



PMA Induction to Prostate Cancer Cell Lines: DU145 and LNCaP

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ABSTRACT

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer mortality in men in the Western World. The effects of androgens are mediated by the Androgen Receptor (AR). Therefore, studies focus on the identification of AR-regulated genes that are also highly expressed in the prostate. STAMP family genes STAMP1/STEAP2 and STAMP2/STEAP4 have only expressed in androgen receptor-positive cells, the role of AR in STAMP family gene expression is an important question. Phorbol 12-myristate 13-acetate, NF- κ B Activator, PMA, TPA PMA is used at 100nM final concentration after transfection of Hismax vector and Hismax- STAMP1 to DU145-brain metastases of prostate cancer cell line. DU145 cell line kinetics were shown. PMA induction to LNCaP cells was done. In summary, the data presented show that STAMP1 is involved in a signaling pathway that is linked to cell cycle progression as well as inhibition of apoptosis. Consistent with its increased expression in prostate cancer, these data suggest that STAMP1 may play important roles in prostate cancer development and progression.

Keywords: DU145, LNCaP, PMA, RT-PCR, STAMP1, AR: Androgen receptor, ARRE: Androgen receptor response element, LNCaP Lymph Node Metastases Prostate Cancer Cell Line, PMA: Phorbol Myristate 13-acetate, STAMP: six transmembrane protein of prostate, RTqPCR: Quantitative polymerase chain reaction TUBA the Turkish Academy of Sciences, TUBITAK The Scientific and Technological Research Council of Turkey

INTRODUCTION

STEAP (Six Transmembrane Epithelial Antigens of Prostate) is the first characterized prostate enriched six transmembrane genes, expressed in metastatic prostate cancer samples, it is tempting to speculate that STAMP/STEAP family genes may be involved in similar functions with a role for both the normal biology and pathophysiology of the prostate.

Phorbol 12-myristate 13-acetate (PMA), also known as 12-O-tetradecanoylphorbol 13-acetate (TPA), is a specific activator of Protein Kinase C (PKC) and hence activates nuclear factor-kappa B (NF- κ B). NF- κ B is a transcription factor that regulates numerous physiological functions and is involved in the pathogenesis of various diseases. It has been identified as a potential therapeutic target in inflammatory processes, cancer, and autoimmune diseases.

PMA is the most common and potent phorbol ester. It is active at nanomolar concentrations and activates NF- κ B in a dose-dependent manner. PMA causes a wide range of effects in cells and tissues and is a very potent mouse skin tumor promoter.

InvivoGen's PMA is designed to study the NF- κ B pathway in cellular assays.

Because knockdown of STAMP1 increased cell death, it was of interest to investigate if there were changes in STAMP1 levels in prostate cancer cells that are undergoing apoptosis.

Previous studies have established that apoptosis in LNCaP cells can be induced by various agents. Prostate cancer is cancer of the prostate. The prostate is a gland in the male reproductive system that surrounds the urethra just below the bladder. Most prostate cancers are slow growing. Cancerous cells may spread to other areas of the body, particularly bones and lymph nodes. It may initially cause no symptoms. In later stages, symptoms include pain or difficulty urinating, blood in the urine, or pain in the pelvis or back. Benign prostatic hyperplasia may produce similar symptoms. Other late symptoms include fatigue, due to low levels of red blood cells.

Factors that increase the risk of prostate cancer include older age, family history, and race. About 99% of cases occur after age 50. A first-degree relative with the disease increases the risk two- to three-fold. Other factors include a diet high in processed meat and red meat, while the risk from a high intake of milk products is inconclusive.

NF-kappa B is a transcription factor found in all cell types. It is inactive in the cytoplasm. When activated, it is transported to the nucleus. There are 5 types: NF-kB1, NF-kB2, RelA, RelB, and c-Rel. It is thought that NF-kappa B has an effect in some autoimmune diseases.

RESULTS

PMA used at 100nM final concentration after transfection of Hismax vector (1A) and Hismax-STAMP1 (1B) to DU145-brain metastases of prostate cancer cell line.

1A DU145 cells transfected with HisMax-vector PMA induced RT-PCR primers amplified.

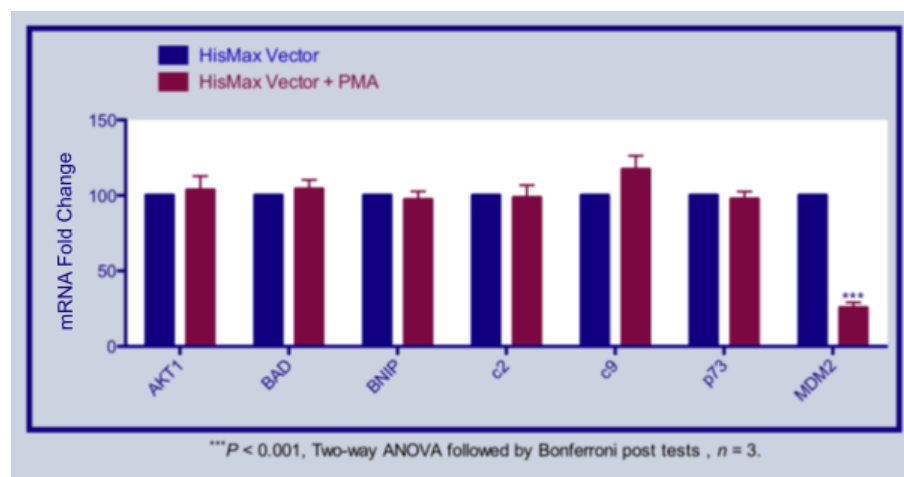
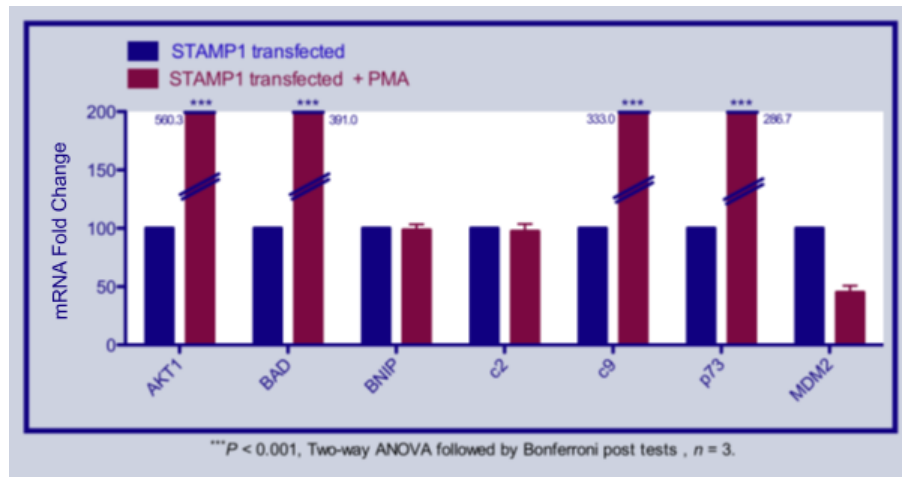


Figure 1 A: DU145 cells transfected with HisMax-vector PMA induced RT-PCR primers amplified.

1B DU145 cells transfected with HisMax-STAMP1 PMA induced RT-PCR primers amplified.



Figuree 1B: DU145 cells transfected with HisMax-STAMP1 PMA induced RT-PCR primers amplified

PMA induction to HisMax vector transfected DU145 cells caused mouse double minute gene2-MDM2 –decrease $p < 0.001$ (1A)

But transfection of STAMP1 changed PMA responses and Akt1, BAD, CASPASE9 and, P73 showed 5.6, 3.9, 3.3, 2.9 fold increase. respectively $p < 0.001$ (1B).

1C: DU145 cell line kinetics: HisMax-Vector- HisMax-STAMP1, HisMax-STAMP2 and HisMax-STAMP3 transfected

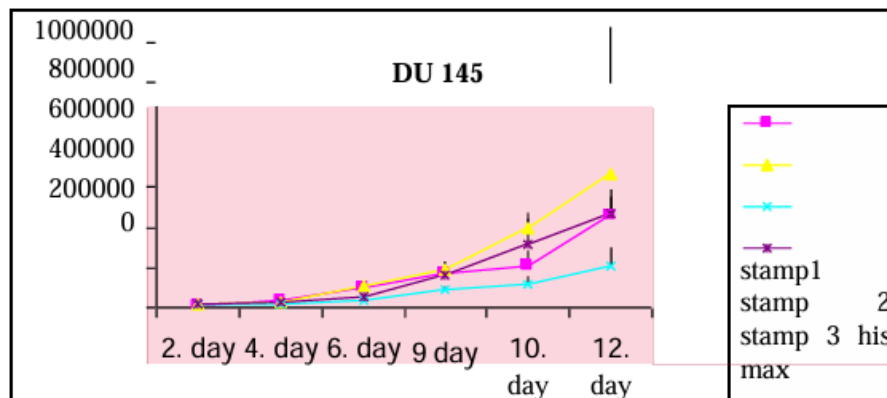


Figure1C: DU145 cells transfected with STAMP constructs (STAMP1, 2 and 3 versus HisMax-vector) growth kinetic result

DU145 cells transfected with STAMP constructs (STAMP1, 2 and 3 versus HisMax- vector)

STAMP/STEAP genes transfection caused gradual growth in brain metastasis of prostate cancer cell line-DU145 cells. Following the day of the transfection done with FuGene HD transfection reagent, cells were taken to 12-well plates as 104 cells per well and dead cells were counted by trypan-blue exclusion assay beginning at day 2 until 12. Six time points were taken that assayed as $n=7$ per point and growth kinetics were drawn (Figure 1C). It was shown that STAMP3 transfection caused relatively lower growth increase compare to control group in the transfected cell group.

PMA induction to LNCaP cells was done.

Caspase7 and 9 showed a decrease $P < 0.001$ and MDM2 gave an increase $p < 0.001$ (FIG2),

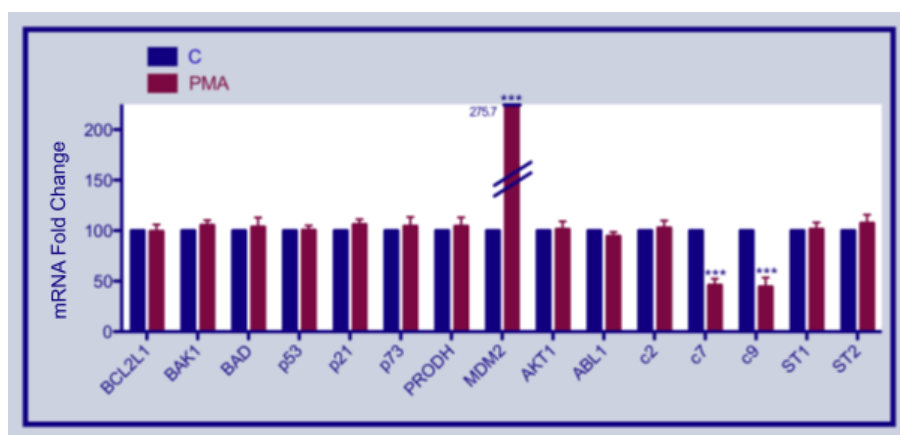


Figure 2: LNCaP cells induced with PMA, RT-PCR primers amplified

DISCUSSION

p53 or tumor protein 53 (TP53), Genome Guardian, anti-tumor p53 is a transcription factor that regulates the cell cycle. It is a very important protein for suppressing cancer in many organisms. It is critical as it inhibits cancer formation in multicellular vertebrates and exhibits tumor suppressive function.

p73 is a protein associated with the p53 tumor protein. Because of its structural similarity to p53, it has also been considered a tumor suppressor. It plays a role in cell cycle regulation and apoptosis induction. Like p53, p73 is characterized by the presence of different isoforms of the protein.

The murine double minute (*MDM2*) oncogene, which codes for the MDM2 protein, was originally cloned, along with two other genes (MDM1 and MDM3) from the transformed mouse cell line 3T3-DM. MDM2 overexpression, in cooperation with oncogenic Ras, promotes transformation of primary rodent fibroblasts, and *MDM2* expression led to tumor formation in nude mice. The human homologue of this protein was later identified and is sometimes called HDM2. Further supporting the role of MDM2 as an oncogene, several human tumor types have been shown to have increased levels of MDM2, including soft tissue sarcomas and osteosarcomas as well as breast tumors. The MDM2 oncoprotein ubiquitinates and antagonizes p53, but may also carry out p53-independent functions. MDM2 supports the Polycomb-mediated repression of lineage-specific genes, independent of p53. MDM2 depletion in the absence of p53 promoted the differentiation of human mesenchymal stem cells and diminished clonogenic survival of cancer cells. Most of the MDM2-controlled genes also responded to the inactivation of the Polycomb Repressor Complex 2 (PRC2) and its catalytic component EZH2. MDM2 physically associated with EZH2 on chromatin, enhancing the trimethylation of histone 3 at lysine 27 (H3K27me3) and the ubiquitination of histone 2A at lysine 119 (H2AK119) at its target genes. Removing MDM2 simultaneously with the H2AK119 E3 ligase Ring1B/RNF2 further induced these genes and synthetically arrested cell proliferation.

AKT1

AKT1 (gene at 14q32.33) is one of three closely related serine/threonine-protein kinases (AKT1, AKT2, and AKT3) that regulate many processes including metabolism, proliferation, cell survival, growth, and angiogenesis by phosphorylating a range of downstream substrates in response to growth factor stimulation (*i.e.*, EGF, IGF). AKT is responsible of the regulation of glucose uptake by mediating insulin-induced translocation of the SLC2A4/GLUT4 glucose transporter to the cell surface. It also regulates the storage of glucose in the form of glycogen. AKT promotes cell survival via the phosphorylation of the apoptosis signal-related kinase MAP3K5. Phosphorylation of "Ser-83" decreases MAP3K5 kinase activity stimulated by oxidative stress and thereby prevents apoptosis. AKT has an important role in the regulation of NF-kappa-B-dependent gene transcription and positively regulates the activity of the cyclic AMP (cAMP)-response element binding protein (CREB1). The phosphorylation of CREB1 induces the binding of accessory proteins that are necessary for the transcription of pro-survival genes.

BAD: Bcl2 Antagonist of Cell Death

Yang et al. (1995) identified a new protein that binds to Bcl2. Antagonist of Bcl2-mediated cell death: It is named Bcl2 antagonist of cell death: BAD. ' This 204-amino acid protein has sequence similarity to the BH1 and BH2 domains of BCL2 family members. Bad shows strong heterodimerization with BclX from this family, weaker binding with Bcl2 but no binding with other members. In functional studies, it has been shown that Bax is removed as a result of dimerization of Bad with BclX and its apoptosis-inducing effect is eliminated.

BNIP

BNip (formerly known as Nip) proteins, including homologues isolated from human, mouse and *Caenorhabditis. elegans*, are a relatively new subgroup of the Bcl-2 family. These proteins are classified into this family based on limited sequence homology with the Bcl-2 homology domain 3 and carboxyl terminal transmembrane domain. BNip proteins were first discovered based on their interaction with the adenovirus E1B 19 kDa/Bcl-2 family protein and since then, their roles in cell death pathways have been actively studied. However, the precise mechanisms by which the BNip proteins induce apoptosis and/or necrosis remain to be determined.

Caspase7

The described roles of caspase-7 in apoptosis and inflammation suggest that interfering with caspase-7 activation may prove beneficial in conditions where excessive cell death and/or inflammation contribute to disease. Therapeutic inhibition can be achieved with synthetic inhibitors or through targeted delivery of natural caspase inhibitors such as XIAP and the baculoviral caspase inhibitor p35. Caspase-7 inhibition seems especially warranted in neurodegenerative disorders such as Alzheimer's disease and Huntington's disease, where increased caspase-7 expression correlates with excessive neuronal cell death. Another potentially promising application is the prevention of lymphocyte cell death in sepsis. Extensive leukocyte apoptosis is commonly observed in sepsis patients and was suggested to contribute to immune suppression and lethality). Synthetic caspase inhibitors and overexpression of the anti-apoptotic protein Bcl-2 have already shown promising results in experimental sepsis models). In addition to the potential roles of caspase-7 in sepsis and neurodegenerative disorders, single nucleotide polymorphisms (SNPs) in the *caspase-7* gene have been linked with rheumatoid arthritis (SNP rs2227309; K249R mutation) Insulin- Dependent Diabetes Mellitus

(SNP rs144692; D251E mutation) Additional studies are required to extend these promising findings and to unravel the mechanisms by which the resulting missense mutations in caspase-7 trigger disease Caspase 9.

Caspase 9 (also known as ICE-LAP6, or mch6) is considered to be a caspase in the upstream of apoptotic signal transduction. When mitochondria release cytochrome c, Caspase 9 can form complex with cytochrome c and Apaf1, and be activated at the same time. Activated Caspase 9 can activate Caspase 3 which is the key enzyme of cell apoptosis, thereby promoting subsequent apoptotic signals. Caspase 9 exists in the form of prozyme in the normal state and has no activity. However, Caspase 9 is activated in the apoptotic stage and participates in the apoptotic process. This kit is used to conjugate Caspase 9 sequence-specific peptides acetyl-Leu-Glu-His-Asp p-nitroanilide (Ac-LEHD-pNA) to yellow group p-nitroaniline (pNA)

Many of the roles played by the tumor suppressor in restraining cancer initiation and progression are well established. These include the ability of to induce cell-cycle arrest, DNA repair, senescence and apoptosis One of the hallmarks of human cancers is the intrinsic or acquired resistance to apoptosis. Evasion of apoptosis can be part of a cellular stress response to ensure the cell's survival upon exposure to stress stimuli. There are two major apoptosis signaling pathways, that is, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway]. Under most circumstances, activation of either pathway eventually leads to proteolytic cleavage and thus activation of caspases, a family of cysteine proteases that act as common death effector molecules. Accordingly, caspases are responsible for many of the biochemical and morphological hallmarks of apoptotic cell death by cleaving a range of substrates in the cytoplasm or nucleus Cytochrome c has been implicated in the redox regulation of apoptosis. Once cytochrome c is released from mitochondria into the cytosol, it triggers the formation of the cytochrome c/Apaf-1/Caspase-9-containing apoptosome, which in turn lead to activation of caspase-9 and downstream effector caspases There is recent evidence that also the redox state of cytochrome c is involved in the regulation of apoptosis. To this end, the oxidized form of cytochrome c ($\text{Fe}(3^+)$) has been reported to induce caspase activation via the apoptosome, while the reduced form of cytochrome c ($\text{Fe}(2^+)$) is unable to do so Response elements of prostate specific antigen (PSA) and NF κ B are located at AR promoter region and suggest that NF κ B may affect AR expression.

Statistical Analysis

All the illustrated results represent one of at least three independent experiments with similar outcomes.

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Experimental Procedures Cell Culture

Cell lines were cultured in DMEM (GIBCO, USA) or RPMI 1640 (GIBCO) with 5% and 10% fetal bovine serum for DU145 and LNCaP cells, respectively. 1% L-glutamine and 1U/ml of each

penicillin/ streptomycin was used. Cells were incubated at 37°C and 5% CO₂ in a humidified atmosphere.

TABLE 1: Primers for RT-PCR amplification

GeneBank/ Symbol	Description		Gen Name	Primers
NM_004322 BAD	BCL2-antagonist of cell death	BBC2/BCL2L8	Forward Reverse	AGGATCCGTGCTGTCTCCTTTG CAAACTTCCGATGGGACCAAG
NM_001188 BAK1	BCL2-antagonist /killer 1	BAK/BCL2L7	Forward Reverse	GGGTGTAGATGGGGAACTGTG AAGACCCTAGGCTGTGCCCAAT
NM_138578 BCL2L1	BCL2-like 1	BCL-X/BCL-XL	Forward Reverse	GTGTGAGGAGCTGCTGGCTTG AGCATCAGGCCGTCCAATCTC
NM_001205 BNIP1	BCL2/adenovirusE1B 19kDa interacting protein 1	NIP1/TRG-8	Forward Reverse	CAGGTTGGATGGAACACAGTGC ATCCCAATGCCAGACCTTCCTC
NM_032982 CASP2	Caspase 2, apoptosisrelated cysteine protease	CASP-2/ICH-1L	Forward Reverse	TCTCCATGGTCCCTAGCAAAA AAGGCTCACAAACCACCCAAAC
NM_001227 CASP7	Caspase 7, apoptosisrelated cysteine protease	CMH-1/ICE-LAP3	Forward Reverse	AAGTGAGGAAGAGTTTATGGCAAA CCATCTTGAAAACAAAGTGCCAAA
NM_001229 CASP9	Caspase 9, apoptosisrelated cysteine protease	APAF-3/APAF3	Forward Reverse	TCCTGAGTGGTGCCAAACAAA AGTGGTTGTCAGGCGAGGAAAG
NM_005157 ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	ABL/C-ABL	Forward Reverse	GGCCTTGAAGACAGAGCAAAGC GGAAGGGACCAGTACCTCATGG
NM_005163 AKT1	V-akt murine thymoma viral oncogene homolog 1	PKB/PRKBA	Forward Reverse	TCCCCCTCAGATGATCTCTCCA CGGAAAGGTTAAGCGTCGAAAA
NM_005427 TP73	Tumor protein p73	P73	Forward Reverse	AGCAGCCCATCAAGGAGGAGTT TCCTGAGGCAGTTTTGGACACA
NM_000546 TP53	Tumor protein p53 (LiFraumeni syndrome)	CYS51STOP/P53	Forward Reverse	AGATGGGGTCTCACAGTGTTGC ATGTTGACCCTCCAGCTCCAC
NM_078467 P21	Homo sapiens cyclindependent kinase inhibitor 1A	CDKN1A	Forward Reverse	GGCAGACCAGCATGACAGATT GCGGCCAGGGTATGTACATGA
NM_002392 MDM2	Homo sapiens Mdm2, transformed 3T3cell double minute 2	HDMX/MGC71221	Forward Reverse	GGGTTGCGACCATTCTCTG GGCAGATGACTGTAGGCCAAGC
NM_016335 PRODH	Homo sapiens proline dehydrogenase (oxidase) 1	PIG6/HSPOX2	Forward Reverse	TTTTTCACCCCACTTGCGAGA TGTCACAGGCAGGTATCAGGTT
NM_001101 ACTB	Homo sapiens actin, beta	PS1TP5BP1, beta-actin	Forward Reverse	CAATGTGGCCGAGGACTTTGAT AGTGGGGTGGCTTTTAGGATGG
NM_002046 GAPDH	Homo sapiens glyceraldehyde 3-phosphate dehydrogenase	G3PD, GAPD	Forward Reverse	CATTGCCCTCAACGACCACTTT GGTGGTCCAGGGGTCTTACTCC

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