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# Co-expression of GH and GHR isoforms in prostate cancer cell lines

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**Summary** Prostate cancer is a significant cause of morbidity and mortality in Western males. While it is known that androgens play a central role in prostate cancer development and progression, other hormones and growth factors are also involved in prostate growth. Insulin-like growth factor-I (IGF-I) plasma levels have been associated with prostate cancer risk, and growth hormone (GH), a major factor regulating IGF levels, also appears to have a role in prostate cancer cell growth. Most significantly, GH has been shown to increase the rate of cell proliferation in prostate cancer cell lines. We have now demonstrated the co-expression of GH and GH receptor (GHR) mRNA isoforms in the ALVA41, PC3, DU145, LNCaP prostate cancer cells by reverse transcription polymerase chain reaction. Sequence analysis has confirmed that these cell lines express the pituitary form of GH mRNA and also the placental mRNA isoform. These prostate cancer cell lines also express the full-length mRNA for the GHR and the exon 3 deleted isoform. We have also demonstrated the presence of GH and GHR proteins in these cell lines by immunohistochemistry. GH expression has not been described previously in human prostate cancer cells. The co-expression of GH and its receptor would enable an autocrine–paracrine pathway to exist in the prostate that would be capable of stimulating prostate growth, either directly via the GHR or indirectly via IGF production. The GH axis in the prostate could therefore be an important additional target for the future development of prostate cancer therapies.



**Key words:** growth hormone, growth hormone receptor, prostate cancer cell lines.

## INTRODUCTION

Prostate cells are dependent on androgens for normal growth, development and function. Other hormones and growth factors are also required for prostate cells to grow and multiply both *in vitro* and *in vivo*.[1] Insulin-like growth factors (IGFs) have a key role in the prostate, and plasma IGF-I levels may be an important indicator of prostate cancer risk[2,3] although this remains controversial.[4] Growth hormone (GH), a major regulator of IGF-I, appears to have an important role in regulating the prostate gland[5] and prostate cancer cell growth,[6] although this role has not been extensively studied. Both GH and IGF-I appear to be required for the normal development of the prostate gland.[7] GH has several prostate-specific effects, with GH treatment increasing the level of transcription of the prostate-specific C3 and probasin genes in the prostate of hypophysectomised castrated rats.[5] GH stimulates amino acid uptake by the epithelium of the ventral rat prostate[8] and most importantly has also been shown to increase the rate of cell proliferation in the LNCaP prostate cancer cell line.[9] In addition GH releasing hormone antagonists inhibit prostate cancer cell growth both *in vitro* and *in vivo*.[10]

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GH could act directly on the prostate via its membrane-bound receptor (GHR) or indirectly, with its effects mediated by IGF-I.[11] GHR is expressed by many tissues, including the human, rat and rabbit prostate glands and in various rat and human prostatic carcinoma cell lines, prostatic carcinoma and benign prostatic hyperplasia.[5,9,11–15]

A recent report of a 1.2 kb GHR mRNA transcript in PC-3 and LNCaP human prostate cancer cell lines[5] has also raised the possibility that the truncated extracellular soluble form of the GHR, the GH binding protein (GHBP), could be transcribed from a separate short mRNA transcript at least in the human prostate. This, however, is contrary to the currently accepted view that GHBP is generated by the cleavage of the GHR in human cells and tissues[16] and has not been independently confirmed. This short mRNA isoform[5] has not been characterized or identified and its protein product has not been described.

The effect of GH releasing hormone (GHRH) antagonists[17] and the finding that GHRH and its receptor are expressed by prostate cancer cell lines[18] suggest that an autocrine–paracrine GHRH–GH–IGF axis may exist in prostate cancer. GH mRNA transcript expression has been described in several human extrapituitary tissues, including mononuclear cells and dermal fibroblasts,[19] and specific isoforms are expressed by the placenta.[20,21] The ability of peripheral tissues to synthesize GH supports the hypothesis that an autocrine–paracrine mechanism of GH action in peripheral tissues may exist.[22] The increasing use of GH and GHRH and the proposed use of GH releasing peptides and secretagogues in the treatment of various conditions, including those in the ageing male, is therefore of potential concern. Accordingly, a fuller understanding of the role of the GH axis in the prostate is required.

The current study describes the expression of GH and GHR mRNA transcripts and protein in the ALVA41, DU145, LNCaP and PC3 prostate cancer cell lines. The co-expression of GH and its receptor would enable an autocrine–paracrine mechanism to affect growth in the human prostate. This study has also characterized GHR mRNA isoforms from these prostate cancer cell lines, using reverse transcription polymerase chain reaction (RT-PCR), 3′ rapid amplification of cDNA ends (RACE) and Northern analysis with a view to providing a more definitive model for the nature of GHR and GHBP production in the prostate.

## MATERIALS AND METHODS

### Cell culture

Androgen-sensitive ALVA41 and LNCaP cell lines and androgen-insensitive PC3 and DU145 cell lines were used in this study. ALVA41 cells, derived from an original ALVA41 clone,[23] inoculated into nude mice were obtained from Dr P. Leedman (Royal Perth Hospital, Perth, Australia); LNCaP, PC3 and DU145 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in 25 or 80 $cm^2$ cell culture flasks (Nagle Nunc International, Roskilde, Denmark). DU145, LNCaP and PC3 cells were grown in RPMI 1640 media (pH 7.4) (Life Technologies, Rockville, MD) with 10% heat-deactivated foetal bovine serum (CSL Biosciences, Melbourne, Australia), ALVA41 cells in RPMI 1640 media (pH 7.4) with 5% non-inactivated foetal bovine serum. HepG2 4464 cells were cultured in DMEM (Life Technologies) with 10% heat-inactivated foetal bovine serum. All cells were grown in media containing 50 units/mL penicillin G and 50 $\mu$L/mL streptomycin sulphate (CSL Biosciences) and incubated at 37 °C in 5% $CO_2$ and 95% air. Cell lines were demonstrated to be free of *Mycoplasma* contamination.

### Reverse transcription polymerase chain reaction

On reaching 70% confluency, cultured cells were detached with trypsin–versene, pelleted by centrifugation and frozen at −80 °C. Total RNA was extracted from these cells using Trizol (Life Technologies) according to the manufacturer's instructions, resuspended in formamide and stored frozen at −80 °C. For reverse transcription 0.5 $\mu$g oligo $dT_{18}$ primer was added to 5 $\mu$g total RNA diluted in DEPC water and incubated for 10 min at 70 °C. The solution was placed on ice for 5 min and briefly centrifuged. First strand buffer, to a final concentration of 50 mM TrisCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$,10 mM dithiothreitol (Life Technologies) and 500 $\mu$M each dNTP (pH 7; Roche, Basel, Switzerland) were added and the tube was equilibrated at 43 °C for 2 min. After the addition of 200 units SuperScript RT II (Life Technologies), the solution was mixed by pipette and incubated at 43 °C for 90 min. The enzyme was inactivated at 70 °C for 10 min. Sterile distilled water (50 $\mu$L) was added to each reaction product and the cDNA stored at −20 °C.

GHR RT-PCRs using primer pairs to exon 2 sense and exon 5 antisense, from exon 5 sense to exon 10 antisense and from exon 7 sense to 10 antisense (Table 1) were performed at 53 °C, 48 °C and 50 °C respectively. To detect GHR mRNA isoforms that lack all (GHR-277) or part (GHR-279) of exon 9, or retain this region (GHRfl),[24] RT-PCR reactions were performed using three different RT-PCRs with specific antisense primers for GHR-277, GHR-279 and GHRfl (Table 1) and using an exon 7 sense primer (exon 7b sense – Table 1) in all three reactions at an annealing temperature of 58 °C.[24]

**Table 1** Primer sequences for PCRs (LT, Life Technologies)

| Primer | Primer sequence |
|---|---|
| GHR exon 2 sense | 5′CAGGTATGGATCTCTGGC3′ |
| GHR exon 5 antisense | 5′CCATTCTTGAGTCCATTCTTGAGTGTTCCTT3′ |
| GHR exon 5 sense | 5′AGGAACACTCAAGAATGGACTCAAAGAATGG3′ |
| GHR exon 10 antisense | 5′TGATGAGAGAAACTCTTTGTCAGGCAAGGG3′ |
| GHR exon 7 sense | 5′TATGGCGAGTTCAGTGAGGTGCTG3′ |
| GHR-277 antisense[24] | 5′CCTCTAATTTTCCTTCCTTTGG3′ |
| GHR-279 antisense[24] | 5′TTGGAACTGGAACTTTGCTG3′ |
| GHR-fl antisense[24] | 5′GGGCAGAATCAGCATTTTAA3′ |
| GHR exon 7b sense[24] | 5′GGATAAGGAATATGAAGTGC3′ |
| Monkey GHBP antisense[25] | 5′GCTATTTTAATCTTTTATTTCTTTTACC3′ |
| GH sense | 5′ATCCAGGCTTTTTGACAACG3′ |
| GH antisense | 5′CAGCCCGTAGTTCTTGAGTA3′ |
| GH nested sense | 5′GCACCAGCTGGCCTTTGACA3′ |
| GH nested antisense | 5′TAGACGTTGCTGTCAGAGGC3′ |
| GHb sense[20] | 5′CTGGCTTCAAGAGGGCA3′ |
| GHb antisense[20] | 5′GTCACAGGGATGCCACC3′ |
| GHb nested sense[20] | 5′CCCATGCATTTCCCAACCATTCCC3′ |
| GHb nested antisense[20] | 5′AGGGCCCGCCCTAGAAGCCACAGCTG3′ |
| Adapter primer (LT) | 5′GGCCACGCGTCGACTAGTAC$(T)_{17}$ 3′ |
| Universal amplification primer (LT) | 5′GGCCACGCGTCGACTAGTAC3′ |
| GHR 3′UTR | 5′CTCTTGCAGGAAGGACTATG3′ |
| $\beta$-Actin sense[26,27] | 5′CGTGGGCCGCCCTAGGCACCA3′ |
| $\beta$-Actin antisense[26,27] | 5′TTGGCCTTAGGGTTCAGGGGGG3′ |
| SP6 antisense | 5′ATTTAGGTGACACTATAGAATA3′ |

In addition, RT-PCRs using a primer designed to detect monkey GHBP[25] using an exon 2 human sense and a monkey GHBP antisense primer pair (Table 1) were performed at a range of annealing temperatures from 45 to 55 °C. Genomic DNA was purified from the ALVA-41, DU-145, PC-3 and LNCaP cell lines using TRIZOL reagent (Life Technologies) and used as a positive control.

PCRs were performed using 5 $\mu$L 10 × PCR buffer II (Roche, Branchburg, NJ) (500 mM KCl, 100 mM TrisCl pH 8.3), a final concentration of 2.5 mM $MgCl_2$ (Roche), 300 $\mu$M each dNTP (Roche), 5 $\mu$L cDNA produced by reverse transcription and 1 unit *AmpliTaq*® polymerase (Roche), in a final volume of 50 $\mu$L in sterile distilled water and overlaid with mineral oil. A total of 100 pmol of each primer was used in each reaction. In negative control reactions, cDNA was replaced with water. Thermal cycling PTC-200 (MJ Research, Watertown, MA), consisted of 5 min at 95 °C initial denaturation, 35 cycles of 1 min denaturation at 95 °C, 2 min at the annealing temperature and 2 min elongation at 72 °C, followed by a final extension of 10 min at 72 °C. cDNA from HepG2 4464 cells, transfected with the coding region of the rabbit GHR,[28] was used as a positive control.

For the detection of GH mRNA expression nested primers were used. GH sense and antisense primers (Table 1) (annealing temperature 42 °C) were used in the initial PCR. Products of the expected size were visualized using a 2% agarose gel, excised from the gel and immersed in 50 $\mu$L sterile water for 24 h. This cDNA (5 $\mu$L) was amplified using GH nested sense and GH nested antisense primers (Table 1) (annealing temperature 42 °C) to increase the sensitivity and specificity of the reaction. In addition RT-PCR was performed according to the method of Boguszewski *et al.*[20] using initial sense and antisense primers (Table 1 – GHb sense and antisense primers) at an annealing temperature of 50 °C and 5 $\mu$L of this product was reamplified in a nested PCR (50 $\mu$L) using nested sense and antisense primers (Table 1 – GHb nested sense and antisense primers) at an annealing temperature of 50 °C. GH PCR was performed using 1 unit Red Hot Polymerase (*Thermus icelandicus*, Integrated Sciences, Melbourne, Australia) with a final buffer concentration of 2.5 mM $MgCl_2$, 300 $\mu$M dNTPs (Roche), 2 $\mu$L cDNA, diluted in sterile

distilled water. Thermal cycling consisted of 5 min at 95 °C initial denaturation, 35 cycles of 30 s denaturation at 95 °C, 30 s at the annealing temperature and 2 min elongation at 72 °C, followed by a final extension of 10 min at 72 °C using a PTC-200 DNA thermal cycler.

#### 3′Rapid amplification of cDNA ends

For GHR 3′ RACE, reverse transcription of total RNA extracted from each cell line was performed as described, with the addition of 10 pmol Adapter Primer (Table 1) (Life Technologies) in the place of oligo dT primer. 3′ RACE was performed with 2 $\mu$L of this cDNA. Conditions were similar to those for RT-PCR (described above) with the addition of 10 pmol Abridged Universal Amplification primer (Table 1) (Life Technologies) as the antisense primer. Gene-specific sense primers (10 pmol) to exon 2, exon 5 or to the 3′ untranslated region of the GHR (Table 1) were used. PCR products were excised from agarose gels, purified using a Jetsorb Gel extraction kit (Genomed, Bad Oeynhausen, Germany) according to the manufacturer's instructions and re-amplified using the same primers. Thermal cycling was performed as described for RT-PCR. Sterile distilled water was substituted for the template in negative controls in all reactions, and mouse liver cDNA (synthesized using the Adapter primer) was used as a positive control.

#### Southern analysis

PCR products separated by agarose electrophoresis were blotted overnight in 20 × SSC (Roche) onto Hybond positively charged membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) for Southern analysis, as described by Roche (DIG user's manual). An oligonucleotide antisense probe for GH (5′TTGGAGGGTGTCGGAATAGAC3′) was synthesized (Genset Pacific Oligos) and labelled using a DIG dUTP/dATP tailing kit using terminal transferase (Roche), according to the manufacturer's instructions. Membranes were prehybridized at 37 °C in DIG Easy-Hyb (Roche) for 2 h and then hybridized with the probe (10 ng/mL) overnight. The membrane was treated for Southern analysis as described by Roche. CDP-star™ diluted 1/100 in detection buffer was added to the membrane and incubated for 5 min in the dark in a sealed bag. The membrane was exposed to Curix film (Agfa-Gavaert, Mortsel, Belgium) for up to 30 min. Films were developed using a Curix 60 automatic processor (Agfa-Gavaert).

#### Sequencing

For sequencing, PCR products were excised from a 2% agarose gel and purified (Concert Rapid Gel PCR purification kit, Life Technologies). Sequencing was performed automatically at the Australian Genome Research Facility (Brisbane, Australia) using the Applied Biosystems 377DN Automated DNA sequencer and an ABI Big Dye Terminator sequencing kit (PE-Biosystems, Foster City, CA). Sequence comparisons were performed using the Fasta and Bestfit programs (Australian National Genome Information Service, Sydney, Australia).

#### Northern analysis

RNA was denatured in 1×MOPS, 5.4% formaldehyde and 45% formamide (pH 7) at 65 °C for 15 min. Total RNA (20 $\mu$g) from each of the four prostate cancer cell lines was separated on a 1.2% agarose, 3% formaldehyde gel in 1 × MOPS, at 75 mA for 2 h under RNase-free conditions. Samples were electrophoresed in conjunction with a DIG-labelled RNA ladder I (50 ng) (Roche). The gel was capillary blotted at 4 °C overnight in 20 × SSC (3 M NaCl, 0.3 M sodium citrate; pH 7) onto a positively charged Hybond filter (Roche). The membrane was air dried and the RNA cross-linked by UV transillumination for 3 min. The integrity of total RNA was assessed by staining the gel with 0.2 $\mu$g/mL ethidium bromide and visualizing 18S and 28S ribosomal bands on the membrane after capillary transfer. Northern analysis was performed using the DIG Easy Hyb detection system using the chemiluminescent substrate CDP-star™ (Roche).

A cRNA probe complementary to the extracellular domain of the hGHR was used. A region spanning from exon 2 to exon 5 of the GHR (Table 1) was amplified by RT-PCR as described above, using HepG2 4464 cDNA as a template. The PCR product was purified using a Qiagen PCR purification kit (Qiagen, Hilden, Germany) and then ligated into a pGEM™ T Easy plasmid vector (Promega) according to the manufacturer's instructions. The vector was introduced into subcloning efficiency DH5$\alpha$™ competent *Escherichia coli* cells (Life Technologies) using the transformation procedure described by the manufacturer. Transformed colonies were selected on IPTG/X-gal/Ampicillin/LB agar plates and the plasmid was purified from an overnight culture (Qiagen miniprep). The insert was sequenced to confirm its identity and orientation. The plasmid was linearized with an *Nco1* restriction digest and a cRNA probe was synthesized using SP6 polymerase and a DIG RNA labelling kit (Roche). The probe was ethanol precipitated and its concentration estimated using the manufacturer's protocol (Roche).

The cRNA probe for GH was synthesized using a PCR method. The nested PCR product (as above) was re-amplified with a GH antisense primer incorporating the promoter for SP6 polymerase

(5′ATTTAGGTGACACTATAGAATAGGTCTGCTTGAA GATCTG3′). Products were purified from a 2% agarose gel and re-amplified using the sense primer and an SP6 antisense primer (Table 1). The product was purified from a 2% agarose gel (Qiagen kit) and cRNA probe synthesized using SP6 polymerase and a DIG RNA labelling kit (Roche).

RT-PCR was used to incorporate DIG-11-dUTP (Roche) into a PCR probe synthesized from a PC3 cDNA template using sense and antisense primers to mouse non-muscle $\beta$-actin[26,28] (Table 1). The reaction included PCR buffer (as above) with a final concentration of 2.5 mM $MgCl_2$, 300 $\mu$M dNTP with 150 mM dTTP and 250 pM DIG-11-dUTP in a total volume of 50 $\mu$L. Thermal cycling was performed as per RT-PCR. The probe was electrophoresed on a 2% agarose gel and purified from the gel slice (JetSorb Gel extraction kit, Genomed). The probe concentration was estimated by comparing the band intensity with DNA marker IX (Roche) on a 2% agarose gel stained with ethidium bromide.

Membranes were prehybridized for 1 h in DIG Easy Hyb (Roche) with 100 $\mu$g herring sperm DNA at 52 °C for the GHR and the GH probes, at 42 °C for the $\beta$-actin probe and 37 °C for the 18S RNA probe. cRNA probes were boiled for 10 min prior to hybridization. Probes were added to fresh hybridization solution at 20 ng probe/mL DIG Easy Hyb and hybridized overnight at the pre-hybridization temperature. The membrane was treated according to the manufacturer's instructions. CDP-star™ diluted 1/100 in detection buffer was added to the membrane in a sealed bag. The membrane was exposed to Curix film (Agfa-Gavaert, Mortsel, Belgium) for up to 30 min. Films were developed using a Curix 60 automatic processor (Agfa-Gavaert).

To assess RNA transfer, membranes were stripped for 10 min at 70 °C in Northern stripping solution (50% formamide, 50 mM TrisCl pH 8 with 1% SDS) and re-hybridized with the DIG-11-dUTP labelled $\beta$-actin PCR probe or an 18S oligonucleotide tailed probe. To detect 18S RNA, an oligonucleotide probe (5′CAATTACAGGGCCTCGAAAG3′) (Genset Pacific Oligos) was labelled with a DIG end-labelling kit (Roche).

### Immunohistochemistry

For immunohistochemistry prostate cancer cells were grown to 50–70% confluency in 96-well plates (Nagle Nunc), washed in phosphate-buffered saline (PBS) and fixed for 5 min in ice-cold 100% methanol. Immunodetection was performed using a Histostain Plus broad spectrum DAB staining kit (Zymed, San Francisco, CA), according to the manufacturer's instructions. For the detection of GH, primary antibody, polyclonal anti-human GH raised in rabbits (Zymed) was diluted 1:50–1:200 in 1% bovine serum albumin (BSA) in 0.01 M PBS and cells were incubated at 4 °C for 24 h. This antibody is specific for human GH, with minimal cross-reactivity (<1%) with prolactin. Negative controls included the abolition of staining by the pre-absorption of primary antibody with 4.0 IU human recombinant GH (somatonorm, kABIvITRUM, Stockholm, Sweden) for GH immunohistochemistry or the replacement of the primary antibody with non-immune serum. Pre-incubation of the antibody with 1 mg/mL human prolactin (National Hormone and Pituitary Program) slightly reduced, but did not abolish, staining. For GHR immunoreactivity, monoclonal antibody Mab 263[27] was diluted from 1:100 to 1:16 000 in 1% BSA in 0.01 M PBS. This antibody has been extensively validated for immunohistochemical studies and does not cross-react with the insulin or prolactin receptors.[15] Cells were counterstained with haematoxylin, photographed using a Nikon inverted microscope and Kodak 200 asa colour film.

### Determination of GHBP levels in the conditioned media of prostate cancer cell lines

On reaching 70–90% confluency, prostate cancer cells were exposed to media deprived of foetal bovine serum for 24 h. The conditioned medium was collected and concentrated (10×) using a Savant speed-vac.® GHBP levels were determined using the LIFA assay[29] and by immunoprecipitation.[30]

## RESULTS

With several primer sets, RT-PCR was used to amplify distinct regions of the entire coding region (exon 2 to exon 10) of the GHR mRNA from ALVA41, DU145, LNCaP and PC3 cells. The RT-PCR amplification of cDNA spanning exon 2 to exon 5 of the GHR revealed two bands, 298 bp and 233 bp in size, in ALVA41, DU145 and PC3 cell lines [Fig. (1a)]. Only one band, 298 bp in length, was amplified from the LNCaP cell lines [Fig. (1a)] and the HepG2 4464 (data not shown) control cell line which has been stably transfected with the entire coding region of the rabbit GHR cDNA. Sequencing of the PCR products from all four prostate cancer cell lines confirmed that the 298 bp band corresponds to the full-length exon 2 to exon 5 region of the human GHR (encompassed by the primers), while the 233 bp band is an exon 3 deleted form in prostate cancer cell lines. RT-PCR, using primers to exon 5 (sense) and exon 10 (antisense) and to exon 7 (sense) and exon 10 (antisense) of the GHR, amplified bands of the expected size (1570 bp and 264 bp respectively) in the prostate cancer cell lines (data not shown). RT-PCRs using primers

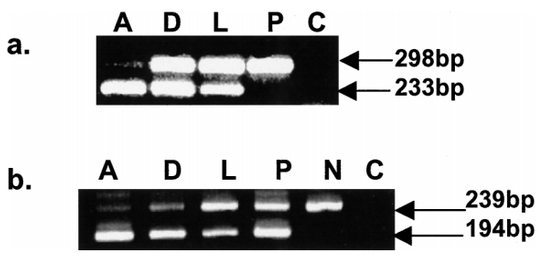


**Fig. 1** (a) Representative ethidium bromide stained 2% agarose gel of RT-PCR products amplified using primers to exon 2 (sense) and exon 5 (antisense) of the GHR in the ALVA41 (A), DU145 (D), LNCaP (L) and PC3 (P) prostate cancer cell lines. Arrows indicate the expected size of PCR products. The 233 bp band represents the exon 3 deleted form and the 298 bp band is the full-length form of the GHR. In the negative control, water was substituted for cDNA template. (b) RT-PCR for GH in prostate cancer cell lines. Arrows indicate the expected size of PCR products. The 239 bp product represents the mRNA isoform encoding the 22 kDa GH-N protein and the smaller 194 bp product has a 45 bp deletion and represents the 20 kDa GH-N protein. N = normal prostate cDNA, C = negative control.

designed to detect specifically previously reported GHR variants – the exon 9 deleted, GHR-277 isoform and the GHR-279 isoform with a 26 bp deletion in exon 9[23] – demonstrated the presence of both isoforms in the PC3 prostate cancer cell line and the full length only of this region (GHRfl) in all prostate cancer cell lines (data not shown).

In the monkey (rhesus macaque), the predicted GHR protein has a 94.1% identity with the human GHR. A 1.7 kb transcript, identified in some monkey tissues by Northern analysis, appears to be an alternatively spliced transcript which could encode a GHBP.[25] An mRNA isoform, isolated using 3′ RACE, is translated into a truncated protein, with the transmembrane and intracytoplasmic domains of the GHR being replaced by a short tail of nine amino acids encoded by the adjacent intron. It has been proposed that this isoform could be secreted and that GHBP could, therefore, be produced both by the proteolytic cleavage of the membrane-bound receptor and by the translation of an alternatively spliced mRNA in the monkey.[25] In the current study, RT-PCR was performed using an antisense primer[25] which shares 96.4% identity with the tail region of the monkey GHBP mRNA sequence and with the corresponding human exon 7–8 intronic sequence.[31] In combination with the GHR exon 2 primer, PCR using the monkey primer did not produce specific bands at various annealing temperatures in the cell lines studied. As a positive control, PCR was performed using genomic DNA from the four prostate cancer cells lines as a template, and bands of the expected size (1353 bp) were amplified (data not shown).

To determine whether a truncated and/or alternatively spliced GHR/GHBP mRNA was expressed, the 3′ end of the GHR mRNA was examined by 3′ RACE. This was performed using cDNA synthesized with a poly A tail Adapter primer, amplified with either GHR exon 2 or exon 5 specific sense primers and the Universal Amplification antisense primer. No bands were produced corresponding to GHR transcripts in the prostate cancer lines studied. As a control for the 3′ RACE technique, cDNA was amplified using the 3′ RACE system over a much shorter 3′ domain of the full-length GHR mRNA using a sense primer to the 3′ untranslated region of exon 10. This gave single bands of the expected size (~470 bp) in ALVA41, DU145 and PC3 cell lines (data not shown). The identity of this band from ALVA41 cells was confirmed by sequencing. Mouse liver cDNA, which contains the truncated GHBP specific alternatively spliced transcript, was also used as a positive control and was amplified with the exon 5 sense primers and Universal Amplification antisense primer. The PCR products were re-amplified with the same primers. Two bands approximately 760 bp and 730 bp in size (expected size ~740 bp) were demonstrated (data not shown).

Northern analysis, using a cRNA probe complementary to the mRNA encoding a region of the extracellular domain of the GHR, gave bands of approximately 5.0 kb, representing full-length mRNA transcripts in the ALVA41, DU145, LNCaP and PC3 prostate cancer cell lines [Fig. (2a)]. No smaller GHBP-like transcripts were observed. The membranes were stripped and re-probed with a DIG-labelled $\beta$-actin PCR product, revealing bands of the expected size (~2.1 kb) in all prostate cancer cell lines, confirming the presence of RNA on the membrane after Northern blotting [Fig. (2b)].

Immunohistochemistry using Mab 263, specific for the extracellular domain of the GHR, detected the GHR

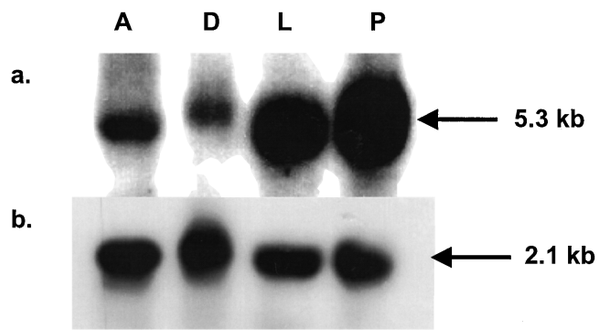


**Fig. 2** GHR Northern analysis of 20 $\mu$g total RNA from ALVA41 (A), DU145 (D), LNCaP (L) and PC3 (P) prostate cancer cell lines. The membranes were exposed to the chemiluminescent substrate for 10 min. Approximate band sizes are represented by arrows. (a) Representative Northern blot hybridized with cRNA DIG-labelled GHR probe spanning exon 2 to exon 5 of the cDNA. (b). The same membrane, stripped and hybridized with a PCR probe to $\beta$-actin.

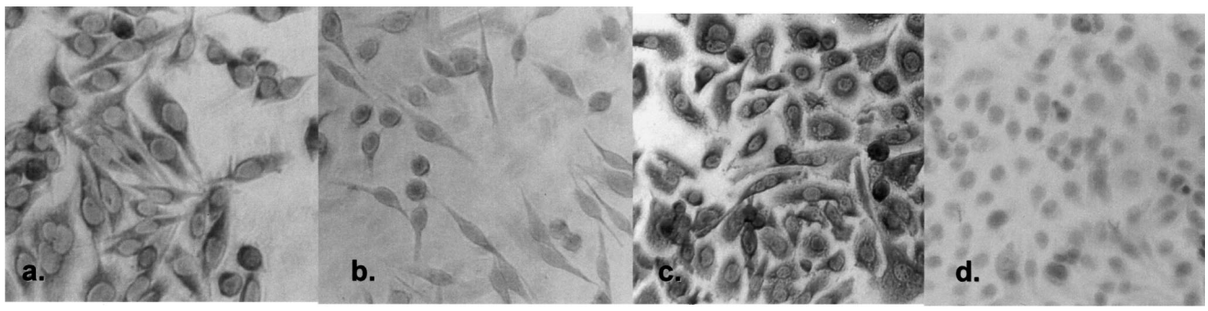


**Fig. 3** Immunohistochemical staining using a Zymed Histostain broad spectrum DAB kit showing representative examples of (a) GH expression in the ALVA41 prostate cancer cell line and (b) a negative control in the ALVA41 cell line. (c) Positive GHR immunostaining in the PC3 cell line and (d) a representative negative control in the PC3 cell line. Non-immunostaining nuclei are counterstained with haematoxylin.

protein in the cytoplasm of the four cell lines in this study (PC3 cells shown in Fig. 3). Negative controls with the omission of primary antibody failed to show specific staining (Fig. 3). These cells exhibited no nuclear staining.

To determine whether prostate cells produce soluble GHBP, concentrated serum-free conditioned media were collected after 24 h from confluent cell lines, and levels of GHBP were determined by ligand immunofunctional assay[29] or immunoprecipitation.[30] No secreted GHBP was detectable (data not shown), indicating that GHBP is not produced by the translation of an alternative transcript or by proteolytic cleavage or is present at very low levels.

In all four prostate cancer cell lines RT-PCR using nested primers specific to GH revealed bands of the expected sizes (239 bp) [Fig. (1b)] and cDNA sequencing confirmed that these products showed 100% homology with the human isoform transcribed from the GH-N gene that encodes the 22 kDa pituitary GH protein. These cells also expressed a smaller splicing isoform (194 bp) that has a 45 bp deletion and encodes for the 20 kDa GH-N isoform [Fig. (1b)]. In addition cDNA products representing the mRNA isoforms encoding the full-length human placental GH variant (GH-V) and an mRNA encoding the 20 kDa (45 bp deleted) form of the GH-V were sequenced from the LNCaP cell line. In addition, a second nested PCR[20] demonstrated the expression of an exon 3 and 4 deleted GH-N isoform in the DU145 cell line. Northern analysis using a GH 234 bp DIG-labelled cRNA probe failed to demonstrate mRNA transcripts.

GH immunohistochemistry performed to determine whether prostate cells express the GH protein in addition to GH mRNA transcript expression showed specific cytoplasmic staining in the four prostate cell lines (ALVA41 cells shown in Fig. 3(a)). Pre-absorption of the antibody with human GH abolished specific staining [Fig. 3(b)]. Pre-absorption of the primary antibody with human prolactin marginally decreased staining (data not shown), confirming that there is minimal cross-reactivity of this antibody with prolactin.

## DISCUSSION

This study has demonstrated the co-expression of GH and the GHR in the ALVA41, DU145, LNCaP and PC3 prostate cancer cell lines. While a single gene encodes the GHR, multiple mRNA transcription products are expressed in most animal species and in humans. GHR mRNA is transcribed in all human prostate cancer cell lines studied, as revealed by cDNA sequencing, and has been demonstrated previously in the LNCaP and PC3 cell lines.[9,24] Using GHR-specific primers spanning exon 2 to exon 5, a full-length form of the receptor and an exon 3 deleted form were demonstrated in ALVA41, DU145 and PC3 cell lines, while the exon 3 deleted isoform was not detected in LNCaP cells or in HepG2 4464 cell control (as expected). The human exon 3 deleted isoform of the GHR mRNA was first demonstrated in the placenta and subsequently in a range of tissues.[32–36] This short isoform, lacking exon 3 which encodes the *N*-terminal extracellular domain of the GHR, results in the translation of a protein 22 amino acids shorter than the full-length GHR.[33] The fact that the GH-binding properties of this short form are the same as the long form of the receptor, and that these isoforms are expressed in an individual-specific manner, casts doubt on their functional significance.[36,37] In this study the GHR (1–277) isoform, which lacks 26 nucleotides in exon 9, and the GHR (1–279) which lacks exon 9[37] were demonstrated in the PC3 prostate cancer cell line as previously reported.[24] These isoforms, however, were not expressed in the ALVA41, LNCaP and DU145 cell lines. The existence of different GHR isoforms could represent a mechanism enabling the multiple biological functions of GH.[34]

The GHBP is a shorter, secreted, soluble form of the GHR, corresponding to the extracellular domain of the GHR.[38,39] The mechanism for the generation of GHBP appears to be species specific. In rats and mice, GHBP is translated from a truncated (~1.2 kb) mRNA isoform,[38,40] resulting in the loss of the transmembrane (exon 8) and intracellular (exons 9 and 10) domains. In the GHBP these domains are replaced by a short hydrophilic tail which allows GHBP secretion from the cell. In contrast, in humans and rabbits, the GHBP appears to arise primarily from the proteolytic cleavage of the full-length GHR protein.[41–45] Although the GHBP could also arise from the expression of a truncated alternatively spliced GHR mRNA transcript in these species,[46] the existence of such mRNA isoforms has not been definitively demonstrated.

The demonstration of a 1.2 kb transcript in human prostate cancer cell lines was suggestive that GHBP could be produced from an mRNA isoform distinct from the one that encodes the full-length GHR, as occurs in rodents.[5] In addition, an apparently GHBP-specific mRNA isoform has been described in the macaque monkey.[25] In the current study, RT-PCR performed using an antisense primer complementary to the alternatively spliced region of the putative monkey GHBP in conjunction with a human GHR mRNA specific primer failed to detect GHR isoforms. The sequence of the tail region of the monkey GHBP corresponds to 26 bp of intron 7 and this region has a 100% homology with the human sequence. Similarly, studies applying a primer to the unique hydrophilic tail region of the rat GHBP mRNA, in conjunction with a human-specific primer, also failed to amplify PCR products (unpublished data). The application of 3′ RACE also failed to detect the presence of any *C*-terminal short GHR mRNA isoforms in the human prostate cancer cell lines. Controls for this system included the amplification of a product of the expected size (~475 bp) from the 3′ untranslated region primer to the Universal amplification primer and the amplification of a 1.2 kb transcript from mouse cDNA. As this system can amplify short mRNA isoforms, a 1.2 kb transcript, if present in human cells (as suggested by Reiter *et al*[5]), should be detected using this method. Therefore, the failure of this system and of RT-PCR methods to reveal a truncated mRNA isoform in PC3, LNCaP, ALVA41 and DU145 cell lines casts doubt on its existence.

Northern analysis was performed in an attempt to determine the expression of GHR mRNA isoforms and their relative abundance in the cell lines studied. Using a cRNA DIG-labelled probe, ~5.0 kb bands were demonstrated in the ALVA41, DU145, LNCaP and PC3 cell lines. These bands correspond to the size of the full-length GHR transcript (4.5 kb–5.1 kb) as described using Northern analysis in human cell lines.[46,47] In stark contrast to a previous report[5] where a 1.2 kb transcript was much more abundantly expressed than the full-length (~4.5 kb) transcript in PC3 and LNCaP cells, no 1.2 kb transcript was apparent in these cell lines in this study. If other isoforms are present in these cell lines, they are less abundant than the full-length form, and this method is not sufficiently sensitive to detect them.

Immunohistochemistry, performed using a monoclonal antibody designed to recognize the extracellular domain of the human GHR,[28] revealed staining in all four cell lines, confirming that these cells are capable of producing the GHR protein. GHR expression has been demonstrated previously in human prostate cancer tissues[12] and in cases of benign prostatic hyperplasia[15] but not in normal prostate tissue.[15]

The liver is believed to be the major site of GHBP production, and the contribution of other cell types to circulating levels of GHBP is unknown.[48] In this study, GHBP levels secreted into the media by the prostate cancer cell lines were negligible. The lack of GHBP measured in conditioned medium reflects the possibility that prostate cancer cell lines produce very low levels of GHBP, if any. GHBP and GHR expression appear to be differentially regulated in rodent tissues.[49] GHBP and GHBP mRNA is present in a wide number of rat tissues, suggesting that they might also be sites of GHBP production.[50] The absence of GHBP suggests that it has a minimal role in the modulation of GH actions in the prostate.

A most novel finding from this study is that GH mRNA is expressed by the ALVA41, DU145, LNCaP and PC3 prostate cancer cell lines. Several isoforms are expressed in these cell lines, with transcripts showing 100% homology to the mRNA isoforms encoding the 22 kDa and the 20 kDa GH proteins. The GH gene cluster encodes five highly related genes, including the normal pituitary GH (GH-N), GH-variant (GH-V) genes.[51] In addition, these genes are both alternatively spliced to produce 22 kDa and 20 kDa forms of the GH-N and GH-V proteins.[20,21] While GH-N transcripts are predominantly expressed by the pituitary, extrapituitary sites, including dermal fibroblasts and lymphoid cell lines, have previously been shown to express GH-N transcripts.[19] GH-V expression has been demonstrated extensively and exclusively in the human placenta.[20,52] In this study, however, expression of mRNA transcripts encoding the alternatively spliced 20 kDa and 22 kDa GH-V placental isoforms have been demonstrated by RT-PCR and cDNA sequencing in the LNCaP cell line. An isoform lacking exon 3 and 4, expressed by the DU145 cell line in this study, has been demonstrated previously in pituitary tissue.[19] Its functional

significance is unknown. None of these five GH mRNA isoforms has previously been detected in human prostate tissue.

GH is expressed primarily by the anterior pituitary, but GH expression also occurs in extrapituitary tissues, including the placenta, dermal fibroblasts and immune cells.[19,21] Immunohistochemical techniques used in this study have shown that the GH protein is also expressed in the cytoplasm of the four prostate cancer cell lines studied. GH expression has not been reported previously in human prostate cancer cell lines; however, GH protein expression has been demonstrated immunohistochemically in canine prostate cancer tissue[53] and in a mouse metastatic prostate cancer model.[54] The co-expression of GH and the GHR in prostate cancer cell lines suggests that an autocrine pathway involving GH could also exist in the prostate, as has been suggested for dermal fibroblasts[19] and breast cancer.[22] Higher circulating GH levels are associated with an increased rate of malignancy.[55] Longitudinal growth, regulated by GH and IGF, is also an indicator of increased prostate cancer incidence, as is high birth weight.[55] Acromegaly results in prostatic hyperplasia,[56] and in one study acromegaly was strongly linked with a significant increase in prostatic cancer.[55] While some studies have failed to demonstrate a link between acromegaly and prostate cancer, many of these patients are also hypogonadal, and low androgen levels are protective against prostate cancer.[57]

This study has characterized GH and GHR mRNA transcripts from human prostate cancer cell lines. A full-length GHR mRNA is expressed by the four cell lines, and the exon 3 deleted form is present in all but the LNCaP cell line. There is no evidence for a short transcript (~1.2 kb) that could encode a GHBP, and GHBP levels secreted into the media by these cell lines are undetectable. Several GH-N isoforms are expressed by these cell lines, and the LNCaP cell line also expresses GH-V transcripts. Immunohistochemical studies have demonstrated the expression of GH and GHR by these cell lines. The novel finding that GH and GHR mRNA and protein are co-expressed in human prostate cancer cell lines indicates that GH is likely to play an important autocrine/paracrine role in prostate cancer. The fact that GH causes a significant increase in cell growth in cell proliferation assays in the LNCaP prostate cancer cell line[9] confirms that this potential mechanism exists.

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