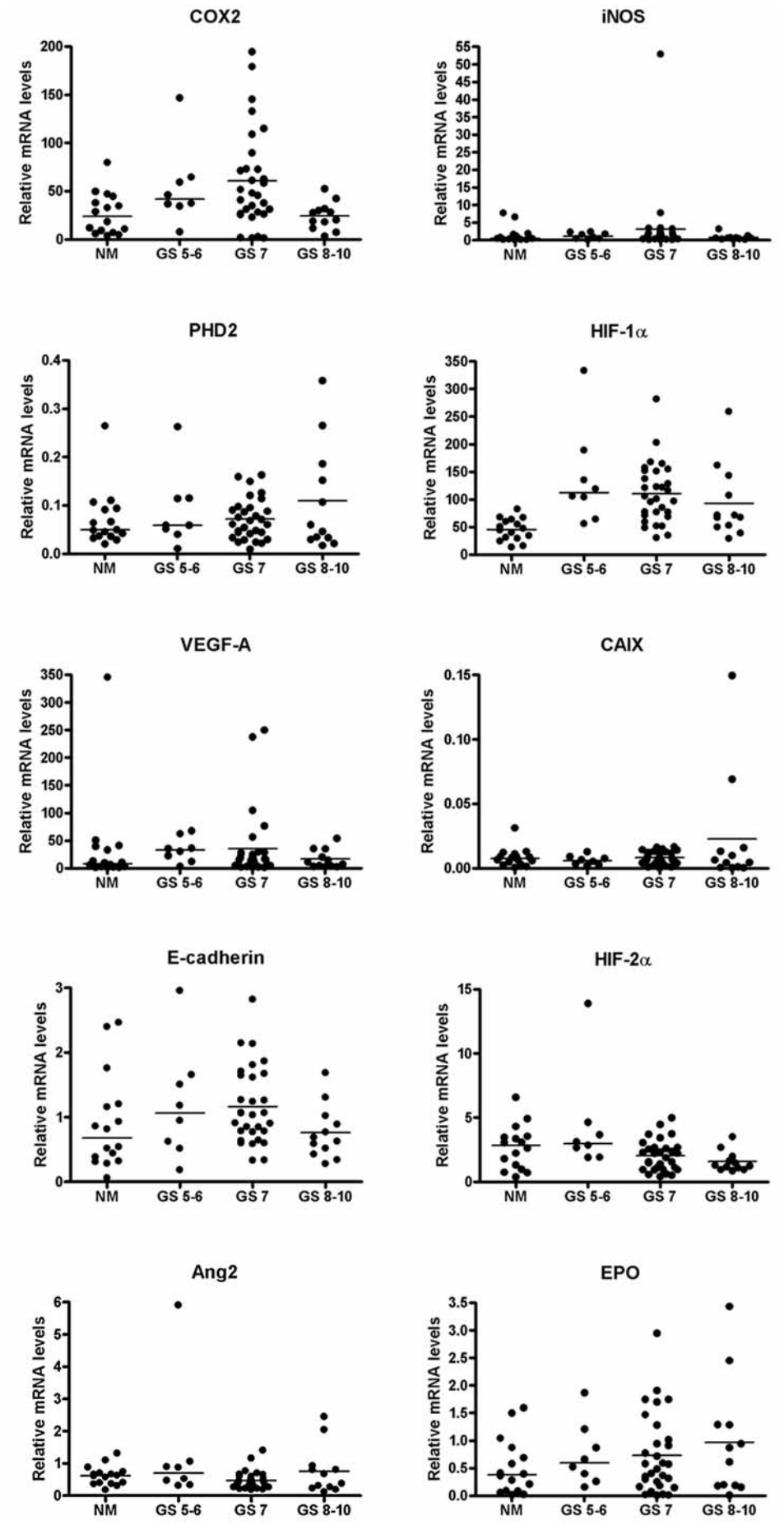

Figure 2. Quantitative RT-PCR analysis of hypoxia-associated genes that did not demonstrate a significant difference between BPH and prostate cancer tissues. Prostate cancer specimens were sorted by Gleason Score (GS) and grouped as those with scores of 5-6, 7 or 8-10. The values of gene output are after normalisation to 18S rRNA. The bars represent the median value. For a summary of the statistics see Table II. NM, non-malignant.

Alternatively, the lack of the expected pattern of HIF-1α mRNA expression may be due to the time lag between biopsy and freezing of biopsy sample following biopsy/surgery. Since, when HeLa cells were incubated at 1% oxygen for 4 h and then returned to 21% oxygen, HIF-lα RNA decreased to below basal levels within 5 min and HIF-1α protein within 15 min (27). Such difficulties suggest that HIF-1α mRNA (and perhaps protein) levels cannot be used as a reliable, routine marker for hypoxia or advanced disease in prostate cancer. Furthermore, these same issues may influence the expression of other hypoxia-associated molecules.

Whereas previous studies have demonstrated HIF-2α immunohistochemical staining correlated with HIF-1α in prostate neoplastic tissue (i.e. increased in neoplasia compared with benign tissue) (28), in the present study HIF-2α mRNA showed a negative correlation with Gleason score. However, there was no significant difference between HIF-2α in BPH and prostate cancer tissue. Further evaluation of HIF-2α protein levels must be made before conclusions can be made about this potential contradiction between HIF-2α mRNA and protein levels.

Many of the genes evaluated in this study have been identified as potential markers of hypoxia *in vitro*, *in vivo* and across different tumour types (1). For example CAIX is a good marker of hypoxia in immortalised prostate cancer cells i.e. showing reliable increases in mRNA expression following hypoxia incubation of PC-3 cells as measured by real-time PCR (21). Furthermore, the results of the present study revealed moderate expression levels of CAIX by both BPH and prostate cancer tissue. However, no significant difference in CAIX mRNA expression was found between BPH and prostate cancer tissue. These results are discordant with previous findings that CAIX is not expressed by primary prostate cancer (29).

The association established in this study of LOX and GLUT-1 with prostate cancer and increasing Gleason score may be independent of oxygen tension and simply a factor of worsening pathology. It has been established that factors other than hypoxia can modulate hypoxia-associated gene expression levels. For example, previous studies showed that HIF-1α expression could be reduced in peri-necrotic tumour by insufficient nutrients (30); similarly, a number of hypoxia-associated genes can be upregulated by perturbations in pH (31). This study did not include any direct methods of measuring hypoxia to correlate with endogenous marker expression. Future studies must assess hypoxia-associated molecules expressed in primary cancer tissue and link expression with intratumoural oxygen levels. However, tumour hypoxia is cyclical having a mix of acute and chronic hypoxia and a constantly changing environment due to a perpetually altering microvascular supply (1). Ultimately, a dynamic method of measuring tumour hypoxia, rather than static measurement of endogenous markers, may be the only way of allowing the tailored treatment of hypoxic prostate cancer, e.g. identify the optimal time to deliver radiotherapy to a man with prostate cancer.

Although the majority of the hypoxia-associated genes analysed in this study were expressed at high levels in prostate tissue most did not show any difference between BPH and cancer or have any correlation with pathological grade. Previous reviews have suggested that rather than considering individual genes a panel of genes may provide a more accurate reflection of the hypoxic state of a tumour (1). From the present study GLUT-1 and LOX would be suitable genes to include in a hypoxia panel.

Acknowledgements

Mr. Grant D. Stewart was supported by the Royal College of Surgeons of Edinburgh, the Prostate Research Campaign UK, the Association for International Cancer Research and the Mason Medical Foundation. The Urological Surgeons at Norfolk and Norwich University Hospital NHS Trust were essential for the recruiting of prostate cancer patients. Thanks to Mr. Krishna Sethia for advice and prostate tissue collection and to Professor Richard Ball for pathological assessment of the prostate tissues prior to mRNA extraction.

References

1. Janssen HL, Haustermans KM, Balm AJ and Begg AC: Hypoxia in head and neck cancer: how much, how important? Head Neck 27: 622-638, 2005.
2. Movsas B, Chapman JD, Hanlon AL, Horwitz EM, Pinover WH, Greenberg RE, Stobbe C and Hanks GE: Hypoxia in human prostate carcinoma: an Eppendorf PO2 study. Am J Clin Oncol 24: 458-461, 2001.
3. Postovit LM, Adams MA, Lash GE, Heaton JP and Graham CH: Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. J Biol Chem 277: 35730-35737, 2002.
4. Young SD, Marshall RS and Hill RP: Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA 85: 9533-9537, 1988.
5. Vaupel P, Kelleher DK and Hockel M: Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. Semin Oncol 28: 29-35, 2001.
6. Overgaard J: Hypoxic radiosensitization: adored and ignored. J Clin Oncol 25: 4066-4074, 2007.
7. Movsas B, Chapman JD, Greenberg RE, Hanlon AL, Horwitz EM, Pinover WH, Stobbe C and Hanks GE: Increasing levels of hypoxia in prostate carcinoma correlate significantly with increasing clinical stage and patient age: an Eppendorf pO(2) study. Cancer 89: 2018-2024, 2000.
8. Riddick AC, Barker C, Sheriffs I, Bass R, Ellis V, Sethia KK, Edwards DR and Ball RY: Banking of fresh-frozen prostate tissue: methods, validation and use. BJU Int 91: 315-323, 2003.
9. Nuttall RK, Pennington CJ, Taplin J, Wheal A, Yong VW, Forsyth PA and Edwards DR: Elevated membrane-type matrix metalloproteinases in gliomas revealed by profiling proteases and inhibitors in human cancer cells. Mol Cancer Res 1: 333-345, 2003.
10. Riddick AC, Shukla CJ, Pennington CJ, Bass R, Nuttall RK, Hogan A, Sethia KK, Ellis V, Collins AT, Maitland NJ, Ball RY and Edwards DR: Identification of degradome components associated with prostate cancer progression by expression analysis of human prostatic tissues. Br J Cancer 92: 2171-2180, 2005.
11. Wall SJ and Edwards DR: Quantitative reverse transcription-polymerase chain reaction (RT-PCR): a comparison of primer-dropping, competitive, and real-time RT-PCRs. Anal Biochem 300: 269-273, 2002.
12. Milosevic M, Chung P, Parker C, Bristow R, Toi A, Panzarella T, Warde P, Catton C, Menard C, Bayley A, Gospodarowicz M and Hill R: Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. Cancer Res 67: 6022-6025, 2007.
13. Denko NC, Fontana LA, Hudson KM, Sutphin PD, Raychaudhuri S, Altman R and Giaccia AJ: Investigating hypoxic tumor physiology through gene expression patterns. Oncogene 22: 5907-5914, 2003.
14. Pouyssegur J, Dayan F and Mazure NM: Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441: 437-443, 2006.
15. Brune B and Zhou J: Hypoxia-inducible factor-1alpha under the control of nitric oxide. Methods Enzymol 435: 463-478, 2007.

16. Kaidi A, Qualtrough D, Williams AC and Paraskeva C: Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. Cancer Res 66: 6683-6691, 2006.
17. Harris AL: Hypoxia - a key regulatory factor in tumour growth. Nat Rev Cancer 2: 38-47, 2002.
18. Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M, Heeboll S, Horwich A, Huddart R, Khoo V, Eeles R, Cooper C, Sydes M, Dearnaley D and Parker C: Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 9: 342-351, 2008.
19. Erler JT and Giaccia AJ: Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res 66: 10238-10241, 2006.
20. Wincewicz A, Sulkowska M, Koda M, Kanczuga-Koda L, Witkowska E and Sulkowski S: Significant coexpression of GLUT-1, Bcl-xL, and Bax in colorectal cancer. Ann NY Acad Sci 1095: 53-61, 2007.
21. Stewart GD, Nanda J, Brown DJG, Riddick ACP, Ross JA and Habib FK: NO-sulindac inhibits the hypoxia response of PC-3 prostate cancer cells via the Akt signalling pathway. Int J Cancer (In press).
22. Gupta S, Srivastava M, Ahmad N, Bostwick DG and Mukhtar H: Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate 42: 73-78, 2000.
23. Baltaci S, Orhan D, Gogus C, Turkolmez K, Tulunay O and Gogus O: Inducible nitric oxide synthase expression in benign prostatic hyperplasia, low- and high-grade prostatic intraepithelial neoplasia and prostatic carcinoma. BJU Int 88: 100-103, 2001.
24. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
25. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59: 5830-5835, 1999.
26. Kallio PJ, Pongratz I, Gradin K, McGuire J and Poellinger L: Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. Proc Natl Acad Sci USA 94: 5667-5672, 1997.
27. Wang GL, Jiang BH, Rue EA and Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci USA 92: 5510-5514, 1995.
28. Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR and Harris AL: 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. Clin Cancer Res 11: 7658-7663, 2005.
29. Thiry A, Dogne JM, Masereel B and Supuran CT: Targeting tumor-associated carbonic anhydrase IX in cancer therapy. Trends Pharmacol Sci 27: 566-573, 2006.
30. Sobhanifar S, Quino-Parsons C, Stanbridge EJ and Olive P: Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. Cancer Res 65: 7259-7266, 2005.
30. Sorensen BS, Alsner J, Overgaard J and Horsman MR: Hypoxia induced expression of endogenous markers in vitro is highly influenced by pH. Radiother Oncol 83: 362-366, 2007.