Biochemistry and Molecular Biology Program

Inhibition of PDZ-Binding Kinase Restores Epigenetically Silenced Tumor Suppressor Gene TIG1 in Human Prostate Cancer

Charles Hinzman and Partha Banerjee

Department of Biochemistry and Molecular Biology

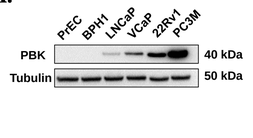
Georgetown University School of Medicine

Georgetown University

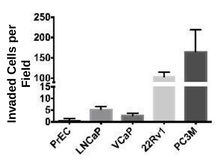
Introduction

PDZ-Binding Kinase (PBK)/Lymphokine-activated killer T-cell-originated protein kinase (TOPK) is an under-studied MAPK family kinase that is over-expressed in a number of aggressive cancers and is hypothesized to be an oncogene[1]. Tazarotene-induced gene-1 (TIG1)/Retinoic Acid Receptor Responder 1 (RARRES1) is a tumor suppressor gene that is downregulated in prostate cancer[2]. Our lab has previously demonstrated an association of PBK with the aggressive behavior of prostate cancer cells (Figures A and B) [3] and, via gene micro-array analysis, that the silencing of PBK results in the upregulation of a number of genes, including some tumor suppressor genes (Figure C) [3].

A.



B.



After a lengthy screening process, we found that inhibition of PBK led to up-regulation of various tumor suppressor genes including RKIP, RASSF1A, MST1 and TIG1. In this study we centered on TIG1. Our goal was to elucidate PBK's regulation of TIG1. We hypothesized that this regulation was mediated through epigenetic silencing, specifically via Enhancer of Zeste Homolog 2 (EZH2), a known transcriptional repressor and the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2). In tandem, we evaluated the biological effect of restored TIG1 expression.

C.

Pathways	p-value	Selected Key Genes
↓Prostate Cancer	3.40E-05	↑ RARRES1, mir-Let7D, VCAN, TFP12, OPRM1, AHRDGI8, BTG3
↓Metastasis	2.20E-05	↑ SERPINE1, MIRLET7D ↓ S100A4, PTGS2, MMP1,3,10,13
↓Invasion	1.09E-05	↑ MIR133B ↓ S100A4,DNMT3B, ELF3, S100P
↓Proliferation of Prostate Cancer Cell Lines	6.16E-04	↓ CTNNBIP1, FGFBP1, LCP1

Methods

Cell Culture: PC-3M cells were cultured in phenol red-free IMEM, penicillin-streptomycin and 10% FBS. PPCL-PDX cells were cultured in KSFM and penicillin-streptomycin. Treated cells were treated with 100nM of OTS-514 for 1-3 days.

Immunoblots: Cell lysates were obtained using RIPA buffer and sonication, protein concentrations standardized via Bradford Assay. Proteins were resolved using standard protocol, 10% SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes, blocked and treated with primary and secondary antibodies. Chemiluminescent reagent was applied to membranes and were visualized in an LAS-1000 imager.

Expression vectors and siRNA: TIG1A and TIG1B expression vectors were transformed into *E.coli* and colonized on agar plates, selected for with kanamycin. Colonies were then cultured and plasmid DNA was purified using a kit (Qiagen). Concentration and purity was measured via NanoDrop. PC-3M cells were transfected using GenJet following manufacturers protocol. Transfection verified via western blot using anti-MycTag antibody. Pooled siRNA's against PBK and EZH2 were purchased and cells were transfected using PepMute, grown for 72 hours with siRNA.

Immunofluorescence: PC-3M cells were stained using anti-TIG1 primary antibody and incubated with secondary antibody conjugated with red fluorescence (Alexa Fluor). Nuclei were stained with DAPI.

Immunohistochemistry: Archival tissue sections from Banerjee Lab. Slides were treated with 1:250 dilution primary antibody overnight at 4° C, followed by treatment with 1:3,000 dilution secondary antibody for 1hr. Slides were incubated in ABC-P Reagent for 30 min. followed by treatment with 3,3'-diaminobenzidine (DAB). Slides were counterstained with hematoxylin, mounted, and photographed.

Colony Formation Assay: PC-3M cells were plated in a 6 well plate, 1,000 cells per well. 3 of the wells were treated. Cells were grown in a 37° C, 5% CO2 incubator for 5 days. Colonies were then stained with .1% Crystal Violet and counted.

Cell Invasion Assay: Performed using a Boyden chamber covered with Matrigel, pore size of 8 microns, according to manufacturer's protocol.

Cell Proliferation Assay: BrdU cell proliferation assay kit was purchased from Roche and performed following manufacturer's protocol.

Quantification and Statistical Analysis: Western blots quantification accomplished using ImageJ and all statistical analysis was performed in GraphPad Prism 7.

Conclusions

We discovered that PBK inhibition restores TIG1 expression in aggressive prostate cancer cells and that TIG1 inhibits the cells growth and invasive potential. These data support the hypothesis that the epigenetic silencing of TIG1 is accomplished, at least in part, by EZH2. Interestingly, PBK may be implicated as having a more significant role in epigenetic regulation, as indicated by the decreased expression of HDACs 5, 8, 9 and 10 and DNMT 3A with PBK inhibition. Finally, we show that PBK inhibition can restore TIG1 expression in various cancer types, not just prostate cancer. Accordingly, PBK may be a promising therapeutic target for the treatment of aggressive prostate cancer.

Results

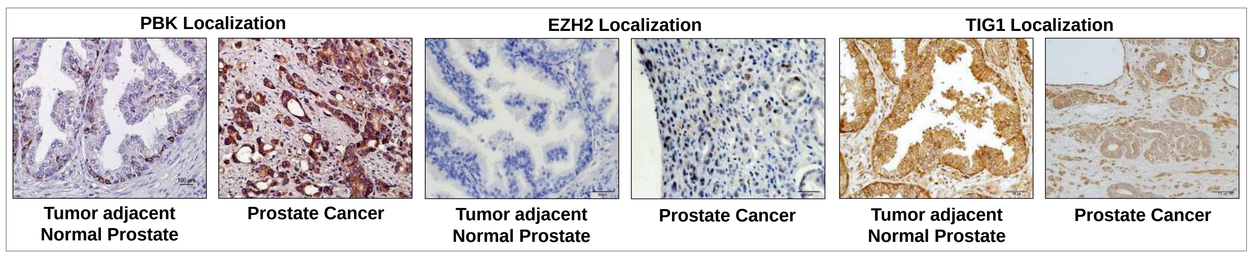


Figure 1. Immunohistochemical staining of PBK, EZH2 and TIG1 in human prostate cancer and tumor adjacent normal prostate tissue.

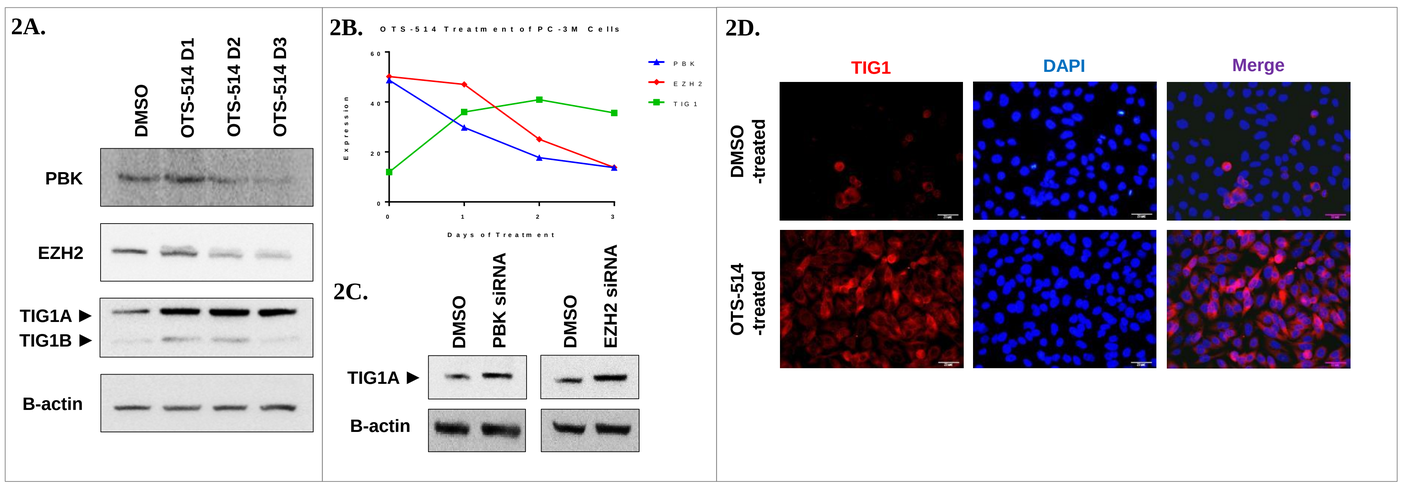


Figure 2. A. Time series immunoblot analysis of PBK, EZH2 and TIG1 in PC-3M cells, comparing expression after treatment with OTS-514 from 1-3 days**. B.** Quantification of immunoblot densities depicting protein expression levels. **C.** PC-3M cells with PBK and EZH2 siRNA to compare rescue of TIG1 expression. **D)** Immunofluorescent microscopy of PC-3M cells after treatment with OTS-514 for 3 days, probing for TIG1.

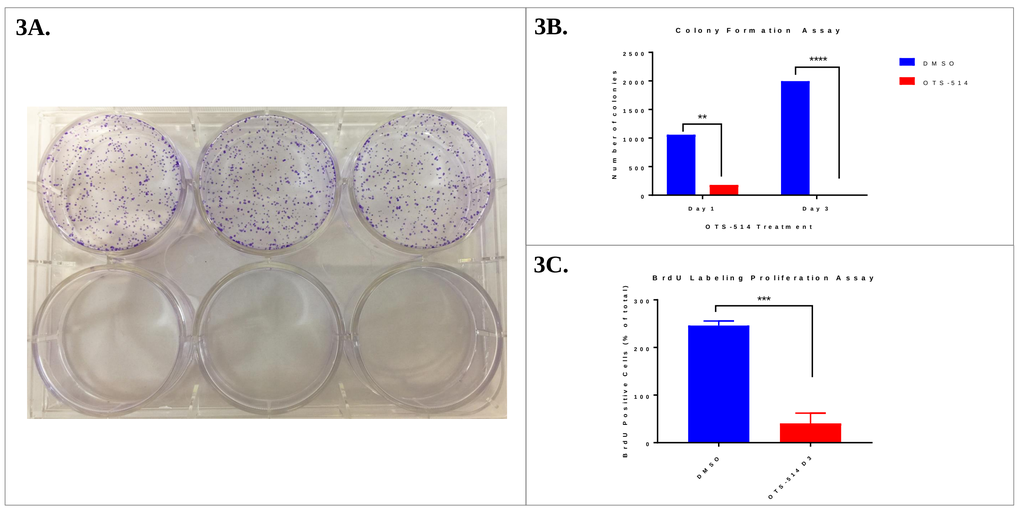


Figure 3. A. Colony Formation Assay 6-well plate, 3 day treatment. Top row PC-3M control cells, bottom row PC-3M cells treated with OTS-514. **B.** Quantification of colony formation assay. Day 1 p = 0.0011, Day 3 p = <0.0001. **C.** BrdU Labeling proliferation assay data, p = 0.0001.

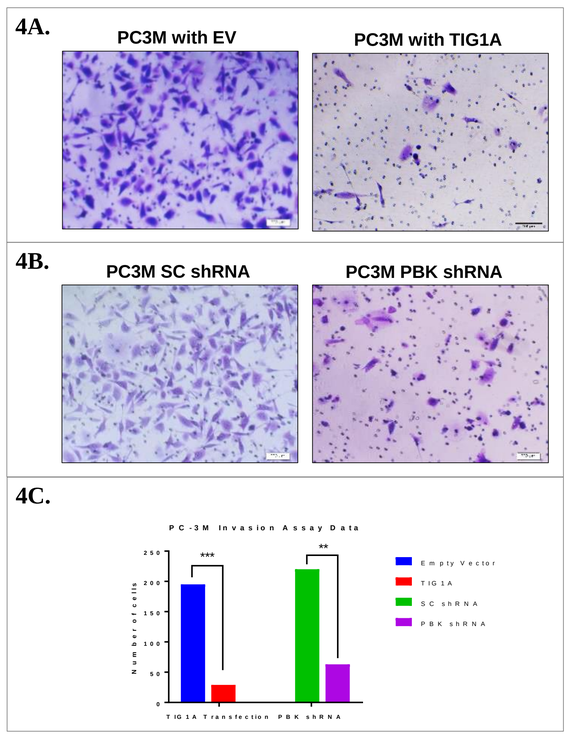


Figure 4. A. Invasion assay data comparing the invasive capability of PC-3M Empty Vector (control) cells to PC-3M cells transfected with a constitutively active TIG1A expression vector. **B.** Invasion assay data comparing the invasive capability of PC-3M control shRNA cells to PC-3M cells transfected with PBK shRNA. **C.** Quantification of invasion assay data.

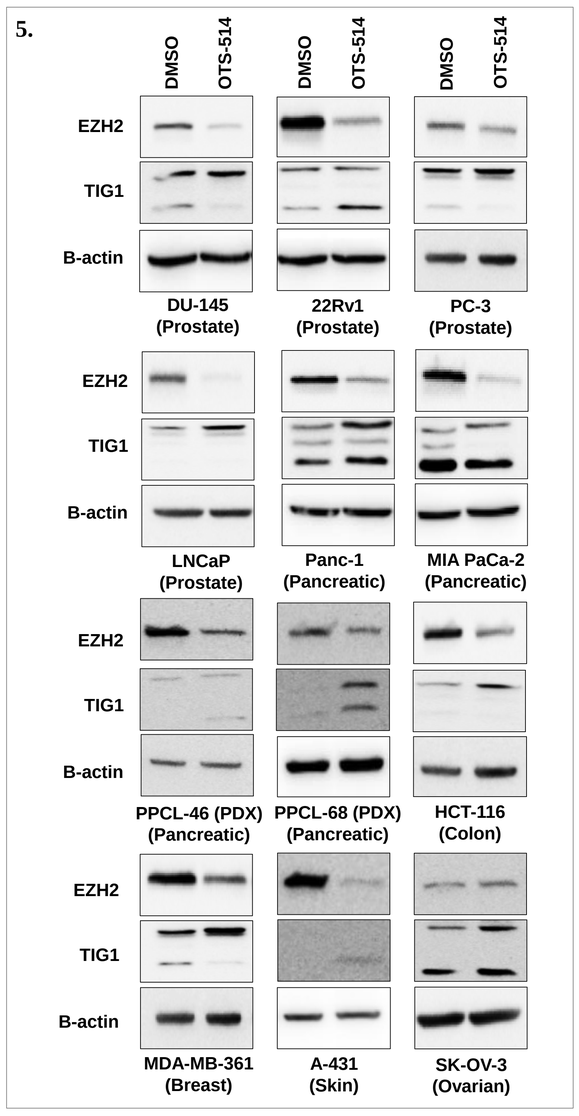


Figure 5. Immunoblot analysis comparing EZH2 and TIG1 expression levels in multiple cancer cell lines after OTS-514 treatment.

Figure 6. A. Immunoblot analysis comparing HDAC expression levels in PC-3M cells treated with OTS-514. **B.** Immunoblot analysis comparing DNMT 3A levels in PC-3M cells treated with OTS-514. **C.** Immunoblot analysis comparing epigenetic regulator inhibitors (HDAC, DNMT and EZH2) and effects on TIG1 expression in PC-3M cells.

Acknowledgments

Special thanks to Dr. Banerjee for his tremendous guidance, Dr. Jose Trevino from the University of Florida for providing the PPCL cell lines and Dr. Shun-Yuan Jiang from Buddhist Tzu Chi General Hospital for the TIG1A and TIG1B expression vectors.

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Fall 2016