Epigenetic regulation of inhibin α -subunit gene in prostate cancer cell lines

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Abstract

Inhibin was first identified as a gonad-derived regulator of pituitary FSH; however, it has subsequently been shown to be a tumour suppressor in the gonad and adrenal glands. Whereas non-malignant regions of human primary prostate carcinomas express inhibin α -subunit (INHA), malignant tissues lack INHA transcript and protein, which is consistent with epigenetic regulation of the inhibin α -subunit gene (*INHA*) promoter. This study investigated whether methylation of the *INHA* promoter was responsible for inactivation of *INHA* transcription and translation in the prostate cancer cell lines, LNCaP, DU145 and PC3. Methylation of the promoter was revealed by bisulphite genomic sequencing and use of inhibitors of methylation and histone deacetylation resulted in reactivation of the *INHA* transcription and translation. Significant ($P < 0.05$) downregulation of a luciferase reporter gene downstream from a methylated *INHA* promoter compared with unmethylated *INHA* promoter occurred *in vitro*. The data demonstrate that promoter methylation is associated with downregulation of the *INHA* gene in prostate cancer cell lines, which is consistent with its tumour suppressive role. Therefore INHA has a significant role in prostate tumorigenesis.

Journal of Molecular Endocrinology (2004) **32**, 55–67

Introduction

Inhibin, a member of the transforming growth factor β superfamily, is a dimeric protein consisting of an α and a β subunit. The α -subunit is synthesised in precursor forms consisting of pre, pro, α_N and α_C components, whereas the β -subunit precursor form consists of only a pro and a β region. The precursor α - and β -subunits link by disulphite bonds to form a 105 kDa inhibin, which forms bioactive 30–31 kDa inhibin α_C - β after post-translational modification (Robertson *et al.* 1989). The β -subunits combine to form homo- or heterodimers called activin, whereas the α -subunit does not dimerise. Inhibin was first identified by its ability for negative regulation of the secretion of follicle-stimulating hormone from the anterior pituitary. Since then it has been demonstrated that the inhibin α -subunit (INHA) is tumour suppressive in INHA knockout mice. Both sexes of these knockout mice developed gonadal sex-cord

tumours with very high penetrance. After gonadectomy of these mice, tumours of the adrenal glands occurred (Matzuk *et al.* 1992).

In contrast to the mouse model, the role of INHA in human cancers is equivocal. Postmenopausal women with granulosa or mucinous epithelial cell ovarian tumours show increased concentrations of INHA in serum and this is used as a valuable clinical marker of ovarian cancer (Healy *et al.* 1993, Robertson *et al.* 1999a,b). In contrast, positive immunostaining for INHA is associated with favourable prognosis in a proportion of patients with ovarian cancer (Ala-Fossi *et al.* 2000). There is also an apparent inconsistency between the observations in human adrenal tumour tissues and the role of INHA as an adrenal tumour suppressor in INHA-null mice. INHA immunoreactivity has been detected in adrenal cortical adenomas and carcinomas. Earlier studies showed positive staining for INHA in hyperplastic tissues and adrenocortical carcinomas (De Jong

et al. 1990), which was later supported by data from larger studies in patients with adrenal cortical neoplasia (Arola *et al.* 1998, 2000, McCluggage *et al.* 1998, Pelkey *et al.* 1998, Fetsch *et al.* 1999, Munro *et al.* 1999). Similar to ovarian cancer, a subgroup of adrenal cortical carcinomas showed loss of INHA immunoreactivity (Munro *et al.* 1999). INHA immunoreactivity is used as a diagnostic marker that can differentiate adrenal cortical tumours from histologically similar tumours. The role of the INHA, if any, is yet to be determined in other cancers such as prostate cancer.

Mellor *et al.* (1998) reported that the INHA transcript (mRNA) and protein were not detected in malignant tissues from men with poorly differentiated prostate cancer. In contrast, the expression of INHA mRNA and protein was observed in tissues from adjacent non-malignant regions of the same patients or in men with benign prostatic hyperplasia (Mellor *et al.* 1998). Cytosine methylation of the INHA gene (*INHA*) promoter region was associated with loss of expression of INHA in malignant regions of prostate cancer specimens, and non-malignant regions adjacent to the cancer cells were less methylated compared with the malignant regions (Schmitt *et al.* 2002). Aberrant cytosine methylation at the CpG sites within the 5' region of a gene has been associated with transcriptional repression and is a common mechanism in the silencing of tumour suppressor genes.

The human *INHA* is located on chromosome 2q33–36 and is composed of two exons separated by a 1.7 kb intron (Stewart *et al.* 1986, Debieve & Thomas 2002). Consistent with the mouse, bovine and rat promoter regions, the human *INHA* promoter lacks an obvious TATA or CCAAT box and is not GC rich (Su & Hsueh 1992). The *INHA* promoter contains several regulatory elements, including a cAMP response element, activator proteins 1 and 2, specificity protein 1, steroidogenic factor 1, a potential Smad binding element, Rnf6 and GATA factors (Feng *et al.* 1998, Ito *et al.* 2000, Lopez *et al.* 2002, Schmitt *et al.* 2002).

In order to evaluate whether methylation is responsible for the inactivation of the expression of *INHA* in prostate cancer, we use androgen-dependent LNCaP and androgen-independent DU145 and PC3 prostate cancer cell lines that lack *INHA* expression (Batres *et al.* 1995, Furst *et al.* 1995, Ying *et al.* 1995), to determine the methylation status of the *INHA* promoter region.

Materials and methods

Cell lines

Human prostate tumour epithelial cell lines LNCaP, DU145 and PC3 and human hepatoma cell line HepG2 were obtained from American Type Culture Collection (Rockville, MD, USA). Cell lines were routinely cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% (v/v) heat-inactivated fetal calf serum (CSL Ltd, Parkville, Victoria, Australia) and antibiotics (100 IU/ml penicillin and 10 µg/ml streptomycin; CSL Ltd, Parkville, Victoria, Australia) in 75 ml culture flasks (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cell lines were passaged every 4 days by trypsinisation.

Bisulphite treatment of cells

Trypsinised cells were recovered in 1 × PBS and 1 µl cell suspension was added to 9 µl molten 1.6% (w/v) low melting point agarose and placed into 1.5 ml Eppendorf tubes. The agarose/cell beads were immediately solidified on ice. Controls containing all reagents, but no cells, were systematically included. Agarose/cell beads were set up in triplicate for each cell line.

Bisulphite treatment of DNA in agarose beads was according to the procedures of Olek *et al.* (1996) and Kerjean *et al.* (2001), with slight modification to suit the requirements of our experiments. Briefly, 200 µl lysis solution (0.5 M EDTA, pH 8; 21 µl 19 mg/ml proteinase K) was deposited over the agarose beads containing the cells. The lysis solution was carefully removed using a syringe after overnight incubation at 50 °C. The beads were then overlaid with 60 µl freshly prepared 0.3 M NaOH for 30 min at room temperature in order to denature the DNA. Aliquots (170 µl) of bisulphite solution (2.5 M sodium metabisulphite (BDH Laboratory Supplies, Poole, Dorset, UK) and 125 mM hydroquinone (BDH Laboratory Supplies) pH 5) were directly added to each reaction tube. The reaction mixtures were incubated for 16 h at 50 °C in the dark. The bisulphite solutions containing the beads were then heated to 80 °C for 10 min to melt the agarose. The modified DNA was purified using Wizard DNA Clean-Up System (Promega Cooperation, Madison, WI, USA) according to the manufacturer's

instructions, and eluted into 50 µl water. The elution mixture was then spun for 2 min at maximum speed to pellet any resin that might have escaped from the DNA Clean-Up System. The supernatant was transferred to new tubes to complete the modifications. Then a 0.1 × volume of 3 M NaOH was added to the DNA and incubated for 15 min at 37 °C, followed by addition of 22 µl of 6 M NH₄OAc, pH 6. The bisulphite-reacted DNA was precipitated for 30 min at -20 °C in 250 µl 100% (v/v) cold ethanol, washed with 75% (v/v) ethanol and resuspended in 50 µl water.

Methylation analysis

Seven CpG sites in a 135 bp region of the *INHA* promoter were the focus of the previous methylation study in primary carcinomas (Schmitt *et al.* 2002). After further analysis of the untranslated region (UTR) of the *INHA* promoter, the region of study was expanded to include an additional two CpG sites. The methylation status of the *INHA* promoter in the three cell lines was determined by analysing the degree of methylation in nine CpG sites in a 500 bp 5' UTR from the start of translation. The new region of study, which included the previously studied CpG sites and two additional CpG sites, was amplified from the bisulphite-treated DNA using nested primers. The primers were designed for the coding strand using sequence published by Schmitt *et al.* (2002) from regions that lacked CpG dinucleotides (Fig. 1). The nested PCR amplifications contained 1 × PCR buffer (inclusive of MgCl₂), 2 mM dNTPs, 10 µM primers (Inha me 1, Inha me 2 for round 1 and Inha me 3, Inha me 4 for round 2; Table 1), 2.5 U Taq polymerase and 3 µl bisulphite-treated DNA in a total volume of 75 µl. After completion of the first round of amplification, 3 µl were withdrawn and used as template for the second round of amplification. Conditions for the first round of amplification were 1 cycle of 94 °C for 2 min; 5 cycles of 94 °C for 1 min, 52 °C for 2 min, 72 °C for 3 min; 25 cycles of 94 °C for 0.5 min, 52 °C for 2 min, 72 °C for 1.5 min and 1 cycle of 72 °C for 6 min. Conditions for the second round of amplification were 1 cycle of 94 °C for 2 min, 25 cycles of 94 °C for 0.5 min, 52 °C for 2 min, 72 °C for 1.5 min and 1 cycle of 72 °C for 6 min. Each bisulphite-treated DNA was set up for PCR in triplicate.

The PCR products were gel purified and the recovered products were analysed by digestion with *Bst*NI to assess if the bisulphite treatment of the DNA was complete. Bisulphite treatment converts unmethylated cytosines (C) to thymidine (T), leaving methylated cytosines unconverted. The recognition site for *Bst*NI is CC/XGG, where X is either A or T. Thus successful conversion of the C to T will change the *Bst*NI recognition to TT/XGG and the enzyme will fail to digest the product. The amplified region contains five *Bst*NI recognition sites (Fig. 1). Only PCR products that were not cleaved by this enzyme were cloned into pGEM-T Easy vector (Promega) and transformed into DH5α-competent cells. Ten colonies from each ligated PCR product were minipreped and sequenced with primer, Inha me 4. Therefore, a total of 90 colonies per cell line were used to determine the methylation status of the *INHA* promoter in each of the respective cell lines.

Treatment of LNCaP, DU145 and PC3 with DNA methylation inhibitor and histone deacetylase inhibitor

The prostate cancer cell lines were seeded at a density of 5×10^5 cells/100 mm tissue culture dish. The cells were allowed to attach to the dishes for 24 h and exposed to different concentrations of the DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-dC) and the histone deacetylase inhibitor, trichostatin A (TSA), both individually and combined. For individual treatment, 5-aza-dC was added every 48 h in aqueous solution at final concentrations of 100, 50 and 10 µM for a total of 96 h and TSA dissolved in DMSO was added 24 h before harvest at final concentrations of 300 and 200 ng/ml. For combined treatment, cells were exposed to 100 µM 5-aza-dC for 96 h and to 300 ng/ml TSA for the last 24 h period.

Total RNA extraction from cell lines

Trizol (2 ml) (Invitrogen) was added to each 100 mm tissue culture dish to lyse the cells. The lysates were transferred into 1.5 ml Eppendorf tubes and 200 µl chloroform was added for every 1 ml of Trizol. This was mixed thoroughly by shaking and incubated at room temperature for 3 min. The Eppendorf tubes were centrifuged at 4 °C for 15 min at maximum speed. The upper

Figure 1 Partial sequence of the *INHA*. The sequence above shows the region used to design primers to amplify a 500 bp promoter region from the bisulphite-treated DNA. The primer sequences shown in Table 1 have all C converted to T. The primers were designed to avoid CpG sites, which are highlighted in bold. The numbers show the positions of the nucleotides in the *INHA* with respect to ATG=1. The *Bst*NI recognition sites are shown in italics.

layer was then transferred into fresh 1.5 ml Eppendorf tubes and 500 µl isopropanol was added for every 1 ml Trizol, to precipitate the RNA. This

was centrifuged at 4 °C for 10 min at maximum speed. All isopropanol was removed and the pellet washed with 75% (v/v) ethanol. After removal

Table 1 Primers for bisulphite experiment and RT-PCR

	Primer name	Primer sequence
Target Inhibin α -promoter	Inh a me 1	TGGTTTAAGTATAGAGTAGGTAGGATTATT
	Inh a me 2	CCTATATATAAAACCCCCAAAACATATCT
	Inh a me 3	TTTTAGAAGGTTTGAGGGGTGATGTATTTTG
	Inh a me 4	CAACAAAAAACAATAAATACAACACCAT
Inhibin α	Inh a ORF F2	CCTGTTCTTGGATGCCTTG
	Inh a ORF R2	AGCTGGGCTGAAGTCACCT
	Inh a ORF F3	TGAGGACAAGTCAGCTGCCAGA
β_2 -microglobulin	b2mg F	CCGTGTGAACCATGTGACTT
β_2 -microglobulin	b2mg R	CAAACATGGAGACAGCACTC

of the ethanol, the pellet was air-dried for a few minutes and resuspended in 30 μ l diethyl pyrocarbonate (DEPC) water.

Total RNA (0.5 μ g/ μ l) was treated with DNA-free kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, with slight modifications. Briefly, 0.1 $\times$ of 10 $\times$ DNase I buffer and 1 μ l DNase I (2 U) were added to the total RNA. This was mixed gently and incubated at 37 °C for 30 min. Then 1 μ l DNase I was added to the total RNA and further incubated for 30 min at 37 °C. The DNase Inactivation Reagent was resuspended by vortexing and a 0.1 $\times$ volume was added to the RNA. The tube was incubated for 2 min at room temperature and then centrifuged at 10 000 *g* for 1 min to pellet the DNase Inactivation Reagent. The total RNA was removed to fresh tubes, care being taken to avoid the resin.

RT-PCR analysis

DNA-free-treated total RNA (2.5 μ g) was added to 1 μ l Random Hexamer (50 μ M; Roche, Mannheim, Germany) and 1 μ l 10 mM dNTP mix, and the volume was made up to 12 μ l with DEPC water. The reaction was incubated at 65 °C for 5 min and chilled on ice after a quick spin. Then 4 μ l 5 $\times$ first-strand synthesis buffer (Invitrogen), 2 μ l 0.1 M dTT (Invitrogen) and 1 μ l RNase inhibitor (40 U/ μ l) (Promega) were added to the tubes and incubated for 2 min at 42 °C. Superscript II (1 μ l) (Invitrogen) was added to positive reverse transcription tubes (+RT) and 1 μ l DEPC water added to control tubes (–RT). Tubes were then incubated at 42 °C for 1 h. The enzymes were inactivated at 70 °C for 15 min.

The resulting cDNA (1 μ l) was used in each PCR. Intron-spanning primers were designed using the Primer3 Output program. Primer sequences for the housekeeping gene, β_2 -microglobulin (β_2 mg) and *INHA* are given in Table 1. The PCR program for β_2 mg was 94 °C for 5 min; 30 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; 72 °C for 7 min. β_2 mg was used to test the quality of cDNA.

The amplification profile for *INHA* expression involved preincubation at 94 °C for 5 min, denaturation at 94 °C for 1 min, and primer annealing at a temperature decreasing from 65 °C to 56 °C by 1 min each cycle for 10 cycles, then 55 °C for 1 min for an additional 30 cycles and extension at 72 °C for 1 min for all cycles. All cDNA amplification for *INHA* expression was achieved using a ThermoHybrid PCR machine (Ashford, Middlesex, UK). All PCR products were run on 1% (w/v) agarose gels.

Southern blotting

INHA expression in the cell lines was analysed by Southern blotting. The gels were transferred onto Hybond XL membrane (Amersham Pharmacia Biotech) using 0.4 M NaOH as the transfer medium. The membranes were washed in 2 $\times$ SSC and incubated in hybridisation buffer [50 $\times$ Denhardt's (2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone); 0.5 M NaH₂PO₄ pH 6.5; 20 $\times$ SSC; 10% (w/v) SDS; deionised formamide; herring sperm (10 mg/ml)] at 42 °C for 1 h. Internal primer, *INHA* open reading frame F3 (Inha ORF F3; Table 1) was labelled with γ ³²P-ATP using trinucleotide kinase and added to the hybridisation buffer with the membranes. The

membranes were probed overnight and washed twice with $2 \times \text{SSC}/0.1\%$ (w/v) SDS and $0.5 \times \text{SSC}/0.1\%$ (w/v) SDS. The membranes were sealed in plastic sleeves and exposed to XOMAT film (Scientific Imaging System, Rochester, NY, USA) and developed after 4 h.

Reporter constructs

A reporter construct consisting of the *INHA* promoter driving a luciferase gene (pGL3-enhancer) was created (S Harris & A Shelling, unpublished observations). The promoter region was subcloned into pGL3-basic backbone (Promega). These aliquots of the constructs were treated individually to have one of three states of methylation: normal, unmethylated and methylated. To maintain the initial characteristics of the *INHA* promoter, DH5 α -competent cells were transformed with the normal construct to retain any methylation of the CpG sites already present on the promoter. SCS110-competent cells (Stratagene Cloning System, La Jolla, CA, USA) do not express the DNA methyltransferase enzymes that are responsible for DNA methylation. These cells were transformed with the *INHA* promoter construct to ensure that the replicating DNA was not methylated. The methylated *INHA* promoter construct was created by treating the *INHA* promoter region with *SssI* methylase to methylate the CpG sites fully, followed by ligation of the methylated promoter into the pGL3-basic vector.

Transient transfections

The mouse adrenal cell line (kindly provided by Dr Paul Farnworth, Prince Henry's Institute of Medical Research, Melbourne, Australia) was plated in 24-well plates, grown to a density of 70–80% and transfected using Superfect (Qiagen Pty Ltd, Clifton Hill, Vic, Australia). Transfection was performed in $n=4$ wells to help control for variation in cell number and culture conditions. Cells were cotransfected with 1.8 μg specific plasmids at optimised concentrations plus 50 ng Renilla luciferase plasmid pRL-TK (as a control for transfection efficiency). The transfection reagent–DNA complexes were incubated with cells for 3 h before the media was replaced with normal growth media. The cell lysates were prepared after 48 h.

Luciferase assays

Luciferase and renilla assays were performed using the Dual-Luciferase reporter assay kit (Promega Corporation) as instructed by the supplier. Briefly, after 48 h the cells were washed with PBS and lysed in 150 μl /well of Passive lysis buffer. The cells were incubated in the lysis buffer for 30 min on a rocker at room temperature and the cell lysates collected. Then 20 μl cell lysate from each transfection well were added to 96-well luminescent solid assay plates (Corning, Acton, MA, USA). Luciferase assay reagent (100 μl) was added and firefly luciferase measured on a LumniCount 96-well plate reader (Packard, Meriden, CT, USA). After a luciferase reading, 100 μl Stop & Glo reagent was added to each well and renilla luciferase was measured. The luciferase activities are represented as relative activities (firefly luciferase activity divided by renilla luciferase activity).

Immunohistochemical staining for the INHA protein

An INHA antibody, monoclonal R1 antibody (kindly provided by Dr Nigel Groome, Oxford Brookes University, Oxford, UK) was used to determine expression of the INHA in the prostate cancer cell lines.

LNCaP, DU145 and PC3 cell lines were seeded in eight-well Lab-Tek II chamber slides with covers (Nalge Nunc International, Naperville, IL, USA). The slides were coated with fibronectin before use to ensure that the cells attached to the slides. Each cell line was set up in duplicate slides. After 24 h of growth, six wells per cell line were treated with 100 μM 5-aza-dC and four wells per cell line were kept as untreated controls for 6 days. After a PBS wash, the cells were fixed on the slides using 70% (v/v) ethanol for 2 min. The chambers were taken off before the immunostaining procedure.

R1 immunostaining was performed by DAKO Autostainer (DAKO, Carpinteria, California, USA). After incubation with 0.03% H_2O_2 (DAKO) for 5 min to block the endogenous peroxidase, the cells were incubated for 60 min with R1 antibody diluted as 7.5 $\mu\text{g}/\text{ml}$. The antibody was detected by incubation with Envision polymer–antimouse–horseradish peroxidase (DAKO) for 15 min and visualised by reaction with diaminobenzidine (DAKO) for 5 min. Concentration-matched mouse IgG1 was used as negative control.

Estimation of the percentage of cells stained for R1 was performed using stereological methods, as described previously (McPherson *et al.* 2001). Briefly, for each cell line, one well for untreated and five wells for demethylated cell lines were examined under $20\times$ magnification using an Olympus microscope (BX50F4). The images were captured by a PULNiX TMC-6 video camera coupled to an IBM computer and projected using a Screen Machine II fast multimedia video adaptor (FAST electronic, GmbH, Germany). The computer program CASTGRID Version 2.1.4 (Olympus, Denmark) was used to generate an unbiased counting frame. The slides were mapped to define well boundaries and sampled at predetermined intervals along X and Y axes using one counting frame. Positively and negatively stained cells were counted and a total of 300–400 cells per well were obtained. The results were expressed as percentage of positive cells of the total number of cells counted.

Statistical analysis

The methylation levels of individual CpG sites were expressed as percentage of 5-methyl-cytosine among the entire population (methylated and unmethylated). A single C at the corresponding CpG site was considered to represent 100% methylation, a single T as no methylation and overlapping C and T as partial methylation. Data are given as means $\pm$ S.E.M. The *t*-test was used for statistical analyses and to determine statistical significance, $** (P < 0.05)$.

Results

Methylation status of the *INHA* promoter in prostate cancer cell lines

The prostate cancer cell lines LNCaP, DU145 and PC3 did not express the *INHA* transcript by RT-PCR as previously demonstrated (data not shown). Methylation of the *INHA* promoter was revealed in these cell lines by bisulphite genomic sequencing. The primers used to identify the regions of methylation were designed to avoid CpG sites, because the methylation status of any of the CpG sites of interest was not known in these cell lines. Nine CpG sites are located in a 500 bp region 5' from the start of translation in the *INHA*

promoter region (Fig. 2a). Sequencing of the bisulphite-treated DNA revealed that methylation was not confined to a specific CpG site, but included all nine CpG sites, spanning the region of interest. The methylation pattern observed ranged from densely to sparsely methylated profiles, with CpG sites 0–3 being undermethylated in DU145 and PC3 cell lines compared with the LNCaP cell line (Fig. 2b).

5-aza-dC and TSA reactivate *INHA* expression in prostate cancer cell lines

To evaluate the role of methylation in the inactivation of the *INHA* promoter in the cell lines, a DNA methyltransferase inhibitor, 5-aza-dC and a histone deacetylase inhibitor, TSA, were used. Treatment of the DU145 and PC3 cell lines with 100, 50 and 10 μ M 5-aza-dC induced expression of *INHA* mRNA (Fig. 3). The level of expression of mRNA in the LNCaP cell line was much lower than that in the DU145 and PC3 cell lines. Treatment with 300 and 200 ng/ml TSA did induce expression of *INHA* mRNA, but at very low levels. A combination of 5-aza-dC and TSA did not change the pattern of expression of mRNA compared with the 5-aza-dC or TSA treatments alone.

A methylated *INHA* promoter represses transcription in a transient system

To determine whether methylation of the *INHA* promoter is sufficient to repress the expression of *INHA* and if an unmethylated *INHA* promoter retains its transcriptional activity, we transfected the mouse adrenal cell (AC) line with *INHA* promoter constructs with various methylation states. The AC cell line expresses high levels of free *INHA* (Farnworth *et al.* 2001) and there is a high sequence homology (70%) between the mouse and human *INHA* promoter region (Su & Hsueh 1992, Schmitt *et al.* 2002). The *INHA* promoter region consisting of the segment required for optimal activity and the CpG sites of interest was cloned into pGL3-basic. Transient transfection of the AC cell line with a methylated *INHA* promoter–pGL3-basic construct resulted in a decrease in the relative luciferase reporter gene activity compared with the normal and unmethylated *INHA* promoter–pGL3-basic constructs (Fig. 4). The relative luciferase

Figure 2 The *INHA* promoter is methylated in prostate cancer cell lines. (a) Map of the CpG sites in the *INHA* promoter in the 500 bp 5' UTR from the start of translation. (b) Mean percentage methylation of each of the nine CpG sites in the *INHA* promoter region studied, in prostate cancer cells. After bisulphite treatment, the *INHA* promoter was PCR-amplified and the product cloned. Each of the experimental steps was performed in triplicate. Methylation status was determined by sequence analysis of 90 clones for each cell line. The percentage methylation shown in (b) was determined for each of the CpG sites identified in (a).

activity showed a significant increase ($P < 0.05$) when the AC cell line was transfected with an unmethylated *INHA* promoter-pGL3-basic construct as compared with transfection with an methylated *INHA* promoter-pGL3-basic construct. The normal *INHA* promoter-pGL3-basic construct showed a non-significant increase in the relative luciferase activity compared with the methylated *INHA* promoter-pGL3-basic construct.

Demethylation of the *INHA* promoter reactivates *INHA* protein expression

To establish whether or not methylation is responsible for the inactivation of *INHA* transcription and subsequently its translation, immunostainings of demethylated and untreated cell lines were carried out using the monoclonal R1 antibody. The percentage of positively stained cells in demethylated LNCaP and DU145 cell lines was low compared with the demethylated PC3 cell line (Table 2). An example of the positive staining for *INHA* expression in the demethylated cell lines is shown in Fig. 5.

Discussion

Similar to malignant regions of primary human prostate carcinomas, the cell lines LNCaP, DU145 and PC3 fail to express *INHA* transcript and protein. These cell lines also demonstrate different degrees of androgen sensitivity, which makes them useful models with which to study different stages of prostate cancer. The previously studied methylation status of primary prostate cancer focused on seven CpG sites (CpG1–CpG7) in a 135 bp region in the UTR of the *INHA* promoter. Further analysis of a 500 bp 5' UTR from the start of translation identified two more CpG sites (CpG0 and CpG8) and transcription factor binding sites that might be important in the downstream processing of the *INHA*. The present data extend our previous observations on human prostate specimens. Schmitt *et al.* (2002), and uses human prostate cell lines to study the implications of changes in the *INHA* promoter in terms of degree of gene silencing, the effects of induced demethylation on gene re-expression at mRNA and protein levels, and the effects of gene silencing at a functional level.

Figure 3 Demethylation, and to a lesser extent deacetylation, reactivates expression of *INHA* in prostate cancer cell lines. The cells were treated with 5-aza-dC and TSA to determine if methylation or acetylation, or both, was responsible for the absence of *INHA* expression. RT-PCR followed by Southern blotting were used to analyse the pattern of expression of *INHA* after treatment. A negative cDNA control (–RT) was run in every alternate lane from the left to confirm the integrity of the cDNA strand. cDNA from HepG2 cell line was used as the positive control for *INHA* expression. β_2 -Microglobulin (β_2 mg) was used as loading control. TSA was dissolved in DMSO vehicle.

The results of our study demonstrate that the *INHA* promoter is extensively methylated in LNCaP, DU145 and PC3 prostate cancer cell lines, which were confirmed to lack expression of *INHA* mRNA. The use of cell lines provides a relatively pure population of cells for such methylation studies and prevents contamination from normal or surrounding tumour cells that may present a major concern because different methylation patterns may exist in this heterogeneous population of primary tumour cells. There is a difference in the degree of methylation at specific CpG sites in the primary prostate carcinoma and that in the current cell line study; nevertheless, together these studies unequivocally demonstrate that the *INHA* promoter is methylated in prostate cancer. The primary prostate carcinomas showed methylation of CpG sites 1–4 and 7 and some methylation of CpG site 6, whereas CpG site 5 remained unmethylated in prostate carcinoma (Schmitt *et al.* 2002). The various patterns of methylation may be the result of random *de novo* methylation, the presence of competition between DNA methyltransferase (DNMT) and transcription factors or the presence of contaminating cell populations. As cells divide, the presence and absence of various factors such as transcriptional activators, repressors

and enzymes such as polymerases have key roles at the molecular level. *De novo* methylation of the DNA by DNMT maintains the CpG sites in a methylated state as the DNA replicates and cells divide. Methyl CpG binding protein preferentially binds to methylated cytosines and in turn participates in complexes that contain active histone deacetylases. Thus the presence of these protein complexes within the promoter region blocks the binding of specific transcription factors and polymerases to their sites (Baylin & Herman 2000). Alternatively, the presence of transcription factors and polymerases at their binding region within the promoter prevents the binding of DNMT to the CpG sites and inhibits methylation at these sites.

There is extensive methylation of the *INHA* promoter in all the three cell lines studied, but the degree of methylation between the cell lines differs. The androgen-dependent LNCaP cell line is highly methylated compared with the androgen-independent DU145 and PC3 cell lines, but whether the sensitivity to androgens contributes to the degree of methylation remains to be explained. The expression of DNMT mRNA has been shown to be increased in cancer cells compared with samples of benign prostatic hyperplasia (Li *et al.*

Figure 4 Methylation status regulates the activity of the *INHA* promoter. The mouse adrenal cell line (AC) was transiently transfected with three forms of *INHA* promoters in pGL3-basic vectors: +, methylated, in which the insert was treated with *Sss1* methylase; +/- normal untreated; -, unmethylated, in which the construct was replicated in SCS110 *E. coli* competent cells lacking DNA methyltransferase enzymes. The pRL-TK renilla luciferase plasmid was cotransfected to adjust for transfection efficiency. bk, cells transfected with empty vector. ** $P < 0.05$, significant difference between methylated *INHA*-pGL3 basic and unmethylated *INHA*-pGL3-basic; ns, no significant difference between methylated *INHA*-pGL3 basic and normal *INHA*-pGL3-basic.

Table 2 Percentage of cells staining positively for RI in untreated and demethylated cell lines. Values are mean $\pm$ S.D.

	Untreated	Demethylated
Cell lines		
LNCaP	0	0.09 $\pm$ 0.15
DU145	0	0.36 $\pm$ 0.22
PC3	0	8.17 $\pm$ 2.04

2000). Recent evidence showed that increased DNMT activity is an early event in carcinogenesis (Belinsky *et al.* 1996, Laird 1997). Our results are consistent with this concept, as LNCaP and PC3 cell lines can respectively represent the early androgen-dependent and late androgen-independent stages of carcinogenesis.

In the prostate cancer cell lines analysed in our study, expression of *INHA* mRNA was correlated well with the methylation status of those cell lines after treatment with the demethylation agent, 5-aza-dC and a histone deacetylase inhibitor, TSA. Induction of mRNA expression by treatment of the LNCaP, DU145 and PC3 cell lines with 5-aza-dC

and TSA demonstrates the presence of all necessary transcription factors for successful transcription of the *INHA* in these cell lines. The results demonstrate that the level of expression achieved by the cell lines is dependent on the degree of methylation within the promoter region. A reciprocal relationship between the degree of methylation and re-expression of the *INHA* was seen after treatment with 5-aza-dC. PC3 cell lines that were the least methylated were easily demethylated and expressed high levels of *INHA* mRNA, whereas LNCaP cell lines that were the most highly methylated showed less expression. The pattern of methylation also reflects on the level of expression of protein. Immunostaining of the demethylated cell lines showed expression of the *INHA* protein, whereas no expression was evident in the untreated cell lines. The percentage of positively stained cells in demethylated LNCaP and DU145 cell lines was low compared with that in demethylated PC3 cell lines. *In vitro* methylation and demethylation of the *INHA*-promoter-driven luciferase reporter construct respectively showed distinct repression and expression of the luciferase reporter activity.

Methylation of CpG sites within the regulatory regions of tumour suppressor genes is a common aberration in human cancers and is often associated with gene silencing. In the current study we have shown evidence that methylation is responsible for the silencing of the *INHA* gene in prostate cancer cell lines. Other genes that have been shown to be selectively methylated in their 5' promoter regions in many prostate cancers include *EDNRD* (Nelson *et al.* 1997), encoding the endothelin B receptor, *CD44* (Lou *et al.* 1999), a cell adhesion molecule encoding a gene with metastasis suppressor activity in rat prostate cancer, oestrogen receptor α and β (Sasaki *et al.* 2002), androgen receptor (Sasaki *et al.* 2002) and glutathione S-transferase P1 (Singal *et al.* 2001). Although methylation studies are important in determining the cause of gene silencing, they have their drawbacks in terms of functional studies. Induced demethylation reactivates several promoters in the model of study, therefore any functional changes cannot be associated with the re-expression of a particular gene.

The data presented in this study demonstrate that methylation of the *INHA* promoter in the prostate is the primary reason for inactivation of

Figure 5 Demethylation induces expression of INHA protein in the prostate cancer cell lines. The immunoreactivity of INHA protein in demethylated cell lines was detected using monoclonal R1 antibody. The cells were treated with 100 µM 5-aza-dC in chambered slides. The untreated LNCaP, DU145 and PC3 cell lines (A–C respectively) displayed no immunoreactivity. Specific staining of INHA protein was observed in demethylated LNCaP, DU145 and PC3 cell lines (D–F respectively). No specific immunoreactivity was recorded in the control cells incubated with mouse IgG1 (inserts). Scale bar represents 25 µm.

INHA expression in prostate cancer cell lines. In this study, repression of the gene activity by methylation had a dominant role over TSA-sensitive histone deacetylase activity. In conclusion, the *INHA* is well characterised for epigenetically mediated aberrant transcriptional silencing in prostate cancer cell lines and therefore this study adds further evidence to support the hypothesis that *INHA* has an important role in prostate tumorigenesis as a putative tumour suppressor. So far, methylation of the *INHA* promoter is evident in both primary and cultured prostate tumours, but whether or not methylation of the *INHA* promoter is a cause or a consequence of malignancy remains to be determined and functional studies of the *INHA* may provide insights into the development of prostate cancer. The present study demonstrated increased methylation of the *INHA* promoter in androgen-dependent cells compared with that in androgen-independent cells. Similar studies can be used as a diagnostic method to detect early forms of prostate cancer and, if diagnosis is made early, 5-aza-dC or its analogues can be used as therapeutic measures to reactivate the expression of those genes of which the inactivation is responsible for prostate cancer development.

Acknowledgements

We thank Dr Jacqueline Schmitt for her contributions, Dr Paul Farnworth for kindly providing the AC cell lines and Dr Nigel Groome for the R1 antibody. The work was supported by National Health & Medical Research Council programme grant.

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Received in final form 29 September 2003

Accepted 28 October 2003