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Impaired expression of protein phosphatase 2A subunits enhances metastatic potential of human prostate cancer cells through activation of AKT pathway.

P Pandey

University of Nebraska Medical Center, ppandey@unmc.edu

Parthasarathy Seshacharyulu

University of Nebraska Medical Center, p.seshacharyulu@unmc.edu

Srustidhar Das

University of Nebraska Medical Center, sdas@unmc.edu

Satyanarayana Rachagani

University of Nebraska Medical Center, srachagani@unmc.edu

Moorthy P. Ponnusamy

University of Nebraska Medical Center, mpalanim@unmc.edu

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Authors

P Pandey, Parthasarathy Seshacharyulu, Srustidhar Das, Satyanarayana Rachagani, Moorthy P. Ponnusamy, Y Yan, Sonny L. Johansson, K Datta, Ming-Fong Lin, and Surinder K. Batra

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Impaired expression of protein phosphatase 2A subunits enhances metastatic potential of human prostate cancer cells through activation of AKT pathway

P Pandey^{1,4}, P Seshacharyulu^{1,4}, S Das¹, S Rachagani¹, M P Ponnusamy¹, Y Yan², S L Johansson^{2,3}, K Datta^{1,2}, M Fong Lin^{1,2} and S K Batra^{*,1,2}

¹Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE 68198-5870, USA;

²Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, USA and

³Department of Pathology and Microbiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE, USA

Background: Protein phosphatase 2A (PP2A) is a dephosphorylating enzyme, loss of which can contribute to prostate cancer (PCa) pathogenesis. The aim of this study was to analyse the transcriptional and translational expression patterns of individual subunits of the PP2A holoenzyme during PCa progression.

Methods: Immunohistochemistry (IHC), western blot, and real-time PCR was performed on androgen-dependent (AD) and androgen-independent (AI) PCa cells, and benign and malignant prostate tissues for all the three PP2A (scaffold, regulatory, and catalytic) subunits. Mechanistic and functional studies were performed using various biochemical and cellular techniques.

Results: Through immunohistochemical analysis we observed significantly reduced levels of PP2A-A and -B γ subunits ($P < 0.001$ and $P = 0.0002$) in PCa specimens compared with benign prostate. Contemporarily, there was no significant difference in PP2A-C subunit expression between benign and malignant tissues. Similar to the expression pattern observed in tissues, the endogenous levels of PP2A-A and B γ subunits were abrogated from the low metastatic to high metastatic and AD to AI cell line models, without any change in the catalytic subunit expression. Furthermore, using *in vitro* studies we demonstrated that PP2A-A α scaffold subunit has a role in dampening AKT, β -catenin, and FAK (focal adhesion kinase) signalling.

Conclusion: We conclude that loss of expression of scaffold and regulatory subunits of PP2A is responsible for its altered function during PCa pathogenesis.

Prostate cancer (PCa) is the second leading cause of cancer-related death among men in United States after lung cancer. In 2012, the estimated number of new cases and deaths caused by PCa are 241 740 and 28 170, respectively (Siegel *et al*, 2012). A majority of PCa patients are older men (over 65 years) and the disease is rarely seen in younger men (below 40 years) (Hankey *et al*, 1999). The median age for PCa diagnosis and death is 67 and 81 years,

respectively (Hoffman, 2011). The growth of PCa remains androgen-dependent (AD) in its initial stage. Hence, during the AD state, androgen deprivation therapy (ADT) leads to regression of the locally advanced PCa (Chuu *et al*, 2011). When PCa relapses after ADT, the disease progresses to an androgen-independent (AI) state and eventually hormone-refractory disease develops (Sharifi *et al*, 2005), which does not effectively respond to the existing

*Correspondence: Dr SK Batra; E-mail: sbatra@unmc.edu

⁴These authors contributed equally to this work.

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therapeutic strategies. Many oncogenes and tumour suppressor genes have been thoroughly investigated and they provide credence to the idea that alteration of their normal physiological actions and regulations can have an adverse impact on gene expression, resulting in various diseases including cancer. Genome-wide expression profiling and pathway prediction associates a number of up- and downregulated genes with cancer progression. Many candidate tumour suppressor genes like *Rb*, *p53*, *PTEN*, *CDKN2A*, *TP73*, *ICAM1*, *SOCS1/2*, *SPRY2*, *PPP2CA*, and *PPP3CA* have been shown to be downregulated in cancer, whereas many proto-oncogenes such as *AKT*, *PKC*, *mTOR*, *c-fos*, *c-jun*, *HGF*, *KRAS*, *MET*, and *EGFR* have been shown to be upregulated (Singh *et al*, 2008).

Uncontrolled cell growth, metabolism, proliferation, migration, and motility of cancer cells are regulated by various kinases and phosphatases (Cohen, 2001). Protein phosphatase 2A (PP2A) is highly conserved across a variety of eukaryotic species and accounts for as much as 1% of the total cellular protein (Wera and Hemmings, 1995). Protein phosphatase 2A is one of the major serine threonine phosphatases and structurally it is composed of three subunits: catalytic (C), scaffold (A), and regulatory (B) subunit, with functional variability (Zhang and Claret, 2012). Protein phosphatase 2A exists in two different forms: core enzyme and holoenzyme (Kremmer *et al*, 1997). The core enzyme is composed of the scaffold subunit (PP2A-A) and the catalytic subunit (PP2A-C) and is present in the heterodimeric form. The spatial and temporal diversity of this enzyme is provided by the regulatory subunit (Virshup and Shenolikar, 2009) and it interacts with the heterodimeric core enzyme to form the heterotrimeric holoenzyme. The PP2A holoenzyme exists in soluble form and gets compartmentalised in the cytosol, nucleus, mitochondria, cytoskeleton, and organelle membranes (Inagaki *et al*, 1994). Both the PP2A scaffold and the catalytic subunit exist in two different isoforms α and β , among which α form is predominant in nature. The regulation of PP2A activity mainly relies on the regulatory B subunit, which determines substrate selectivity, subcellular localisation, and regulates the catalytic activity in a wide range of biological processes (Yang *et al*, 2003; Yang and Phiel, 2010). The regulatory subunit is complex and diverse in nature and constitutes four different families: B (B55/PR55), B' (B56/PR61), B'' (PR48/PR72/PR130), and B''' (PR93/PR110). To provide further complexity, each member of the regulatory subunit is subdivided into various isoforms like α , β , γ , δ , and ϵ (Kamibayashi *et al*, 1994; Shi, 2009).

Chen *et al* (2004) have demonstrated that suppression of a specific regulatory subunit PP2A-B' γ is sufficient for human cellular transformation by displacing PP2A-B' γ from the AC dimer altering the PP2A-specific phosphatase activity in human embryonic kidney epithelial cells (Chen *et al*, 2004). Somatic alterations of PP2A-A α , A β , and B' γ are associated with breast, lung and colorectal cancer, and soft tissue sarcomas (Wang *et al*, 1998; Ruediger *et al*, 2001; Esplin *et al*, 2006; Perrotti and Neviani, 2008; Grochola *et al*, 2009). Functional haploinsufficiency of PP2A-A α or loss of PP2A-A α are defective in binding with B' γ , resulting in complete loss of heterotrimeric complex formation, eventually leading to cellular transformation (Chen *et al*, 2005), thus supporting the tumour suppressor nature of PP2A (Mumby, 2007). In addition, earlier reports of Cho *et al* (2007) and Xu *et al* (2006) have explained about the holoenzyme interaction of PP2A-A/PP2A-C with the specific regulatory B subunit B56 γ . Protein phosphatase type 2A has an important role in DNA replication, cell cycle, signal transduction, and cytoskeleton dynamics (Janssens and Goris, 2001). It positively regulates apoptosis and negatively regulates the mitogenic pathway, suggesting that loss of it might be involved in cancer development and progression (Perrotti and Neviani, 2008). In various malignancies, such as lung, breast, colon, gastric, and leukaemia, it has been shown that PP2A is downregulated and its role in transformation clearly defines PP2A

as a tumour suppressor gene (Westermarck and Hahn, 2008). In prostate epithelia, various growth factors, receptors, and androgens coordinately regulate cell proliferation by altering the equilibrium between phosphorylation and dephosphorylation. Studies have shown that the post-translational modifications of the androgen receptor, such as active phosphorylation, facilitates androgen-binding capacity and aids in the activation of the DNA-binding domain during transformation (Brinkmann, 2001). Previous studies have shown that the androgen receptor is regulated by phosphorylation and dephosphorylation. Insufficient dephosphorylation of the androgen receptor leads to continuous activation of the androgen receptor and enhances the androgen receptor-mediated signalling pathways.

The aim of this study was to elucidate the expression pattern at transcriptional and translational level of the scaffold, catalytic and regulatory subunits of PP2A in AD and AI PCa cells, and in benign and cancerous tissues. The methods in this study included immunohistochemical analysis, quantitative real-time PCR, and western blot assay to investigate the expression pattern of PP2A subunits in PCa progression. Furthermore, we extended our study by knocking down of PP2A scaffold subunit to identify whether the loss of PP2A scaffold subunit expression may be one of the mechanisms contributing to PCa progression. Overall, our results suggest that even if there is no significant change in the expression level of the catalytic subunit of PP2A, the differential expression or loss of expression of the scaffold and regulatory subunits may lead to altered function during the pathogenesis of PCa.

MATERIALS AND METHODS

Cell culture. Human PCa cell lines were obtained from various sources: LNCaP cells having passage number less than 33 were designated as clone 33 (C-33), which are generally AD cells, and more than 80 passaged cells were designated as clone 81 (C-81, AI) (Igawa *et al*, 2002; Singh *et al*, 2008), PC-3 (low metastatic, AI) cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and PC-3M (AI), a highly metastatic subline derived from liver metastasis of PC-3 cells, was obtained from Dr Fidler University of Texas MD Anderson Cancer Center, Houston, TX, USA. (Stephenson *et al*, 1992). PC-3 and PC-3M cell lines were maintained in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS) and antibiotics (100 $\mu\text{g ml}^{-1}$ penicillin and streptomycin) and LNCaP C-33 and C-81 cells were grown in RPMI 1640 culture medium supplemented with 5% FBS and antibiotics (penicillin and streptomycin 100 $\mu\text{g ml}^{-1}$). All cell lines were grown at 37°C with 5% CO₂ in a humidified atmosphere and media were changed every other day.

Antibodies and transfection reagents. The antibodies used are very specific for three different subunits. Protein phosphatase 2A catalytic subunit (catalogue no. 1512-s; Epitomic, Burlingame, CA, USA) reacts to both α - and β -isoform of catalytic subunit. The antibody does not crossreact among other family members of PP2A subunits. Antibody directed against PP2A-A scaffold subunit (81G5 rabbit monoclonal antibody, catalogue no. 2041-s; Cell Signalling Technology, Danvers, MA, USA) detects both α - and β -isoform of PP2A scaffold subunit. Protein phosphatase type 2A-B56- γ antibody (H-40, rabbit polyclonal, catalogue no. SC67038; Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognises the epitope corresponding to amino acids 431–470 near the C terminus of PP2A-B56- γ . Other antibodies used for signalling studies include: phospho-AKT, total AKT, active β -catenin, phospho-FAK (focal adhesion kinase) (Y576/577), total FAK, and β -actin (obtained from Cell Signalling). Horseradish peroxidase-coupled anti-mouse and anti-rabbit IgG secondary antibodies, and enhanced chemiluminescence reagent was

purchased from Amersham Biosciences (Buckinghamshire, UK). Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Two small-interfering RNA (siRNA) oligos targeted against human PP2A-A α (PPP2R1A), NM 014225 (Si-1 '5'-AGGCGGAACUUCGACAGUA-3') and Si-2 '5'-AAACUUAACUCCUUGUGCA-3') and scramble (SCR) oligo were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA) and were used for the transient knockdown study.

Immunohistochemistry analysis. Prostate tissue array having normal prostate tissues and multiple PCa (Gleason grade 6–10) tissue spots were obtained from Biomax (Bethesda, MD, USA). First, the paraffin-embedded TMA arrays were deparaffinised in xylene for 4 \times 10 min, each followed by rehydration through graded ethanol. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 30 min. Antigen retrieval was accomplished using 0.01M preheated citrate buffer (pH = 6.0, 90 °C) for 15 min and was allowed to cool at room temperature. The slides were then washed with phosphate-buffered saline (PBS) 2 \times 5 min each. Binding of nonspecific proteins were blocked by incubating the tissue slides with 2.5 U horse serum (ImmPRESS kit; Vector Labs, Burlingame, CA, USA) for 2 h. The sections were incubated overnight at 4 °C with primary antibodies, PP2A-C (1 : 500), PP2A-A α / β (1 : 50), and PP2A-B' γ (1 : 75) diluted in PBS. Slides were then washed with PBS 4 \times 10 min and incubated with the appropriate secondary antibody (peroxidase-labelled, universal anti-mouse/anti-rabbit IgG ImmPRESS kit; Vector Labs) for 1 h at room temperature. The slides were then washed using PBS 4 \times 10 min and the tissue sections were treated with DAB reagent to develop colour as per the manufacturer's instruction (DAB substrate kit; Vector Labs) and the sections were counterstained with haematoxylin (Vectors Lab). Finally, the slides were washed in tap water and were dehydrated in increasing grades of alcohol (20–100%), and then washed with xylene for 5 min and dried at 37 °C. After air drying, slides were mounted in paramount mounting medium (Fisher Scientific, Fair Lawn, NJ, USA). Slides were observed and photographs were taken using Nikon Eclipse E400 light microscope (Kawasaki, Japan).

Immunohistochemistry scoring. Stained tissue arrays were observed and graded by pathologist Dr Sonny L Johansson at UNMC (Omaha, NE, USA). The grading of PP2A-A-, B' γ -, and -C-stained intensity was scored on a 0–3+ scale and defined as follows: 0, no staining; 1+, weakly positive; 2+, moderately positive; and 3+, strongly positive. The percentage of the area stained was based on: 0–25% = 1, 26–50% = 2, 51–75% = 3, and 76–100% = 4. The composite score is the product of the percentage of positive cells per area and intensity of staining. The composite score is used to define the positivity and negativity of staining. A composite score of 0 is considered negative staining, whereas ≥ 1 is positive staining. Photographs of the representative area were taken.

RNA isolation and quantitative real-time PCR. Total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). The concentration of RNA was determined using Nano-Drop ND 1000 Spectrophotometer (Wilmington, DE, USA) and cDNA was synthesised by reverse transcription as described previously (Moniaux *et al*, 2008). Quantitative reverse transcription real-time PCR was carried out as described previously (Rachagani *et al*, 2011). Real-time PCR assay was performed on Roche Light Cycler 480 systems (Roche, Indianapolis, IN, USA). The primer sequences were designed using the Primer 3 software (Rozen and Skaletsky, 2000) and the sequences are given in Table 1. Polymerase chain reaction was then performed in 10 μ l reaction containing 5 μ l 2 \times SBYR Green Master Mix, 3.2 μ l of autoclaved nuclease free water, 1 μ l diluted RT product (1 : 10), and 0.2 μ l each of forward and reverse primers (5 pmol) for all three subunits. The amplification conditions used for all three subunits were a two-step cyclic process (95 °C for 10 min, followed by 45 cycles for 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s). The real-time PCR reactions were performed in triplicate and template controls were run for each assay under similar conditions. β -Actin was used as an internal control. The fold change of PP2A-A, -B' γ , and -C was calculated by Delta Delta CT method.

Protein isolation and western blot assay. Cells were washed two times with PBS and lysed in RIPA buffer (Tris-HCl 0.25 M, SDS 0.2%, pH 6.8, glycerol 20%, 1 mM NaF, 10 mM β -glycerol phosphate, and 1 μ l ml⁻¹ protease inhibitor cocktail; Sigma-Aldrich Corp., St. Louis, MO, USA). Scraped lysate was passed through a 25-G 7/8 needle and centrifuged at 4 °C for 25 min at 13 000 r.p.m. The protein concentration of each sample was quantified by using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Total proteins (40 μ g) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Blocking was performed in 5% dry milk in PBS for 2 h, and incubated with primary antibodies (at 1 : 1000 dilution) against PP2A-A, PP2A-B' γ , and PP2A-C) overnight, and then washed with PBST 4 \times 10 min. The corresponding secondary antibodies (at 1 : 2000 dilution) were added and incubated at room temperature for 1 h. After washing with PBST 4 \times 10 min, the signals were detected using enhanced chemiluminescence (ECL) reagents and membranes were exposed to ECL-sensitive X-ray films (Molecular Technologies, St. Louis, MI, USA).

Transfection of PCa cells with siRNA specific for PP2A-A α . For transient knockdown of PC-3, C-33 and C-81 PCa cells, we used 100 bp of siRNA oligonucleotides of human PP2A-A α -specific siRNA and SCR oligos using Lipofectamine 2000 transfection reagent. Cells that have been transfected for 72 h were considered for experimental studies such as migration assay and immunoblotting analysis. To demonstrate the effect of PP2A-A α knockdown on AKT phosphorylation, whole-cell lysates were collected for further analysis after 72 h of transfection and proteins were fractionated on 10% SDS-PAGE and blotted on PVDF membranes. Following blotting, membranes were probed with antibodies specific for PP2A-A α , phospho-AKT, total AKT, active

Table 1. The oligonucleotide primers sequence used for detecting the expression of PP2A-C, -B' γ , and -A α / β , in the RT-PCR analysis

Gene	Primer sequences	Amplified length (bp)	Annealing temperature (°C)
PP2A-C	Forward: 5'-TCGTTGTGGTAACCAAGCTG-3' Reverse: 5'-AACATGTGGCTCGCCTCTAC-3'	100	58
PP2A-B' γ	Forward: 5'-ACAGTGAAGGACGAGGCTCA-3' Reverse: 5'-CTTCCAAGGCTTCTTGGTG-3'	112	54
PP2A-A α / β	Forward: 5'-GCTTCAATGTGGCCAAGTCT-3' Reverse: 5'-GGTCTGGGTGCTCAGCTTCT-3'	102	55

Abbreviations: PP2A = protein phosphatase 2A; RT-PCR = Real Time -polymerase chain reaction.

β -catenin, phospho-FAK (Y576/577), and total FAK. The blots were re-probed with β -actin antibody to confirm equal loading of the samples in all the lanes.

Cell migration assays

Transwell migration and wound healing assay. Assays were adapted with slight modification as described previously (Senapati *et al*, 2010). For both procedures, PP2A-A α knockdown PC-3 were trypsinised and used to perform assays. First, to investigate the role of PP2A-A α in cell migration, we used Boyden chambers with 8- μ m pore size. Transient knockdown of PC-3 cells were seeded at the density of 1×10^5 cells per well in the upper chamber of the insert, while the lower chamber was filled with 10% FBS, which is used as a chemoattractant. After 24 h of incubation, the PC-3 cells remaining above the insert membrane were removed by gentle scraping with a sterile cotton swab. Cells that invaded through the pores to the bottom of the insert were fixed and stained with crystal violet for 10 min. Invading cells on representative sections of each membrane were counted under light microscopy. Under each experimental condition, the number of invading cells was calculated as the percentage of invaded cells compared with the SCR control cells. The experiment was performed in triplicate and *t*-tests were used to determine the statistical significance in migration between the SCR and knockdown cells. For wound healing assay, post-transfected PC-3 cells were trypsinised,

counted, and seeded at a density of 1×10^5 cells in 60-mm Petri dishes with 10% RPMI, with and without knockdown of PP2A-A α , for 48 h. After overnight incubation and with >90% confluence, a wound was induced with a sterile pipette tip. Images of wound areas were randomly chosen and images were obtained at 0 and 12 h, and the motility of the cells was compared between the SCR and knockdown cells.

RESULTS

Immunostaining of the scaffold subunit of PP2A-A α / β in benign and PCa tissues. The expression pattern of the PP2A-A α / β scaffold subunit in PCa and benign tissues was studied using IHC. A total of 175 tissue spots were stained for PP2A-A α / β , among which 29 ($n=29$) were benign and 146 ($n=146$) were cancer tissue spots. In benign prostate tissue, a strong expression of PP2A-A α / β in the epithelial cells was observed, while no staining was observed in the surrounding stroma (Figure 1A). Out of the 146 total cancer tissues analysed, 12.3% (18 out of 146) were positive for PP2A-A α / β . In the benign tissue, 48.2% (14 out of 29) were positive (Table 2). Protein phosphatase type 2A-A α / β exhibited both cytoplasmic and nuclear staining patterns and immunohistological staining was stronger in benign tissues compared with malignant tissues and the difference was

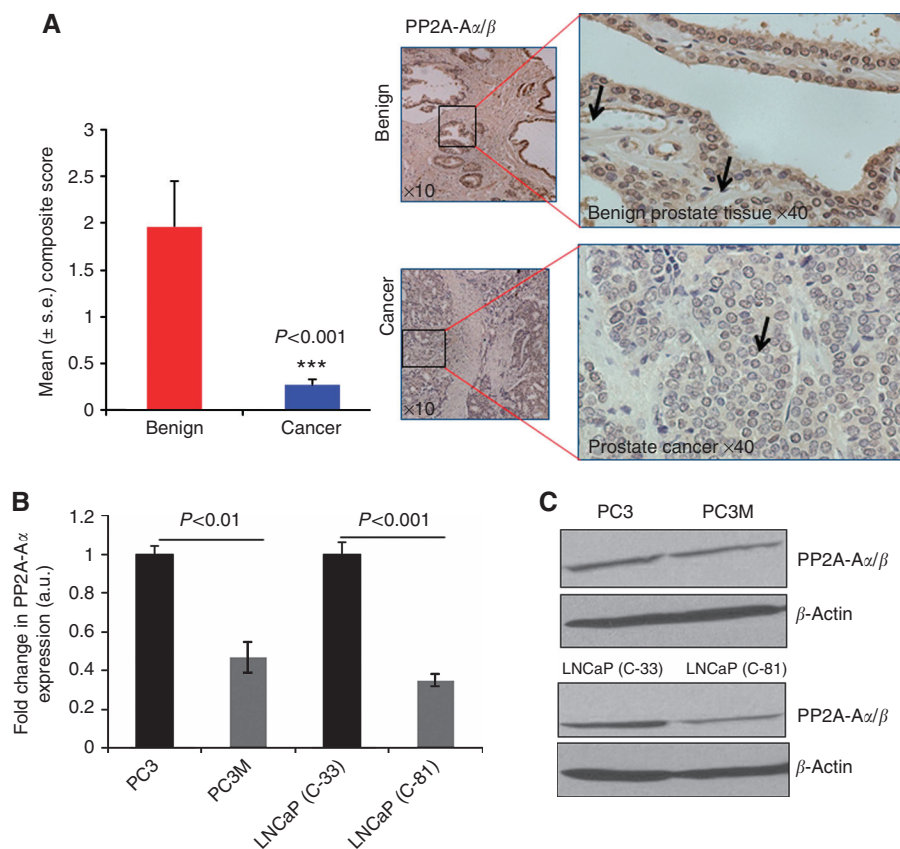


Figure 1. Immunohistochemical, real-time PCR, and western blot analysis of the PP2A scaffold subunit (PP2A-A α / β) in prostate tissues and cancer cells. **(A)** The mean composite score is plotted and a significant ($P < 0.001$) downregulation of the scaffold subunit (PP2A-A α / β) was observed in the prostate cancer tissues compared with benign prostatic tissue. The representative figure shows immunoreactivity of PP2A-A α / β in benign prostate and prostate cancer tissues. A distinct nuclear to diffused cytoplasmic staining was observed in benign tissue, whereas almost no staining was observed in the cancerous prostate tissues (black arrows). **(B)** Real-time PCR analysis using primers specific for PP2A-A α / β in LNCaP C-33, C-81, PC-3, and PC-3M shows downregulation of PP2A-A α / β in AI-LNCaP C-81 compared with AD-C-33 cells. Similarly, the PC-3 cells show a higher expression of PP2A-A α / β than in PC-3M. β -Actin was used as an internal control. **(C)** Western blot analysis shows a decreased expression of PP2A-A α / β in high metastatic cells (PC-3M, C-81) compared with low metastatic cells (PC-3, C-33).

Table 2. Comparison of the composite scores for positivity and negativity of PP2A-C, -B'γ, and -Aα/β staining between benign prostatic tissue and prostate cancer.

Tissue type	PP2A-C				PP2A-B'γ				PP2A-Aα/β			
	Pos.	Neg.	Total	P-value (χ ² test)	Pos.	Neg.	Total	P-value (χ ² test)	Pos.	Neg.	Total	P-value (χ ² test)
Benign	28 (90.3%)	3 (9.7%)	31	0.6	10 (52.6%)	9 (47.4%)	19	0.0002	14 (48.2%)	15 (51.8%)	29	<0.001
Cancer	58 (90.6%)	6 (9.4%)	64		22 (13.4%)	141 (86.6%)	163		18 (12.3%)	128 (87.7%)	146	
	86	9	95		32	150	182		32	143	175	

Abbreviations: Neg. = negative; Pos. = positive; PP2A = protein phosphatase 2A.

statistically significant (P -value <0.001) between benign and malignant prostate tissue (Figure 1A and Table 2). On the basis of the staining analysis, we observed that there is a gradual loss of PP2A-Aα/β expression during PCa pathogenesis, strongest being in normal and benign and very weak expression in the cancer tissue sections.

Expression of the PP2A-Aα/β scaffold subunit in low and high metastatic PCa cells. Upon performing quantitative real-time PCR, we observed that the expression of PP2A-Aα/β was downregulated at the mRNA level (2.1-fold less) in the highly metastatic PC-3M cells when compared with the low metastatic PC-3 cells (P <0.01) (Figure 1B). Similar results were observed in high metastatic LNCaP (C-81) cells as compared with low metastatic LNCaP (C-33) cells (P <0.001). In the LNCaP (C-81) cells, the PP2A-Aα/β was 2.8-fold less than the LNCaP (C-33) cells (Figure 1B). At the protein level, both in the PC-3M and LNCaP (C-81) cells, a significantly low expression of PP2A-Aα/β was observed compared with PC-3 and LNCaP (C-33) cells, which implies a downregulation of PP2A-Aα/β in androgen-insensitive and highly metastatic PCa cells (Figure 1C).

Downregulation of PP2A-Aα/β scaffold subunit leads to increase of phosphorylated Akt in PCa cells. On the basis of the fact that AKT is one of the major substrate of PP2A and AKT activation is dependent on the phosphorylation at serine residue 473. We further assessed AKT phosphorylation in the low metastatic PC-3 and high metastatic PC-3M cells, as well as in AD LNCaP (C-33) and AI LNCaP (C-81) cells by western blotting. Here, we compared the endogenous levels of phosphorylation on serine 473 of AKT in relation to that of PP2A-Aα/β downregulation in PCa cells. Phosphorylation of AKT-Ser473 increases in PC-3M and LNCaP (C-81) cells as compared with PC-3 and LNCaP (C-33) cells (Supplementary Figure 1).

Immunohistochemistry of the regulatory subunit PP2A-B'γ in benign and PCa tissues. In this analysis, 182 prostate tissue spots were stained. Out of 163 cancer tissue spots, 13.4% (22 out of 163) were positive for PP2A-B'γ, whereas 52.6% (10 out of 19) benign tissue were positive (Figure 2A and Table 2). Localisation of PP2A-B'γ was mostly in the nucleus and was also present in the cytoplasm of epithelial cells. No expression of PP2A-B'γ was observed in the surrounding stroma. Upon comparing prostatic adenocarcinoma and benign prostatic tissue, the expression of PP2A-B'γ was observed to be significantly decreased in the adenocarcinoma tissues (P -value = 0.0002), while it was uniformly lost in the highly metastatic cancer cells (Figure 2A and Table 2).

Expression of the regulatory subunit PP2A-B'γ in PCa metastatic cells. Regulatory subunit PP2A-B'γ was significantly downregulated at both the transcript and protein levels in highly metastatic PCa cells vs low metastatic PCa cells. The expression of PP2A-B'γ mRNA was significantly low in LNCaP (C-81) cells (3.33-fold less) when compared with LNCaP (C-33) cells

(P <0.05). In the PC-3 model, PC-3M cells (highly metastatic) had 1.5-fold less expression of PP2A-B'γ mRNA than in PC-3 cells (low metastatic) (Figure 2B) (P =0.6). At the protein level, the expression of PP2A-B'γ was low in the highly metastatic PC-3M and LNCaP (C-81) cells when compared with low metastatic PC-3 and LNCaP (C-33) cells (Figure 2C), indicating a gradual loss of PP2A-B'γ expression with the progression of PCa.

Immunoreactivity of PP2A-C catalytic subunit in benign and malignant PCa tissue sample. We demonstrated PP2A-C expression pattern in benign prostatic hyperplasia (BPH) and PCa tissue sample by performing IHC on commercially available PCa tissue microarrays. We screened a total of 95 tissue spots where 64 spots were malignant and 31 were benign (BPH) spots. Protein phosphatase type 2A-C was equally expressed in both BPH and cancer (Figure 3A and Table 2). With respect to localisation, PP2A-C was primarily expressed in the cytoplasm and the nucleus (Figure 3A). The positivity of PP2A-C expression in BPH/benign and malignant tissues were 90.3% (28 out of 31) and 90.6% (58 out of 64), respectively, and the difference was not statistically significant (P -value = 0.6) (Figure 3A and Table 2).

Expression of PP2A-C at mRNA and protein levels. In addition to performing immunohistochemical analysis in various pre-malignant and malignant PCa tissues, the expression pattern of PP2A-C was analysed at the mRNA and protein levels in PC-3 (low and high metastatic cell lines) and LNCaP (C-33 and C-81) PCa cell line models. For quantitative analysis, PP2A-C expression at the mRNA level was assessed by real-time PCR, where both PC-3 and PC-3M cells had comparable expression (P =0.3) with nonsignificant P -value and a similar expression pattern was obtained in LNCaP C-33 (AD) and LNCaP C-81 (AI) cell lines (Figure 3B) (P =0.9). Western blot studies further confirmed the mRNA expression pattern, as there was no difference in the level of PP2A-C protein between LNCaP (C-33) and LNCaP (C-81) cells, as well as between PC-3 and PC-3M cells (Figure 3C). Thus, the expression of PP2A-C is constant at both the mRNA and protein levels in those paired cell lines.

Effects of transient knockdown of PP2A-Aα on Akt signalling and invasive potential of PCa cells. By extending our previous observation on the scaffold subunit, two siRNA oligos against the PP2A-Aα were used for transient knockdown of PP2A-Aα in PC-3 and LNCaP cells. Scramble oligo-transfected cells were used as a control. Lysates were collected and probed with the respective antibodies. We looked at the impact of PP2A-Aα knockdown on the activity and protein levels of Akt, β-catenin, and FAK protein levels in PC-3 cells. As a result, the transient knockdown of PP2A-Aα in PCa cells led to a remarkable increase in the level of phospho-Akt, active β-catenin, and phospho-FAK compared with the SCR siRNA-transfected control (Figure 4A). Similar results were also demonstrated in LNCaP (C-33 and C-81) cell line model system, which showed that transient knockdown of PP2A-Aα

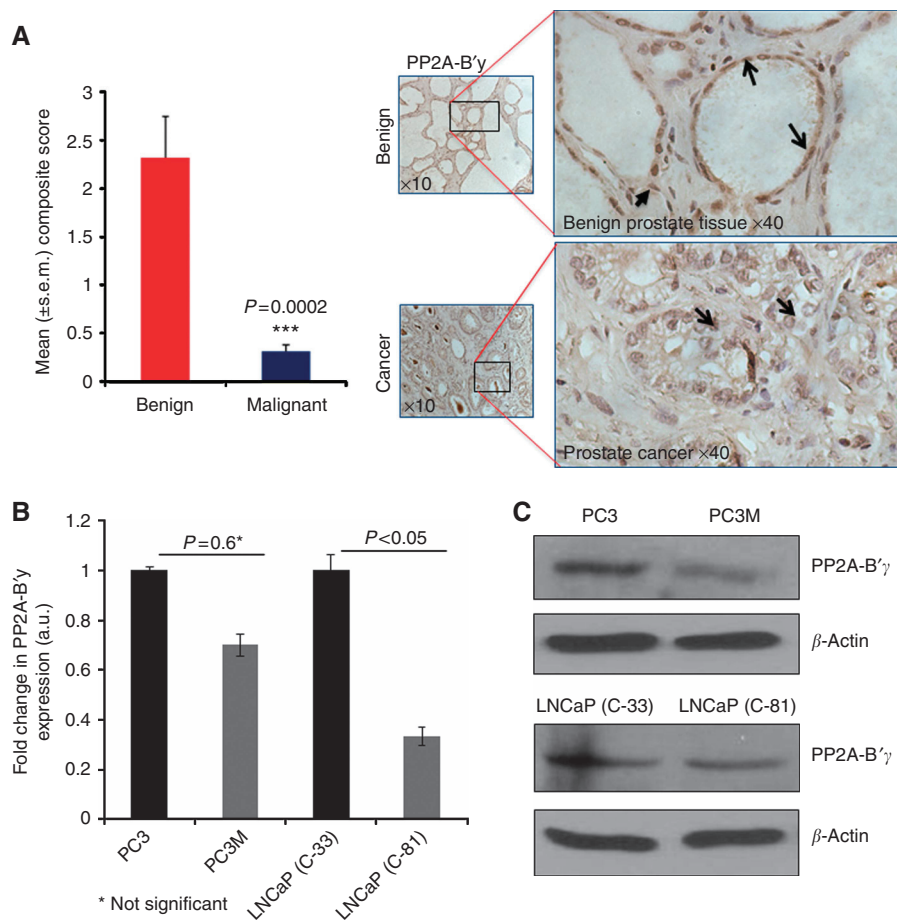


Figure 2. Protein phosphatase 2A regulatory subunit (PP2A-B' γ) expression pattern in benign and prostate cancer tissues and cell lines. **(A)** Immunohistochemical analysis of PP2A regulatory subunit (PP2A-B' γ) in benign and prostate cancer tissues. The mean composite score shows statistically significant ($P < 0.001$) downregulation of the regulatory subunit (PP2A-B' γ) in prostate cancer tissues compared with benign prostatic tissue. Representative tissue spots show prominent nuclear and cytoplasmic staining in benign tissue, whereas almost no staining was observed in the cancerous tissue (black arrows). **(B)** Quantitative real-time RT-PCR shows that regulatory (PP2A-B' γ) subunit mRNA was significantly downregulated in AI LNCaP (C-81) PCa cells compared with AD LNCaP (C-33) PCa cells in the LNCaP model. No significant downregulation of PP2A-B' γ mRNA was observed between PC-3 and its metastatic variant PC-3M cells. β -Actin was used as an internal control. **(C)** Immunoblotting analysis shows a significant decrease of PP2A-B' γ protein in LNCaP (C-81) and PC-3M cells compared with LNCaP (C-33) and PC-3 cells, respectively. β -Actin was used as loading control.

resulted in enhanced AKT-Ser473 phosphorylation with no change in the total AKT levels (Supplementary Figures 2 and 3).

To investigate the effect of PP2A-A α on cell motility, migration and wound healing assays were performed in PC-3 cells in which PP2A-A α was transiently knocked down for 48 h. As shown in Figure 4B, the migration potential of PP2A-A α knockdown cells significantly increased at 24 h in transwell migration assay. Similarly, quantification of the migration distance from wound healing assays showed that wound closure in PP2A-A α knockdown cells was significantly higher than the control cells transfected with SCR siRNA (Figure 4C). These results indicate that specific knockdown of PP2A-A α results in increased migration and motility property of PCa cells.

DISCUSSION

It has been postulated that phosphorylation and dephosphorylation are coordinated events that are governed and balanced by the activity of kinases and phosphatases (Bononi *et al*, 2011). Protein phosphatase type 2A is a ubiquitously expressed serine/threonine

phosphatase, which acts as key dephosphorylating enzyme for various signal-transduction pathways in eukaryotic cells (Millward *et al*, 1999). Although numerous studies have shown the role of PP2A as a tumour suppressor, the regulatory role of individual subunits, which is deregulated in cancer, remains to be defined. Recent evidence from the genomic and proteomic data clearly defines the critical role of PP2A subunits under both normal physiological and oncogenic conditions by regulating particular substrates (Muneer *et al*, 2002; Sablina *et al*, 2007). Further, because of the existence of several isoforms for each subunit, their ability to form a variety of heterotrimeric (70 different forms) could explain the diversity of the PP2A holoenzyme complex and its functional distinction (Janssens and Goris, 2001; Mumby, 2007; Janssens *et al*, 2008). Thus, decreased activity of any of these subunits might contribute to the proliferation of cancer cells. This study focuses on the differential expression of various PP2A subunits in benign and malignant prostatic tissues as well as how this differential expression favours proliferation in the PCa cell model system. To study the specific role of various subunits of PP2A, we have compared the differential expression pattern of PP2A-A α / β , -B' γ , and -C in benign prostatic tissue and malignant PCa tissue spots on a prostate TMA and in the clinically relevant

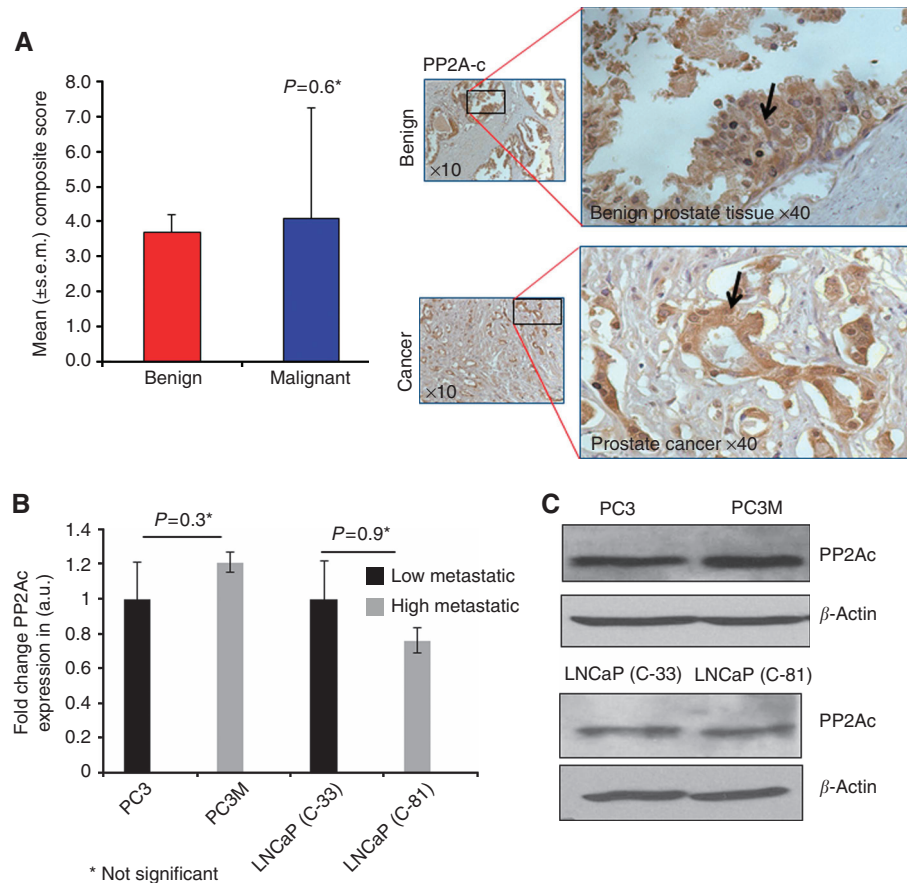


Figure 3. Expression of PP2A catalytic subunit (PP2Ac) in benign and prostate cancer tissues and cell lines. **(A)** Immunohistochemical analysis of PP2Ac staining between benign and prostate cancer did not show any significant difference using a composite score method. Representative images of prostate tissues stained for PP2Ac show cytoplasmic and nuclear staining (black arrows) in both BPH and prostate tumour. **(B)** Real-time PCR-based measurement of PP2A catalytic (PP2Ac) subunit in prostate cancer cells. Expression of PP2Ac at the transcript level is similar in both the prostate cancer cell models (PC-3 and LNCaP). **(C)** Western blot analysis showing an equal expression of PP2Ac in both the prostate cancer cell model systems (PC-3 and LNCaP cells) and β -actin was used as loading control.

PCa cell model system PC-3 and LNCaP cells, to explore the mechanism in both the AD and the AI status of PCa.

Previous work in various cancers such as breast, lung, and colon have shown that the cancer-derived mutant form *PPP2R1B* (PP2A structural subunit) may have an impaired function because of its inability to form a complex with the catalytic and regulatory subunits (Calin *et al*, 2000; Ruediger *et al*, 2001; Tamaki *et al*, 2004). Furthermore, Sablina *et al* (2007) have reported that the loss of the PP2A-A (scaffold) subunit can induce oncogenic transformation through activation of small GTPase RalA (Sablina *et al*, 2007). In our study, we observed a statistically significant reduction of the PP2A-A α / β scaffold subunit in cancer specimens when compared with benign tissues. Similarly, the endogenous level of PP2A-A α at both the protein and mRNA levels were significantly downregulated in C-33 and PC-3 cells as compared with C-81 and PC-3M high metastatic cell lines. Therefore, our study is in support of the somatic mutations of the PP2A-A α and - β subunits observed in colon, breast, and lung cancers, stating that alterations or loss of any of PP2A-A α and - β subunits can lead to an inhibitory effect by interfering with the interaction of the PP2A scaffold subunit and the PP2A catalytic subunit or regulatory subunit (Calin *et al*, 2000; Takagi *et al*, 2000; Tamaki *et al*, 2004). Also, earlier reports indicate that the loss of expression of the PP2A structural subunit may be an important factor for the failure of integrin dephosphorylation in breast cancer cells (Suzuki and Takahashi, 2003).

Second, we looked at the possible involvement of the regulatory (B) subunit in both tissues as well as in the cell line model systems.

Previous reports from many researchers have provided their ideology of PP2A interacting with small t (SV40 small t-antigen) protein, implying that small t might be directly interacting with the dimer or it displaces the third regulatory subunit from the holoenzyme complex. Among the PP2A-B family members, B56 or B' will participate in several regulating mechanisms, such as signalling, cell cycle check point, development, and apoptosis (Seeling *et al*, 1999; Li *et al*, 2002; Yang *et al*, 2003; Chen *et al*, 2004; Van Kanegan *et al*, 2005; Margolis *et al*, 2006). Studies from Chen *et al* (2004) have provided evidence that suppressing B56 γ seemed to mimic the ST-induced cell transformation, rather than the B55 regulatory subunit, thus inhibiting the PP2A-specific phosphatase activity (Chen *et al*, 2004). Our results show that immunohistochemical expression for the PP2A-B' γ subunit displayed a higher expression in benign tissues as compared with the malignant prostate tissue. Similarly, the mRNA and the endogenous level of the PP2A-B' γ subunit was strongly expressed in both AD C-33 and PC-3 (AI) cells when compared with AI C-81 and high metastatic PC-3M cells. This finding is consistent with the observation of Sablina *et al* (2010), who demonstrated that the suppression of specific regulatory subunits B56 α , B56 γ , and PR72/130, as well as PTPA, induced a transformed phenotype, implicating their role in cell transformation. Also, it is suggested that PP2A-B' γ was involved in the regulation of WNT signalling by interacting with APC tumour suppressor protein, thereby interfering with the APC-Axin complex formation, which may have a destabilising effect on β -catenin, and thus indirectly prevent

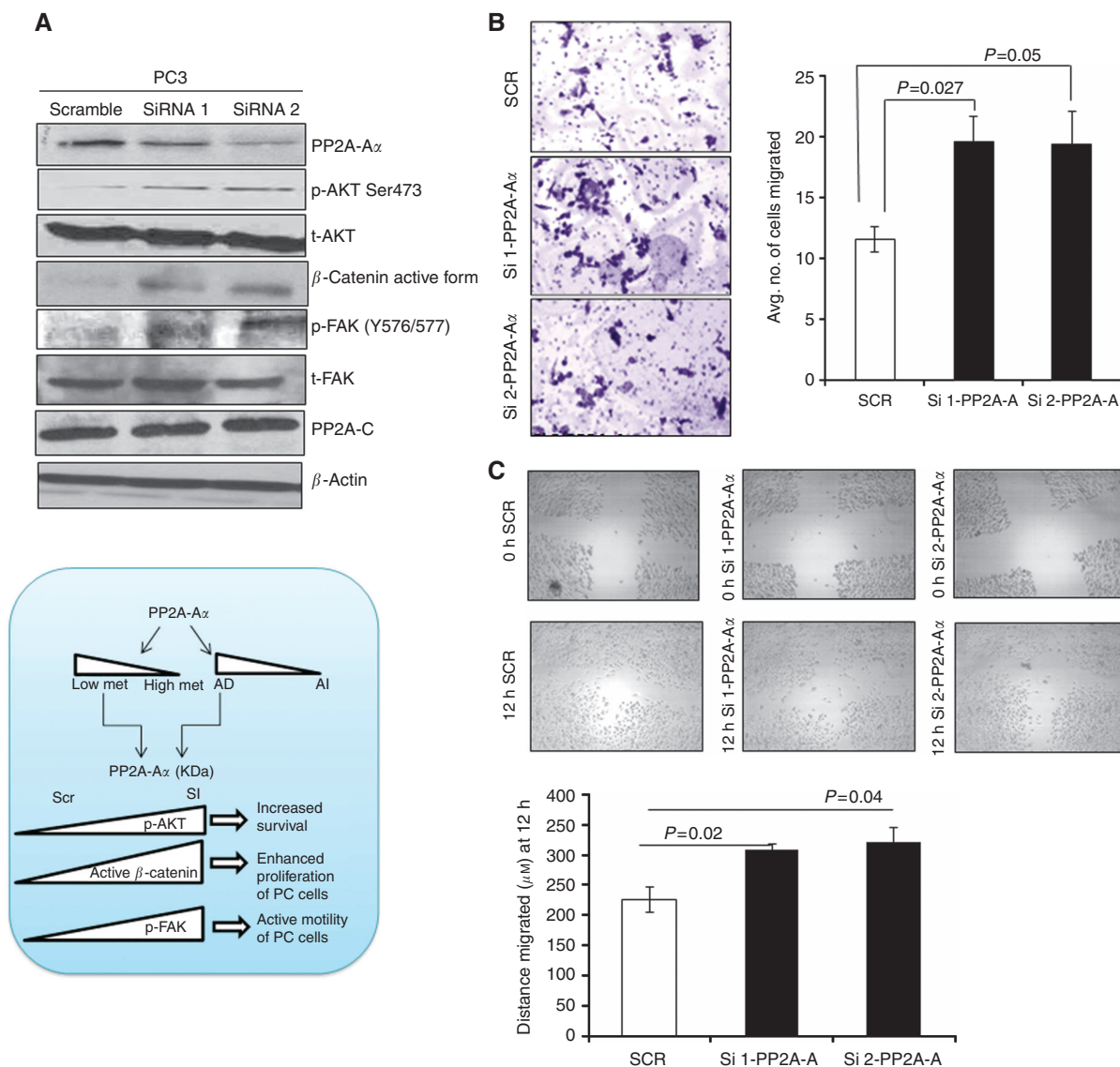


Figure 4. Effect of transient knockdown of PP2A-A α on Akt, β -catenin, and FAK signalling and cell motility assays in prostate cancer (PC) cells. **(A)** Upon knockdown of PP2A-A α , phosphorylation of AKT-Ser473 in PC-3 cells was increased. In addition, immunoblot analysis of PC-3 cells demonstrates increased phosphorylation of FAK-Tyr576/577 along with β -catenin activation after transient knockdown of PP2A-A α . **(B)** Also, the migration potential of PC cells was increased after transient knockdown of PP2A-A α at 48 h. Cells that are migrated through the 8- μ m pores of a polyethylene terephthalate (PET) membrane were quantified in 10 random fields and plotted. **(C)** Protein phosphatase type 2A-A α was transiently knocked down in PC-3 PC cells and grown for 48 h. Cells were then trypsinised and reseeded at the cell density of 1×10^5 per plate. PC-3 PC cells were grown until they achieved a confluent monolayer and a scratch was made using a sterile pipette tip. Introduced wounds were photographed at 0 and 12 h. The distance migrated by the PC-3 cells were quantified by measuring the distance migrated from the 0 to 12 h. Thus, the knock down of the scaffold subunit (PP2A-A α) demonstrated the increasing metastatic potential as a consequence of reduced levels. Thus, western blot analysis showing that phospho (p)-Akt, active β -catenin, and phospho-FAK protein levels directly correlates with cell survival, proliferative capacity, and invasive potential of PC cells but no change in the respective total proteins. Furthermore, the cell motility analysis through transwell migration and wound closure analysis on PC-3 cells after transient knockdown of PP2A-A α at 48 h suggests that the knockdown of PP2A-A α may have a role in increased migration capacity of PC cells.

transcriptional activation of β -catenin target genes such as *MYC* (*c-myc*) and *CCND1* (*Cyclin D1*) (Seeling *et al*, 1999). The alteration/loss of expression of PP2A-B γ observed in our study may contribute to enhanced β -catenin activation, leading to the transcription of β -catenin into the nucleus and resulting in malignant transformation. Further, Li *et al* (2007) was the first to report that specific regulatory subunit B56 γ -containing complexes of PP2A function as tumour suppressors by dephosphorylating p53 at Thr55, which further leads to p53 degradation. Specifically, these reports suggest that the loss of B56 γ subunit

expression would result in a loss of PP2A phosphatase activity and thereby induce cellular transformation (Li *et al*, 2007).

In our earlier study, we have demonstrated that *PPP2CA*, which encodes for the catalytic subunit (α -isoform) of the PP2A-C, was found to be downregulated in AI PCa cell line as compared with AD (Singh *et al*, 2008). In addition, Prowatke *et al* (2007) also showed the downregulation of the β -isoform of the PP2A catalytic subunit in prostate carcinoma using TMA (Prowatke *et al*, 2007). In the present communication, we have demonstrated that there was no significant difference in the expression of PP2A-C between

benign and PCa tissue spots, with regard to AD and AI cells (LNCaP) and between low metastatic and high metastatic (PC-3 and PC-3M) cells both at the protein and mRNA levels. The available oncomine data in gastric cancer also supports that the equal existence of catalytic subunit (remained unchanged) in both normal and cancerous tissues. Although there was a slight difference in the PP2A-C expression pattern between benign and malignant prostate tissues, the difference was statistically insignificant. The results in this study support the notion that the synergistic involvement of the catalytic, regulatory, and scaffold subunits are required for the entire phosphatase activity of PP2A. This study supports the mechanism of autoregulation of PP2A (Baharians and Schonthal, 1998), which exerts a constant level of PP2A-C nonspecific expression in tissues that are independent of hormone status. Early investigations demonstrated that post-translational modification of PP2A catalytic subunit modified by methylation or phosphorylation (at tyrosine and threonine residues) is a crucial determinant for PP2A regulation and holoenzyme complex formation. These modification are governed by leucine carboxyl methyltransferase 1 and phosphatase methyl esterase. Like the catalytic subunit, the regulatory subunit of PP2A PR61/B' is also subjected to post-translational modification (phosphorylation) (Longin *et al*, 2008). However, this modification is a context-dependent process. Serine or proline phosphorylation of PR61/B' subunit by ERK would lead to its dissociation from the catalytic or holoenzyme complex (Letourneux *et al*, 2006; Cho and Xu, 2007). In contrast, phosphorylation of PR61/B' subunit at serine 37 by Chk1 would specifically favour formation of holoenzyme complex assembly (Margolis *et al*, 2006). Thus, in the holoenzyme regulation apart from the catalytic subunit post-translational modifications of the other regulatory and scaffold subunits are equally important.

Finally, it is now well established that deregulation of PP2A signalling is considered to be a prerequisite for the development of malignant cells. More recently, it has been demonstrated that inhibition of PP2A activity leads to proliferation and survival of AI PCa cells through activation of AKT and ERK signalling (Bhardwaj *et al*, 2011). In addition, the direct evidence of PP2A as a tumour suppressor has been shown in a knock-in and knockout mouse model expressing E64D and E64G mutations (Ruediger *et al*, 2011). However, little is known about the nature of phospho-signalling networks associated with this malignant transformation of prostate (Westermarck and Hahn, 2008). Moreover, the enzymatic activity of a protein can be altered by reversible phosphorylation (Nolan *et al*, 1964). Considering the distinct role of PP2A, which can regulate the phosphorylation of particular substrates involved in neoplastic transformation, further work was extended in the expressional analysis of the phosphorylation status of one of the PP2A substrate AKTs. The role of PP2A as a major negative regulator of the AKT pathway is well established in PCa (Li *et al*, 2005). Previous work has shown that the partial suppression of the endogenous α subunit leads to the activation of the AKT kinase (Chen *et al*, 2005). Furthermore, hyperactivation of AKT signalling has been shown in prostate cells as well as in advanced PCa tissues (Kreisberg *et al*, 2004). To determine whether the AKT pathway was altered in PCa (*in vitro*), we transiently knocked down PP2A- α in PC-3 and C-33 cells for 48 h. We found that transient knockdown of PP2A- α in PC-3 and C-33 cells produced a more significant increase in the phosphorylated AKT level at the serine 473 residue as compared with the SCR-transfected control cells. In addition, through functional analysis, we observed a knock down of the scaffold subunit using PP2A- α siRNA or antisense oligodeoxynucleotides, which significantly enhanced cell migration and invasion in PC-3 cell line. Our results imply the direct involvement of PP2A subunits in regulating growth and proliferation of AI PCa cells. Moreover, we observed that there is a perfect correlation between

the level of AKT phosphorylation and the loss of expression of endogenous levels of the PP2A- α subunit. This implies that the loss of expression or suppression of the PP2A scaffold subunit may contribute to the activation of the PI-3 kinase/AKT pathway and thus initiate oncogenesis and transformation of benign cells into PCa cells. We believe that alterations in the level of PP2A catalytic or the regulatory subunit in PCa cells may be critical in regulating the downstream targets (Seeling *et al*, 1999; Suzuki and Takahashi, 2003; Margolis *et al*, 2006; Li *et al*, 2007; Bhardwaj *et al*, 2011). The influence of PP2A on cellular dynamics such as cell adhesion, migration, and cytoskeleton dynamics were poorly understood. A series of events such as lamellipodium formation, actin-rich extension (filopodia), and focal adhesion formation are indicative of cellular migration (Ross *et al*, 2012). Protein phosphatase 2A has been shown to colocalise with β 1-integrin, which is required for FAK regulation (Young *et al*, 2002, 2003). Our studies corroborate earlier findings, which have demonstrated that PP2A inhibition resulted in enhanced hyperphosphorylation of FAK leading to cellular migration, enhanced motility, and cellular spreading activity (Slack *et al*, 2001; Young *et al*, 2002). These observations were substantially demonstrated in BL6 mouse melanoma cells, head and neck cancer, lung cancer, and other malignancies (Jackson *et al*, 1997; Meisinger *et al*, 1997; Ito *et al*, 2000). The data obtained in our study demonstrate that downregulation of PP2A scaffold subunit in PCa cells leads to enhanced cell spreading, migration, survival signaling, and increased viability. Furthermore, the FAK activation in the phospho-form of Y576/577 will define the crucial role of PP2A in adhesion and migration. In addition, the role of PP2A scaffold subunit in cellular communication can be evidenced by active β -catenin expression. Taken together, the results revealed the mechanistic insight into the functional role of PP2A subunit, specifically the scaffold subunit in promoting progression to androgen independence. Thus, PP2A is likely to

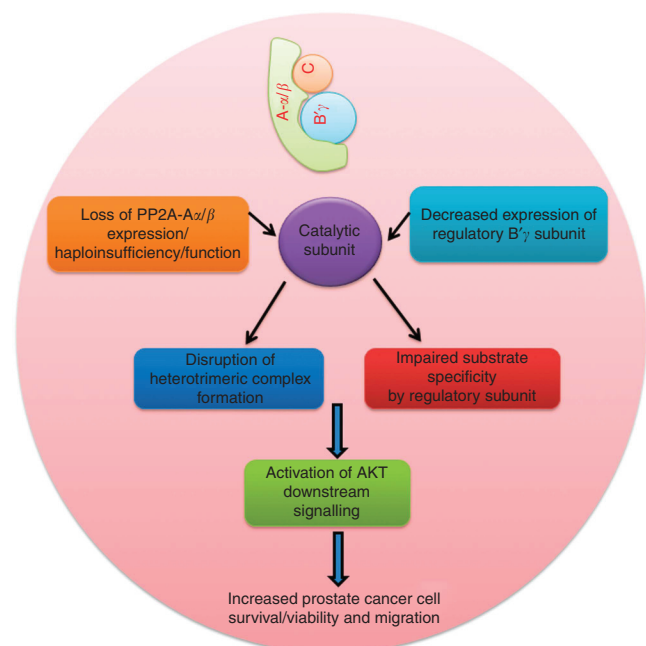


Figure 5. Diagrammatic representation of the loss of PP2A subunits in prostate cancer pathogenesis. Schematic representation of loss/reduced expression levels of the scaffold subunit (PP2A- α / β) and the regulatory subunit (PP2A- $B'\gamma$), thereby leading to the disruption of heterotrimeric complex formation and impaired substrate specificity. Thus, the regulatory and scaffold subunit of PP2A may have a role in prostate cancer progression. Mechanistically, transient knockdown of PP2A- α leads to increased phospho-AKT level in prostate cancer cells.

play an integral role in tumour suppressor network, which is partially regulated by AKT/FAK/ β -catenin in promoting AI growth of PCa.

In conclusion, based on our studies we report that the expression of the PP2A was downregulated during PCa progression primarily due to the downregulation of the regulatory and scaffold subunits and not the catalytic subunit. The downregulation of the scaffold subunit further emphasises the fact that it was unable to organise both the regulatory and catalytic subunit together for its functional role of cellular transformation (Figure 5). Further, based on this study, it is very clear that even if there is no change in the constitutive expressional level of the C subunit, a differential expression or loss of expression on A and B subunits may have an influential role in PCa progression. Thus, this study on the downregulation of the PP2A subunits has a critical role in PCa pathogenesis and progression.

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AUTHOR CONTRIBUTIONS

SKB, PP and PS: conceived and designed the experiments; PP, PS, SD and SR: performed experiments; SKB, PP, PS, PPM, KD, ML and YY: analysed the data; SKB: contributed reagents/materials/analysis tools; and SLJ: served as the certified pathologist for scoring of haematoxylin and eosin, as well as scoring of IHC slides. PS and PP wrote the paper. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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