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Clinical Study

# The HIF-1α C1772T polymorphism may be associated with susceptibility to clinically localized prostate cancer but not with elevated expression of hypoxic biomarkers

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**Key words:** prostate cancer, hypoxia, polymorphism

**We investigated the role of the C1772T polymorphisms in exon 12 of the Hypoxia-inducible factor-1 alpha (HIF-1α) gene C1772T genotype in prostate cancer (PCa) and amplification of the hypoxic response. We identified the heterozygous germline CT genotype as an increased risk factor for clinically localized prostate cancer (Odds ratio = 6.2; p < 0.0001). While immunostaining intensity for HIF-1α and VEGF was significantly enhanced in 75% of PCa specimens when compared to matched benign specimens (p < 0.0001), the CT genotype did not modulate the kinetics of HIF-1α protein expression in hypoxia in vitro and was not associated with enhanced expression of hypoxic biomarkers. This study provides the first evidence of an increased risk for clinically localized prostate cancer in men carrying the C1772T HIF-1α gene polymorphism. Although our results did not suggest an association between expression of hypoxic biomarkers and genotype status, the correlation may merit further investigation.**

## Introduction

Since its discovery,[1] the link between hypoxia-inducible factor-1 α (HIF-1α) expression and treatment outcome has been well documented in a variety of human malignancies, including PCa.[2,3] Under normoxic conditions, HIF-1α is rapidly degraded by ubiquitination through the von Hippel-Lindau tumor suppressor protein (pVHL) in the proteasome, in order to control its cytoplasmic levels. The inhibition of prolyl hydroxylase domain-containing (PHDs) proteins enzymatic activity by poor oxygen availability results in accumulation of HIF-1α and in its translocation to the nucleus, where it binds to constitutively expressed HIF-1β to form HIF-1. It is the binding of this heterodimer to hypoxia response elements located in the promoter region of target genes, such as Vascular Endothelial Growth Factor (VEGF) that dictates the hypoxia-induced cellular response.[4] As a result, both HIF-1α and VEGF have been proposed as molecular markers of tumor hypoxia.

Single nucleotide polymorphisms (SNPs) in the oxygen-dependent degradation domain of the HIF-1α gene have been identified in many cancers. The C1772T SNP corresponds to a P582S proline to serine amino acid substitution.[5] While no association between the C1772T genotype and prostate cancer risk was reported from a large case-control cohort study,[6] this genotype was identified at an elevated frequency in androgen-independent PCa patients[7] and as a somatic mutation in two of 20 advanced prostate tumors.[8,9] The P582S substitution was shown to stabilise HIF-1α in normoxia in vitro[8] and enhance its activity as a transcription factor in both normoxia and hypoxia,[10] suggesting that the C1772T polymorphism offers a selective advantage to cells in which it occurs. To date, HIF-1α overexpression has been reported in HGPIN and primary PCa, compared with normal prostate epithelium.[11] However, while HIF-1α upregulation has been proposed as an early event in PCa and correlated with increased VEGF expression (reviewed in ref. 12), the role of the C1772T SNP in the HIF1α pathway in vivo remains to be elucidated.

If hypoxia is a factor of disease progression,[13] we proposed that the C1772T SNP may participate in prostatic tumorigenesis through amplification of the hypoxic response. We initially correlated the frequency of this polymorphism in peripheral blood with prostate cancer risk in a cohort of radical prostatectomy patients and controls. To evaluate the mechanistic function of this SNP, genotype status

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was correlated with hypoxia-induced HIF-1α protein expression in a panel of normal and malignant prostate cell lines. Finally, the potential role of C1772T SNP in the hypoxic response was addressed by correlating genotype status with HIF-1α and VEGF immunostaining in matched tumor and histologically normal tissue specimens obtained from patients undergoing radical prostatectomy.

## Results

**C1772T genotype frequencies in peripheral blood.** We initially investigated the C1772T genotype frequencies in peripheral blood samples obtained from 95 patients with prostate cancer undergoing radical prostatectomy and from healthy age-matched controls. The genotype distribution amongst the patient population was 30/95 (31.6%) CT and (65/95, 68.4%) wild type CC ($\chi^2$, $p < 0.0001$). In comparison, almost all of the control subjects 175/188 (93.1%) showed the CC genotype. The TT genotype was not observed in either cohort. The CT genotype was significantly more common (4.6-fold) in patients (31.6%) than in controls (6.9%) (OR = 6.21 [C.I. 95% 3.053 < OR < 12.64], two tail $\chi^2$, $p < 0.0001$). Age at surgery, pre-operative serum PSA and Gleason score did not differ significantly with genotype (Table 3). However, 56% (19/34) of patients carrying the CT genotype had a high Gleason score (7–9) compared with 38% (29/76) of patients harbouring the CC genotype.

**C1772T genotype frequencies in normal and malignant prostate cell lines.** We next determined the genotype status of a panel of normal (PWR1E, RWPE1) and malignant (22Rv1, DU145, RC58/T) prostate cell lines and investigated its effect on HIF-1α protein expression in lysates of hypoxia (4, 8, 16, 24 and 48 hours) and aerobic cells. Of the five cell lines tested, only the benign prostate cell line RWPE-1 was heterozygous for the CT genotype. However, this variant genotype was not associated with detectable aerobic HIF-1α expression (Fig. 1B), in contract to previous report.[8] Moreover HIF-1α was no longer detected at 48 hours in hypoxia, in all cell lines tested.

**HIF-1α and VEGF expression patterns in prostate tissue specimens.** We next set out to identify hypoxic regions in prostate tissue specimens by immunohistochemical analysis of HIF-1α. Given that it may be argued that the post-operative prostate gland may be hypoxic, resulting in artifactual HIF-1α upregulation, tissue sections were also stained for the HIF-1α-regulated gene VEGF. To correlate HIF-1α expression patterns and disease progression, matched specimens of HGPIN were also evaluated. Representative slides are presented in Figures 2 and 3.

As shown in Table 3, the HIF-1α scores in benign tissue ranged from zero (none) to three (10–50%). The majority of samples (28/40 (70%) scored 1 (1–10%) ($\chi^2$, $p < 0.001$). In PCa patients, HIF-1α staining was generally enhanced (29/40 (72.5%)), but the distribution of the scores amongst samples was not statistically significant ($\chi^2$, $p = 0.111$). Nonetheless, intense HIF-1α staining (>50%) was significantly more frequent in PCa (14/40 (35%)) than HGPIN (2/18 (11.1%)) and benign tissue (0/40) (Fisher's exact, $p < 0.0001$). Specimens with strong VEGF staining were more likely to be PCa (Fisher exact, $p < 0.0001$). Only one PCa sample showed weak HIF-1α expression (<1%) and strong VEGF staining. Similarly, benign specimens with weak HIF-1α also stained weakly for VEGF.

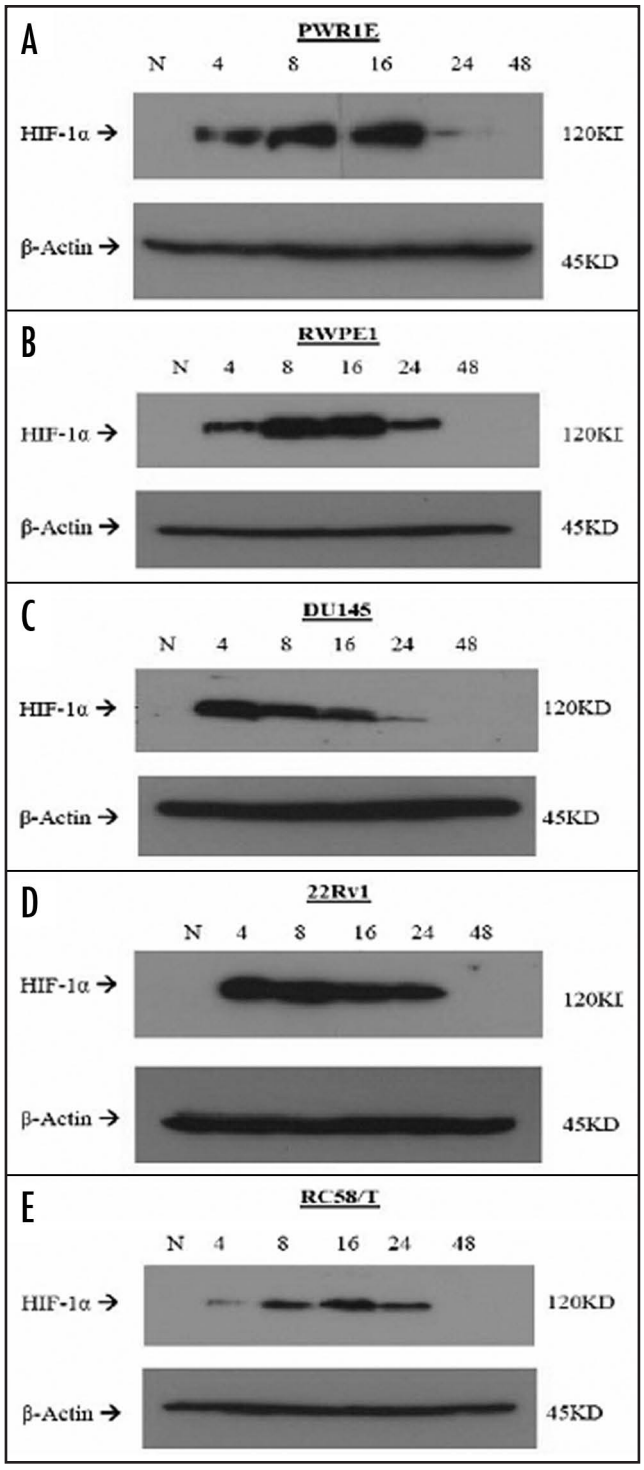


Figure 1. Time Course of HIF-1α induction in a panel of prostate cell lines. Normal (PWR-1E, RPWE-1) and malignant (22Rv1, DU145, RC58/T) cells were exposed to normoxia or hypoxia (4, 8, 16, 24 and 48 hours). Representative HIF-1α and β-actin (used for normalisation) immunoblots are presented.

Staining intensity of both markers was variable between HGPIN specimens (Tables 3 and 4). The correlation between HIF-1α and VEGF staining intensity was also not evident. Indeed, while 10/18 (55.6%) of samples stained ≤ score 2 (<10%) for HIF-1α, only 6/10 (60%) of these specimens were associated with strong VEGF expression.

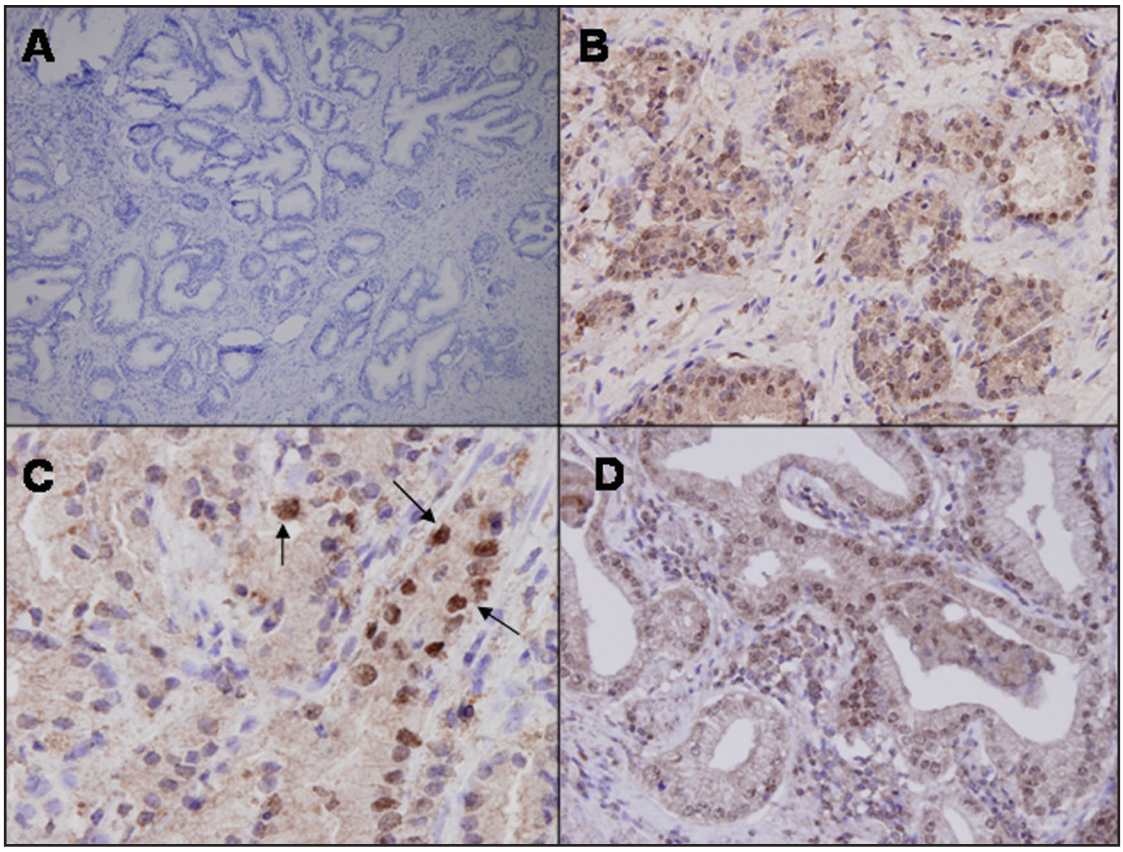


Figure 2. HIF-1α staining in prostate specimens. (A) Negative control (no staining). (B) Nuclear HIF-1α staining in PCa specimens, 20x magnification, score 2. (C) PCa, 40x magnification, score 3 (black arrows = positive nuclei). (D) Occasional HIF-1α staining (score 1) in adjacent benign prostatic tissue.

We next tested whether staining intensity for these hypoxia biomarkers was increased in PCa specimens, compared with their matched benign tissue in each individual patient. BMS was calculated for each benign, HGPIN and PCa specimen. BMS scores were significantly different (Krustal-Wallis, $p < 0.0001$) (Fig. 4A). The difference in BMS between PCa and benign tissue was then computed for each patient and ranged from -2 to 5. While the majority of patients (75%) scored higher in their PCa specimen than their benign counterpart (BMS difference >0), four patients had a difference of zero, while five patients showed reduction in their PCa BMS score (BMS difference <0) (Fig. 4B). Moreover, patients with BMS ≤ 0 had significantly lower PSA values (5.02 ± 0.76 ng/ml) than those with BMS > 0 (9.09 ± 1.26 ng/ml) (Mann-Whitney, $p = 0.008$).

**Influence of the C1772T polymorphism on HIF-1α and VEGF expression patterns in prostate tissue specimens.** We further examined the consequence of the CT genotype on HIF-1α and VEGF protein levels in these specimens. We determined the C1772T genotype in 19 of these matched benign and malignant prostate tissue specimens. The benign and malignant tissue genotypes were identical in all patients. The CT genotype was observed in 4/19 (21.1%) of PCa patients, with the remainder having the CC genotype. CT genotype status was not significantly associated with HIF-1α overexpression (>10%) (Fisher's exact, $p = 0.3$), increased PSA (Mann-Whitney, $p = 0.8$) or high Gleason score (≥7) (Mann-Whitney, $p = 0.7$). Similarly, BMS in PCa specimens between genotype groups were not significantly different (Mann-Whitney, $p = 0.46$).

## Discussion

The role of SNPs in the oxygen-dependent degradation domain of the HIF-1α gene in prostate carcinogenesis appears complex. While recent studies failed to find a link between overall and advanced PCa risk and HIF-1α polymorphism,[6,16,17] the CT genotype was also associated with increased susceptibility to PCa.[7-9] A study of 402 prostate cancer patients found no significant difference in C1772T genotype distribution between patients and controls, although it must be noted that the controls selected in their study were healthy women, who could not have had prostate cancer regardless of genetic predisposition.[17] A large case-control cohort also found no association between C1772T genotype and prostate cancer risk, with 20% of 1072 cases and 18% of 1271 controls displaying the CT genotype.[6] In our cohort of peripheral blood donors however, the CT genotype was significantly more represented in clinically localized prostate cancer patients (30/95 (31.6%)), compared to healthy male controls (6.9%). Although our patient numbers are smaller, the CT genotype was more frequently observed in our cohort than in that of Li et al.[6] ($p = 0.047$). The patients in our study and those of Li et al. were similar in disease stage and Gleason score, although the mean age of diagnosis was 69 compared to 60 at radical prostatectomy in our cohort. To our knowledge, the C1772T genotype has not previously been identified as a risk factor for clinically localized prostate cancer.

Despite evident association between HIF-1α and hypoxia, previous studies have not addressed the link between the C1772T

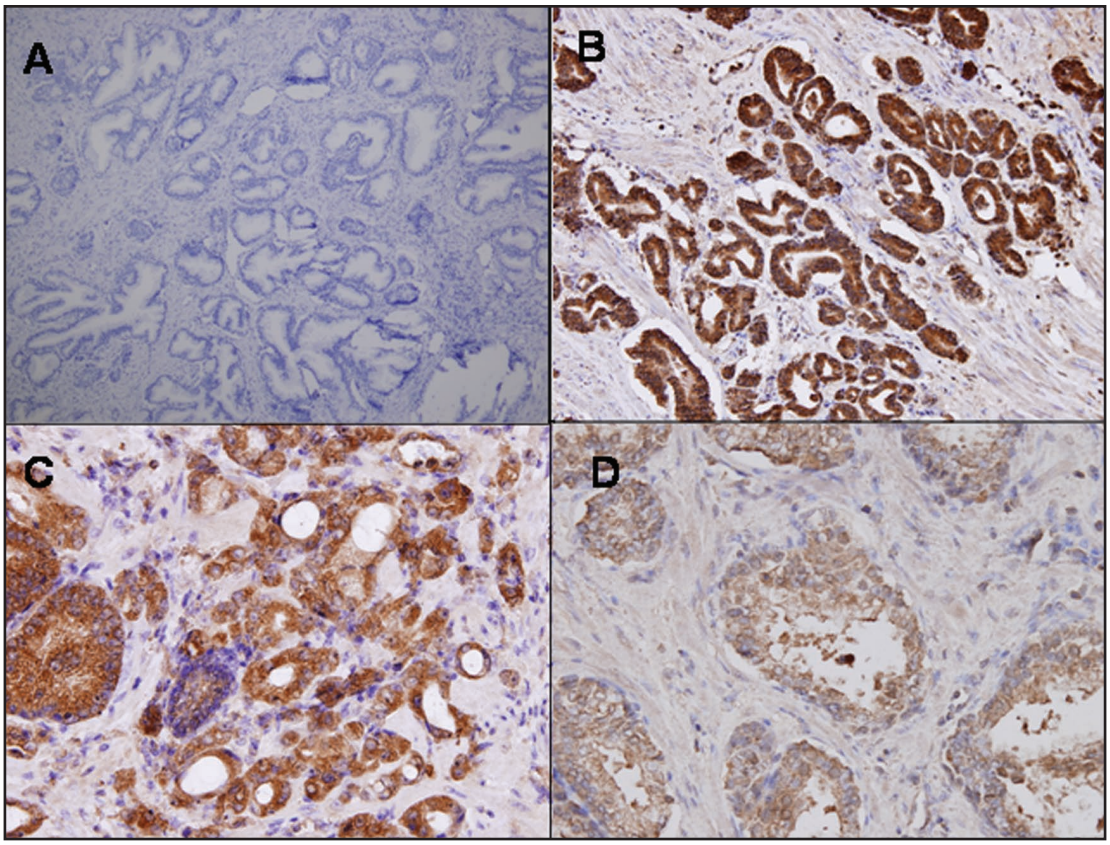


Figure 3. VEGF staining in prostate specimens. (A) PCa—negative control. (B and C) Strong VEGF staining in PCa epithelium (20x, 40x magnification). (D) Weak VEGF staining in benign prostatic epithelium.

polymorphism and the presence of hypoxic regions in prostate tumors. An important mediator of the hypoxic response is HIF-1α, whose enhanced expression in PCa has been observed by us and by others.[2,11] HIF-1α overexpression has, as a result, been proposed as a marker of tumor hypoxia and associated aggressive phenotype.[2,18] While HIF-1α protein expression was hypoxia selective in all cell lines tested, our in vitro results suggest that the potential of HIF-1α protein expression as a marker of tumor hypoxia in prostate specimens may be limited, because the protein was no longer detected in the lysate of chronically hypoxic cells, suggesting areas not staining for HIF-1α may not be as well-oxygenated as currently hypothesised.

Immunomolecular imaging, physical $PO_2$ measurements and positron emission tomography of [$^{18}$F] fluoro-misonidazole have been used to confirm the presence of hypoxic regions in prostate tumors.[19,20] We chose to use the combination of HIF-1α and HIF-regulated gene VEGF immunostaining to identify hypoxic regions in our tumor samples. We compared the combined intensity scores for these two biomarkers in paired benign and PCa specimens. Slides associated with high scores were more likely to contain tumor and our results suggest the presence of hypoxic regions in the majority of our tumors (75%). HIF-1α was overexpressed in 9/18 (50%) of HGPIN specimens, which is similar to previous reports.[11]

Hormonal therapy has been shown to reduce the hypoxic fraction of prostate tumors.[21] However, hypoxia was also recently shown to enhance PSA expression,[22] increase the activity of the androgen receptor, sensitizing tumor cells to low androgen levels[23] and proposed as a limiting factor to the therapeutic ratio of androgen-deprivation therapies. While a clinical link between PSA levels and hypoxic fraction remains to be determined, in our cohort, a positive BMS difference was associated with enhanced PSA, compared with negative controls. Somatic mutation of C1772 to the T allele has previously been reported in one of five androgen-independent prostate tumors[9] and one of 15 bone metastases of prostate cancer.[8] In our cohort of 19 patients with clinically localized prostate cancer, no somatic C to T mutation was observed. It may be that such mutations are more likely to occur in advanced disease. Moreover, the CT genotype was twice as frequent in 196 androgen-independent prostate cancer patients as in 196 healthy controls (15% versus 7%).[7] Further studies may thus be required to investigate whether hypoxia may induce this somatic mutation.

We compared DNA isolated from malignant and adjacent histologically benign prostate tissue from 19 prostate cancer patients. The CT genotype was observed in the paired specimens of four patients. Our results thus identify for the first time a subset of patients carrying the T allele as a germline mutation. If heterozygosity for the C1772T mutation is associated with HIF-1α overexpression, we next hypothesised that patients carrying this genotype may exhibit high HIF-1α staining scores. However, these specimens were not associated with enhanced HIF-1α and VEGF expression, compared with the wild type, suggesting that HIF-1α overexpression in cancer involves more complex pathways rather than this particular genetic aberration alone. Although we did not find an association between the C1772T phenotype and enhanced expression of hypoxia biomarkers, further studies on the role of the C1772T polymorphism on the transition to androgen independence in hypoxic prostate tumors may be required.

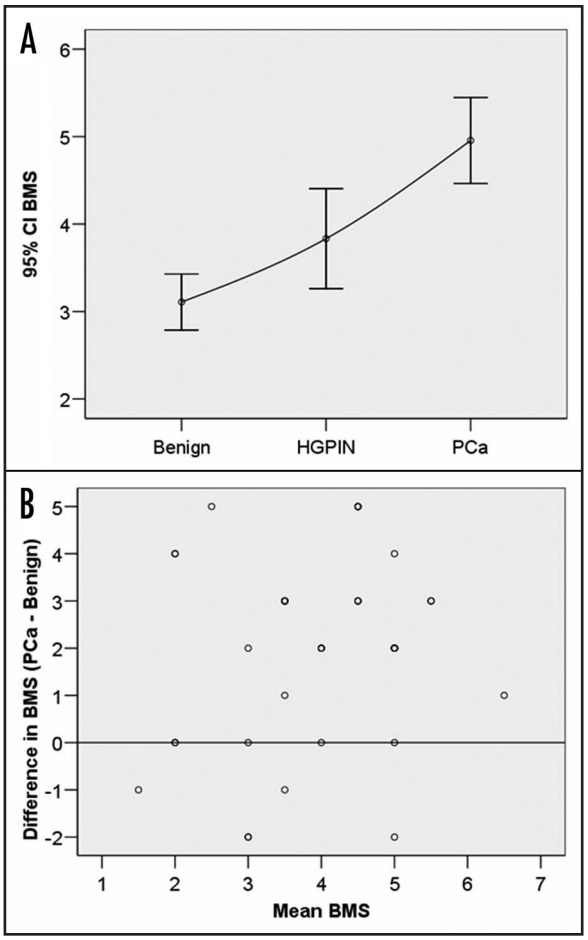


Figure 4. Biomarker scores (BMS). (A) 95% confidence intervals for the BMS scores in benign, HGPIN and PCa specimens. (B) The difference in BMS between PCa and benign, for each patient is represented by a single dot on a Bland-Altman plot. An increase in BMS in PCa specimens results in a positive difference.

Table 1 **Clinicopathological features of prostate cancer patients**

| | Peripheral blood PCa cohort Mean (range) | Prostate tissue cohort Mean (range) |
|---|---|---|
| N | 95 | 40 |
| Age (years) | 60.1 (44–71) | 61.2 (44–83) |
| Gleason score | 6.4 (4–9) | 6.3 (6–9) |
| | 4 (n = 1) | 6 (n = 24) |
| | 5 (n = 11) | 7 (n = 14) |
| | 6 (n = 39) | 9 (n = 2) |
| | 7 (n = 37) | |
| | 8 (n = 5) | |
| | 9 (n = 2) | |
| PSA (ng/ml) | 7.9 (2.4–16.5) | 6.4 (0.7–16) |

Table 2 **PCR primers and extension primers used in genotyping patient and control DNA for the C1772T HIF-1α polymorphism**

| Primer | Cohort | Sequence (5'–3') |
|---|---|---|
| PCR forward | Patients | AGGACACAGATTTAGACTTG |
| PCR reverse | Patients | CAGACTCAAATACAAGAACC |
| PCR forward | Controls | ACGTTGGATGCTTTGAGGACTTGCGCTTTC |
| PCR reverse | Controls | ACGTTGGATGCTTCCAGTTACGTTCCTTCG |
| Extension, C allele | Controls | CCTTCGATCAGTTGTCAC |
| Extension, T allele | Controls | CCTTCGATCAGTTGTCATC |

## Materials and Methods

**Peripheral blood collection.** Peripheral blood samples (n = 95) were obtained prospectively from prostate cancer patients (T1–T3) undergoing radical prostatectomy in St. James' Hospital and the Mater Misericordiae Hospital, Dublin, with approval from the hospitals' Ethics Committees as part of the Prostate Cancer Research Consortium Bio-Resource. Clinicopathological features of the patients are summarized in Table 1. Control samples (n = 196) for genotyping were obtained from a biobank of peripheral blood samples of healthy European Caucasian men aged 50–69 years. DNA was extracted from peripheral blood samples using the automated Gentra™ system (Gentra Systems Inc., USA).

**Prostate tissue collection.** Samples of adjacent PCa (n = 40), histologically normal (n = 40) and HGPIN (n = 18) tissue were obtained retrospectively from patients with primary disease (T1–T3) treated at the Adelaide and Meath incorporating the National Childrens Hospital, (AMNCH) Dublin, Ireland, as previously described.[14] The clinicopathological data associated with the samples are summarized in Table 1. DNA was extracted from within pathologically reviewed areas using the QIAamp DNA micro kit (Qiagen, UK).

**Genotyping.** The C1772T HIF1α polymorphism was analysed by PCR amplification (primers shown in Table 2) and subsequent sequencing on an Applied Biosystems 3100 Genetic Analyser. The sequences were determined using SeqMan II software (DNAstar, USA).

**Cell culture.** Human PCa cell lines (22Rv1, DU145) (ATCC, UK) were maintained in RPMI 1640 medium (Gibco, UK) supplemented with 10% foetal calf serum (Globepharm, UK) and 1% streptomycin-penicillin (Gibco, UK). Human RC58/T prostate cancer cells were kindly provided by Prof. Rhim, Center for Prostate Disease, Bethesda, USA. These cells, along with normal human prostate cell lines PWR-IE and RPWE-1 were routinely maintained in Keratinocyte SFM medium (Gibco, UK) supplemented with Bovine Pituitary Extract, recombinant Epidermal Growth Factor and 1% streptomycin-penicillin (Gibco, UK). DNA was extracted from cell lines using a QIAamp DNA mini kit (Qiagen, UK).

**Hypoxic exposure.** Hypoxic conditions (0.5% oxygen) were achieved in a 1000 in vivo Hypoxic Chamber (BioTrace, UK). Culture media was replaced at the start of each hypoxic exposure (4, 8, 16, 24 and 48 hours) with fresh pre-hypoxia-conditioned (24 hrs) media.

**Western blotting.** The cells were scraped from the incubation dishes under hypoxic conditions and stored on ice. The pellet was resuspended in lysis buffer.[15] Protein was extracted, subjected to polyacrylamide gel electrophoresis and transferred to nylon/nitrocellulose membranes. The membranes (Amersham, UK) were then

Table 3 **Distribution of HIF-1alpha & VEGF staining in prostate samples**

| HIF-1 | 1 = <1% | 2 = 1–10% | 3 = 10–50% | 4 = >50% | Total |
|---|---|---|---|---|---|
| Benign | 28 | 7 | 5 | 0 | 40 |
| HGPIN | 7 | 3 | 6 | 2 | 18 |
| PCa | 4 | 10 | 12 | 14 | 40 |
| **VEGF** | **0 = None** | **1 = Weak** | **2 = Moderate** | **3 = Strong** | **Total** |
| Benign | 5 | 18 | 9 | 8 | 40 |
| HGPIN | 1 | 8 | 4 | 5 | 18 |
| PCa | 1 | 6 | 13 | 20 | 40 |

Table 4 **Upregulation of HIF-1α and VEGF in HGPIN and PCa specimens**

| | | HIF-1α | VEGF |
|---|---|---|---|
| HGPIN | N | 18 | 18 |
| | Upregulation (R > 1) | 9/18 (50%) | 7/18 (39%) |
| | Sustained (R = 1) | 4/18 (22%) | 2/18 (11%) |
| | Downregulation (R < 1) | 5/18 (28%) | 9/18 (50%) |
| PCa | N | 40 | 40 |
| | Upregulation (R > 1) | 29/40 (73%) | 29/40 (57.1%) |
| | Sustained (R = 1) | 4/40 (10%) | 16/40 (40%) |
| | Downregulation (R < 1) | 7/40 (17%) | 4/40 (10%) |

R = Staining intensity score (HGPIN: benign or PCa: benign).

probed with mouse monoclonal anti-HIF-1α antibody (1:250, BD Biosciences, UK) and Horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (1:1000, Amersham, UK). The Pierce Luminal kit (Pierce, UK) was used for protein detection. Membranes were stripped prior to reprobing with a mouse monoclonal anti-actin antibody (1:10000, Sigma-Aldrich, UK).

**Immunohistochemistry.** 4-μm sections were cut from the corresponding formalin fixed paraffin specimens. Sections were immunostained with HIF-1α antibody (1:3000, Santa Cruz Biotechnology, USA) on an automated platform (BondMax™ system—Leica Microsystems, Australia). Immunodetection of VEGF (1:100, Abcam, UK) was performed using the Envision® Plus System-HRP (Dako, Ireland). Human placenta tissue was used as a positive control. For negative controls specimens, the primary antibody was substituted with TBST buffer. Sections were next incubated with a peroxidase anti-mouse HRP labelled polymer (Envision Kit, Dako).

Expression of HIF-1α and VEGF was determined by two separate observers by assessing semi-quantitatively the percentage of stained tumor cells and staining intensity. Five regions per slide were selected as counting areas of representative positive immunoreactivity. Because active HIF-1 is located only in the nucleus, only cells with completely and darkly stained epithelial nuclei were regarded as positive. This nuclear staining was interpreted as an increased level. The results were then scored for extent as follows: 0 = None; 1 = <1%; 2 = 1–10%; 3 = 10–50%; 4 = >50%. In contrast to HIF-1α, VEGF expression is cytoplasmic and immunohistochemical staining observed for VEGF protein within prostatic epithelial cells was scored for intensity as either 0 = None, 1 = Weak, 2 = Moderate or 3 = Strong.

**Statistics.** Analyses were performed using the SPSS software. Values are represented as mean ± Standard Error, unless otherwise specified. The significance of the differences between genetic variation frequencies in patients and controls was tested using Chi-square ($\chi^2$) tests or Fisher's exact test, where more appropriate. Odds ratios and associated 95% confidence intervals were calculated as an estimation of risk amongst mutation carriers. Finally, biomarker scores (BMS), defined as the sum of the HIF-1α and VEGF scores were calculated for each matched benign and PCa specimen. HIF-1α upregulation was calculated as the ratio of HGPIN to Benign HIF-1α staining scores. The mean scores in benign, HGPIN and PCa were tested using a Krustal-Wallis test. The differences between benign and PCa BMS were calculated as a measure of the role of these biomarkers in disease progression. BMS in PCa specimens were tested according to CT genotype using a Mann-Whitney test. A p-value of <0.05 was considered statistically significant.

## Conclusion

We identified for the first time the C1772T polymorphism as a risk factor in clinically localized prostate cancer. We found no direct correlation between C1772T polymorphism status and expression of common hypoxia biomarkers in radical prostatectomy specimens.

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