

*Original Article*

# **Activated androgen receptor and cyclin dependent kinase 1 are highly expressed in cases of Malaysian prostate cancer and are associated with high Gleason's score; expression is attenuated in prostate cancer cells following treatment with curcumin but not after treatment with diosgenin.**

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**Running title:** The androgen receptor in prostate cancer and the *in vitro* effects of curcumin and diosgenin.

## Abstract

**Ethnopharmacological relevance:** The incidence of prostate cancer in Asia is rising but traditionally occurs at a lower rate than western countries, and differences in diet and lifestyle are thought to play a major role. Malaysia is representative of the three most populous ethnicities in Asia; Malay, Chinese and Indian. In this study we investigate whether curcumin, a natural colouring and flavouring of Malaysian curries, derived from the rhizome of turmeric, and diosgenin isolated from fenugreek and wild yam, extensively used in Chinese traditional medicines, have the ability to suppress prostate cancer cell growth through their effects on the expression of the androgen receptor (AR). Moreover, in clinical samples from Malaysian men with prostate cancer we investigate whether expression of the AR is related to clinical features of the disease.

**Aim of the study:** To determine the clinical relevance of AR expression in Malaysian men with prostate cancer, and whether curcumin and diosgenin, components of the Malaysian diet and traditional medicine, respectively, are able to suppress prostate cancer cell growth through suppression of the AR.

**Materials and Methods:** Fifty-nine cases of adenocarcinoma of the prostate were tested for expression of AR to include its phosphorylation at serine 81, and expression of cdk1 by immunohistochemistry. Expression was related to clinical and pathological features of the disease, to include tumour stage and grade (Gleason Score). The effects of treatment with curcumin, diosgenin and Casodex on prostate cancer cell growth, AR expression, AR regulated gene expression and cyclin dependant kinase-1 (cdk1) expression were investigated in the androgen dependant and independent prostate cancer cell lines, LNCaP and PC-3, respectively.

**Results:** AR was highly expressed in Malaysian cases of prostate cancer and was significantly associated with the most aggressive grades with high Gleason scores ( $p=0.034$ ). In addition, AR expression was significantly associated with its phosphorylation on serine 81 ( $p<0.001$ ) and the phosphorylation of cdk1 at threonine-161 ( $p<0.001$ ). Treatment of prostate cancer cell lines with curcumin significantly attenuated prostate cancer cell line viability and expression of the AR, Cdk1 and the AR regulated gene, TMPRSS2. In contrast, diosgenin significantly increased prostate cancer cell line viability and expression of AR and TMPRSS2.

**Conclusions:** AR expression is highly expressed in prostate cancer and is associated with high tumour grade. Curcumin, a common ingredient of the Malaysian diet may protect against aggressive forms of the disease by down regulating AR expression and its activation. In contrast, diosgenin, commonly used in Malaysian traditional medicines, increases prostate cancer cell viability and AR expression and therefore is unlikely to be protective against the aggressive forms of the disease..

## Graphical Abstract:

**Keywords:** Androgen Receptor, Prostate Cancer, Curcumin, Diosgenin

### Abbreviation:

AR, Androgen Receptor; Cdk1, Cyclin Dependent Kinase; DMSO, Dimethyl sulfoxide; HE, haematoxylin and eosin; IHC, immunohistochemistry; MTS, tetrazolium3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium; pAR<sup>S81</sup>, AR phosphorylated at Ser-81; pCdk1<sup>161</sup>, Cdk1 activated through Thr-161; PSA, prostate-specific antigen; TBS, tris-buffered saline.

### Chemical compounds studied in this article:

Curcumin, Diosgenin

### Introduction

There is increasing evidence that diet and lifestyle plays a major in prostate cancer tumorigenesis and this, at least in part, accounts for differences in prostate cancer incidence between Western and Asian countries (Ha Chung et al., 2018; Lassed et al., 2016; Muir et al., 1991; Rose et al., 1986; Shimizu et al., 1991) However, prostate cancer incidence rates are also rising sharply in developed Asian countries such as China, Korea, Japan and Singapore (Zhang et al., 2012). These increases are said to be due to a nutritional transition during the same period of time, defined as a steady shift towards a more Westernized diet by consumption of increased amounts of fat, and red meat and lower amounts of dietary fibre (Zhang et al., 2012).

Whilst there still exist differences in the clinical incidence and mortality in men of different ethnicities and nationalities, the pathological incidence of quiescent latent prostate cancer, as revealed by autopsy studies, appears to be the same irrespective of geography and ethnicity (Haas et al., 2008). If dietary factors, such as those common to an Asian diet are responsible for preventing indolent cancers developing into clinically relevant disease, then it is important to identify what these dietary factors are.

Diosgenin (molecular formula, C<sub>27</sub>H<sub>42</sub>O<sub>3</sub>; molecular weight, 414.61 Da) is a plant steroidal saponin that is isolated from fenugreek (*Trigonella foenum-graecum*) and Wild Yam (*Dioscorea* species) (Acharya et al., 2008), the latter having been extensively used in traditional Chinese medicines. Diosgenin is an important raw material in the synthesis of sex hormones, cortisone and oral contraceptives (Chaudhary et al., 2015). Preclinical and mechanistic studies have demonstrated that diosgenin possesses anti-tumour (He et al., 2014; Yang et al., 2013) and anti-diabetic (Kalailingam et al., 2014; Sato et al., 2014) properties, reduces blood lipid content (McKoy et al., 2014), is anti-inflammatory (Ebrahimi et al., 2014) a vasodilator (Manivannan et al., 2014), and protects the myocardium (Badalzadeh et al., 2014). In the context of anti-tumour activity, diosgenin has been demonstrated to inhibit the growth of multiple tumour types, including breast, oesophageal, liver and gastric cancer (Ding et al., 2015; Ghosh et al., 2015; Li et al., 2015; Mao

et al., 2012). However, few studies have looked at the effect of diosgenin on prostate cancer and the mechanism underlying its reported antitumor activity remains unclear.

Curcumin (diferuloylmethane) [1,7-bis (4-hydroxy-3-methoxyphenyl)-1, 6-hepta-diene-3,5-dione], a natural colouring and flavouring component of curry, is a lipophilic phenolic compound that is derived from the rhizome of turmeric (*Curcuma longa*), a spice widely used in Asian cuisine. Traditionally, turmeric has been used to treat various ailments, including coughs, sinusitis, hepatic disease and anorexia. The potential effectiveness of turmeric has been associated with the active compound, curcumin, well-known for its anti-inflammatory activities (Chuang et al., 2000; Cole et al., 2007; Garodia et al., 2007; Lim et al., 2001; Thiyagarajan and Sharma, 2004). Studies in the past have demonstrated pharmacology safety of curcumin and its anti-cancer effects in several types of cancer, which are attributed to its ability to modulate numerous targets and particularly cell kinases associated with cell survival, cell proliferation and cell cycle regulation (Lin et al., 2007; LoTempio et al., 2005; Wang et al., 2008). Although the precise mechanisms by which curcumin inhibits prostate cancer tumorigenesis prostate cancer have yet to be elucidated, it has been shown to downregulate the expression of the androgen receptor (AR) and AR-binding activity (Dorai et al., 2000; Nakamura et al., 2002; Tsui et al., 2008).

Prostate cancer cell growth depends upon an active AR even at the castrate resistant stage (Isaacs and Isaacs, 2004). Following the binding of androgen, the AR undergoes phosphorylation at serine residues (Gioeli and Paschal, 2012) which is thought to inhibit AR proteolytic degradation and stabilize AR homo-dimers (Blok et al., 1998). Phosphorylation of the AR may also influence transactivation of the AR, independent of androgen (Blok et al., 1998). The AR protein undergoes phosphorylation at serine/threonine and tyrosine residues, with phosphorylation of serine 81 (Ser81) at the AR N terminus being the most intensely phosphorylated site in response to androgen binding (Gioeli et al., 2002; Gioeli and Paschal, 2012). Ser81 phosphorylation has been shown to mediate chromatin binding and transcriptional activation (Chen et al., 2012) and has been associated with AR promoter selectivity and cell growth (Gordon et al., 2010).

Several kinases have been linked to AR phosphorylation. In particular, cyclin dependant kinase-1 (Cdk1) phosphorylates the AR on Ser81 and is associated *in vitro* with increased AR expression (Chen et al., 2006). Cdk1 is a member of the Cdk family with a fundamental role in cell cycle regulation and is reported to be upregulated in prostate cancer tissue (Kallakury et al., 1997).

In this study, we aimed to determine whether AR phosphorylation at Serine 81 and cdk1 expression are associated with clinical and pathologic features of a Malaysian cohort of patients diagnosed with adenocarcinoma of the prostate. Moreover, we investigate whether the phytochemicals curcumin and diosgenin, which are commonly consumed in Asia either as a dietary component (curcumin) or in traditional Chinese medicine (diosgenin), can abrogate prostate cancer cell line growth and expression of activated AR and Cdk1.

## **MATERIALS AND METHODS**

### ***Patients and Tissue Samples***

From a prospective PSA screening study initiated by Urologists from the Department of Surgery, University of Malaya, men shown to have elevated PSA levels subsequently underwent prostatic tissue biopsy. A total of 89 cases were shown to have adenocarcinoma of the prostate, with samples from 59 cases of these been available for the current study. All the tissue biopsies had been fixed in neutral buffered formalin for 24 hours, prior to tissue processing and embedding in paraffin wax. The patients' age at diagnosis, serum PSA level, Gleason score and TNM tumour stage based on The American Joint Committee on Cancer recommendations were retrieved from the pathology records. Serial sections of each case were stained with haematoxylin and eosin (HE) for routine histological examination and immunohistochemically tested for AR (to include its phosphorylation AR at serine-81) and phosphorylated Cdk1 at Threonine-161. Ethics approval for tissue collection was obtained from the University Malaya Ethics Committee (MREC ID No: 982.12).

### ***Immunohistochemistry***

Immunohistochemistry (IHC) was conducted on the tissue biopsies for the following proteins: Cdk1 activated through Thr-161 (pCdk1<sup>161</sup>), AR and AR phosphorylated at Ser-81 (pAR<sup>S81</sup>). Briefly, tissues were dewaxed in xylene and rehydrated through graded alcohol. The AR and pCdk1<sup>161</sup> antigen retrieval was performed in pH 9 Tris EDTA-buffer (10mM Trizma Base, 0.25mM EDTA) in a pre-heated water bath at 99°C for 30 minutes, whilst the same procedure was used for staining with the pAR<sup>S81</sup> antibody but using citrate buffer (pH6). Sections were cooled in buffer for 20 minutes before immersing in 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase. Non-specific secondary antibody binding was blocked using 5% Goat Serum in TBS before primary antibody incubation overnight at 4°C. Antibodies for pCdk1<sup>161</sup> and AR were diluted at 1:100 and pAR<sup>S81</sup> at 1:8000, in tris-buffered saline (TBS) with 0.05% Tween 20. Bound antibody complex was detected using the Real EnVision kit (Dako UK Ltd), and the horse radish peroxidase label visualized by the application of H<sub>2</sub>O<sub>2</sub> substrate and the chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako UK Ltd). Nuclei were counterstained with Harris's haematoxylin (Leica, Germany) and blue in running tap water before air drying and mounting in Di-N-Butyl Phthalate in Xylene (DPX).

### ***Scoring of immunohistochemistry***

The immunohistochemically stained slides were blinded and assessed independently of clinical features. Only nuclear expression was scored as this represents the active form of the protein. Staining was recorded as positive when either part or the whole of the tumour compartments showed positive staining.

### ***Statistical analysis***

Statistical analysis was performed using SPSS version 20.0 for Windows (IBM SPSS, Chicago, IL, USA). Spearman's rank correlation coefficients (CC) was used to assess associations between protein expression. Mann-Whitney *U*-test or Kruskal Wallis test was used to assess relationships between protein expression and clinical and-pathological criteria. A <0.05 significance level was used and Bonferroni correction was used where applicable.

### ***Reagents and antibodies***

The anti-androgen drug, Bicalutamide (Casodex) was obtained from the pharmacy, University of Malaya Medical Centre. Curcumin was a kind gift from the Department of Pharmacology, University of Malaya. Diosgenin was purchased from Chemfaces (Hubei, China). Both phytochemicals were stored as stock solution in DMSO at -20°C. The primary antibodies were obtained from the following suppliers; AR (mouse monoclonal AR441, Dako UK Ltd), pAR<sup>S81</sup> (rabbit polyclonal, 07-1375, Merck Millipore, CA), pCdk1<sup>Thr161</sup> (rabbit polyclonal, ab47329, Abcam, Cambridge, UK).

### ***Cell lines and cell culture***

Androgen-sensitive, LNCaP cells were purchased from American Type Culture Collection (Rockville, MD, USA) whereas the androgen-independent PC-3 cell was a kind gift from Dr. Jason Lee (Faculty of Health and Medical Sciences, Taylor's University, Malaysia). All experiments were conducted in less than 6 months of continuous passage. Both cell lines were cultured in RPMI-1640 supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin (all purchased from Gibco, Thermo Scientific, Inc. Country). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and incubated for 48h, after which the medium was removed and replaced with fresh RPMI-1640 containing either 20µM curcumin, 5µM Diosgenin, 30µM Casodex or DMSO (untreated control) for 48h. A final concentration of less than 0.1% (v/v) DMSO was used in the culture medium. DMSO (0.1%) treated cells served as controls.

### ***Morphologic changes and Cell Viability Assays determinations MTS Assay***

LNCaP and PC-3 cells were plated onto a 96-well plate at a density of 1x10<sup>4</sup> cells/well and curcumin, diosgenin and Casodex were added at a final concentration of 20, 40 and 80uM; 5, 10 and 20uM; and 10, 30uM respectively. The cells were exposed to treatment for 72 hours and a phase-contrast microscope was used to observe morphologic changes in examined cells at the end of this time.

Cell viability was measured using a colorimetric proliferation assay using tetrazolium3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) using the CellTiter 96 Aqueous One Solution Proliferation Assay system (Promega, Tokyo, Japan) according to the manufacturer's instruction. Briefly, prostate cancer cells were harvested and seeded in a 96-well microtiter plate. The cells were treated with different concentrations of curcumin, diosgenin and Casodex for up to 72 hours whereas the control cells were untreated. After incubation, 20µl of MTS reagent was added to each well and incubated for 1–4 hours. Subsequently, the cells were placed in a 96-well microplate reader (Biotek, Winooski, VT, USA) for analysis and the absorbance were recorded at 490nm. Cell viability was evaluated using the following formula: Cell viability (%) = [1 - (OD of the samples/OD of the control)] x 100%.

### ***Extraction of total RNA***

Total RNAs were isolated from cultured cells using the RNeasy Plus Mini Kit (Qiagen). The concentration of RNA was calculated as µg/µl by Nanophotometer and the quality was evaluated by absorbance ratio of 260/280 nm. If not used immediately, the RNA was stored at -80°C until analysis.

### ***Real time PCR analysis***

RNA was converted to cDNA and analysed with the SYBR Green System using the One-Step BrightGreen qRT-PCR kit (ABM) according to manufacturer's protocol. Briefly, qPCR was performed in a reaction mixture containing 1X BrightGreen qPCR Master Mix (ABM), 1X qRT-PCR Enzyme Mix and 0.5 $\mu$ M of each primer. Cycling conditions were 1 cycle at 42°C for 15 minutes, 1 cycle at 95°C for 10 min, 40 cycles through a denaturing step at 95°C for 15 sec and 1 annealing-elongation step at 60°C for 1 min. The StepOne Plus Sequence Detection Software Version (Applied Biosystems) was used for the estimation of the C<sub>T</sub> parameter (threshold cycle). The 2<sup>- $\Delta\Delta$ C<sub>T</sub></sup> method was applied for the quantification and GAPDH was used as a housekeeping gene for the normalisation. All experiments were performed in duplicated and normalised with respect to GAPDH levels.

The sequences for primers are as follows: Androgen Receptor (Forward: 5'-CCTGGCTTCCGCAACTTACAC -3' and Reverse: 5'-GGACTTGTGCATGCGGTACTIONCA -3'), TMPRSS2 (Forward: 5'- CAGGAGTGACGGGAATGTGATGGT -3' and Reverse: 5'-GATTAGCCGTCTGCCCTCATTTGT -3'), Human Cdk1 (Forward: 5'-TGGATCTGAAGAAATACTTGGATTCTA -3' and Reverse: 5'- CAATCCCCTGTAGGATTTGG -3') and GAPDH (Forward: 5'- GAAGGTGAAGGTCGGAGTC -3' and Reverse: 5'-GAAGATGGTGATGGGATTTTC -3')

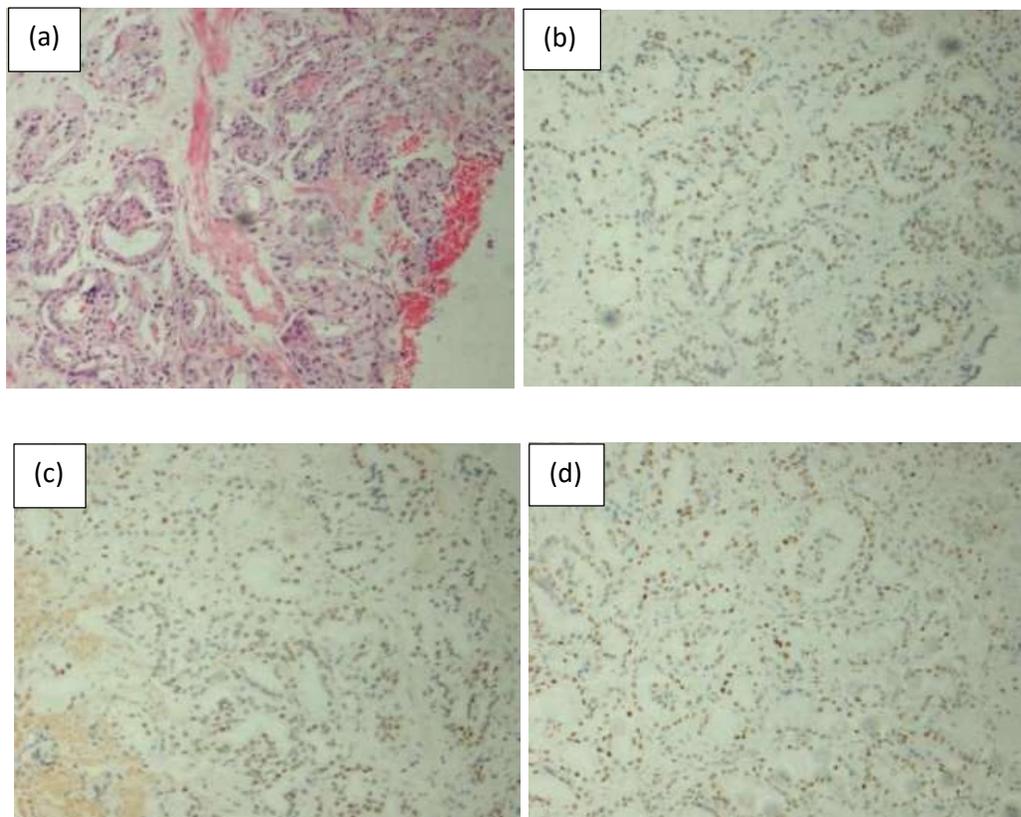
### ***Quantitative measurement and statistical analysis***

Mean and SD were calculated statistically from three experiments with triplicates and analysed by one-way analysis of variance followed by the Tukey's post-hoc test. Comparison between the groups was made by analysing data using a post-hoc method. Student's t-test or analysis of variance was used to statistically analyse the results between the control and treatment groups. GraphPad Prism 5 software (GraphPad Software, Inc, La Jolla, CA, USA) were used to perform all statistical analyses.  $P < 0.05$  was considered significant. Asterisks shown in figures indicate significant differences of experimental groups in comparison with the corresponding control conditions.

## RESULTS

### *Immunohistochemical results for AR, AR81 and CDK-1.*

Expression of AR, pAR<sup>s81</sup>, pCdk1<sup>161</sup> was predominantly nuclear in localization. Whilst there was some staining of adjacent stromal cells, the levels were relatively low compared to the strong positive staining of tumour nuclei (**Figure 1**). Using the scoring system described, 51/59 (86.4%) of the cases were positive for AR, 51/59 (86.4%) for pAR<sup>s81</sup> and 53/59 (89.8%) for pCdk1<sup>161</sup>. (**Table 1**). AR expression was significantly associated with its activation on pAR<sup>s81</sup> ( $p < 0.001$ ), which in turn was significantly associated with expression of kinase pCDK1<sup>161</sup> ( $p < 0.001$ ).



**Figure 1.** A representative case of prostatic adenocarcinoma stained with; haematoxylin and eosin (a), androgen receptor (b), pAR<sup>s81</sup> (c) and pCdk1<sup>161</sup> (d). Magnification 10x.

### *Correlation of immunohistochemical expression of AR, pAR<sup>s81</sup>, pCdk1<sup>161</sup> with clinical and pathological parameters*

Immunohistochemical expression of AR, was significantly associated with high grade prostate cancer (a high Gleason score,  $p = 0.034$ ), but not with other clinical or pathological variables (**Table 1**).

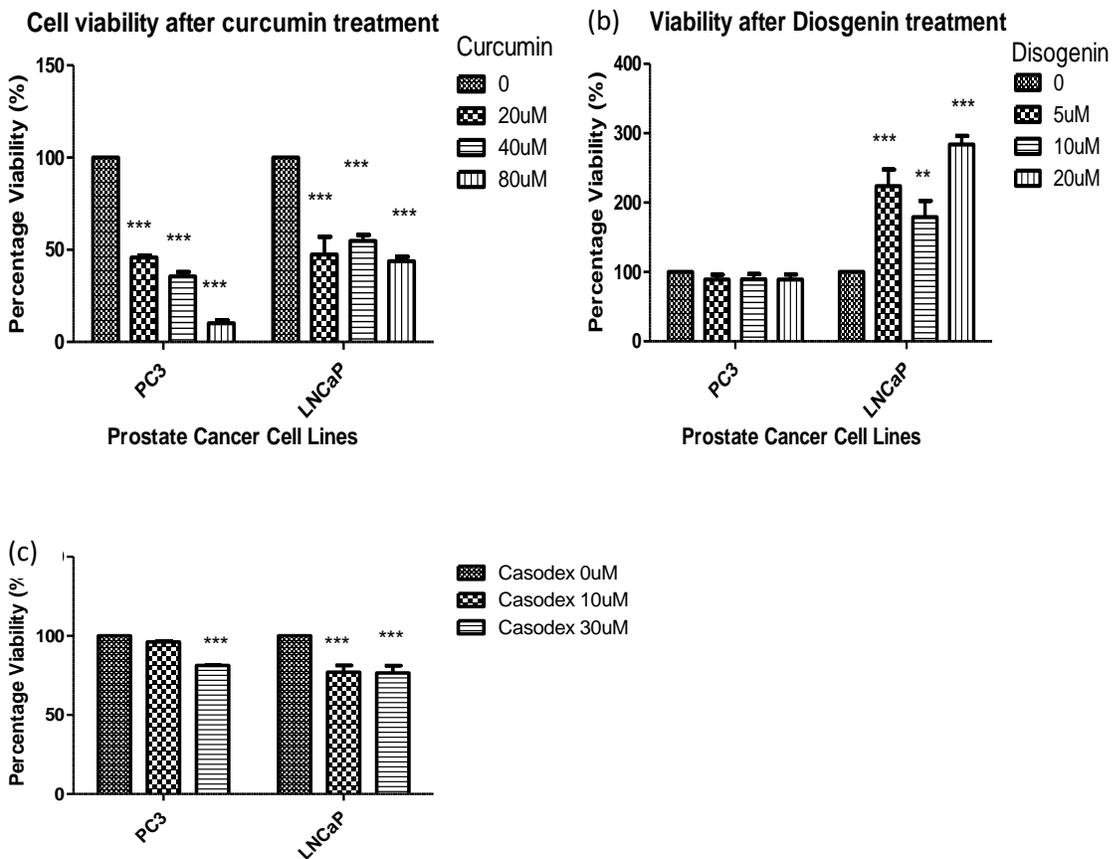
**Table 1.** Correlation of immunohistochemical *AR*, *pAR<sup>s81</sup>*, *pCdk1<sup>161</sup>* expression with clinical and pathological parameters on needle biopsy

	<i>AR</i>		<i>p</i>	<i>pARS81</i>		<i>p</i>	<i>pCdk1</i>		<i>p</i>
	-ve (n=8)	+ve (n=51)		-ve (n=8)	+ve (n=51)		-ve (n=6)	-ve (n=53)	
Mean age (range)	67.5 (53-82)	68.92 (52-81)	0.66	69.63 (53-80)	68.57 (52-82)	0.744	62.5 (53-76)	69.45 (52-82)	0.053
PSA (ng/ml)			0.393			0.287			0.335
(mean; range)	80 (8.6-357.6)	225.5 (1.5-2603)		37 (4.4-80)	228.7 (1.5-2603)		39.2 (6.6-99)	224.6 (1.5-2603)	
Gleason Score			<b>0.034</b>			0.218			1
<7	3 (37.5%)	9 (17.6%)		0 (0%)	12 (23.5%)		1 (16.7%)	11 (20.8%)	
7	0 (0%)	18 (35.3%)		12 (50%)	14 (27.5%)		2 (33.3%)	16 (30.2%)	
≥8	5 (62.5%)	24 (47.1%)		12 (50%)	25 (49.0%)		3 (50%)	26 (49.1%)	
TNM Staging			0.167			0.516			0.503
T1/T2	5 (62.5%)	15 (30.6%)		3 (37.5%)	17 (34.7%)		3 (50%)	17 (33.3%)	
T3	1 (12.5%)	7 (14.3%)		2 (25%)	6 (12.2%)		1 (16.7%)	7 (13.7%)	
T4, N1/Mx, M1	2 (25%)	27 (55.1%)		3 (37.5%)	26 (53.1%)		2 (33.3%)	27 (52.9%)	

### ***Cell viability after curcumin and diosgenin treatment of prostate cancer cell lines***

Both the androgen-dependent (LNCaP) and androgen-independent (PC3) cell lines were treated with varying concentrations of curcumin and diosgenin for 72 h and assessed for cell viability. Curcumin inhibited the growth of both LNCaP and PC3 prostate cancer cell lines in a dose dependent manner (Figure 2a). In contrast, diosgenin treatment increased the cell viability LNCaP cells but had no effect on PC3 cell viability (Figure 2b). For comparison, we compared the cell viability of the two cell lines following treatment with an established androgen antagonist, Casodex (bicalutamide) (Figure 2c). At 10µM, (the steady state concentration of medication used for treatment of men with prostate cancer) the inhibitory effect of bicalutamide was different in LNCaP and PC3 cells. Casodex inhibited growth of the androgen dependant LNCaP cells at the lower dose of 10uM but only showed inhibitory effects on the androgen independent PC3 cell line at a concentration of 30Um. The androgen sensitive cell line, LNCaP, was used for the remaining studies.

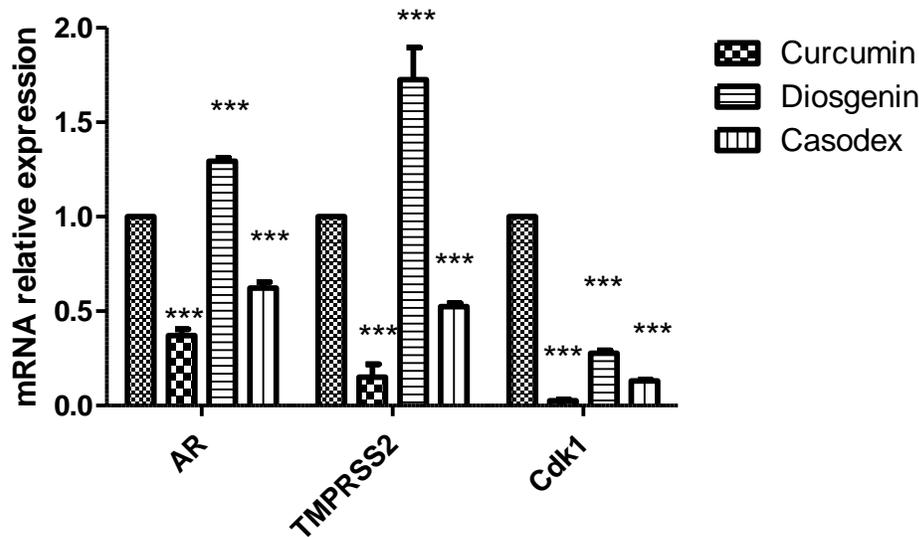
(a)



**Figure 2.** Effects on viability of the androgen dependent cell line (LNCaP) and androgen independent cell line (PC3) following treatment for 72 hours with the phytochemicals curcumin (a), Diosgenin (b) and the androgen antagonist Casodex (c). The percent cell proliferation was determined using an MTS assay by normalizing the proliferation of treated cells against proliferation of control treated cells. Mean  $\pm$  SE; n=3, \*p<0.05.

### ***AR gene expression and AR regulated gene expression after curcumin and diosgenin treatment***

We next evaluated the effect on AR, cdk-1 and an AR regulated gene (TMPRSS2) expression in the androgen dependant LNCaP prostate cancer cell line following treatment with curcumin, diosgenin or Casodex for 72 hours. Curcumin decreased levels of AR mRNA expression whilst diosgenin increased AR mRNA levels. Curcumin also decreased the expression of the AR regulated gene TMPRSS2, whilst diosgenin increased its expression. All 3 treatments significantly decreased the expression of cdk-1 (Figure 3).



**Figure 3.** The effect of curcumin, diosgenin and Casodex treatment at 72 hours on AR, TMPRSS2 and Cdk1 mRNA expression.

## DISCUSSION

Prostate cancer development and progression to metastatic disease have been known to be dependent on androgen signalling since the 1940's (Huggins and Hodges, 2002). Despite the development of numerous AR antagonists, advanced prostate cancer continues to depend on AR signalling by constitutive activation of the AR and its downstream pathways through multiple mechanisms such as overexpression of the AR protein, which enables AR activation even when there is only low levels of androgens (Waltering et al., 2009).

Mass spectrometry studies identify the most highly phosphorylated site on the AR after androgen stimulation to be Ser-81 (pAR<sup>S81</sup>) (Chen et al., 2006; Gioeli et al., 2002). The AR can be phosphorylated at Ser81 even at very low levels of androgen (Chen et al., 2006) and has the highest stoichiometric phosphorylation on AR in response to androgen (Gioeli et al., 2002) and in addition plays a role in AR transactivation. Loss of Ser-81 phosphorylation in LNCaP cells reduces AR transactivation and subsequent expression of the AR regulated genes PSA and TMPRSS2 (Chen et al., 2012). This finding indicates a role for pAR<sup>S81</sup> in regulating AR promoter selectivity.

The current study shows AR to be highly expressed in the prostate cancers of a cohort of Malaysian men and that there is significant association between AR expression and high Gleason score. Moreover, AR expression was significantly associated with its activation Ser-81 phosphorylation site on the AR and with the activated form of Cdk1 (pCdk1). Our study suggests that Cdk1 may drive this phosphorylation of the AR at Ser-81, resulting in increased tumour growth and consequently select for more aggressive forms of prostate cancer, reflected in a high Gleason score. Increased cdk1 activity could sensitize prostate cancer cells to low levels of androgen and may thereby contribute to AR activity in advanced disease (Chen et al., 2006).

Curcumin is extensively used as a spice or pigment in Asian cuisine, especially in curries. An epidemiological study suggested that the high intake of curcumin could partly explain the lower incidence of colon cancer in the Indian population (Mohandas and Desai, 1999). The chemo-

preventive potential of curcumin has been previously described in prostate cancer (Teiten et al., 2010). Curcumin has been shown to cause apoptosis and cell-cycle arrest with inhibited cell growth, activation of signal transduction, and transforming activities in both androgen-dependent and independent prostate cancer cells (Dorai et al., 2000; Goel et al., 2008; Salvioli et al., 2007); whereas studies on the effects of diosgenin on prostate cancer are still lacking.

Curcumin has been demonstrated to inhibit numerous molecules in prostate epithelial cells important to cancer formation and progression. Among these targets are transcription factors, receptors, intracellular kinases, cytokines and growth factors (Aggarwal, 2008). The effect of curcumin on the AR and its target PSA has been demonstrated by several independent investigators using endogenously expressed AR in LNCaP and ectopically expressed AR in PC-3 cells (Nakamura et al., 2002; Tsui et al., 2008). However, in these studies, curcumin was used at relatively high concentrations, typically  $>30\mu\text{M}$  which contra-indicates the attainable physiological concentration due to its poor bioavailability as reported in animal models and humans (Anand et al., 2007). The present study was undertaken to evaluate the potential efficacy of curcumin and diosgenin as prostate cancer preventive agents even under low concentrations, reflecting the attainable levels through dietary intake. The hypothesis that curcumin impacts on AR function in LNCaP cells is supported by other studies showing that curcumin inhibits AR expression mRNA (Choi et al., 2010; Teiten et al., 2012).

We then explored the potential signalling following treatment with curcumin and diosgenin on androgen-dependent prostate cancer LNCaP cells. qRT-PCR was performed to determine TMPRSS2 expression RNA expression levels. TMPRSS2 is a prostate specific, androgen responsive, transmembrane serine protease and its fusion with the coding sequence of the erythroblastosis virus E26 (Ets) gene transcription family member ERG, is reported to be the most prevalent gene fusion occurring in prostate cancer (Tomlins et al., 2008). The result of the present study demonstrates that the mRNA expression levels of TMPRSS2 in cells treated with curcumin decreased significantly.

Diosgenin, a steroidal sapogenin has been investigated in many studies to explore its potential interest in a wide range of medical conditions (Chen et al., 2015; Jayachandran et al., 2009; Mao et al., 2012; Raju and Mehta, 2009). Although studies have reported its therapeutic potential in cancer, studies of diosgenin on prostate cancer are still lacking. The present study demonstrated the ability of diosgenin to increase cell viability, especially on the androgen-responsive LNCaP cell line but had no effect on the androgen-unresponsive PC3 cell line. This finding is in contrast to what has been previously reported on PC3 and DU-145 cells (Chen et al., 2011). However, a study carried out on primary Sertoli cells demonstrated an increase in proliferation (Wu et al., 2015). It is also in keeping with its known steroidal characteristics (Chaudhary et al 2015) Following treatment with diosgenin, the current study shows an increase in AR gene expression, and increased expression of the androgen regulated gene, TMPRSS2. This is the first *in vitro* study of its kind to report these findings using the androgen dependant prostate cancer cell line LNCaP. The results reflect those of Wu et al.(2017), which demonstrated that treatment with *Tribulus terrestris* (TT) extracts, of which the most abundant constituents are tigogenin and diosgenin, caused an increase in AR protein levels in rats (Wu et al., 2017).

In summary, In a series of PSA detected prostate cancers reported on biopsy samples, we show that AR and its phosphorylation at serine 81 is highly expressed and is significantly correlated with expression of the active form of the cyclin dependant kinase -1 (pCdk-1) which is able to bring about the phosphorylation of AR at serine 81. Moreover, the increased AR expression is associated with aggressive forms of the prostate cancer with high Gleason Grade.

We then investigated the potential for common components in the Asian diet to inhibit the viability of prostate cancer cell lines by their attenuation of AR expression, Cdk-1 expression and an AR regulated gene, TMPRSS2.

The results of the study show that prostate cancer cell growth, in the LNCaP androgen dependent cell line, is significantly inhibited following treatment with the phytochemical curcumin. As this is accompanied by significantly reduced expression of; cdk-1, AR and the androgen regulated TMPRSS2, it suggests that this growth attenuation may act through the AR axis, and its activation by Cdk1. However, further mechanistic studies are required to confirm this. In contrast, the phytochemical diosgenin increased prostate cancer cell viability in the androgen dependant cell line, LNCaP, in addition to significantly increasing the expression of AR and TMPRSS2. However, Cdk1 levels were diminished suggesting that whilst diosgenin increases growth through the AR axis, this may not be via increased Cdk1 levels. Again, further studies are required to determine the mechanisms by which diosgenin increases prostate cancer cell viability.

### **Author contributions**

Conceived the study and designed the experiments: JSJ Tan, KC Ong, A Rhodes; Performed the experiments; JSJ Tan, KC Ong, Analysis of pathological data; DB-L Ong, Analysis of clinical data; A Razack, S Kuppusamy;, Analysis of cell line data: JSJ Tan, KC Ong; Wrote the paper: JSJ Tan, A Rhodes

### **Conflicts of interest**

All authors have declared no conflict of interest.

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