Androgen-regulation of the protein tyrosine phosphatase PTPRR activates ERK1/2 signalling in prostate cancer cells

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Abstract

Background: Androgens drive the onset and progression of prostate cancer (PCa) via androgen receptor (AR) signalling. The principal treatment for PCa is androgen deprivation therapy, although the majority of patients eventually develop a lethal castrate-resistant form of the disease, where despite low serum testosterone levels AR signalling persists. Advanced PCa often has hyper-activated RAS/ERK1/2 signalling thought to be due to loss of function of key negative regulators of the pathway, the details of which are not fully understood.

Methods: We recently carried out a genome-wide study and identified a subset of 226 novel androgen-regulated genes (PLOS ONE 6:e29088, 2011). In this study we have meta-analysed this dataset with genes and pathways frequently mutated in PCa to identify androgen-responsive regulators of the RAS/ERK1/2 pathway.

Results: We find the PTPER4 and TSPYL2 genes are up-regulated by androgen stimulation and the ADCY1, OPKR1, TRIB1, SPRY1 and PTPRR are down-regulated by androgens. Further characterisation of PTPRR protein in LNCaP cells revealed it is an early and direct target of the androgen receptor which negatively regulates the RAS/ERK1/2 pathway and reduces cell proliferation in response to androgens.

Conclusion: Our data suggest that loss of PTPRR in clinical PCa is one factor that might contribute to activation of the RAS/ERK1/2 pathway.

Keywords: PTPRR, RAS/ERK1/2, MAP Kinase, Androgens, Prostate cancer

Background

Prostate cancer (PCa) is the most commonly-diagnosed malignancy in men [1], and is driven by androgen hormones acting via their cognate nuclear androgen receptor (AR) transcription factor. The AR exerts its transcriptional effects by binding to DNA sequences termed androgen response elements (AREs) within promoter regions of a number of androgen-regulated genes, including genes encoding cell cycle regulators and regulators of central metabolism and biosynthesis [2]. An important feature of PCa is prognostic heterogeneity: while some prostate cancers can remain indolent for many years others can become much more rapidly aggressive. Distinguishing key signatures between these different cancer types is a key goal. Androgen deprivation therapy (ADT) is the principal treatment for advanced PCa, although, over time, the disease becomes castration-resistant (CRPCa) with limited treatment options [3]. Persistence of AR signalling and reprogramming of the AR transcriptional landscape may underlie progression to CRPCa [4,5], and highlights the importance of AR biology in advanced PCa. Hence, increasing our understanding of the AR signalling in PCa cells should lead to more effective treatment strategies for advanced PCa.

Recently, reciprocal cross-talk between the PI3K pathway and AR signalling has been highlighted as a potential mechanism underlying CRPCa [6]. Alterations in PI3K signalling in advanced PCa are predominantly driven by loss of the tumour suppressor gene PTEN which contributes to the progression to invasive disease.
Another common feature of advanced PCAs is hyper-activation of the RAS/ERK1/2 pathway [10-12] thought to be driven by loss of function of key negative regulators of the pathway [13]. Although RAS/ERK1/2 activation alone cannot initiate PCA development, it can serve as a potentiating second hit to loss of PTEN to accelerate PCA progression [13].

Because of its established importance in clinical prostate cancer, the identification of new mechanisms through which the RAS/RAF/MAPK/ERK pathway is regulated is of great interest. We recently carried out genome-wide exon-specific profiling of PCa cells to identify novel androgen-regulated transcriptional events [14]. As well as identifying a number of alternative mRNA isoforms [15], we also identified a subset of 226 novel androgen-regulated genes [14]. In the light of evidence implicating cross-talk with AR, we searched this dataset for novel androgen-regulated genes associated with RAS/ERK1/2 signalling.

**Methods**

**IPA Pathway analysis**

Gene lists from Rajan* et al.* [14] were uploaded to the web-based Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software programme, and the “Core Analysis” function was used to study direct and indirect regulatory relationships between genes and their known biological functions.

**Antibodies**

The following antibodies were used in the study: anti-PTPRR rabbit polyclonal (17937 Proteintech), anti-phospho-p44/42 MAPK mouse monoclonal (Erk1/2 Thr202/Tyr204) (Cell signalling 9106), anti-ERK2 mouse monoclonal (1647 Santa Cruz), anti-actin rabbit polyclonal (A2668, Sigma), anti-AR mouse antibody (BD Bioscience, 554226), anti-FLAG mouse monoclonal (F3165, Sigma), anti-PTEN rabbit polyclonal (Cell Signalling 138G6), anti-α-Tubulin mouse monoclonal (Sigma T5168), normal rabbit IgG (711-035-152 Jackson labs) and normal mouse IgG (715-036-150 Jackson labs). The specificity of the PTPRR antibody was confirmed by blocking with the immunising peptide (ag12145 Proteintech) (Additional file 1: Figure S2).

**esiRNA**

esiRNAs PTPRR and AR were obtained from Sigma-Aldrich (EHU078991 and EHU025951).

**DNA constructs**

*PTPRR* cloned into pCDNA3.1 was a kind gift from Mirco Menigatti, University of Zurich. The *PTPRR* open reading frame was subsequently cloned into pCDNA5 using *NotI* and *XhoI* for creation of the Flp-In™-293 stable cell line.

**Cell culture**

Cell culture and androgen treatment of cells was as described previously [14,15]. All cells were grown at 37°C in 5% CO₂. LNCaP cells (CRL-1740, ATCC) were maintained in RPMI-1640 with L-Glutamine (PAA Laboratories, R15-802) supplemented with 10% Fetal Bovine Serum (FBS) (PAA Laboratories, A15-101). For androgen treatment of LNCaP cells, medium was supplemented with 10% dextran charcoal stripped FBS (PAA Laboratories, A15-119) to produce a steroid-deplete medium. Following culture for 72 hours, 10 nM synthetic androgen analogue methyltrienolone (R1881) (Perkin-Elmer, NLP005005MG) was added (Androgen +) or absent (Steroid depleted) for the times indicated. Where indicated, LNCaP cells were pre-treated for 1 hour with vehicle (dimethylsulfoxide; DMSO) (Sigma, C1988) or 1 µg/ml cycloheximide (Sigma, D2438) prior to addition of 10 nM R1881 for 24 hours as previously described [16]. Similarly, LNCaP cells were pre-treated with with 10 µM bicalutamide (Casodex, AstraZeneca) or ethanol (vehicle) for 2 hours prior to addition of 10 nM R1881 for 24 hours.

PC-3 (CRL-1435, ATCC), PC-3 M [17], CWR22Rv1 (CRL-2505, ATCC), DU145 (HTB-81, ATCC), and BPH-1 cells [18] were maintained in RPMI-1640 with L-Glutamine supplemented with 10% FBS. LNCaP-AI and LNCaP-cdxR were derived from LNCaP parental cells and maintained as previously described [19,20].

Stable LNCaP cell lines were generated by transfecting cells using Lipofectamine 2000 (11668-027, Invitrogen), followed by selection with 300 µg/ml Geneticin (Invitrogen, 10131019) (reduced to 150 µg/ml following the death of untransfected cells) for at least four weeks. Flp-In™-293 cells (R750-07, Invitrogen) were maintained in DMEM GlutaMax (Invitrogen, 10566-040), supplemented with 10% FBS (PAA Laboratories, A15-101) and stable cell lines generated using the Flp-In T-Rex Core Kit (K6500-01, Invitrogen) according to the manufacturer’s instructions. Protein expression was induced using 1 µg/ml tetracycline (T7660, Sigma) for 72 hours.

**RT-qPCR**

Cells were harvested and total RNA extracted using TRIzol (Invitrogen, 15596-026), according to manufacturer’s instructions. RNA was treated with DNase 1 (Ambion) and cDNA was generated by reverse transcription of 1 µg of total RNA using the Superscript VILO cDNA synthesis kit (Invitrogen, 11754-050). Quantitative PCR (qPCR) was performed in triplicate on cDNA using SYBR® Green PCR Master Mix (Invitrogen, 4309155) using Applied Biosystems 7900HT. Samples were normalised
using the average of three reference genes: GAPDH, β-tubulin and actin. All primer sequences are listed in Additional file 2: Table S2.

**Proliferation assay**

EdU incorporation was measured over 6 hours using the Click-iT™ EdU Alexa Fluor® 488 Imaging Kit (Invitrogen, C10337) and counted using ImageJ. At least 3000 cells were counted for each cell line across 3 coverslips. MTT cell proliferation assay was carried out as per the manufacturer’s instructions (Cayman, 10009365) starting with 20,000 cells per well, with 9 replicates per sample.

**Clinical samples**

Six protein lysates from primary clinical prostate tumours were used in this study. Full ethical approval was obtained for human sample collection from the Northumbland, Tyne and Wear NHS Strategic Health Authority Local Research Ethics Committee (Ref: 2003/11) and written informed consent for the use of surgically obtained tissue was provided by all patients.

**Results**

**Genes encoding components of RAS/ERK1/2 signalling pathways are regulated by androgens in PCa cells**

Complete gene lists from our ExonArray dataset [14] were manually curated for androgen-regulated changes within genes associated with RAS/ERK1/2 signalling. We identified potent down-regulation of SPRY1 expression in response to androgens (Log_FC = −2.37 p < 0.001). Full gene lists were then uploaded to the web-based Ingenuity Pathway Analysis (IPA; Ingenuity Systems) software programme, and the IPA ‘Core Analysis’ function was used to identify novel androgen-regulated genes within pathways associated with SPRY1 (Figure 1A & Additional file 3: Table S1). This network analysis identified a number of novel androgen-regulated genes previously linked to the RAS/RAF/MAPK/ERK signalling pathway. We confirmed androgen regulation of these genes in LNCaP cells using real-time PCR (Figure 1B). Two genes, PTGER4 and TSPY1L2 were up-regulated in response to androgens, whereas five others, ADCY1, OPKR1, TRIB1, SPRY1 and PTPRR were repressed.

**PTPRR is an early and direct target of the AR at the mRNA and protein level**

The above network analysis suggested that the PTPRR gene is a novel androgen regulated target in the MAPK/ERK signalling network. The genomic loci of AR binding sites mapped by ChIP in LNCaP cells [4] were uploaded onto the UCSC genome browser. Three known AR binding sites were identified in the vicinity of the PTPRR gene, one of which was less than 5 kb upstream, and another within an internal intronic region (Additional file 4: Figure S1). To test whether the PTPRR gene might be under direct control of androgens through AR regulation, we examined PTPRR expression in LNCaP cells grown in steroid depleted medium and in cells treated with 10 nM of the synthetic androgen analogue R1881 (methytrienolone) by real-time qPCR over a 24 hour period (Figure 2A), and at the protein level over 48 hours by western blotting (Figure 2B). The specificity of the PTPRR antibody used was confirmed by peptide blocking, detection of over-expressed protein and detection of esiRNA mediated protein depletion (Figure 2B, Figure 3A and D and Additional file 1: Figure S2). PTPRR expression was rapidly reduced by 10 nM R1881 treatment at both the mRNA and protein level. Repression of the PTPRR gene and protein was also observed with a range of R1881 concentrations from 0.1 nM to 100 nM (Figure 2C). To test whether androgen-mediated suppression of PTPRR expression was a direct result of AR activity, we treated LNCaP cells with 10 nM R1881 in the presence and absence of cycloheximide to inhibit de novo protein synthesis. Androgen-mediated down-regulation of PTPRR mRNA expression was still observed in the presence of the protein synthesis inhibitor cycloheximide indicating that PTPRR repression might be directly mediated by the AR (Figure 2D). Confirming this, we found androgen-mediated PTPRR protein reduction was prevented by the AR antagonists casodex (Figure 2E), and flutamide (Figure 2F), and when cells are depleted of AR using esiRNA (Figure 2G). Immunofluorescent staining of LNCaP cells grown in the absence of androgens indicates that PTPRR protein localises to the cytoplasm (Figure 2H). The structure of the PTPRR gene and protein are illustrated in Figure 2I.

**Re-expression of PTPRR in androgen treated LNCaP cells reduces phosphorylation of ERK1/2 and regulates downstream oncogenic transcription factors**

The above data predicted that AR-regulated PTPRR suppression in PCa cells may contribute to modulation of RAS/ERK signaling in response to androgens. To test this prediction, we created a stable LNCaP cell line in which PTPRR was expressed under the control of the CMV promoter and a control stable cell line transfected with empty vector. This CMV promoter is active independent of androgen stimulation. In the stable cell line made with PTPRR, increased PTPRR gene expression was detected at both the RNA level (by qRT-PCR relative to three housekeeping genes) and protein level (by western analysis, relative to actin) compared to the control cell line made with empty vector (Figure 3A left and middle panels).

Consistent with stable expression of PTPRR being sufficient to dampen activity of the MAPK/ERK network, LNCaP cells over-expressing PTPRR protein also showed reduction in phosphorylated ERK1/2 in the presence of
Figure 1 (See legend on next page.)
androgens (Figure 3A middle panel). As a parallel loading control, no change in total ERK1/2 levels was detected (Figure 3A right panel). Correlating with this modulation of the RAS/ERK pathway in response to PTPRR expression, we also observed parallel repression of three oncogenic transcription factors (c-fos, fra1 and c-jun) known to be downstream regulated targets of ERK1/2 [21] (Figure 3B).

Prostate cancer cells can be genetically heterogeneous, and this can have important implications for prognosis and treatment. We recently found a synergetic effect between loss of Pten and Spry2 in murine PCa progression [22]. To test if the ability of PTPRR to negatively regulate MAPK signalling might depend on cellular background we generated a second stable cell line over-expressing PTPRR in the HEK293 cell background which are not derived from PCa cells but are PTEN positive (Figure 3C top left panel). Consistent with an important role for cell background in the response to PTPRR expression, over-expression of PTPRR did not reduce either phosphorylation of ERK1/2 or the expression of c-fos, fra1 or c-jun in the HEK293 cell background (Figure 3C). To test whether endogenous levels of PTPRR alone were sufficient to repress phosphorylation of ERK in the absence of other signals in androgen-treated LNCaP cells, we depleted PTPRR from androgen-depleted cells. Even though over-expression of PTPRR in androgen treated LNCaP cells was sufficient to repress ERK1/2 phosphorylation, depletion of PTPRR alone by esiRNA in steroid depleted LNCaP cells was insufficient to restore ERK1/2 phosphorylation (Figure 3D). This result suggests that although PTPRR has an important role in this pathway, additional androgen-regulated proteins are involved in modulating ERK1/2 phosphorylation in response to androgens. These additional genes likely include other members of the RAS/ERK1/2 pathway (identified by our IPA pathway analysis in Figure 1).

**Over-expression of PTPRR reduces LNCaP cell proliferation**

The down-regulation of c-fos, fra1 and and c-jun expression in stable LNCaP cells over-expressing PTPRR suggested that androgen-mediated down-regulation of PTPRR expression might be important for proliferation of LNCaP cells. To test for such an effect on cell proliferation, we measured the proportion of cells in S-phase in our stable LNCaP cell line expressing PTPRR, and control LNCaP cells, using incorporation of EdU. Consistent with a reduced rate of proliferation, LNCaP cells over-expressing PTPRR in the presence of androgens had a reduced amount of cells in S phase over a six hour period (from 21.2% to 18.8%) relative to control cells (p < 0.04) (Figure 3E). A decrease in LNCaP cell growth in response to PTPRR expression was confirmed using an MTT assay, where there was a significant reduction in cell proliferation in PTPRR-expressing cells (p < 0.003) (Figure 3F).

**Low expression of PTPRR in Invasive PCa cell lines and Clinical PCa tissue**

We next examined PTPRR mRNA expression in a panel of PCa cell lines of different invasive capabilities, and with differing expression of PTEN (Figure 4A). Consistent with AR-dependent down-regulation, there was reduced PTPRR expression in the casodex resistant LNCaP derivative cell line relative to the androgen sensitive LNCaP cells (p < 0.003) (Figure 4A). Consistent with potential changes in PTPRR expression over disease progression, in these cell line models there was a low level of expression of PTPRR in PC3 cells, but expression was undetectable in their metastatic derivative PC3M.

The above data show that decreased PTPRR expression can be important to prostate cancer cell proliferation through modulation of the MAPK/ERK pathway, and this might be modulated dependent on cellular background including PTEN status. We examined PTPRR gene expression in prostate cancer clinical samples using previously published datasets that are publically available. Comparison of PTPRR expression by Affymetrix Array of 14 samples reported a 3.381 fold reduction in prostate cancer relative to normal tissue [23], and a 4.686 fold reduction in metastatic versus primary prostate cancer [24] (Figure 4B). PTPRR expression was also significantly reduced in 3 additional previously published datasets [25-27] with 2 other datasets showing no significant changes in PTPRR gene expression [11,28].

We tested PTPRR protein expression in a small panel of clinical PCa samples. Although this was a small sample
Figure 2 (See legend on next page.)
set, the data suggested PTPRR has a heterogeneous expression profile in prostate tumour clinical samples. PTPRR expression was detected in half the samples but either low or undetectable in 3/6 samples (samples 3, 4 and 6) despite high expression of four other control proteins (not shown). PTEN expression was also detected in 5/6 of these clinical PCa samples, but one tumour (sample 3) had low levels of both PTPRR and PTEN. Although this sample of tumours is small, these data suggest individual PCa patients have heterogeneous patterns of PTPRR expression relative to other potential modifier genes (Figure 4C, western blot with patient information shown below), and might contribute to the known heterogeneity of prostate tumours.

Discussion

A common feature of aggressive PCa is hyperactivation of the RAS/ERK1/2 pathway [10,11]. ERK1/2 signalling is known to play an important role in PCa development [29] and activation of ERK1/2 has been correlated with malignancy [10-12]. One possible mechanism for RAS/ERK1/2 hyper-activation is loss of function of key negative regulators of the pathway, such as the Sprouty genes. SPRY1 and SPRY2 function primarily as physiological negative regulators of the RAS/ERK1/2 pathway and act to suppress prostate tumourigenesis [13,30]. SPRY1 and SPRY2 are commonly inactivated in PCa where they are linked to disease progression [22,31,32]. Using network analysis and a meta-analysis of prior exon array data, we identify concomitant down-regulation of a novel androgen-regulated gene, phosphatase receptor type R (PTPRR) as well as the known RAS/ERK1/2 pathway negative regulator SPRY1.

Here we show data to suggest that PTPRR is a direct AR target gene, and is rapidly repressed by androgens in LNCaP cells. We also demonstrate that over-expression of PTPRR in androgen stimulated cells is sufficient to decrease phosphorylation of ERK1/2 and reduce both the expression of oncogenic transcription factors and proliferation of prostate cancer cells. The PTPRR gene encodes a classical transmembrane protein-tyrosine phosphatase (PTP) receptor type R (PTP RR). PTPRR is normally expressed in the brain, placenta, small intestine, stomach, uterus and weakly in the prostate [33]. Mouse gene Ptprr encodes multiple protein tyrosine phosphatase receptor type R (PTP RR) isoforms, which display distinct patterns of expression during neural development [34,35], and negatively regulate mitogen-activated protein kinase (MAPK) signalling pathways; both ERK1 and ERK2 are hyperphosphorylated in the brains of mice deficient for PTPRR [36]. In cell lines PTPRR has been shown to dephosphorylate p44/42 ERK1/2 in response to growth factors [37,38]. Repression of PTPRR expression via methylation has been detected in pre-cancerous colorectal lesions and in cervical adenocarcinoma [39,40], and in cervical cancer PTPRR may have a role in metastasis and be a biomarker of invasive cervical cancer [21]. PTPRR expression has also recently been identified as a prognostic indicator in oral squamous cell carcinoma [41].

Prostate tumours show huge biological heterogeneity, with some patients living for 20 years with organ confined disease, while others progress to lethal metastatic disease within 2 years of diagnosis. A deeper understanding of this genomic diversity will help identify genomic changes which can help distinguish indolent from aggressive PCa. The RAS/ERK1/2 signalling pathway is mutated in 43% of primary PCa tumours and 90% of PCa metastases [11]. Although PTPRR is only mutated in 1% of PCa tumours [11], our data demonstrates that it is a key component of the clinically important RAS/ERK1/2 signalling pathway, and that its expression level can have a clear affect on the activity of this pathway. Recent work has shown that although RAS/ERK1/2 activation alone cannot initiate PCa development, RAS/ERK1/2 and PTEN loss cooperate to promote EMT and metastasis initiated from PCa progenitor cells [12]. Our data also indicate that the importance of PTPRR expression on the ERK1/2 pathway activity is cell line and context dependent, suggesting cell background is important. One potential source of this cell background
Figure 3 Expression of *PTPRR* in androgen treated LNCaP cells reduces ERK1/2 signalling and decreases cell proliferation in the presence of androgens. (A) Over-expression of PTPRR in our LNCaP stable cell lines was confirmed by real-time PCR and by western blotting (A, left and middle panels). Analysis by western blot revealed that cells over-expressing PTPRR had decreased levels of phospho-ERK1/2 (pERK1/2). Actin was used as a loading control (A, middle panels). Total levels of ERK2 did not change in these cells (A, right panel). (B) Analysis of three transcription factors: fra1, c-fos and c-jun, in our stable LNCaP cells by real-time PCR in the presence of androgens revealed decreased expression levels for all three targets in cells over-expressing PTPRR. fra1, c-fos and c-jun are downstream targets of pERK1/2. (C) Western blotting confirmed our LNCaP cells to be PTEN negative and our HEK293 cells to be PTEN positive (C, upper left panels). In contrast to what was seen for LNCaP cells, there is no change in pERK1/2 levels in HEK293 cells over-expressing PTPRR. There was no change in total ERK2 levels. α-tubulin was used as a loading control (C, lower left and lower right panels). There was also no change in the expression of fra1, c-fos or c-jun expression as detected by real-time PCR (C, upper right panel). (D) esiRNA mediated depletion of PTPRR in steroid depleted LNCaP cells. Despite loss of PTPRR, there is no effect on the levels of pERK1/2 (upper panel lanes 3-6) or on the expression of fra1, c-fos or c-jun (lower panel, real-time PCR). (E) LNCaP cells over-expressing PTPRR and grown in the presence of androgens, had a lower percentage of cells in S phase, as detected by EdU incorporation (*p* = 0.039), and also had an impaired rate of proliferation as measured by MTT assay (*p* < 0.003). (F)
might be PTEN. We recently found a synergistic effect in murine PCa progression between loss of PTEN and SPRY2 [22]. We speculate that similar to SPRY2 the effects of PTPRR on ERK1/2 signalling might only be exerted in clinical prostate cancer in the absence of PTEN (e.g. patient 3 in Figure 4C). Further in vitro and in vivo studies are required to determine whether loss of PTPRR is a consistent event in PCa, and whether reintroduction of PTPRR expression may limit the progression of CRPCa.

Conclusions
In summary, our study has identified the protein tyrosine phosphatase PTPRR as an early and direct target of the androgen receptor that is rapidly repressed by androgens in prostate cancer cells. Further characterisation of PTPRR protein in LNCaP cells revealed it negatively regulates the RAS/ERK1/2 pathway and reduces cell proliferation in response to androgens. Our data suggest that loss of PTPRR protein might contribute to hyperactivation of the RAS/ERK1/2 pathway and has important implications for the development of therapies targeting this pathway.

Additional files

Additional file 1: Figure S2. The specificity of the PTPRR antibody used was confirmed by peptide blocking with the corresponding immunising peptide.

Additional file 2: Table S2. Primer sequences used.

Additional file 3: Table S1. IPA Pathway analysis scores.
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Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
JM carried out the majority of the experiments, participated in experimental design and interpretation of results, and drafted the manuscript. NPL assisted with some of the molecular experiments and was involved in the conception of the study and the interpretation of results. GK and PR carried out the IPA pathway analysis. CNR and HYL were involved in the analysis and interpretation of data, and were involved in critically revising the manuscript. DJE and JAF conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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