Elevated level of inhibin-α subunit is pro-tumourigenic and pro-metastatic and associated with extracapsular spread in advanced prostate cancer

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The biological function of inhibin-α subunit (INHα) in prostate cancer (PCa) is currently unclear. A recent study associated elevated levels of INHα in PCa patients with a higher risk of recurrence. This prompted us to use clinical specimens and functional studies to investigate the pro-tumourigenic and pro-metastatic function of INHα. We conducted a cross-sectional study to determine a link between INHα expression and a number of clinicopathological parameters including Gleason score, surgical margin, extracapsular spread, lymph node status and vascular endothelial growth factor receptor-3 expression, which are well-established prognostic factors of PCa. In addition, using two human PCa cell lines (LNCaP and PC3) representing androgen-dependent and -independent PCa respectively, we investigated the biological function of elevated levels of INHα in advanced cancer. Elevated expression of INHα in primary PCa tissues showed a higher risk of PCa patients being positive for clinicopathological parameters outlined above. Over-expressing INHα in LNCaP and PC3 cells demonstrated two different and cell-type-specific responses. INHα-positive LNCaP demonstrated reduced tumour growth whereas INHα-positive PC3 cells demonstrated increased tumour growth and metastasis through the process of lymphangiogenesis. This study is the first to demonstrate a pro-tumourigenic and pro-metastatic function for INHα associated with androgen-independent stage of metastatic prostate disease. Our results also suggest that INHα expression in the primary prostate tumour can be used as a predictive factor for prognosis of PCa.

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Inhibins A and B are members of the transforming growth factor-β (TGFβ) superfamily. Inhibins are heterodimers of an 18 kDa α-subunit disulphide linked to one of two 13 kDa β-subunits (βA and βB) resulting in inhibin A (αβA) and B (αβB) respectively. Primarily of gonadal origin, inhibins regulate pituitary follicle-stimulating hormone secretion by feedback inhibition. In humans, women produce both inhibin A and inhibin B (Welt et al, 1999; Welt and Schnyder, 2001), whereas in adult men inhibin B is the primary form (Marchetti et al, 2003). Inhibins have also been shown to be expressed in adrenal cortex, pituitary and prostate (reviewed in Risbridger et al, 2001). Other endocrine and paracrine functions of inhibins involve regulating members of the TGFβ superfamily, such as TGFβ itself, activins and bone morphogenic protein (BMP), by competing for their receptors (Wiater and Vale, 2003; Farnsworth et al, 2007). This antagonistic effect of inhibin is amplified in the presence of the inhibin receptor, TGFβ receptor III (TGFβRIII), also known as betaglycan.

The first study that linked inhibin to reproductive cancers showed that serum inhibin increased in women with granulosa cell tumours of the ovary (Lappohn et al, 1989) and inhibin currently serves as a robust biomarker for this cancer. A direct biological function for inhibin in carcinogenesis was demonstrated by Matzuk et al (1992) when they created the inhibin-α subunit (INHα) knockout mouse. Both sexes developed gonadal sex-cord stromal tumours with high penetrance and developed tumours in their adrenal glands after castration, showing that INHα can act as a tumour suppressor. Subsequent mouse model studies revealed a complex network of interactions involving inhibin, activin and other modifiers in the development and progression of gonadal and adrenal tumours in INHα-deficient mice (reviewed in Risbridger et al, 2001). Although it is clear that total inhibin as a serum test has utility in the initial detection and prognosis of certain types of ovarian cancers, the biological function of inhibin in tumourigenesis is far from clear.

We have observed both up- and down-regulation of INHα expression in prostate cancer (PCa) tissues dependent on the stage...
of disease. Studies from our laboratory on metastatic PCa epithelial cell lines demonstrated that high concentrations (400 ng ml−1) of recombinant inhibin A inhibited growth of androgen-responsive LNCaP cells, but not androgen-independent DU145 cells, suggesting that the effect of inhibin may be dependent on cell phenotypes related to specific stages of PCa (McPherson et al, 1997). Furthermore, we have shown loss of heterozygosity or epigenetically regulated loss or down-regulation of INH in PCa patient samples and LNCaP, DU145 and PC3 cell lines (Mellor et al, 1998; Schmitt et al, 2002; Balanathan et al, 2004). Collectively, these data suggest a tumour suppressive function for INH in the prostate. In contrast, new insights into the role of INH in the prostate came from a recent study using a large cohort of PCa patient tissues that showed that INH was frequently over-expressed in high-grade PCa (Risbridger et al, 2004a,b). The intensity of INH immunoreactivity was associated with a higher risk of recurrence of PCa. These variable clinical and experimental observations provide equivocal evidence for a role of INH in PCa. Thus it was proposed that like TGFβ (Roberts and Wakefield, 2003), INH has tumour suppressive activity in normal epithelial cells, which changes to tumour promoting in cancer cells (Ball et al, 2004; Risbridger et al, 2004a).

To date there is no proof that INHα can increase tumourigenesis and metastasis. Thus the primary aim of this study was to examine the pro-tumourigenic and pro-metastatic function of INHα in advanced PCa. Specifically, the expression profile of INHα and clinicopathological parameters were examined in primary PCa tissues including specimens from patients with organ-confined disease and those with metastasis to the lymph nodes. In addition, human metastatic PCa cell lines, LNCaP and PC3, were used in 2D and 3D functional studies to determine the biological function of elevated levels of INHα on migration, invasion, tumour growth and metastasis.

MATERIALS AND METHODS

Analysis of clinical material

Relationship between INHα expression and clinicopathological parameters in primary prostate adenocarcinomas This study was conducted in accordance with Australian National Health and Medical Research Council (NHMRC) guidelines. Archival formalin-fixed paraffin-embedded tissue blocks were retrieved from 37 patients with prostate carcinoma who underwent radical prostatectomy. The clinicopathological characteristics, vascular endothelial growth factor-C (VEGF-C) expression, lymphatic vessel density (LVD) and lymph node status of this cohort have been described previously (Zeng et al, 2004, 2005). We conducted a cross-sectional study to determine whether INHα expression was associated with clinicopathological parameters (Gleason score, surgical margins, extracapsular spread, VEGF receptor-3 (VEGFR-3) expression and lymph node status) and/or linked to well-established prognostic factors in prostate adenocarcinoma. The PO 12 antibody (kindly provided by Dr Nigel Groome) was used to determine the expression pattern of INHα in primary prostate tissues as previously described (Risbridger et al, 2004b). Each immunostained tissue section was assessed and staining intensity in benign epithelial, cancer (Gleason grade G3/G4) and stromal regions was scored from 0 to 4 with 0 representing negative staining and 4 representing very strong positive staining. The relative risk of PCa patients being positive for the respective parameters was determined.

Stable transfection of LNCaP and PC3 cell lines LNCaP and PC3 were obtained from American Type Culture Collection (Rockville, MD, USA) and routinely cultured as described previously (Balanathan et al, 2004). Expression vectors pcDNA3.1 (empty vector, EV) and human INHα cDNA subcloned into pcDNA3.1 (pcDNA3.1 (INHα)) were purchased from Invitrogen (Mount Waverley, Victoria, Australia) and prepared for transfection according to the manufacturer's instructions. LNCaP and PC3 cells were transfected using Lipofectamine plus (Invitrogen) and Superfect (Qiagen, Doncaster, Victoria, Australia) respectively according to the manufacturer's instructions. Individual colonies surviving after 2–3 weeks selection were picked and propagated for analysis.

Confirmation of mRNA expression in INHα-transfected LNCaP and PC3 cell lines Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription (RT) was performed as previously described (Balanathan et al, 2004). Intron-exon microarray (β2, 2-microglobulin (β2-mg), actin) was used as a housekeeping gene for block PCR. Primer sequences were: INHA, forward: CAGTGGTCTGAGGTGGCTCTT; reverse: AGCTGGGCTGAA GTCACTT and β2-mg, forward: CGGTGTTGAACCATGTGACTT; reverse: CAAACATGGGAGACACGGCT. Absolute quantitative real-time analysis was used to assess the levels of TGFβRIII mRNA expression in the clones. The analysis was performed on a LightCycler real-time PCR machine (Roche Diagnostic, Mannheim, Germany) using LightCycler Fast Start DNA Master SYBR Green 1 (Roche Diagnostic) according to the manufacturer’s instructions. All experiments were carried out twice and duplicate readings were taken for each replicate. The quantity of mRNA was determined using a standard curve and all values were normalised using the housekeeping gene, hypoxanthine ribosyl transferase (HPRT). Primer sequences were: TGFβRIII, forward: TCTCGGTTCACCC GACCTGAAAT; reverse: CGTCAGGAGGACACAGATTA and HPRT, forward: TGAATGACCAGTCAAAGGG; reverse: TGGCTTATAT CCAACACTTCG.

Confirmation of protein expression by ELISA Cell lysates and conditioned media (conditioned for 24 h) were prepared from EV- and INHα-transfected clones. Total protein (1 μg μl−1) was used for further analysis. Inhibin A and B and activin A concentrations were measured in triplicate using specific ELISA according to the manufacturer’s instructions (Diagnostic Systems Laboratories, Webster, TX, USA). VEGF-A and VEGF-C ELISAs were measured in duplicate using specific ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Two biological replicates were examined.

Direct cell counting: proliferation assay LNCaP and PC3 cells were seeded at a density of 1 × 105 cells per well and 5 × 104 cells per well, respectively, in 24-well plates and incubated at 37 °C. Triplicate wells were harvested by trypsinisation on days 1, 2, 3, 4 and 5, and numbers of cells per well were counted using haemocytometer. Each experiment was repeated twice. The results obtained from individual clones (EV and INHα) were pooled for each treatment.

Scratch wound assay: motility assay Cells were plated in triplicate in 6- or 12-well plates and grown until approximately 70–80% confluence. The cell monolayer was then wounded and analysed over a maximum of 2 weeks as previously described (Sharp et al, 2004). Each experiment was repeated twice. The results obtained from individual clones (EV and INHα) were pooled for each treatment.

Intra-prostatic inoculation of LNCaP and PC3 cells The experiments were in accordance with NHMRC of Australia guidelines. LNCaP (2 × 106) or PC3 (5 × 105) clones were injected orthotopically into the ventral lobe of the prostate gland (10 animals per clone) of male SCID mice as previously described (Zeng et al, 2006). After 7–9 weeks, mice were killed and primary prostate tumours were removed and weighed. In addition, regional lymph nodes were removed for analysis. Monoclonal human mitochon-
determine the presence of human cells in the tumours as previously described (McCulloch et al, 2005). The monoclonal R1 antibody (7.5 μg ml⁻¹), kindly provided by Dr Nigel Groome, was used to determine INHα expression in tumours as previously described (Balanathan et al, 2004).

Lymph node volumes were determined using stereological analysis as previously described (McPherson et al, 2001). The lymph nodes were serially sectioned at 5 μm thickness and using a random sampling scheme, every 20th section was chosen for analysis. Briefly, the computer program newCAST component (version 2.14; Visiopharm, Hørsholm, Denmark) was used to generate a point grid, and volumes of the lymph nodes were determined. Each section was examined using ×20 magnification and tissue sections were mapped to define tissue boundaries and were sampled at predetermined intervals along x- and y-axes using a single grid-counting frame. The volume was then determined using the equation = no of points for each tissue × area per point × distance; in this case the distance was defined by thickness of the sections (5 μm) plus (5 μm × 20 for every 20th section).

LVD in the intra-prostatic tumours Lymphatic vessels were identified using lymphatic vascular endothelial hyaluronan receptor (LYVE-1), a marker of lymphatic endothelium (Banerji et al, 1999). Invasion of tumour cells into lymphatics was monitored by the presence of human mitochondrial protein-stained cancer cells in lymph vessels. Double immunostaining for LYVE-1 and mitochondria was performed on a Dako Autostainer (Dako, Glostrup, Denmark). The sections were incubated with LYVE-1 antibody (Fitzgerald, Boston, MA, USA) diluted at 0.5 μg ml⁻¹ for 2 h. LYVE-1 was detected by incubation with Envision polymer anti-rabbit-HRP (Dako) for 15 min and visualised with diamino-benzidine (Dako). Sections were then incubated with Double Staining Enhancer (Zymed, San Francisco, CA, USA) for 15 min and exposed to mitochondrial antibody (Chemicon) diluted at 1:200 for 2 h. Secondary antibody, biotinylated rabbit anti-mouse IgG1 (Zymed) was applied and the immunoreactivity was detected by ExtrAvidin-Alkaline phosphatase (Sigma, St Louis, MO, USA) and visualised by reaction with Vector Red (Vector Laboratories, Burlingame, CA, USA). The sections were counterstained with haematoxylin (Dako) and immunolocalisation was examined using an Olympus BX-60 microscope (Tokyo, Japan).

Lymphatic vessels were counted using stereological methods as previously described (Balanathan et al, 2004). Lymphatic vessels were counted within tissue sections (of randomly selected INHA-positive prostate tumours, n = 15 and EV tumours, n = 11; using n = 2 randomly selected sections per tumour) to assess the LVD within the tumour (intra-tumoural) region, the region in contact with both the tumour and the stroma (peritumoural) and the region away from tumour. LVD was expressed as the number of lymph vessels per mm².

Statistical analyses All statistical analyses were performed and results were analysed by ANOVA or t-tests as specified. The relationships between INHα expression and clinicopathological parameters were evaluated by Fisher’s exact test. The mean staining intensity of patients positive for each of the respective clinicopathological parameter was compared to the mean staining intensity (reference) of those patients who were negative. The relative risks and 95% confidence intervals (CI) were estimated.

RESULTS

Relationship between INHα expression and clinicopathological parameters in primary prostate adenocarcinomas

Immunostaining revealed differential expression of INHα in benign epithelial, G3/G4 cancer regions as well as in the stroma of primary PCa tissues from patients with organ-confined disease (Figure 1A–D) and those with metastasis to the lymph nodes (Figure 1E–H). Association between clinicopathological prognostic factors and INHα expression is shown in Table 1. Elevated expression of INHα in the benign epithelial regions of the primary PCa tissues showed a higher relative risk in PCa patients positive for extracapsular spread (P = 0.01). Similarly, elevated expression of INHα in the stroma of the primary PCa tissues showed a higher risk of PCa patients positive for extracapsular spread (P = 0.0011), surgical margins (P = 0.0006), VEGFR-3 expression (P = 0.00067) and lymph node metastasis (P < 0.0001). Further analysis showed that there was a significant increase in INHα staining in benign epithelial (P = 0.018) and stromal (P < 0.0001) regions but not in G3/G4 cancer regions in tissues from patients with lymph node metastasis compared to patients with organ-confined disease (data not shown).

Isolation and characterisation of cells over-expressing INHα

The INHα expression profile in clinical specimens suggests protumourigenic and pro-metastatic function for INHα in advanced PCa.
were a heterogeneous population of INH-a chemistry (data not shown). Immunohistochemistry of antibiotic protein was also validated by western blot and immunohisto-
a INH-transfected LNCaP cells and both inhibin A and inhibin B in the clones increase in expression of inhibin B, but not inhibin A in INH-a EV-transfected clones. bINH-over-expressing clones.

**Table 2** Inhibin A, inhibin B and activin A protein expression in INH-a clones

| Cell lines | LNCaP | PC3 |
|------------|-------|-----|
|            |       |     |
|            | Inhibin A (ng ml^{-1}) | Inhibin B (ng ml^{-1}) | Activin A (ng ml^{-1}) | Inhibin A (ng ml^{-1}) | Inhibin B (ng ml^{-1}) | Activin A (ng ml^{-1}) |
| L16        | <10   | <10 | <10 |
| L17        | <10   | <10 | <10 |
| L18        | <10   | <10 | <10 |
| L1         | <10   | <10 | <10 |
| L5         | <10   | <10 | <10 |
| L8         | <10   | <10 | <10 |

PCa. Thus, to elucidate the effect of elevated levels of INH-a expression on PCa cells, we stably transfected LNCaP and PC3 cell lines with an expression vector containing an INH-a cDNA or with a control EV and confirmed the expression of the transgene by PCR (Supplementary Figure 1). Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. PCR (Supplementary Figure 1). Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA. Expression of dimeric proteins (inhibin A, inhibin B and activin A) was examined by ELISA.

**Table 1** Relationships between the expression of INH-a and clinicopathological parameters in prostate adenocarcinoma (n = 37)

| Parameters               | No. of specimens | Mean intensity | Relative risk (95% CI) | P-value | Mean intensity | Relative risk (95% CI) | P-value | Mean intensity | Relative risk (95% CI) | P-value |
|--------------------------|------------------|----------------|------------------------|---------|----------------|------------------------|---------|----------------|------------------------|---------|
| Combined Gleason grade   |                  |                |                        |         |                |                        |         |                |                        |         |
| 6                        | 16               | 0.7            | NA                     |         | 1.88           | NA                     |         | 0.30           | NA                     |         |
| 7                        | 14               | 1.51           | NA                     |         | 2.21           | NA                     |         | 1.21           | NA                     |         |
| 8                        | 7                | 1.29           | NA                     |         | 1.99           | NA                     |         | 2.00           | NA                     |         |
| Extracapsular spread     |                  |                |                        |         |                |                        |         |                |                        |         |
| Positive                 | 24               | 1.43           |                        |         | 2.13           |                        |         | 1.41           |                        |         |
| Negative                 | 13               | 0.65 (reference) | 2.07 (1.04 – 4.13) | 0.01*  | 1.87 (reference) | 1.27 (0.76 – 2.11) | NS*    | 0.08 (reference) | 2.55 (1.39 – 4.65) | 0.0011* |
| Surgical margins         |                  |                |                        |         |                |                        |         |                |                        |         |
| Positive                 | 16               | 1.15           |                        |         | 1.84           |                        |         | 1.47           |                        |         |
| Negative                 | 21               | 1.12 (reference) | 1.15 (0.68 – 3.08) | NS*    | 2.18 (reference) | 0.71 (0.32 – 1.53) | NS*    | 0.50 (reference) | 4.75 (1.62 – 13.93) | 0.0006* |
| VEGFR3+ vessels          |                  |                |                        |         |                |                        |         |                |                        |         |
| Positive                 | 18               | 1.29           |                        |         | 2.07           |                        |         | 1.53           |                        |         |
| Negative                 | 19               | 0.97 (reference) | 1.27 (0.62 – 2.60) | NS*    | 2.00 (reference) | 1.20 (0.00 – 2.39) | NS*    | 0.33 (reference) | 2.85 (1.28 – 6.37) | 0.0067* |
| Lymph node metastasis    |                  |                |                        |         |                |                        |         |                |                        |         |
| Positive                 | 16               | 1.15           |                        |         | 1.84           |                        |         | 1.47           |                        |         |
| Negative                 | 21               | 1.01 (reference) | 1.62 (0.67 – 3.97) | NS*    | 2.00 (reference) | 0.84 (0.48 – 1.46) | NS*    | 0.15 (reference) | 13.22 (1.94 – 90.00) | <0.0001* |

Abbreviations: VEGFR = vascular endothelial growth factor receptor; NA = not applicable; NS = not significant; CI = confidence interval. *Fisher’s exact test.

Table 2 Inhibin A, inhibin B and activin A protein expression in INH-a clones

| Cell lines | Conditioned media |
|------------|-------------------|
|            |                   |
| Inhibit A  | Inhibit B         | Activin A |
| (pg ml^{-1}) | (pg ml^{-1}) | (pg ml^{-1}) |
| LNCaP      |                   |           |
| L16        | <10               | <10       | <10 |
| L17        | <10               | <10       | <10 |
| L18        | <10               | <10       | <10 |
| L1         | <10               | <10       | <10 |
| L5         | <10               | <10       | <10 |
| L8         | <10               | <10       | <10 |
| PC3        |                   |           |
| P128       | 50.1              | 67.2      | 1.96 |
| P129       | 56                | 64.1      | 1.78 |
| P130       | 48.1              | 73        | 1.72 |
| P20        | 228.1             | 149.7     | 1.28 |
| P103       | 194.1             | 186       | 1.69 |
| P104       | 221.6             | 187.8     | 1.67 |

Table 2 Inhibin A, inhibin B and activin A protein expression in INH-a clones

| Cell lines | Conditioned media |
|------------|-------------------|
|            |                   |
| Inhibit A  | Inhibit B         | Activin A |
| (pg ml^{-1}) | (pg ml^{-1}) | (pg ml^{-1}) |
| LNCaP      |                   |           |
| L16        | <10               | <10       | <10 |
| L17        | <10               | <10       | <10 |
| L18        | <10               | <10       | <10 |
| L1         | <10               | <10       | <10 |
| L5         | <10               | <10       | <10 |
| L8         | <10               | <10       | <10 |
| PC3        |                   |           |
| P128       | 50.1              | 67.2      | 1.96 |
| P129       | 56                | 64.1      | 1.78 |
| P130       | 48.1              | 73        | 1.72 |
| P20        | 228.1             | 149.7     | 1.28 |
| P103       | 194.1             | 186       | 1.69 |
| P104       | 221.6             | 187.8     | 1.67 |

**Effect of INH-a over-expression on tumour growth and metastasis**

The influence of INH-a on tumour growth and metastasis was determined in vivo. The clones were injected at the orthotopic site (prostate) and after 7 – 9 weeks primary prostate tumours as well as the adjacent lymph nodes were harvested. Primary prostate tumour size was determined and the incidence of lymph node metastasis was scored. Positive immunostaining for human mitochondrial protein confirmed that the primary and secondary tumours originated from intra-prostatic injection of human cells. INH-a immunostaining was used to confirm INH-a expression in tumours (Figure 3A – D, left). Immunostaining in the INH-a tumours showed that INH-a expression was not uniform within the tumour, which is consistent with a heterogeneous population of INH-a-positive and -negative cells. Because primary PCa cells are known for their heterogeneity and the aim of this study was to determine the effect of elevated levels of INH-a in cancer, it was concluded the heterogeneous population was not going to bias the outcomes of the functional assays.

**Growth characteristics of INH-a-transfected LNCaP and PC3 clones in vitro**

To evaluate the effect of over-expression of INH-a on the growth of LNCaP and PC3 cell lines, we examined the proliferative capacity and motility of the various clones. Direct cell count demonstrated reduced proliferation of INH-a over-expressing LNCaP cells (Figure 2A) and increased proliferation of PC3 cells (Figure 2B) when compared to their respective EV clones in the later stages of the growth curve. Monolayer wound healing assays showed INH-a over-expressing LNCaP cells had a reduced rate of wound closure at earlier time points (Figure 2C) whereas INH-a over-expressing PC3 cells demonstrated a trend towards increased rate of wound closure (Figure 2D). Because the significant changes in proliferation and motility observed in this study occur at different time points, it suggests that they are independent from each other.
LVD and invasion of tumour cells into lymphatics in INH\textsubscript{x} over-expressing primary prostate tumours

Changes to LVD and lymphangiogenesis are often associated with metastatic spread of cancer cells to the lymph nodes (Mattila et al., 2002; Zeng et al., 2005). To understand the mechanisms and to provide proof of metastatic spread observed in the mice injected with INH\textsubscript{x}-positive cells, we stained LNCaP and PC3 INH\textsubscript{x} and EV orthotopic tumours for LYVE-1, and human mitochondrial antibody to determine LVD and the degree of invasion of tumour cells into lymphatic vessels (lymphatic invasion) in the tissues. Consistent with our previous study (Zeng et al., 2006), the analysis of LNCaP tumours in the present study was complicated by the significantly larger size of the tumours compared to PC3 tumours. This larger size resulted in numerous necrotic areas in the LNCaP tumours, making it difficult to consistently define the different tumour peripheries and the intra- and inter-tumoural regions. For this reason stereological analysis of the LNCaP tumours was not possible. However, histological evaluation of the LNCaP tumours demonstrated no LYVE-1-positive lymphatic vessels within the tumour with lymphatic vessels only present in the regions away from the tumour (Figure 4A and B). In contrast, PC3 tumours had lymphatic vessels distributed throughout the tumour (Figure 4C and D). Stereological analysis of the PC3 tumours revealed a significant increase ($P = 0.0023$) in total LVD in the intra-tumoural regions with no difference in LVD in peritumoural regions and regions away from tumour in INH\textsubscript{x}-positive tumours compared to the controls (Figure 4E). A significant increase in lymphatic invasion in the intra-tumoural ($P = 0.0002$), peritumoural ($P = 0.0225$) and regions away from tumour ($P = 0.0077$) in INH\textsubscript{x}-positive tumour tissues compared to the controls was evident (Figure 4F).

Factors inducing tumour growth and metastasis in INH\textsubscript{x} over-expressing PC3 tumours

The observed increase in LVD in INH\textsubscript{x}-positive PC3 tumours suggests that the metastatic spread of the cancer cells from the primary tumour site to the lymph nodes occurs through the process of lymphangiogenesis. Because VEGF-A and VEGF-C have been implicated in inducing/promoting metastasis to the lymph nodes in PCa (Karkkainen et al., 2002; Weis and Cheresh, 2005; Zeng et al., 2006), we went on to determine the expression of VEGF-A and VEGF-C proteins by ELISA (Table 3) in INH\textsubscript{x}- and EV-transfected clones in vitro. There was no significant change in the amount of VEGF-A and VEGF-C secreted by the different LNCaP clones. VEGF-C protein was significantly increased ($P = 0.0011$) in the INH\textsubscript{x} over-expressing PC3 clones compared to the controls.
to their EV clones, however there was no significant change in secreted VEGF-A levels.

**Expression of TGFβRIII in LNCaP and PC3 transfected clones**

Although being a receptor for TGFβ, TGFβRIII is also a receptor for inhibins (Wiater and Vale, 2003), and recent studies have reported loss of TGFβRIII as a common and important event in human PCa (Turley et al, 2007; Sharifi et al, 2007a). Down-regulation of TGFβRIII in PCa has been suggested to reflect loss of sensitivity to tumour suppressive inhibin by the PCa cells. To test this hypothesis, we determined the levels of TGFβRIII mRNA expression in the clones. Consistent with a recent study (Sharifi et al, 2007a), real-time analysis of PCa cell lines revealed LNCaP cells to have more TGFβRIII mRNA expression compared to PC3 cells. There was no change in TGFβRIII expression in INHα-transfected LNCaP or PC3 clones compared to EV-transfected clones (Figure 5).

**DISCUSSION**

Although inhibin is used as a biomarker for ovarian cancer, the biological function of elevated levels of INHα expression in PCa and certain types of ovarian cancer is unclear. This study is the first functional study to link up-regulation of INHα expression in androgen-independent prostate disease and progression of primary prostate and secondary tumours and metastasis.

We conducted a cross-sectional study to determine a link between INHα expression and a number of clinicopathological parameters: Gleason score, surgical margin status, extracapsular...
spread, lymph node status and VEGFR-3 expression, which are well-established prognostic factors of PCa (Wheeler et al, 1998; Cheng et al, 1999, 2001, 2005; Li et al, 2004). This study showed that an elevated level of INH \( \alpha \) expression in the primary prostate tumour can be used as a predictive factor for prognosis in PCa. Univariate analysis showed a significant association between elevated levels of INH \( \alpha \) in primary PCa tissues and extracapsular spread, surgical margins, VEGF-3 expression and lymph node status. This finding is supported by our previous study that reported elevated levels of INH \( \alpha \) in PCa patients to be associated with a higher risk of recurrence, although this association was not statistically significant (Risbridger et al, 2004b). The proposed cellular site for INH \( \alpha \) expression and action is epithelial cells, however our immunostaining and analysis of the primary prostate tumour showed a significant association of elevated levels of INH \( \alpha \) in both benign epithelial cells and stromal cells to extracapsular spread. There is increasing evidence that the surrounding microenvironment also has a major function in cancer cell growth, survival, invasion and metastatic progression, further supporting the pro-tumourigenic and pro-metastatic function of INH \( \alpha \). Further analysis of our clinical data reported elevated levels of INH \( \alpha \) expression in both epithelial and stromal cells with VEGF-C-linked metastasis. Further analysis of our clinical data showed that INH \( \alpha \) expression is stronger in benign epithelium and stroma but not the G3/G4 regions of the prostate in patients with lymph node metastasis compared to those with organ-confined spread. There is increasing evidence that the surrounding microenvironment also has a major function in cancer cell growth, survival, invasion and metastatic progression, further supporting the pro-tumourigenic and pro-metastatic function of INH \( \alpha \). Further analysis of our clinical data showed that INH \( \alpha \) expression is stronger in benign epithelium and stroma but not the G3/G4 regions of the prostate in patients with lymph node metastasis compared to those with organ-confined spread. There is increasing evidence that the surrounding microenvironment also has a major function in cancer cell growth, survival, invasion and metastatic progression, further supporting the pro-tumourigenic and pro-metastatic function of INH \( \alpha \). 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Role of INH in advanced PCa

P Balanathan et al.

Figure 5 TGF-βRIII expression in LNCaP and PC3 clones. Total RNA (2.5 μg) was reverse transcribed (RT) and absolute quantitative real-time analysis was performed to determine the levels of TGF-βRIII mRNA expression in the EV- and INH-transfected clones. The bars represent EV-transfected LNCaP and PC3 clones in grey, INH-transfected LNCaP and PC3 clones in black. Data are shown as mean ± S.E. of the mean.

Because the proposed cellular site for pro-tumourigenic and pro-metastatic action of INH is cancer epithelial cells, we over-expressed INHA in metastatic epithelial PCa cell lines, LNCaP and PC3, to evaluate functionally the effect of elevated levels of INH in PCa. A key event in the progression of PCa is the transition from an androgen-dependent to -independent stage, where subpopulations of tumour cells either gain resistance to, or adapt to, an androgen-depleted environment, and begin to proliferate resulting in the progression to highly aggressive and metastatic androgen-independent disease. Furthermore, when PCa is advanced or metastatic it is usually incurable and tends to metastasise to bone and lymph nodes. LNCaP cells, originally isolated from a lymph node metastasis (Horozewicz et al., 1980, 1983), have retained their ability to respond to androgens, whereas PC3 cells, originally derived from a bone metastasis (Kaighn et al., 1979), are androgen independent. Thus these cell lines represent early and late stages of metastatic prostate disease.

INH over-expression in LNCaP cells demonstrated reduced cell proliferation, migration and primary prostate tumour growth whereas INH over-expression in these cells did not change the cells’ ability to metastasise to the lymph nodes nor did it influence the growth of the lymph node tumours. The lack of change in VEGF-C expression in INH-transfected LNCaP cells is consistent with the unchanged metastatic ability of LNCaP cells in vivo. In contrast, INH over-expression PC3 cells demonstrated increased cell proliferation, migration, primary prostate tumour and lymph node tumour growth. These cells showed increased metastasis to the lymph node, which was accompanied by an elevation of LVD and tumour cell invasion into lymphatics. These effects were associated with up-regulation of VEGF-C. Similarly, Karpanen et al. (2001) reported that VEGF-C expression in a mouse tumour model strongly promoted the growth of tumour-associated lymphatic vessels, which in the tumour periphery were commonly infiltrated with the tumour cells. Although limited to the use of only two cell lines, the current study is the first to functionally show a pro-tumourigenic and pro-metastatic function for INH in an androgen-independent model of metastatic prostate disease.

Another explanation for the different effects of INH observed in the current study is the loss or gain of a yet-to-be-identified signalling pathway for INH in either tumour suppression or promotion. We have shown that LNCaP cells express more TGF-βRIII mRNA expression compared to PC3 cells and that the level of TGF-βRIII mRNA expression is maintained after INH over-expression. Loss of TGF-βRIII during PCa progression has been suggested to be a reason for loss of sensitivity to the tumour suppressive effect of inhibin (Turley et al., 1997; Sharifi et al., 2007a, b). Whether androgen status, different cell types and/or levels of TGF-βRIII expression are responsible for the different effects of inhibin observed in the present study is an important area of future investigation.

Inhibins are known to be involved with regulating members of the TGF-β superfamily, including TGF-β itself, as well as activins and BMP, by competing for their receptors or co-receptors (Wiater and Vale, 2003; Farnworth et al., 2006, 2007). However, unlike other members of the TGF-β superfamily (Pangas and Woodruff, 2000; Shi and Massagué, 2003), the signalling pathway(s) for inhibin have not yet been defined. Although it remains unclear how INH mediates downstream cellular events leading to prostate disease, the observed effect of INH in the present study suggests and supports an effect of inhibin which is independent of TGF-β/activin in PCa. Owing to the presence of both βA and βB subunits in LNCaP and PC3 cells, the clones were able to produce dimeric inhibin A and inhibin B. The presence of inhibin B in both LNCaP and PC3 cells is significant as inhibin B is the pre-dominant form of inhibin in men and therefore more relevant to the study of PCa. Although the presence of inhibin A in PC3 may be of relevance to the observed phenotypes of PC3 cells, further investigation into the functional difference resulting from inhibin A vs inhibin B production in these cells was beyond the scope of this project. The level of inhibin A and inhibin B produced by the cells reflects the level of the β-subunits expressed and secreted by the respective cell lines (McPherson et al., 1999). Our study showed no change in the levels of activin A (βA/βA), which indicates that observed effects are specific to inhibin and not a response to activin levels. Therefore, although TGF-β/activins have been shown to induce expression of angiogenic/lymphangiogenic factors through PI3K and/or SMAD2 pathways (Wagner et al., 2004; Kang, 2006) and TGF-β/activins inhibit growth of PCa cells (Wilding et al., 1989; Wang et al., 1996; McPherson et al., 1997), the different responses of the PCa cell lines to elevated levels of INH we have observed cannot be simply explained by abrogation of TGF-β/activin signalling. Thus, the effect of inhibin on LNCaP cells observed in this study supports a role for inhibin in PCa independent of TGFβ/activin. Also, the increased metastatic ability of INH observed in INH over-expressing PC3 cells cannot be due to antagonism of activin action because of the simultaneous expression of follistatin in these cells (McPherson et al., 1999). Follistatin is an activin-binding protein that binds to activin with a high affinity thereby inhibiting activin bioactivity (Phillips and de Kretser, 1998). Taken together, the current study demonstrates a direct action of INH in PCa that is context and/or cell-type dependent.

In summary, this study showed both benign epithelial and stromal regions of primary prostate tumours to be the sites of INH expression. We have also demonstrated a strong association of elevated levels of INH to well-established prognostic factors of PCa. This finding has identified INH as a very important predictive factor that can be used to identify patients at increased risk for disease progression and cancer death after radical prostatectomy, so that appropriate therapy can be selected. Finally, the current
study has shown a pro-tumourigenic and pro-metastatic role for INH in an androgen-independent model of metastatic prostate disease. This suggests that in the absence of androgens, elevated levels of inhibin may be driving the more aggressive and metastatic phenotypes of PCa tumour.

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Role of INHs in advanced PCa
P Balalanathan et al

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