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Promoter Region Analysis of STAMP1/STEAP2 Gene-Silencing of STAMP1/STEAP2 Gene Triggers P53 Upregulation

Ceren Gönen

Department of Pharmacology, Faculty of Pharmacy, Ege University, Izmir, Türkiye
Email: gonenceren72@gmail.com

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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. In the initial stages, prostate cancer is dependent on androgens for growth which is the basis for androgen ablation therapy. 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. STEAP (Six Transmembrane Epithelial Antigen of Prostate) is the first characterized prostate of 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 the 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 on the p53- and caspase-related pathways were characterized.

Keywords

LNCaP, STAMP1/STEAP2, P53, RT-PCR

1. Significance Statement

In silico analysis of the promoter of STAMP1/STEAP2 gene and target gene response element regions were examined using the Genomatix website, and the 1.2

kilobase promoter regions of STAMP1/STEAP2 (see Figure 1).

2. Introduction

Prostate cancer is an important issue in men due to the prolonged life expectancy in modern society. The mechanisms involved in the pathology, which causes morbidity and mortality, have not yet been fully understood. It is a type of cancer that cannot be clarified and therefore cannot be fully treated. In the initial phase of the disease, tumor growth occurs androgen-dependent forms the basis of androgen ablation therapy currently in use [1].

However, in many cases, prostate cancer reverts to an androgen-independent phenotype and there is currently no successful treatment that results in mortality [2]. With the studies carried out in recent years, androgen-regulated and prostate-specific genes have been identified. Prostate-Specific Antigen (PSA)/Kallikrein 3 (KLK3) clinical as a tumor marker, it is routinely used to detect prostate cancer and monitor its development [3]. Many other androgen-regulated genes are also implicated in the prostate. May be listed as KLK2 [4], KLK4 [4] [5], NKX3.1 [6] [7] [8].

It is regulated positively/negatively by PCGEM1 [9] and many more androgens. The gene is identified and characterized by multiple cell-based analysis methods, studies continue [10] [11]. We searched for prostate-specific genes expressed in the early stages of prostate cancer. In one project, we came across a gene with six transmembrane domains at its C-terminus (Six Transmembrane Protein of Prostate 1, STAMP1) [12] [13] and later STAMP2 [14] [15] and STAMP3 were identified.

3. 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).

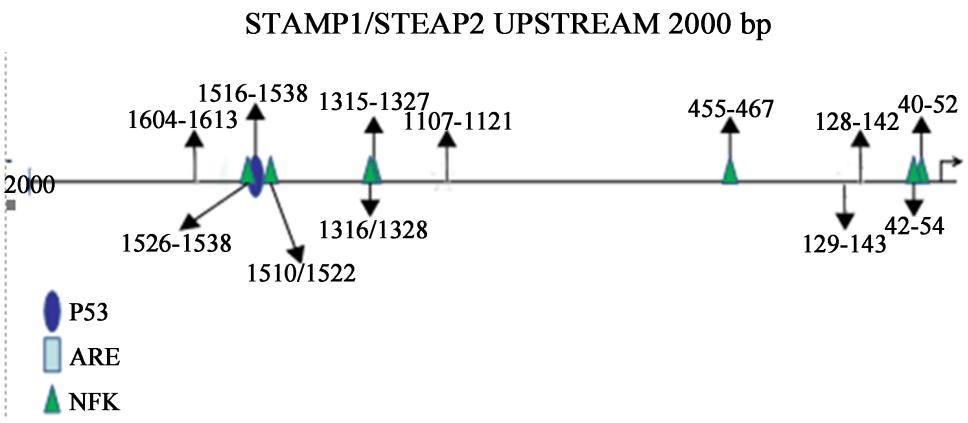

Figure 1. Promoter region of STAMP1/STEAP2 gene.

siRNA-mediated knockdown of genes LNCaP cells were transfected with either scrambled control siRNA (sc-37007) or p53-specific siRNA (sc-29435), STAMP1 specific siRNA: (sc-76587) 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.

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 p53-siRNA-transfected cells. The primers used are shown in **Table 1**. 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.

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 used to compare groups of data. $P \leq 0.05$ was considered to indicate a statistically significant result. GraphPad Software, Version 4.03 (San Diego, CA, USA) was used for the statistical analysis (see **Table 2**).

4. Results

Silencing of the apoptotic gene p53-siRNA administration:

LNCaP cells have the mutant p53 gene, although standard p53 siRNA has been developed by Santa Cruz Biotechnology Inc. (Bergheimer, Germany). In amplifications using primers specific for p53 1, 2, and 4 siRNA samples versus the control, day 4 was considered the day with optimal silencing for apoptosis panel RT-PCR –amplification (see **Figure 2** and **Figure 3**).

With suppression of STAMP1, 2.8 fold in BCL2L1 compared to control, at a

relative expression of 3.67-fold in p53, 2.86-fold in caspase 7 and 3.48-fold in caspase 9 expression increased and decreased in MDM2, AKT1 were shown (see Figure 4).

Table 1. Genes and primers used as an apoptosis panel for quantitative Polymerase Chain Reaction (qPCR) analysis.

GENE BANK	SYMBOL	DEFİNİTİON	GENE NAME	LEFT PRİMER	RİGHT PRİMER
NM_0043 22	BAD	BCL2-antagonist of cell death	BBC2/BCL2L8	AGGATCCGTGCTGT CTCCTTTG	CAAAACTTCCGATG G GACCAAG
NM_0011 88	BAK1	BCL2-antagonist/killer 1	BAK/BCL2L7	GGGTGTAGATGGGG GAACTGTG	AAGACCCTAGGCTG T GCCCAAT
NM_1385 78	BCL2L 1	BCL2-like 1	BCL-X/BCL-XL	GTGTGAGGAGCTGC TGGCTTG	AGCATCAGGCCGTC C AATCTC
NM_0012 5	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1	NIP1/TRG-8	CAGGTTGGATGGAA CACAGTGC	ATCCCAATGCCAGA C CTTCCTC
NM_0329 82	CASP2	Caspase 2, apoptosis-related cysteine protease	CASP-2/ICH-1L	TCTCCCATGGTCCC TAGCAAAA	AAGGCTCACAAACC A CCCAAAC
NM_0012 27	CASP7	Caspase 7, apoptosis-related cysteine protease	CMH-1/ICE-LAP3	AAGTGAGGAAGAGT TTATGGCAA A	CCATCTTGAAAACA A AGTGCCAAA
NM_0012 29	CASP9	Caspase 9, apoptosis-related cysteine protease	APAF-3/APAF3	TCCTGAGTGGTGCC AAACAAAA	AGTGGTTGTCAGGC GAGGAAAG
NM_0051 57	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	ABL/C-ABL	GGCCTTGAAGACAG AGCAAAGC	GGAAGGGACCAGTA CCTCATGG
NM_0051 63	AKT1	V-akt murine thymoma viral oncogene homolog 1	PKB/PRKBA	TCCCCCTCAGATGA TCTCTCCA	CGGAAAGGTTAAGC GTCGAAAA
NM_0054	TP73	Tumor protein p73	P73	AGCAGCCCATCAAG GAGGAGTT	TCCTGAGGCAGTTT T GGACACA
NM_0005 46	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	CYS51STOP/P53	AGATGGGGTCTCAC AGTGTTGC	ATGTTGACCCTTCC A GCTCCAC
NM_0784 67	P21	Homosapiens cyclin-dependent kinase inhibitor 1A	CDKN1A	GGCAGACCAGCATG ACAGATT	GCGGCCAGGGTATG TACATGA
NM_0023 92	MDM2	Homo sapiens Mdm2, transformed 3T3 cell double minute 2	HDMX/MGC71221	GGGTTCGCACCATT CTCCTG	GGCAGATGACTGTA GGCCAAGC
NM_0163 35	PRODH	Homo sapiens proline dehydrogenase (oxidase) 1	PIG6/HSPOX2	TTTTTCACCCCACA CTTGCAGA	TGTCCCAGGCAGGT ATCAGGTT
NM_0011 01	ACTB	Homo sapiens actin, beta	PS1TP5BP1, beta-actin	CAATGTGGCCGAGG ACTTTGAT	AGTGGGGTGGCTTT T AGGATGG
NM_0020 46	GAPDH	Homo sapiens glyceraldehide 3-phosphate dehydrogenase	G3PD, GAPD	CATTGCCCTCAACG ACCACTTT	GGTGGTCCAGGGGT CTTACTCC

Table 2. STAMP1/STEAP2 promoter region response element sequence.

Further Information	Opt.	Position	Str.	Core Sim.	Matrix Sim.	Sequence
NF-kappaB (p50)	0.83	315 - 327	(+)	1.000	0.994	cagGGGAtcccct
NF-kappaB (p50)	0.83	316 - 328	(−)	1.000	0.994	cagGGGAtcccct
NF-kappaB	0.82	510 - 522	(+)	1.000	0.862	gaGGGAgttttca
Tumor Suppressor p53	0.66	527 - 547	(−)	0.750	0.671	taaAATGttcggaaatgccct
Tumor Suppressor p53	0.66	528 - 548	(+)	0.750	0.689	gggCATTtccgaacattttat
Tumor Suppressor p53	0.66	1083 - 1103	(−)	0.750	0.676	ctgCGTGcccggacagctcgt

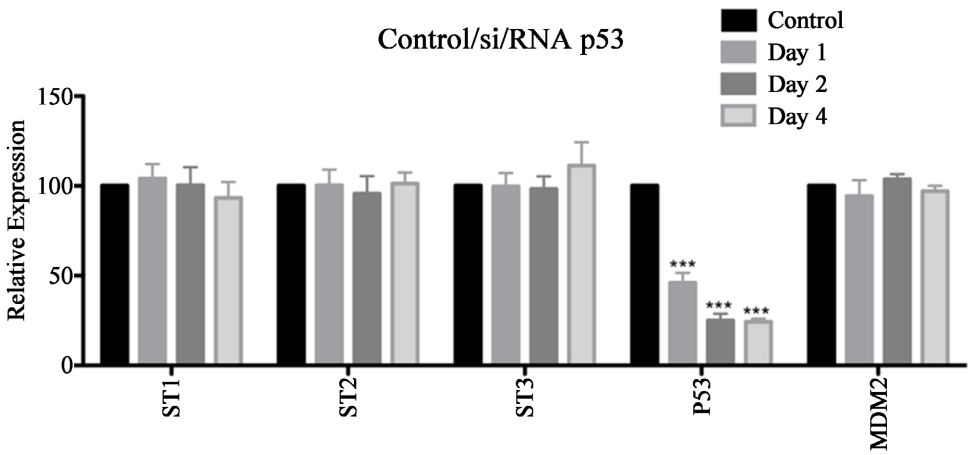

Figure 2. LNCaP cells time-dependent p53 siRNA administration, 2 way ANOVA followed by Bonferroni's post test. ***P < 0.001 (vs Control).

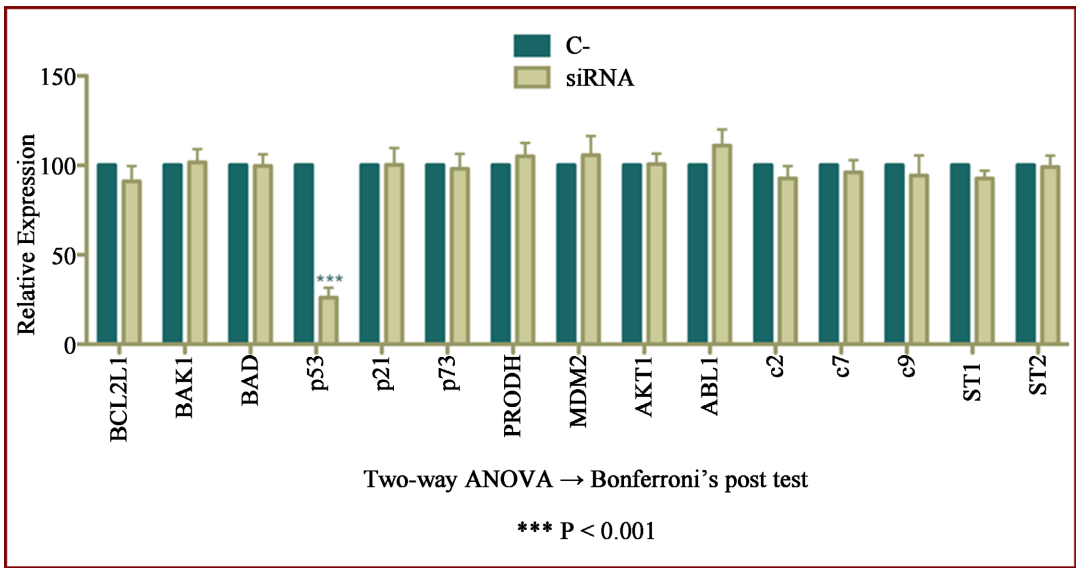

Figure 3. P53 siRNA day 4 samples were subjected to apoptosis panel RT-PCR amplification.

Important Note: In all gene silencing-siRNA experiments, inductions were initiated within the last 2 hours of transfection time.

Following transfection, induction of apoptosis and confirmation of their

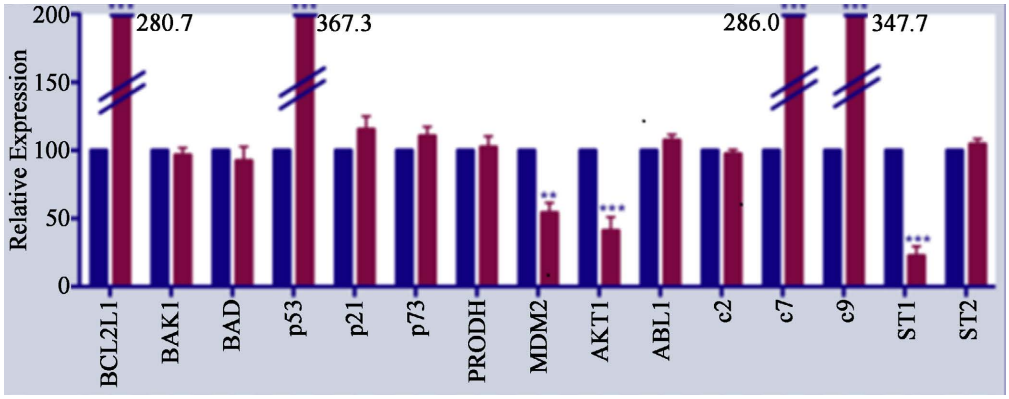

Figure 4. LNCaP cells STAMP1-siRNA 5th day administration apoptosis panel (n = 3). ***P < 0.001.

Table 3. Stimuli and processes known to influence STAMP1 expression.

Stimuli/Disease/Process	Effect	Tissue-cell Line
Cancer	Increase	Prostate Cancer
Adipogenesis	Decrease	3T3-L1 Murine Preadipocytes, Murine Mesenchymal Stem Cell
TNF-alpha	Decrease	LNCaP Cell Line
Androgen Receptor	Sustain	Prostate Cancer Cell Lines

expression RNA isolations from cells (RNeasy, QIAGEN) were performed. RNA samples are complementary DNA-cDNA synthesis (cDNA Synthesis Kit, Roche) was performed. Conventional PCR (MasterMix, Qiagen) once again confirmed the existence of STAMP genes.

A test containing apoptotic and anti-apoptotic genes by uptake of STAMP gene amplifications, RT-PCR in apoptosis panel (LC480, Roche-Ege University, Faculty of Engineering, Bioengineering Department) application was made.

5. Discussion

Prostate 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 (see Table 3). 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 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 ar-

rest in the G_0 - G_1 phase. The proliferative activities of STAMP1 appear to be related to the ERK (extracellular signal-regulated kinase) pathway.

Other members of the STAMP family include pHyde, a rat homologue that has been implicated in the apoptosis of prostate cancer cells [16], and its human homologue TSAP6 (also known as STEAP3), a p53-inducible gene involved in apoptosis and the cell cycle in prostate cancer and HeLa cells [17].

Studies reported that STAMP family members have metalloreductase activities associated with iron and copper uptake into HEK-293T cells [18], though mentioned activities have been shown for prostate cells [19].

Although STAMP1/STEAP2 gene promoter region does not have Androgen Receptor Response Element (ARRE), the second identified STAMP2/STEAP4 gene has ARRE and is shown by ChIP. As a final point, both genes have a p53 response element, but siSTAMP1/STEAP2 application causes p53 gene: GENOME GARDİAN up-regulation.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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Appendix

AR	Androgen Receptor
ARRE	Androgen Receptor Response Element
ChIP	Chromatine Immunoprecipitation
LNCaP	Prostate Adenocarcinoma, Lymph Node Metastatic Site
MDM2	E3 Ubiquitin Ligase
NFkB	Nuclear Factor Kappa B
siRNA	Small Interfering RNA
STEAP	Six Transmembrane Epithelial Antigen of Prostate
STAMP	Six Transmembrane Protein of Prostate
RTqPCR	Quantitative Polymerase Chain Reaction
TUBA	Turkish Academy of Sciences
TUBITAK	Scientific and Technological Research Council of Türkiye