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Abstract:

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The authors declare no conflicts of interest or any commercial relationship which might be considered related to the submitted article.

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Take Home Message:

Hedgehog signalling is active in androgen independent prostate cancer and PTCH positive circulating prostate tumour cells can be identified. Systemic anti-hedgehog medicines have potential to become a new therapeutic strategy in the treatment of advanced prostate cancer.

Hedgehog Signalling in Androgen Independent Prostate Cancer

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Abstract**Objectives**

Androgen deprivation therapy effectively shrinks hormone naïve prostate cancer, both in the prostate and at sites of distant metastasis. However prolonged androgen deprivation generally results in relapse and androgen independent tumour growth which is inevitably fatal. The molecular events which enable prostate cancer cells to proliferate in reduced androgen conditions are poorly understood. Here we investigate the role of Hedgehog signalling in androgen independent prostate cancer.

Methods

The activity of the Hedgehog signalling pathway was analysed in cultured prostate cancer cells and circulating prostate tumour cells isolated from blood samples of patients with androgen independent prostate cancer.

Results

Androgen independent prostate cancer cells were derived through prolonged culture in reduced androgen conditions, modelling hormone therapy in patients and expressed increased levels of Hedgehog signalling proteins. Exposure of cultured androgen independent prostate cancer cells to cyclopamine, which inhibits Hedgehog signalling, resulted in inhibition of cancer cell growth. The expression of the Hedgehog receptor PTCH and the highly prostate cancer specific gene $DD3^{PCA3}$ was significantly higher in circulating prostate cancer cells isolated from patients with androgen independent prostate cancer compared with samples prepared from normal individuals. There was an association between PTCH and $DD3^{PCA3}$ expression and the length of androgen ablation therapy.

Conclusions

Our data are consistent with reports implicating hedgehog signalling overactivity in prostate cancer and suggest that Hedgehog signalling contributes to the androgen independent growth of prostate

cancer cells. As systemic anti-hedgehog medicines are developed the Hedgehog pathway becomes a potential new therapeutic target in advanced prostate cancer.

1. Introduction

Prostate cancer is the second leading cause of male cancer related deaths and the incidence of prostate cancer diagnosis is also increasing as life expectancy increases, awareness improves and PSA measurement is performed more frequently [1]. Androgen deprivation therapy can be used to shrink androgen dependent tumours, both in the prostate and at distant sites. Eventually androgen deprivation leads to androgen independent prostate cancer (AIPC) which progresses and undergoes metastasis despite low levels of serum testosterone [2]. Once the cancer becomes androgen independent the median survival is 20-24 months with the most effective chemotherapeutic regimes [3,4]. AIPC may arise as a consequence of amplification or mutations affecting the activity of the androgen receptor or cell signalling pathways that enable prostate cancer cells to proliferate in reduced androgen conditions [5]. Developing diagnostic and therapeutic approaches that target AIPC therefore has significant potential for improving survival and quality of life for prostate cancer patients.

Hedgehog pathway activity is important in organogenesis and the plant steroidal alkaloid cyclopamine, a hedgehog pathway inhibitor is a profound teratogen [6]. In those organs in which Hedgehog signalling affects organogenesis it is frequently also involved in carcinogenesis, the prostate is one such organ [7]. The Hedgehog pathway has recently been implicated in prostate cancer development and metastasis [8]. Patched (PTCH) is the receptor for all three vertebrate Hedgehog proteins Sonic (SHH), Indian (IHH) and Desert (DHH). In the absence of Hedgehog, PTCH inhibits Smoothed (SMO), a G protein coupled-like receptor. When Hedgehog binds to PTCH, SMO is disinhibited and initiates a signalling cascade that results in activation of GLI transcription factors and increased expression of target genes (that include PTCH and GLI1) [9,10]. Hedgehog pathway overactivity has been shown to render prostate epithelial cells tumorigenic, while inhibition of the Hedgehog pathway induces apoptosis and decreases invasiveness of prostate cancer cells [8]. Recent studies have shown a high prevalence of Hedgehog activity in high grade or

metastatic prostate cancers [8,11,12], but the contribution of Hedgehog signalling to AIPC is unclear.

To investigate the role of Hedgehog signalling in AIPC we used a cell line model to demonstrate that Hedgehog signalling is active and required for the growth of AIPC cells. To correlate our findings with clinical samples we used a published protocol to isolate prostate cancer circulating tumour cells (CTCs) from peripheral blood samples of patients with AIPC. CTCs are cells which have become detached from the primary prostate tumour and are migrating via the circulation to sites suitable for the development of metastatic lesions, and their detection has been associated with poor prognosis in breast and prostate cancer patients [13,14]. Our results suggest the Hedgehog signalling pathway represents a potential new therapeutic target in AIPC.

2. Methods

2.1. Cell and Tissue Culture

Androgen dependent LNCaP cells were obtained from the American Type Culture Collection (LGC Promochem, Teddington, UK). Androgen independent LNCaP C4-2, and C4-2B cells were purchased from Viomed Laboratories, Minnetonka, USA. LNCaP AR2 cells were generated through prolonged (9 months) culture of LNCaP cells in androgen free media. All cells were grown at 37 °C in RPMI media without phenol red (Sigma-Aldrich, Gillingham, UK) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Paisely, UK) or 10% (v/v) charcoal stripped FBS (Hyclone, Utah, USA), 2.4mM glutamine (Sigma-Aldrich, Gillingham, UK), 1% (v/v) pyruvate (Sigma-Aldrich, Gillingham, UK), penicillin and streptomycin (50U and 50µg/ml) (Invitrogen, Paisely, UK). Cells were treated with cyclopamine (Sigma-Aldrich, Gillingham, UK) as detailed.

2.2. MTT assay for cell proliferation

LNCaP cells were seeded onto 96 well plates at $2-4 \times 10^4$ /well in 200µl media and synchronized in G₀ (quiescent) phase of the growth cycle by culture in serum free medium. After 12 hours this serum free medium was replaced with RPMI containing serum and cyclopamine. After 48 hours

20µl of the 5mg/ml MTT substrate (Sigma-Aldrich, Gillingham, UK) was added to each well, incubated for 1 hour, washed and absorbance measured spectrophotometrically at a wavelength of 570 nm. All experiments were performed in triplicate.

2.3. Isolation of Circulating Tumour Cells (CTCs)

Peripheral blood samples were obtained from 15 patients (named according the format ABC 12) and 5 normal male subjects (named NORM 1-5, all under 40 years of age with no significant intercurrent illness). Two 10ml peripheral blood samples were collected in EDTA coated tubes (Greiner Bio-One, Stonehouse, UK), kept on ice and processed within three hours of collection. Samples were processed through Oncoquick (Greiner Bio-One, Stonehouse, UK) tubes, as per the manufacturer's instructions. In order to further purify the CTCs the Oncoquick enriched samples were depleted of CD45 positive cells and positively selected for EpCAM expressing cells using AutoMACS™ (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The study was fully approved by the Local Research Ethics Committee (reference number 05/Q0602/48).

2.4. RNA isolation, amplification and Quantitative PCR (QPCR)

RNA was isolated from the samples using the RNeasy Mini kit (Qiagen, Crawley, UK). The MessageAmp™ II RNA amplification kit (Ambion, Foster City, USA) was used to amplify and prepare CTCs cDNA from CTCs RNA samples. LNCaP cDNA was synthesized following DNase treatment by reverse transcription using Superscript III (Invitrogen, Paisely, UK) with oligo-dT priming. QPCR was performed in triplicate on CTC and LNCaP cDNA using Platinum SYBR®Green qPCR Supermix-UDG (Invitrogen, Paisely, UK) on an Opticon DNA Engine 2™. The primer sequences used to determine the relative expression of PTCH, GLI1, DD3^{PCA3} and housekeeping gene β_2 -microglobulin were: PTCH-f CTCCAAGCAAATGTACGAGCA; PTCH-r TGAGTGGAGTTCTGTGCGACAC; GLI1-f GAAGACCTCTCCAGCTTGGA; GLI1-r GGCTGACAGTATAGGCAGAG; DD3^{PCA3}-f GGTGGGAAGGACCTGATGATAG; DD3^{PCA3}-r GGGCGAGGCTCATCGAT; β_2 -microglobulin-f TGAATTCGTATGTGTCTGGGT; β_2 -microglobulin-r CCTCCATGATGCTGCTTACAT. In order to confirm that our cDNA samples

were not contaminated with genomic DNA we used GREX/INTRON primers which amplify a sequence across an intron/exon boundary. No PCR products were observed in the cDNA cell line samples indicating an absence of genomic DNA, whereas the appropriate sized product was amplified from genomic DNA (data not shown). To validate the specificity of the PTCH, GLI1, DD3^{PCA3} and β_2 -microglobulin primer sets standard curves for each primer set were generated using LNCaP C4-2B derived cDNA and single appropriately sized single products detected following agarose gel electrophoresis of the PCR products (data not shown). All cDNA samples expressed the housekeeping gene β_2 -microglobulin and this was used for normalization.

2.5. Antibodies and immunostaining

Primary antibodies used were as follows: PTCH (#sc-6149; Santa Cruz Biotechnology, Santa Cruz, USA), GAPDH (#9484; Abcam, Cambridge, UK) and EpCAM (#ab20160; Abcam, Cambridge, UK). Antibodies were used at concentrations recommended by the manufacturers. For westerns primary antibodies were incubated overnight at 4°C with blocking and visualised using an appropriate HRP-conjugated secondary antibody using enhanced chemiluminescence (GE Healthcare UK Ltd, Little Chalfont, UK). For immunofluorescence staining primary antibodies were incubated overnight in blocking buffer and detected using appropriate secondary antibodies.

Nuclear counterstaining was performed using DAPI. Stained preparations were analysed on a Zeiss 510 confocal microscope.

2.6. Statistics

Statistical analysis was performed using Student's t-test and Pearson's correlation coefficient. *p* values <0.05 were considered significant (***).

3. Results

Androgens are essential for the optimal growth of the prostate cancer cell line LNCaP despite expression of a promiscuous gain-of function mutant AR [15]. We generated the LNCaP subline AR2 following 9 months of culture of parental LNCaP cells in androgen free media (AFM). We

also used androgen independent sublines LNCaP C4-2 and C4-2B which were originally isolated and characterised following growth in castrated athymic mice from the site of grafting (C4-2) or bony metastases (C4-2B) [16]. The derived cell lines showed a continuum of increasing growth rates in androgen free conditions (Table 1). To investigate the contribution of Hedgehog signalling to androgen independent cell growth we determined the mRNA expression levels of components of this pathway in LNCaP (androgen dependent LNCaP cells maintained in androgen containing medium), LNCaP-A (androgen dependent LNCaP cells maintained in androgen free medium for five days), LNCaP AR2, LNCaP C4-2 and LNCaP C4-2B cells (maintained in androgen free medium). QPCR was performed to determine the expression level of PTCH and GLI1 mRNA. There was significant up regulation of PTCH and GLI1 expression in LNCaP AR2, LNCaP C4-2 and LNCaP C4-2B cells (Fig.1A) and the PTCH RNA changes correlated with increased expression of PTCH protein (Fig.1B), which is indicative of active Hedgehog signalling [10]. To verify the importance of the Hedgehog pathway to AIPC cell growth we used the SMO inhibitor cyclopamine which blocks Hedgehog signalling [17]. PTCH and GLI1 are both constituents of the hedgehog pathway and targets that are also up regulated as a consequence of Hedgehog signalling. Application of cyclopamine for 48 hours to androgen independent LNCaP C4-2B cells resulted in a significant decrease in expression of PTCH and GLI1 (Fig.1C), consistent with inhibition of Hedgehog signalling activity. The growth of androgen independent LNCaP cells was also reduced by treatment with the Hedgehog pathway inhibitor. Using cyclopamine between 0.01-1000 μ M there was minimal affect at the lowest dose and up to 90% growth inhibition at higher concentrations (Fig.1D).

To investigate whether the Hedgehog pathway contributes to clinical cases of AIPC we isolated CTCs from 20ml peripheral blood samples of patients with advanced prostate cancer who (with the exception of SUY25) are on second line treatment having failed primary hormone therapy (with a rising PSA in the face of castrate serum testosterone levels) and are therefore androgen independent, (clinical characteristics: age 53-89 years, median 76; last PSA 12-667ng/ml, median

64; testosterone less than 0.7nmol/L and duration of hormone therapy 1.2-13.9 years, median 4.4, as detailed in Table 2). The use of CTCs is advantageous as biopsies are rarely performed on patients at this cancer stage and obtaining CTCs is a low risk minimally invasive technique compared to biopsy. CTCs were not detected in the normal controls whereas each prostate cancer patient sample contained more than 5 nucleated CD45 negative, EpCAM positive cells (Fig.2), which has been associated with poor prognosis in breast and prostate cancer patients [13,14]. We established the expression levels of the PTCH receptor RNA and the prostate cancer specific marker DD3^{PCA3} [18,19] in CTCs preparations from healthy controls and prostate cancer patients (Fig.3). PTCH and DD3^{PCA3} were expressed in all cancer patients CTCs and we observed very good discrimination between the normal subjects and the patient groups for PTCH and DD3^{PCA3}. Although a low level of DD3^{PCA3} expression was found in one of the controls. Heterogeneity of expression was observed within the cancer patient samples and this has also been previously reported for other markers using PCR profiling of CTCs [20]. The expression of the androgen responsive prostate cancer marker DD3^{PCA3} in CTCs isolated from patients receiving androgen ablation therapy and having low levels of serum testosterone is consistent with the presence of AIPC cells in the CTCs samples. PTCH showed the most discriminatory power between prostate cancer patients and normal subjects due to the absence of detectable expression in normal subjects. No correlation was found between PTCH or DD3^{PCA3} expression levels and last PSA serum level (Fig.3), age (data not shown), or PSA velocity (calculated by gradient of best fit with at least 3 readings in the past 18 months; data not shown). PATCH and DD3^{PCA3} expression was very low in two patients (SUY25 and BAP20) who do not show radiologically detectable metastasis.

There was a strong correlation between the CTCs expression levels of PTCH and DD3^{PCA3}, (Fig.4). Interestingly, we also observed that expression of PTCH protein tended to be highest in CTC isolated from patients who had received androgen ablation therapy for the longest periods of time (Fig.2) and a highly significant increase in the CTC expression levels of DD3^{PCA3} and PTCH RNA was found when the patients were dichotomised about the median duration of androgen

deprivation (Fig.5). In our cancer patient group there is 85% survival 12 months after CTCs analysis, though there is no difference in survival between high or low expression of PTCH, further follow up will be required to determine long term survival affects.

4. Discussion

Hedgehog signalling is required for normal prostate development and several studies have recently reported that active Hedgehog signalling occurs in localized and metastatic prostate cancer [8,11,12,21]. PCR analysis has shown that while hedgehog ligands are expressed abundantly in localized and metastatic prostate cancer, in metastatic lesions the expression of PTCH and GLI1 is dramatically increased and treatment of an aggressive prostate xenograft model with cyclopamine prevented metastasis to the lung, whereas overexpression of Gli1 caused lung metastasis [8]. This is consistent with our observation that PATCH expression was very low in two patients (SUY25 and BAP20) who do not show radiologically detectable metastasis. On this basis inhibiting Hedgehog activity has been described as having “tremendous promise for controlling advanced prostate cancer” [22]. However, whether Hedgehog activity is also of importance in the development and progression of AIPC is unclear. In this study, we show that Hedgehog signalling is active in AIPC. We observed increased expression of components of the Hedgehog signalling pathway in androgen independent LNCaP sub-lines and in CTC isolated from patients with AIPC. In addition targeting Hedgehog activity also inhibited growth of AIPC cells, consistent with the antiproliferative results recently reported following treatment of a spontaneously arising separate androgen independent LNCaP C81 subline with cyclopamine [5].

We found significant expression in CTC isolated from patients with AIPC of PTCH, consistent with active Hedgehog signalling and this correlated with expression of the prostate cancer specific marker DD3^{PCA3}. This is the first reported expression of PTCH and DD3^{PCA3} RNA in prostate cancer CTCs. Interestingly, there was heterogeneity in the expression of PTCH in the isolated AIPC patient CTCs, similar to the relative expression reported for other markers using PCR

profiling of CTCs [20]. We also found a significant association between the duration of patient androgen deprivation therapy and the level of PTCH expressed. With patients who had received androgen deprivation for longer periods of time expressing higher levels of PTCH. Increased Hedgehog signalling could further promote the growth or metastasis of AIPC cells in these patients. However, as there is 85% survival 12 months after CTCs analysis in our prostate cancer patient group, additional follow up will be necessary to determine if Hedgehog signalling affects long term survival.

Profiling of CTCs has the potential to identify prostate patients where the Hedgehog signalling pathway is active. Thus enabling targeted treatments in prostate cancer, similar to breast cancer where Herceptin therapy is targeted to the 25% of advanced or metastatic breast cancers that are ErbB2 (HER2) positive. At present, there is no clinically available treatment that specifically targets the Hedgehog signalling pathway. However the SMO inhibitor cyclopamine, which we show can be used to inhibit AIPC cell proliferation, along with other Hedgehog signalling targeting compounds are currently being developed (Curis, USA) and a Phase I clinical trial of a systemically administered small molecule Hedgehog antagonist for testing in advanced cancer has been initiated (Genentech, USA). A combination of EGFR receptor gefitinib and cyclopamine has also recently been shown to improve the cytotoxic effects of docetaxel on cultured metastatic prostate cancer cells [23] and we have found strong synergistic effects on AIPC cell growth from combination treatments of cyclopamine with the ErbB signalling inhibitors gefitinib or lapatinib (unpubl. data., Shaw and Prowse). The synergy may occur through a direct effect of EGF signalling selectively enhancing Hedgehog activity [24]. The use of small molecule Hedgehog antagonists as adjuvant therapies may therefore improve the survival rate observed with current regimes which are ineffective against AIPC and prove to be a useful addition to the armamentarium.

5. Conclusion

The Hedgehog signalling pathway is active in AIPC and PTCH positive CTC can be identified in patients with metastatic AIPC. As systemic anti-hedgehog medicines are developed targeting the Hedgehog pathway has potential to become a new therapeutic strategy in the treatment of advanced prostate cancer.

6. Acknowledgements

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Table 1. Androgen independent growth of LNCaP sub-lines.

Cell type	Androgen independent growth. (growth after 5 days in AFM/ growth in regular medium)
LNCaP	0.3
LNCaP AR2	0.7
LNCaP C4-2	1.0
LNCaP C4-2B	1.2

Table 2. Clinical characteristics of AIPC prostate cancer patients. Samples were anonymised according to the format ABC 12. ND=not determined; RT=radiotherapy; RP=radical prostatectomy; MAB=maximum androgen blockade; DA=dexamethosone and aspirin; CLE=cisplatin, lomustine, etoposide; LHRH=leutenising hormone therapy and Orchid= castration by bilateral subcapsular orchidectomy.

ID	Age	Radiological Metastasis	Previous Treatment	Years of Androgen Deprivation	Type of Medication	PSA Velocity	Last PSA	Castrate Testosterone Level
BAP 20	87.08	N	-	7.7	MAB	0.22	56.3	Y
PEJ 21	89.72	Y	-	2.8	MAB	0.23	159.3	Y
BLR 22	53.26	Y	-	1.6	MAB	ND	19.0	Y
BRL 22	82.10	Y	RT	7.9	MAB	0.60	199.6	Y
RYR 22	73.19	Y	-	4.4	MAB	0.02	23.2	Y
FLR 23	72.67	Y	RP	7.9	MAB	0.00	51.8	Y
ROA 23	76.01	Y	-	2.4	DAS	3.73	471.0	Y
LAD 24	76.97	Y	-	2.5	DA	0.25	152.5	Y
SUY 25	55.27	N	RT	1.8	LHRH	0.03	43.6	N
GIP 28	63.38	Y	RT	12.1	CLE	26.60	666.9	Y
MUH 30	86.67	Y	-	10.3	MAB	ND	164.1	Y
TUK 31	71.36	Y	-	3.2	MAB	3.86	153.4	Y
FOJ 33	76.72	Y	-	1.2	MAB	-0.11	11.8	Y
HOD 34	77.88	ND	-	13.9	Orchid	0.00	64.0	Y
LAP 34	68.91	Y	-	5.1	LHRH	0.16	63.9	Y

Figure Legends

Figure 1. (A). QPCR showing relative expression of PTCH and GLI by LNCaP cells cultured in RPMI (LNCaP); LNCaP cells cultured in AFM for 5 days (LNCaP-A) and LNCaP AR2, C4-2 and C4-2B cultured in AFM. (B). Immunoblot showing expression of PTCH in LNCaP cells and sublines. C. 14 μ M cyclopamine treatment inhibits expression of PTCH and GLI1 RNA in AIPC LNCaP C4-2B cells: control cells (lanes 1 and 3); 48hrs cyclopamine treatment (lanes 2 and 4). D. Concentration effect curve showing cyclopamine treatment inhibits growth of AIPC LNCaP C4-2B cells. The means \pm SD are shown, *** indicates significant difference in expression ($p < 0.05$).

Figure 2. Representative immunofluorescence of CTC from patient < 4 years ADT (BLR22: ADT 1.6 years) or > 4 years ADT (HOD34: HT 13.9 years) with PTCH and EpCAM antibodies.

Figure 3. QPCR showing relative expression of DD3^{PCA3} and PTCH in control (Norm1-5) and prostate cancer patient (ABC12) CTC samples. The expression of DD3^{PCA3} and PTCH is significantly higher ($p < 0.05$) in the prostate cancer patient group. Patient samples are shown sorted left to right in order of last serum PSA measurement (FOJ33 lowest PSA-HOD34 highest PSA). No association exists between PSA measurement and expression of DD3^{PCA3} (Pearson's $r = 0.318$, $p > 0.05$) or PTCH (Pearson's $r = 0.191$, $p > 0.05$).

Figure 4. QPCR showing relative expression of DD3^{PCA3} and PTCH in prostate cancer patient CTC samples sorted in order of relative DD3^{PCA3} expression (PEJ21 lowest-RYR22 highest).

A significant association exists between the two expression levels (Pearson's $r = 0.832$, $p < 0.05$).

Figure 5. Box and whisker plots showing smallest and largest observation, median, 25th and 75th centile values for DD3^{PCA3} and PTCH RNA expression in control and prostate cancer patient CTC samples, dichotomised for patient samples by duration of androgen deprivation therapy (ADT). Patients <4 years ADT: FOJ33, BLR22, SUY25, ROA23, LAD24, PEJ21, TUK31; Patients >4 years ADT: RYR22, LAP34, BAP20, FLR23, BRL22, MUH30, GIP28, HOD34. *** indicates statistically significant difference between dichotomised groups $p < 0.05$.

Figure 1A

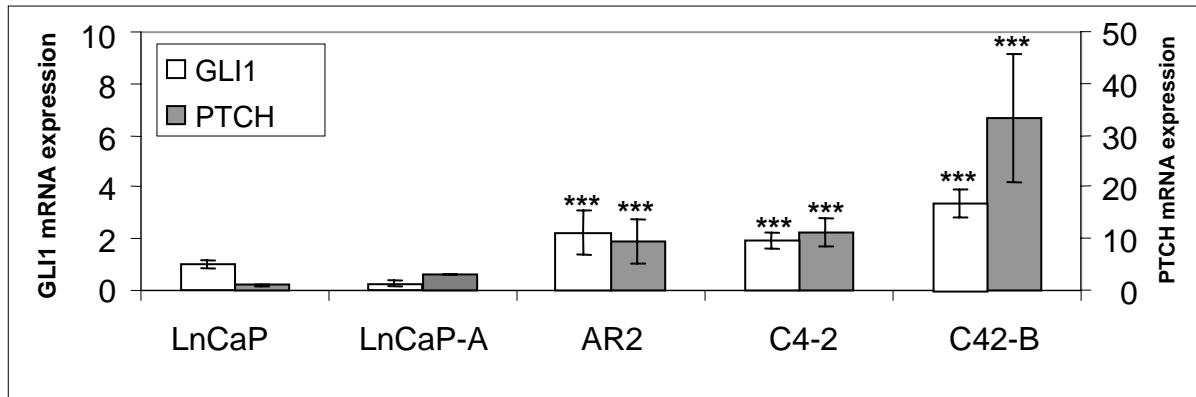


Figure 1B

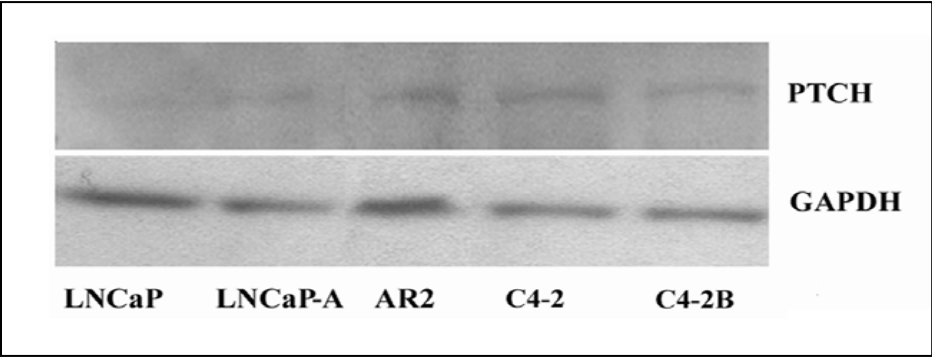


Figure 1C

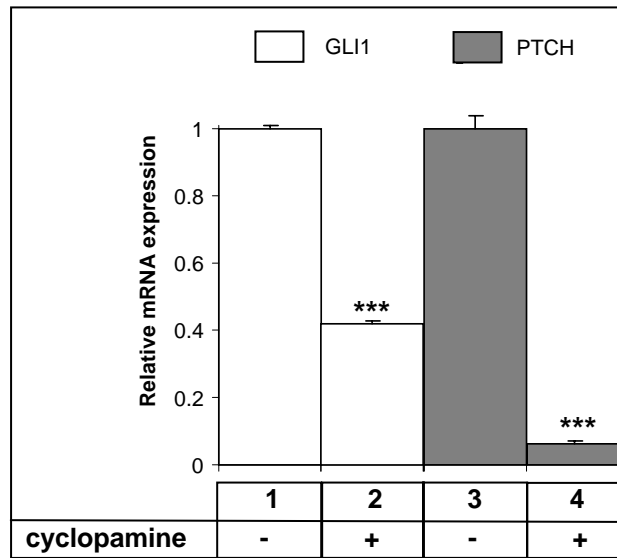


Figure 1D

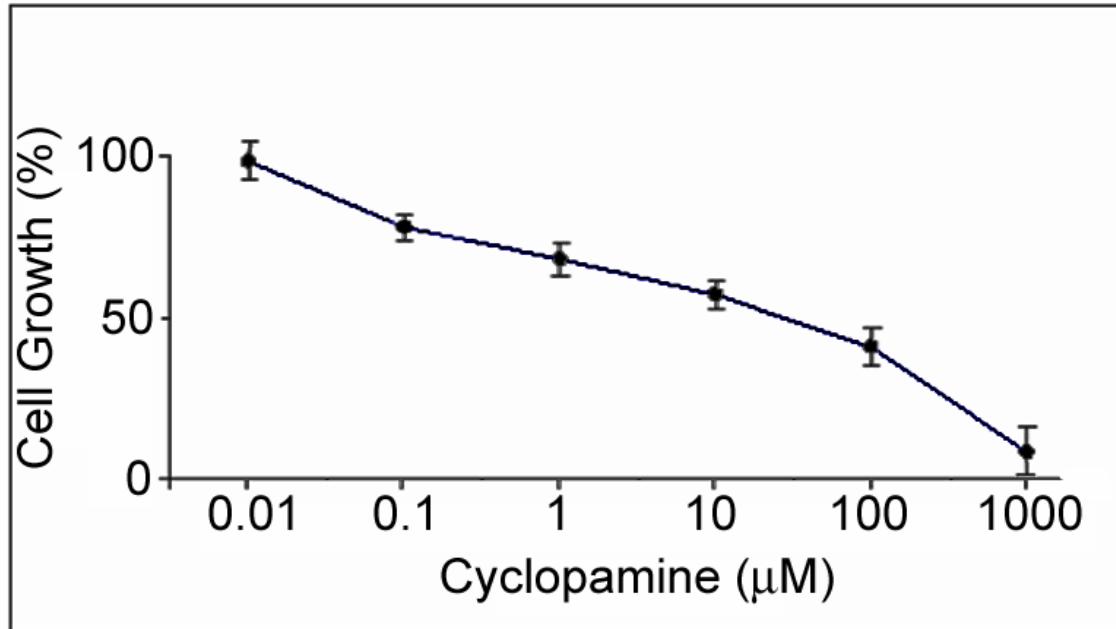


Figure 2

BLR22: Androgen deprivation therapy 1.6 years HOD34: Androgen deprivation therapy 13.9 years

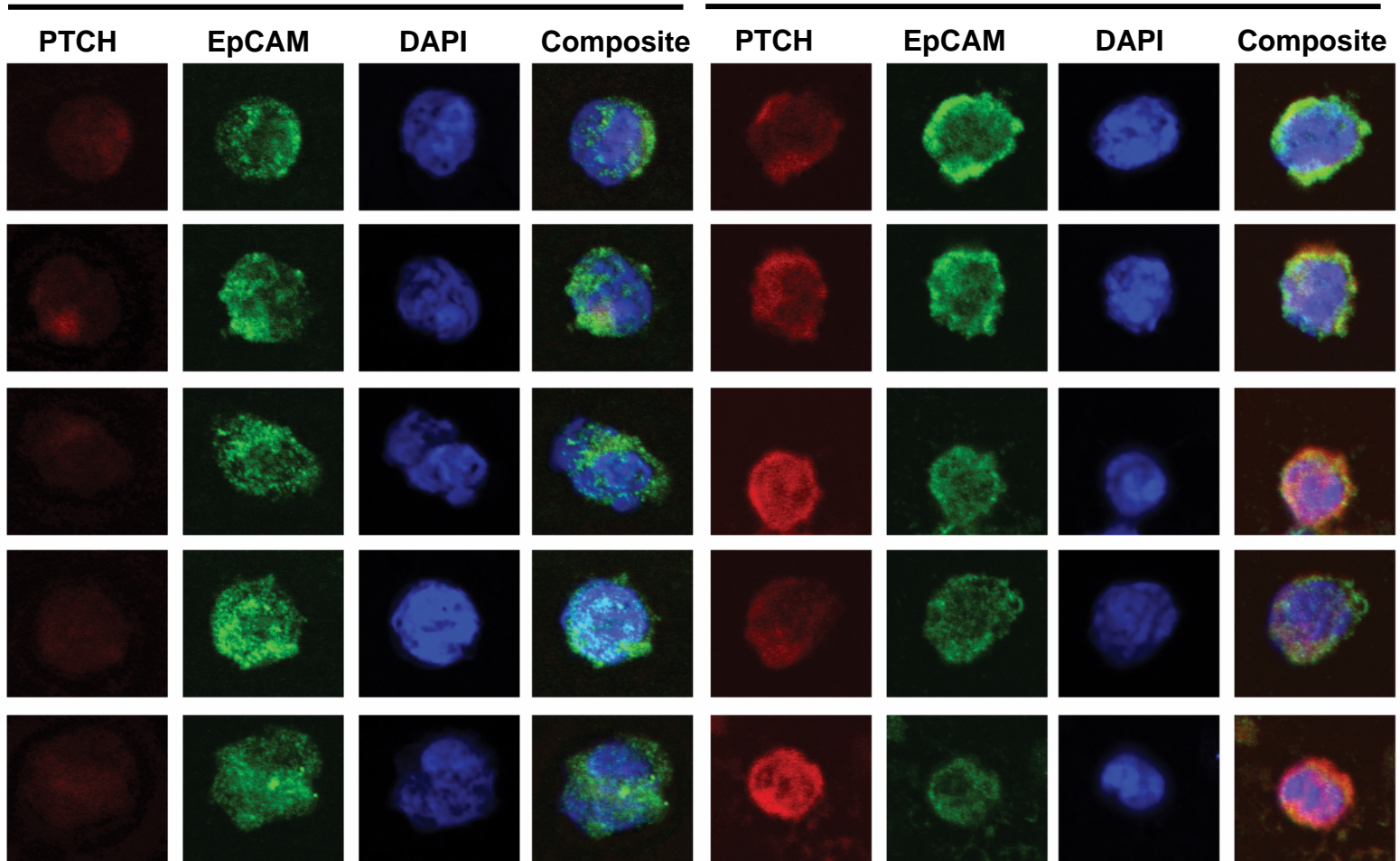


Figure 3

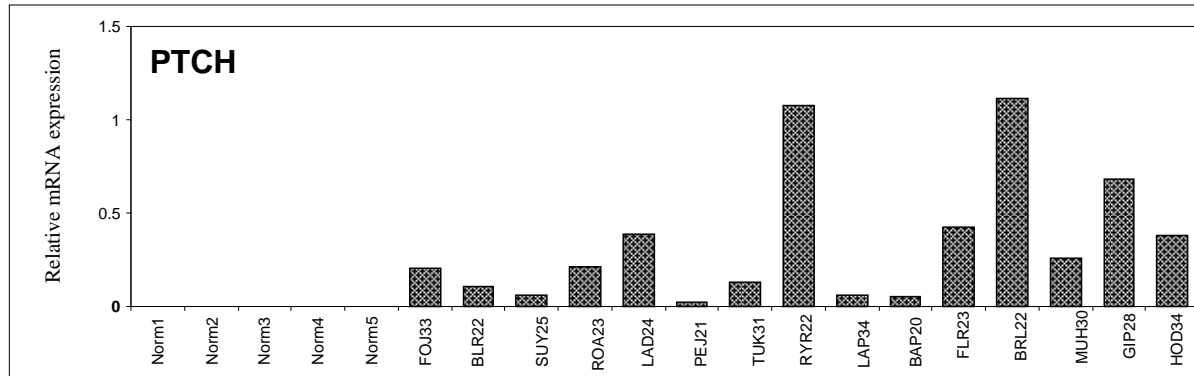
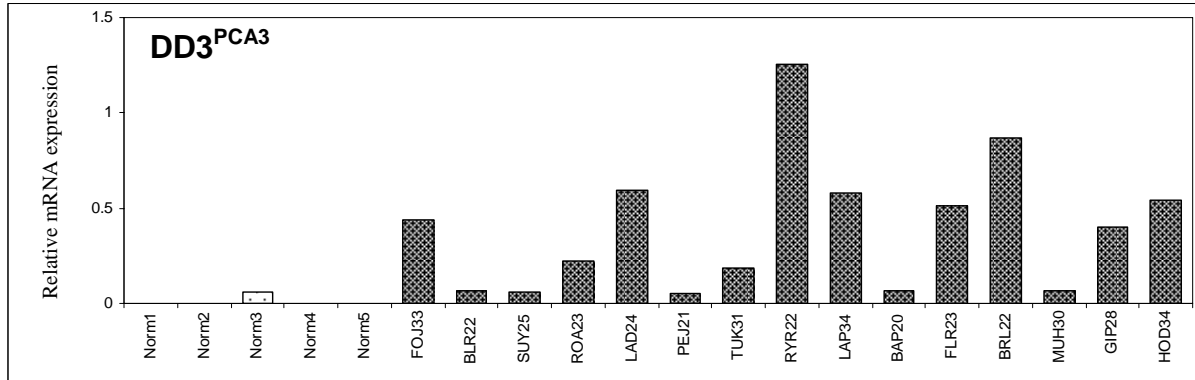


Figure 4

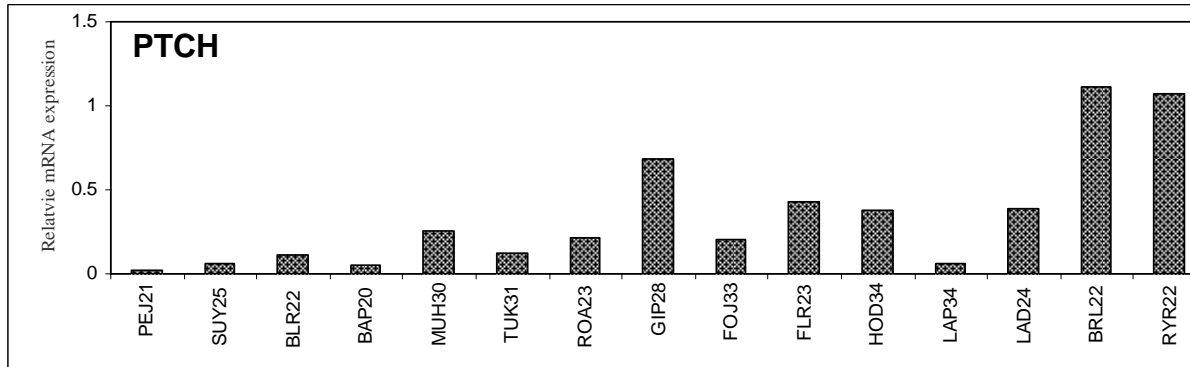
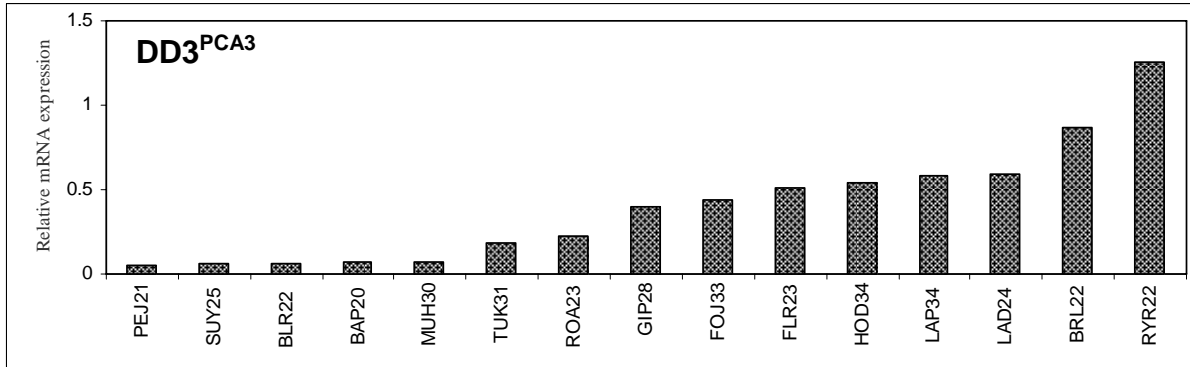


Figure 5

