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Silencing of STAMP1/STEAP2 and Amplification with Apoptosis Panel Primers*

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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 identification of AR regulated genes that are also highly expressed in the prostate. STAMP family genes STAMP1/STEAP2 (Korkmaz KS., 2002) and STAMP2/STEAP4 (Korkmaz CG., 2005) are only expressed in androgen receptor positive cells, the role of AR in STAMP family gene expression is an important question. STEAP (six transmembrane epithelial antigen of prostate) (Hubert RS., 1999) is the first characterized prostate enriched six transmembrane gene, 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 prostate. Using siRNA technology in LNCaP cells expressing STAMP genes *per se*, an apoptosis panel including pro-apoptotic and/or apoptotic molecules was assayed by RT-PCR, By this research project, prostate-specific STAMP gene family and its regulatory effects were characterized.

Keywords: silencing of STAMP1/STEAP2, LNCaP, P53, RT-PCR, AR: Androgen receptor, ChIP: Chromatine Immunoprecipitation, RTqPCR: Quantitative polymerase chain reaction, TUBITAK: The Scientific and Technological Research Council of Turkey, STAMP: six transmembrane protein of prostate, TUBA: The Turkish Academy of Sciences.

INTRODUCTION

Cancer Models

Scientists have established prostate cancer cell lines to investigate disease progression. LNCaP, PC-3 (PC3), and DU-145 (DU145) are commonly used prostate cancer cell lines. The LNCaP cancer cell line was established from a human lymph node metastatic lesion of prostatic adenocarcinoma. PC-3 and DU-145 cells were established from human prostatic adenocarcinoma metastatic to bone and to brain, respectively. LNCaP cells express AR, but PC-3 and DU-145 cells express very little or no AR.

The proliferation of LNCaP cells is androgen-dependent but the proliferation of PC-3 and DU-145 cells is androgen-insensitive. Elevation of AR expression is often observed in advanced prostate tumors in patients. Some androgen-independent LNCaP sublines have been

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developed from the ATCC androgen-dependent LNCaP cells after androgen deprivation for study of prostate cancer progression. These androgen-independent LNCaP cells have elevated AR expression and express prostate specific antigen upon androgen treatment. Paradoxically, androgens inhibit the proliferation of these androgen-independent prostate cancer cells.

MATERIALS AND METHODS

Cell culture. LNCaP cells were cultured in RPMI-1640 (Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS), 1% L-glutamine and 1 U/ml each of penicillin/streptomycin. Cells were incubated at 37˚C with 5% CO_2 in a humidified atmosphere. The cell lines were purchased from ATCC (Manassas, VA, USA).

siRNA-mediated knockdown of genes LNCaP cells were transfected with either scrambled control siRNA (sc-37007) or STAMP1specific siRNA: (sc-76587) and STAMP2 specific siRNA (sc-89820) purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The sequences were provided by the manufacturer.

A total of 100 pmol siRNA (final concentration, 50 nM) was used to transfect cells with the aid of 10 µl FuGENE HD transfection reagent and the cells were incubated with the siRNA construct for 1 and 4 days, respectively, in accordance with the manufacturer's instructions.

Table I: Genes and primers used as an apoptosis panel for quantitative polymerase chain reaction (qPCR) analysis

GeneBank	SEMBOL	TANIM	GEN ADI	SOL PRİMER	SAĞ PRİMER
NM_004322	BAD	BCL2-antagonist of cell death	BBC2/BCL2L8	AGGATCCGTGCTGTCTCCTTTG	CAAAACTTCCGATGGGACCAAG
NM_001188	BAK1	BCL2-antagonist/killer 1	BAK/BCL2L7	GGGTGTAGATGGGGGAACTGTG	AAGACCCTAGGCTGTGCCCAAT
NM_138578	BCL2L1	BCL2-like 1	BCL-X/BCL-XL	GTGTGAGGAGCTGCTGGCTTG	AGCATCAGGCCGTCCAATCTC
NM_001205	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	NIP1/TRG-8	CAGGTTGGATGGAACACAGTGC	ATCCCAATGCCAGACCTTCCTC
NM_032982	CASP2	Caspase 2, apoptosis-related cysteine protease	CASP-2/ICH-1L	TCTCCCATGGTCCCTAGCAAAA	AAGGCTCACAAACCACCCAAAC
NM_001227	CASP7	Caspase 7, apoptosis-related cysteine protease	CMH-1/ICE-LAP3	AAGTGAGGAAGAGTTTATGGCAAA	CCATCTTGAAAACAAAGTGCCAAA
NM_001229	CASP9	Caspase 9, apoptosis-related cysteine protease	APAF-3/APAF3	TCCTGAGTGGTGCCAAACAAAA	AGTGGTTGTCAGGCGAGGAAAG
NM_005157	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	ABL/C-ABL	GGCCTTGAAGACAGAGCAAAGC	GGAAGGGACCAGTACCTCATGG
NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1	PKB/PRKBA	TCCCCCTCAGATGATCTCTCCA	CGGAAAGGTTAAGCGTCGAAAA
NM_005427	TP73	Tumor protein p73	P73	AGCAGCCCATCAAGGAGGAGTT	TCCTGAGGCAGTTTTGGACACA
NM_000546	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	CYS51STOP/P53	AGATGGGGTCTCACAGTGTTGC	ATGTTGACCCTTCCAGCTCCAC
NM_078467	P21	Homosapiens cyclin-dependent kinase inhibitor 1A	CDKN1A	GGCAGACCAGCATGACAGATT	GCGGCCAGGGTATGTACATGA
NM_002392	MDM2	Homo sapiens Mdm2, transformed 3T3 cell double minute 2	HDMX/MGC71221	GGGTTCGCACCATTCTCCTG	GGCAGATGACTGTAGGCCAAGC
NM_016335	PRODH	Homo sapiens proline dehydrogenase (oxidase) 1	PIG6/HSPOX2	TTTTTCACCCCACACTTGCAGA	TGTCCCAGGCAGGTATCAGGTT
NM_001101	ACTB	Homo sapiens actin, beta	PS1TP5BP1, beta-actin	CAATGTGGCCGAGGACTTTGAT	AGTGGGGTGGCTTTTAGGATGG
NM_002046	GAPDH	Homo sapiens glyceraldehide 3-phosphate dehydrogenase	G3PD, GAPD	CATTGCCCTCAACGACCACTTT	GGTGGTCCAGGGGTCTTACTCC

Statistical analysis. All results represent one of at least three independent experiments with similar outcomes. All data are expressed as the mean ± standard error of mean. One-way analysis of variance (ANOVA) and Tukey post hoc test were usBCLed to compare groups of data. P≤0.05 was considered to indicate a statistically significant result. GraphPad Software, Version 4.03 (San Diego, CA, USA) was used for the statistical analysis.

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Reverse transcription quantitative polymerase chain reaction (RT-qPCR) using a panel of apoptosis-related gene primers. qPCR was performed using a Light Cycler® 480 (Roche Diagnostics) instrument and Light Cycler 480 SYBR Green 1 Master kit (Roche Diagnostics). Briefly, the reactions were performed in a 20-µl volume with 5 pmol of each primer and 1 µl of cDNA template derived from reverse-transcribed RNA of scrambled siRNA (control) and NFkB-siRNA-transfected cells. The primers used are shown in Table I. GAPDH, a human housekeeping gene, was used as an endogenous control and reference gene for relative quantifications. The same thermal profile was optimized for all primers: pre-incubation for 5 min at 95˚C for 1 cycle, followed by 40 cycles of denaturation at 95˚C for 10 sec, primer annealing at 64˚C for 20 sec, and primer extension at 72˚C for 10 sec. Water was included as a no-template control. Melting curves were derived after 40 cycles by a denaturation step at 95˚C for 10 sec, followed by annealing at 65˚C for 15 sec, and a temperature rise to 95˚C with a heating rate of 0.1˚C/sec and continuous fluorescence measurement. Final cooling was performed at 37˚C for 30 sec. Melting curve analyses of each sample were performed using LightCycler 480 Software version LCS480 (Roche Diagnostics). The analysis step of relative quantification was a fully automated process accomplished by the software, with the efficiency set at 2 and the cDNA of untreated cells defined as the calibrator. Silencing time course assay is done with 1, 2 and 5 day samples. Since the 5 day sample gave the most severe silencing 5th day samples are taken to apoptosis panel amplification.

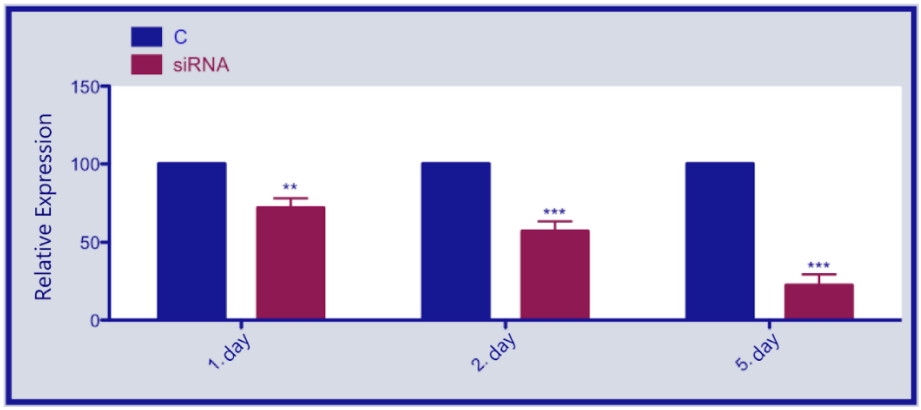

Figure 1: Silencing of STAMP1/STEAP2 time course.

Apotosis panel primers are used with 5 th day samples. BCL2L1, P53, Caspase 7 and 9 gave higher amplification, MDM2 and Akt1 gave lower as STAMP1/STEAP2, but BAK1, BAD, P21,P73, PRODH, ABL1 and caspase2 and STAMP2 showed no change.

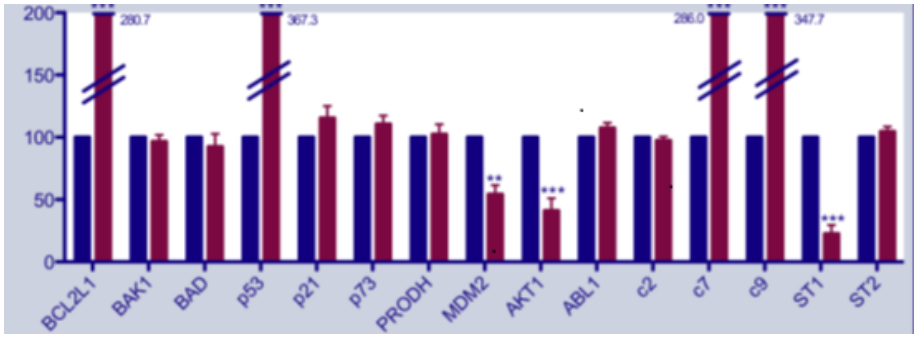

Figure 2: Apoptosis panel amplificatio

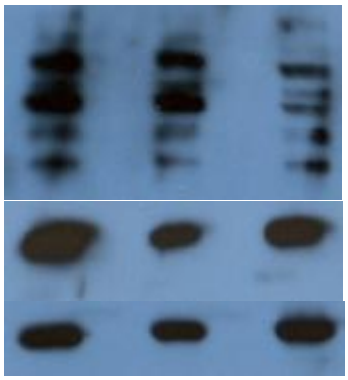

Figure 3: oxidation pathway p47,PHOX, Cu/Zn SOD results of siSTAMP1 and siSTAMP2 samples.

Western signals for the oxidative stress-related proteins p47 PHOX and Copper/Zinc Superoxide Dismutase indicate that, when STAMPs are silenced, antioxidant responses are reduced, particularly for p47 PHOX at STAMP2, and for Cu/ZnSOD at STAMP1.

These results support the increased total antioxidant responses observed in the TÜBİTAK 1001 project. Western studies with p67 PHOX, which also belongs to the same family, are ongoing to strengthen these results.

In summary, we can say that STAMP genes have anti-apoptotic properties and exert this effect through increased antioxidant capacity. They suppress apoptotic p53, working in the same direction as MDM2 in this suppression.

Significance Statement

Since the 5 day sample gave the most severe silencing 5th day samples are taken to apoptosis panel amplification.

DISCUSSION

BCL2L1: The protein encoded by this gene belongs to the BCL-2 protein family. BCL-2 family members form hetero- or homodimers and act as anti- or pro-apoptotic regulators that are involved in a wide variety of cellular activities. The proteins encoded by this gene are located at the outer mitochondrial membrane, and have been shown to regulate outer mitochondrial membrane channel (VDAC) opening. VDAC regulates mitochondrial membrane potential, and thus controls the production of reactive oxygen species and release of cytochrome C by mitochondria, both of which are the potent inducers of cell apoptosis. Alternative splicing results in multiple transcript variants encoding two different isoforms. The longer isoform acts as an apoptotic inhibitor and the shorter isoform acts as an apoptotic activator.

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.

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TP53 maintains genome stability by preventing mutations in the genome. It is also called the "genome guard" because it prevents the genome from being corrupted or changed by preventing mutation. p53 is functional in the cell with a tetramer bond.

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.

STAMP1/STEAP2 Metalloreductase STEAP2 is an enzyme that in humans is encoded by the STEAP2 gene. This gene is a member of the STEAP family and encodes a multi-pass membrane protein that localizes to the Golgi complex, the plasma membrane, and the vesicular tubular structures in the cytosol.

Cancer is the second most common type of cancer in men worldwide today. Despite advances in diagnosis, follow-up and treatment, prostate cancer is a highly heterogeneous disease. STAMP1 is extensively expressed in normal and malignant prostate cells. It is usually associated with the trans-golgi network in the plasma membrane of prostate epithelial cells and the golgi complex. Apart from the prostate, STAMP1 is found in the heart, brain, pancreas, ovary, skeleton, muscle, mammary gland, testis, uterus, kidney, lung, trachea, and liver. No reduction in STAMP1 levels occurred after castration in androgen-dependent CWR22 tumors in mice. STAMP1 expression is unaffected by androgen stimulation, but responds to the to the androgen receptor. STAMP1 is localized in the cytosol and cell membrane of prostate epithelial cells. Knockout of the STAMP1 gene in mice results in a dramatic reduction in tumor size. Studies suggest that STAMP1 may be an important target in new treatment strategies.

By regulating some genes involved in the cell cycle, STAMP1 causes cycle arrest in the G_0-G_1 phase. The proliferative activities of STAMP1 appear to be related to the ERK (extracellular signal-regulated kinase) pathway.

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Authorship Contributions
Designed research: Gonen
Conducted experiments: Gonen
Contributed new reagents or analytic tools: Gonen
Performed data analysis: Gonen
Wrote the manuscript: Gonen

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