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Responses Taken by Silencing of NFkappaB, STAMP1 and STAMP2 Genes and Expression of NFkB, Act-1, p53 and p73 at −/+ TNFalpha Induced LNCaP Cells

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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. 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. 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. In this research project, the prostate-specific STAMP gene family and its regulatory effects on the nuclear factor kappa B and caspase-related pathways were characterized. Considering that the beta-actin response in the control group was high in the immunolabeling studies, an increase in the induction of Tumor Necrosis Factor (TNF) was detected in the signals received with the vital proteins NFkB and akt, which were silenced by siRNA, which means that STAMP genes potentiate vital proteins.

Keywords

Promoter Analysis, RNA/siRNA, Regulation of Gene Expression

1. Introduction

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 [1]. Other factors include a diet high in processed meat and red meat, while the risk from a high intake of milk products is inconclusive. An association with gonorrhea has been found, although no reason for this relationship has been identified. Increased risk is associated with *BRCA* mutations. Diagnosis is by biopsy. Medical imaging may be done to assess whether metastasis is present.

Prostate cancer screening, including Prostate-Specific Antigen (PSA) testing, increases cancer detection but whether it improves outcomes is controversial. Informed decision-making is recommended for those 55 to 69 years old. Testing, if carried out, is more appropriate for those with a longer life expectancy. Although 5α-reductase inhibitors appear to decrease low-grade cancer risk, they do not affect high-grade cancer risk, and are not recommended for prevention. Vitamin or mineral supplementation does not appear to affect risk.

Many cases are managed with active surveillance or watchful waiting. Other treatments may include a combination of surgery, radiation therapy, hormone therapy, or chemotherapy. Tumors limited to the prostate may be curable. Pain medications, bisphosphonates, and targeted therapy, among others, may be useful. Outcomes depend on age, health status, and how aggressive and extensive the cancer is. Most men with prostate cancer do not die from it. The United States five-year survival rate is 98%.

Globally, it is the second-most common cancer. It is the fifth-leading cause of cancer-related death in men. In 2018, it was diagnosed in 1.2 million and caused 359,000 deaths. It was the most common cancer in males in 84 countries, occurring more commonly in the developed world. Rates have been increasing in the developing world. Detection increased significantly in the 1980s and 1990s in many areas due to increased PSA testing. One study reported prostate cancer in 30% to 70% of Russian and Japanese men over age 60 who had died of unrelated causes.

2. Cancer Models

Scientists have established prostate cancer cell lines to investigate disease progression. LNCaP, PC-3 (PC3), and DU-145 (DU145) are commonly used pros-

tate 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 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.

However, in many cases prostate cancer reverts to an androgen-independent phenotype and there is currently no successful treatment and results in mortality. 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. Many other androgen-regulated genes are also implicated in the prostate. May be listed as KLK2, KLK4 [2], NKX3.1.

It is regulated positively/negatively by PCGEM1 and many more androgens. The gene is identified and characterized by multiple cell-based analysis methods, studies continue.

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) and later STAMP2 [3] 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).

siRNA-mediated knockdown of genes LNCaP cells were transfected with either scrambled control siRNA (sc-37007) or *NFkB*-specific siRNA (sc-29410, 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.

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 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 Light Cycler 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 (Table 2 and Table 3).

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 Türkiye 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.

4. Results

1) Performing promoter region analyzes of stamp genes

In silico analysis of the promoters of STAMP genes and target gene, response element regions were examined using the Genomatix website, and the 1.2 kilobase promoter regions of STAMP genes were scanned to list the sequences containing the p53 and NFkappaB response element (Figures 1-3).

With the chromatin-immunoprecipitation (Chromatine-Immunoprecipitation, ChIP) procedure, this region where the androgen receptor binds was degraded with androgen receptor antibody. Likewise, ChIP technique was applied with antibodies specific for p53 and NFkappaB.

2) Silencing of nuclear factor kappa B

With silencing of NFkappaB following TNF alpha induction given genes p53, p73, Act1, caspase 7, capase 9 and STAMP1 got decreased by each new day of silencing, but not MDM2 and STAMP2 showed a change at relative amplification by each new day.

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 PRİNER
NM_0043 22	BAD	BCL2-antagonist of cell death	BBC2/BCL2L8	AGGATCCGTGCTGTC TCCTTTG	CAAAACTTCCGATGG GACCAAG
NM_0011 88	BAK1	BCL2-antagonist/ killer 1	BAK/BCL2L7	GGGTGTAGATGGGGG AACTGTG	AAGACCCTAGGCTGT GCCCAAT
NM_1385 78	BCL2L 1	BCL2-like 1	BCL-X/BCL-XL	GTGTGAGGAGCTGCT GGCTTG	AGCATCAGGCCGTCC AATCTC
NM_0012 5	BNIP1	BCL2/adenovirus E1B 19 kDa interacting protein 1	NIP1/TRG-8	CAGGTTGGATGGAAC ACAGTGC	ATCCCAATGCCAGAC CTTCCTC
NM_0329 82	CASP2	Caspase 2, apoptosis-related cysteine protease	CASP-2/ICH-1L	TCTCCCATGGTCCCTA GCAAAA	AAGGCTCACAAACCA CCCAAAC
NM_0012 27	CASP7	Caspase 7, apoptosis-related cysteine protease	CMH-1/ICE-LAP 3	AAGTGAGGAAGAGTT TATGGCAA A	CCATCTTGAAAACAA AGTGCCAAA
NM_0012 29	CASP9	Caspase 9, apoptosis-related cysteine protease	APAF-3/APAF3	TCCTGAGTGGTGCCA AACAAAA	AGTGGTTGTCAGGC GAGGAAAG
NM_0051 57	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1	ABL/C-ABL	GGCCTTGAAGACAGA GCAAAGC	GGAAGGGACCAGTA CCTCATGG
NM_0051 63	AKT1	V-akt murine thymoma viral oncogene homolog 1	PKB/PRKBA	TCCCCCTCAGATGATC TCTCCA	CGGAAAGGTTAAGC GTCGAAAA
NM_0054	TP73	Tumor protein p73	P73	AGCAGCCCATCAAGG AGGAGTT	TCCTGAGGCAGTTTT GGACACA
NM_0005 46	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	CYS51STOP/P53	AGATGGGGTCTCACA GTGTTGC	ATGTTGACCCTTCCA GCTCCAC
NM_0784 67	P21	Homosapiens cyclin-dependent kinase inhibitor 1A	CDKN1A	GGCAGACCAGCATGA CAGATT	GCGGCCAGGGTATG TACATGA
NM_0023 92	MDM2	Homo sapiens MDM2, transformed 3T3 cell double minute 2	HDMX/MGC712 21	GGGTTCGCACCATTCT CCTG	GGCAGATGACTGTA GGCCAAGC
NM_0163 35	PRODH	Homo sapiens proline dehydrogenase (oxidase) 1	PIG6/HSPOX2	TTTTTCACCCCACACT TGCAGA	TGTCCCAGGCAGGT ATCAGGTT

Continued

NM_0011 01	ACTB	Homo sapiens actin, beta	PS1TP5BP1, beta-actin	CAATGTGGCCGAGGA CTTTGAT	AGTGGGGTGGCTTTT AGGATGG
NM_0020 46	GAPDH	Homo sapiens glyceraldehide 3-phosphate dehydrogenase	G3PD, GAPD	CATTGCCCTCAACGA CCACTTT	GGTGGTCCAGGGGT CTTACTCC

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

Further Information	Op.	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

Table 3. STAMP2/STEAP4 promoter region response element sequences.

Further Information	Opt.	Position	Str.	Core Sim.	Matrix Sim.	Sequence
NF-kappaB	0.89	743 - 755	(−)	1.000	0.896	ggGGGAttaacct
Androgen Receptor Binding Site	0.80	790 - 808	(−)	1.000	0.801	atggttctcacTGTTtttc
Prostate-specific Homeodomain Protein NKX3.1	0.84	1083 - 1097	(−)	1.000	0.936	taggttAAGTattat

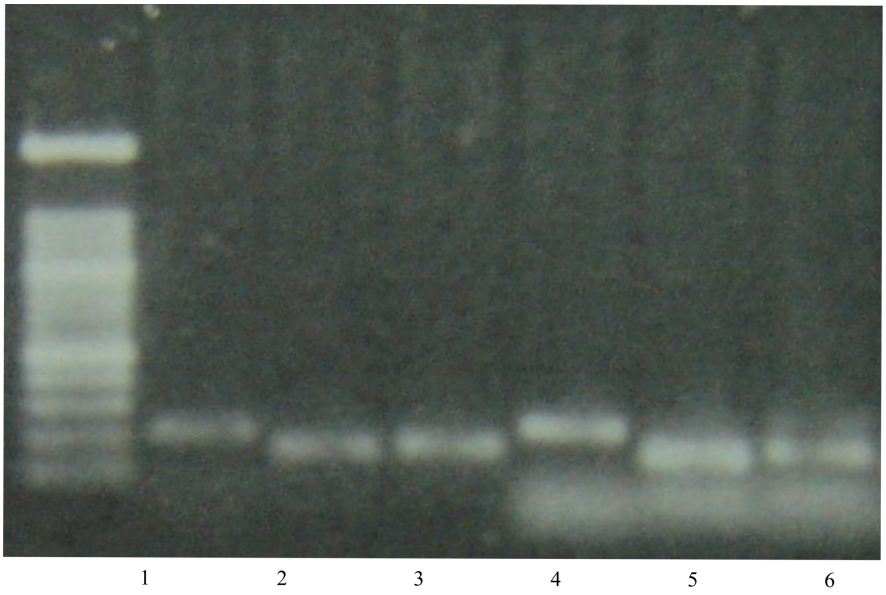

Figure 1. Primer control designed for the promoter region. 1. STAMP1 P53-53 kb; 2. STAMP1NFK-B-50 kb; 3. STAMP1 NFK-B-50 kb; 4. STAMP2 P53-53 kb; 5. STAMP2 NFK-B-50 kb; 6. STAMP2 NFK-B-50 kb.

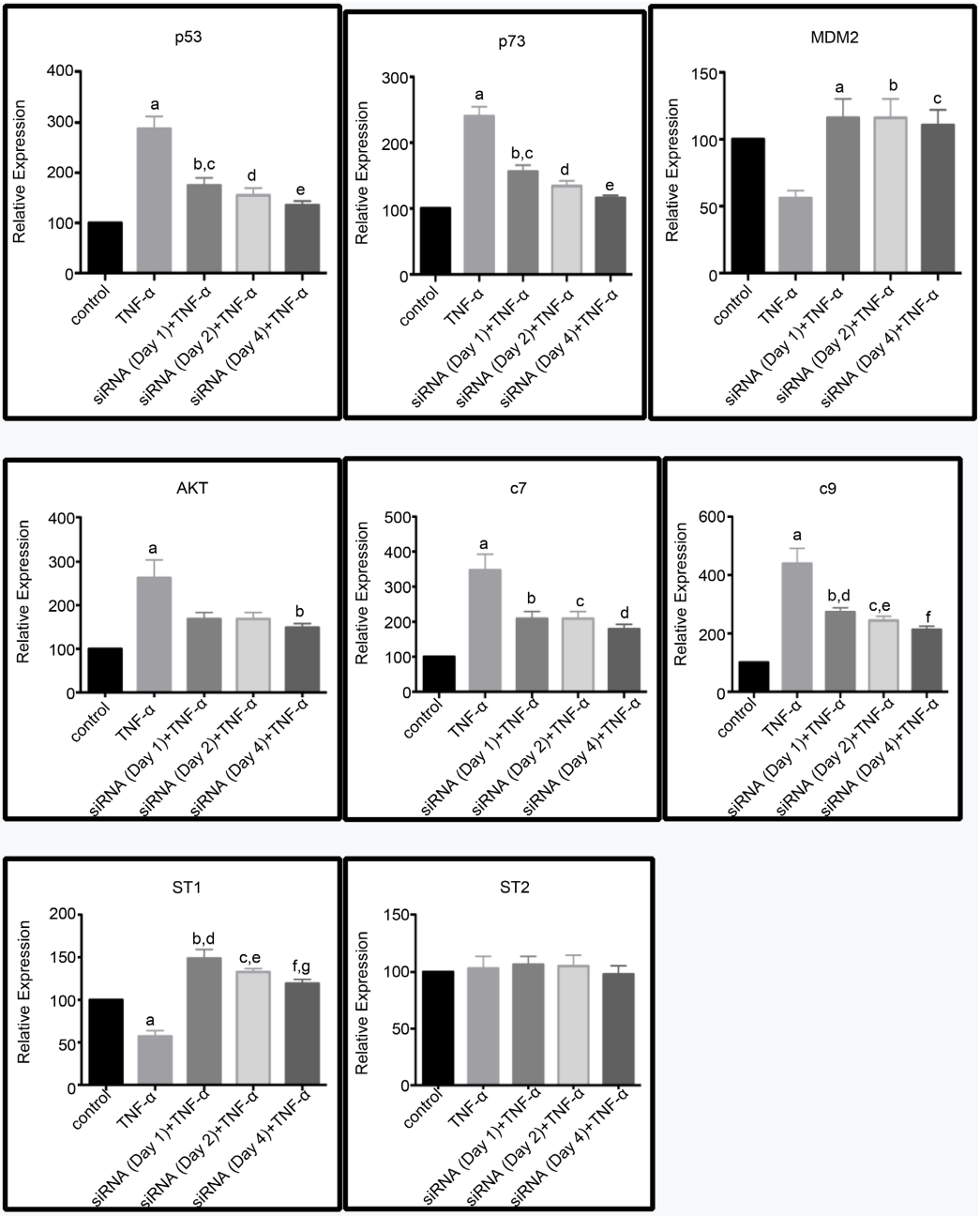

Figure 2. LNCaP cells time-dependent NFkB siRNA administration, 2 way ANOVA followed by Bonferroni's post test. ***P < 0.001 (vs Control). A. P53 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. B. P73 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. C. MDM2 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. D. ACT1 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. E. Caspase7 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. F. Caspase 9 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. G. STAMP1 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha. H. STAMP2 amplification at control, TNFalpha, siNFkappaB silencing for 1 day+TNF alpha, siNFkappaB silencing for 2 days+TNF alpha, siNFkappaB silencing for 4 days+TNF alpha.

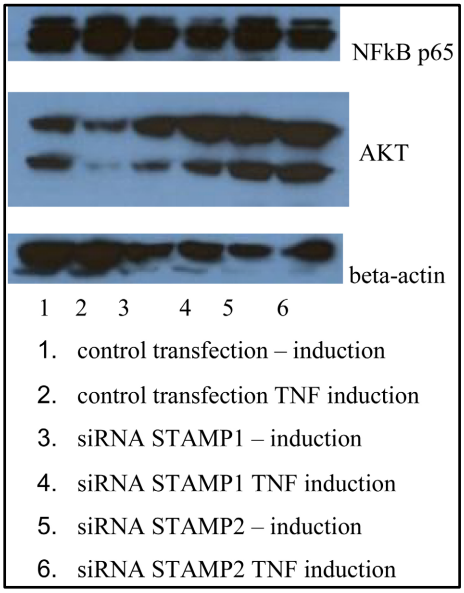

Figure 3. STAMP1 and STAMP2 siRNA transfected LNCaP cells, NFkB p65, AKT and beta-actin western after TNF induction.

Considering that the beta-actin response in the control group was high in the immunolabeling studies, an increase in the induction of Tumor Necrosis Factor (TNF) was detected in the signals received with the vital proteins NFkB and akt, which were silenced by siRNA, which means that STAMP genes potentiate vital proteins.

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 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

1) 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 [4] [5] [6] [7].

2) 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.

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.

The TP53 gene encodes at least 15 protein isoforms, ranging in size from 3.5 kDa to 43.7 kDa. All these p53 proteins are called p53 isoforms. The TP53 gene is the most frequently mutated gene in human cancers, which indicates that the p53 gene has critical importance in preventing cancer development.

- It has many anti-cancer functions.
- Activates DNA repair proteins when DNA is damaged.
- It initiates "apoptosis" (programmed cell death) when DNA is damaged beyond repair.
- Human TP53 Gene.
- This gene is located on the 17th chromosome of humans (17p13.1). Substituting arginine for proline at codon position 72 is a common polymorphism in humans. Although many studies have examined that this change is associated with cancer susceptibility, the results are controversial and a clear decision has not been reached. A meta-analysis conducted in 2009 failed to show such a link for the cervix [1]. In a study conducted in 2011, it was found that TP53 proline mutation had a profound effect on the risk of male pancreatic cancer [2]. In a study conducted on Arab women, it was stated that proline homozygosity reduced the risk of breast cancer [3]. In another study, it was suggested that TP53 codon 72 polymorphism collectively may be associated with susceptibility to MDM2 SNP309 and A2164G non-oropharyngeal cancer, and MDM2 SNP309 and TP53 codon 72 increase the risk of non-oropharyngeal cancers.
- Again, in a study conducted in 2011, it was found that codon 72 polymorphism was associated with an increased risk of lung cancer.
- Meta-analyses conducted in 2011 did not find a significant relationship between the TP53 codon 72 polymorphism and both colorectal cancer risk and endometrial cancer risk.
- Functions.
- DNA damage and repair.
- p53 controls progression or regulation through cell cycle, apoptosis, and genome robustness through several different mechanisms:
- Activates DNA repair proteins when DNA is constantly damaged. It can be an important factor in aging.
- When DNA damage is detected, it can pause the cell cycle at the G1/S checkpoint. At this point, if enough time is waited, DNA repair proteins will be able to fix the damage, and when the damage is removed, cell cycle arrest ends and

continues.

- If DNA damage is beyond repair, it may initiate apoptosis.
- Required in the response of cell aging to short telomeres.

WAF1/CIP1 produces p21 and hundreds more inhibitory/down-regulatory/activity-reducing proteins. p21 binds to the G1-S/CDK (CDK4/CDK6, CDK2, CDK1) complex (molecules important in the transition from the G1 to S in the cell cycle) and inhibits its activity. When p21 combines with CDK2, the cell cannot transition to the next cell cycle stage. A mutated p53 cannot bind effectively to DNA and as a result, the p21 protein will no longer be used as a "stop" signal. Cancer development may occur as a result of the absence of p53 and therefore not being able to make p21 do the desired work. p53 expression can be induced by UV rays that cause DNA damage. In this case, the events that initiate tanning develop. It is also seen in other animals:

- Mouse: 11th chromosome.
- Rat: chromosome 10.
- Dog: 5th chromosome.
- Pig: 12th chromosome.

3) 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.

An additional MDM2 family member, Mdm4 (also called MdmX), has been discovered and is also an important negative regulator of p53.

MDM2 is also required for organ development and tissue homeostasis because unopposed p53 activation leads to p53-overactivation-dependent cell death, referred to as podoptosis. Podoptosis is caspase-independent and, therefore, different from apoptosis. The mitogenic role of MDM2 is also needed for wound healing upon tissue injury, while MDM2 inhibition impairs re-epithelialization upon epithelial damage. In addition, MDM2 has p53-independent transcription factor-like effects in nuclear factor-kappa beta (NFκB) activation. Therefore, MDM2 promotes tissue inflammation and MDM2 inhibition has potent anti-inflammatory effects in tissue injury. So, MDM2 blockade had mostly anti-inflammatory and anti-mitotic effects that can be of additive therapeutic efficacy in inflammatory and hyperproliferative disorders such as certain cancers or lymphoproliferative autoimmunity, such as systemic lupus erythematosus or crescentic glomerulonephritis.

Ubiquitination target: p53.

The key target of MDM2 is the p53 tumor suppressor. MDM2 has been identified as a p53 interacting protein that represses p53 transcriptional activity. MDM2 achieves this repression by binding to and blocking the N-terminal trans-activation domain of p53. MDM2 is a p53 responsive gene—that is, its transcription can be activated by p53. Thus when p53 is stabilized, the transcription of MDM2 is also induced, resulting in higher MDM2 protein levels.

E3 ligase activity.

The E3 ubiquitin ligase MDM2 is a negative regulator of the p53 tumor suppressor protein. MDM2 binds and ubiquitinates p53, facilitating it for degradation. p53 can induce transcription of MDM2, generating a negative feedback loop [8]. MDM2 also acts as an E3 ubiquitin ligase, targeting both itself and p53 for degradation by the proteasome (see also ubiquitin). Several lysine residues in p53 C-terminus have been identified as the sites of ubiquitination, and it has been shown that p53 protein levels are downregulated by MDM2 in a proteasome-dependent manner. MDM2 is capable of auto-polyubiquitination, and in complex with p300, a cooperating E3 ubiquitin ligase, is capable of polyubiquitinating p53. In this manner, MDM2 and p53 are the members of a negative feedback control loop that keeps the level of p53 low in the absence of p53-stabilizing signals. This loop can be interfered with by kinases and genes like p14arf when p53 activation signals, including DNA damage, are high.

Structure and function.

The full-length transcript of the MDM2 gene encodes a protein of 491 amino acids with a predicted molecular weight of 56 kDa. This protein contains several conserved structural domains including an N-terminal p53 interaction domain, the structure of which has been solved using x-ray crystallography. The MDM2 protein also contains a central acidic domain (residues 230 - 300). The phosphorylation of residues within this domain appears to be important for regulation of MDM2 function. In addition, this region contains nuclear export and import signals that are essential for proper nuclear-cytoplasmic trafficking of MDM2.

Another conserved domain within the MDM2 protein is a zinc finger domain, the function of which is poorly understood.

MDM2 also contains a C-terminal RING domain (amino acid residues 430 - 480), which contains a Cis3-His2-Cis3 consensus that coordinates two ions of zinc. These residues are required for zinc binding, which is essential for proper folding of the RING domain. The RING domain of MDM2 confers E3 ubiquitin ligase activity and is sufficient for E3 ligase activity in MDM2 RING autoubiquitination. The RING domain of MDM2 is unique in that it incorporates a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins, as well as a nucleolar localization sequence. The RING domain also binds specifically to RNA, although the function of this is poorly understood.

4) Regulation

There are several known mechanisms for regulation of MDM2. One of these mechanisms is phosphorylation of the MDM2 protein. MDM2 is phosphorylated at multiple sites in cells. Following DNA damage, phosphorylation of MDM2 leads to changes in protein function and stabilization of p53. Additionally, phosphorylation at certain residues within the central acidic domain of MDM2 may stimulate its ability to target p53 for degradation. HIPK2 is a protein that regulates MDM2 in this way. The induction of the p14arf protein, the alternate reading frame product of the p16INK4a locus, is also a mechanism of negatively regulating the p53-MDM2 interaction. p14arf directly interacts with MDM2 and leads to up-regulation of p53 transcriptional response. ARF sequesters MDM2 in the nucleolus, resulting in inhibition of nuclear export and activation of p53, since nuclear export is essential for proper p53 degradation.

Inhibitors of the MDM2-p53 interaction include the cis-imidazoline analog nutlin [9]-[14].

Levels and stability of MDM2 are also modulated by ubiquitylation. MDM2 auto ubiquitylates itself, which allows for its degradation by the proteasome. MDM2 also interacts with a ubiquitin specific protease, USP7, which can reverse MDM2-ubiquitylation and prevent it from being degraded by the proteasome. USP7 also protects from degradation the p53 protein, which is a major target of MDM2. Thus MDM2 and USP7 form an intricate circuit to finely regulate the stability and activity of p53, whose levels are critical for its function.

Overview of signal transduction pathways involved in apoptosis.

MDM2 has been shown to interact with MDM2 p53-independent role.

MDM2 overexpression was shown to inhibit DNA double-strand break repair mediated through a novel, direct interaction between MDM2 and Nbs1 and independent of p53. Regardless of p53 status, increased levels of MDM2, but not MDM2 lacking its Nbs1-binding domain, caused delays in DNA break repair, chromosomal abnormalities, and genome instability. These data demonstrated MDM2-induced genome instability can be mediated through MDM2:Nbs1 interactions and independent from its association with p53.

5) 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.

6) Caspase-7

Caspase-7, an apoptosis-related cysteine peptidase, also known as CASP7, is a human protein encoded by the CASP7 gene. CASP7 orthologs have been identified in almost all mammals for which full genome data are available. Unique orthologs are also found in birds, lizards, lissamfibians, and teleosts., a 303 aa long, 34 kDa protein, encoded by the gene mapped on chromosome 10, is acts as effector enzyme in apoptosis activation after cleaved and…

7) Caspase-9 is an enzyme that in humans is encoded by the CASP9 gene. It is an initiator caspase, critical to the apoptotic pathway found in many tissues. Caspase-9 homologs have been identified in all mammals for which they are known to exist, such as *Mus musculus* and *Pan troglodytes*.

Caspase-9 belongs to a family of caspases, cysteine-aspartic proteases involved in apoptosis and cytokine signalling. Apoptotic signals cause the release of cytochrome c from mitochondria and activation of apaf-1 (apoptosome), which then cleaves the pro-enzyme of caspase-9 into the active dimer form. Regulation of this enzyme occurs through phosphorylation by an allosteric inhibitor, inhibiting dimerization and inducing a conformational change.

Correct caspase-9 function is required for apoptosis, leading to the normal development of the central nervous system. Caspase-9 has multiple additional cellular functions that are independent of its role in apoptosis. Nonapoptotic roles of caspase-9 include regulation of necroptosis, cellular differentiation, innate immune response, sensory neuron maturation, mitochondrial homeostasis, corticospinal circuit organization, and ischemic vascular injury. Without correct function, abnormal tissue development can occur leading to abnormal function, diseases and premature death. Caspase-9 loss-of-function mutations have been associated with immunodeficiency/lymphoproliferation, neural tube defects, and Li-Fraumeni-like syndrome. Increased caspase-9 activity is implicated in the progression of amyotrophic lateral sclerosis, retinal detachment, and slow-channel

syndrome, as well as various other neurological, autoimmune, and cardiovascular disorders.

Different protein isoforms of caspase-9 are produced due to alternative splicing.

8) 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.

STAMP2/STEAP family member 4 is a protein that in humans is encoded by the STEAP4 gene.

6. Function

The protein encoded by this gene belongs to the STEAP (Six Transmembrane Epithelial Antigens of Prostate) family, and resides in the Golgi apparatus. It functions as a metalloreductase that has the ability to reduce both Fe(3+) to Fe(2+) and Cu(2+) to Cu(1+), using NAD(+) as an acceptor. Studies in mice and humans suggest that this gene may be involved in adipocyte development and metabolism, and may contribute to the normal biology of the prostate cell, as well as prostate cancer progression. Alternatively, spliced transcript variants encoding different isoforms have been found for this gene.

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. 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 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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Conflicts of Interest

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

References

[1] Albright, F., Stephenson, R.A., Agarwal, N., Teerlink, C.C., Lowrance, W.T., Farnham, J.M. and Albright, L.A.C. (2015) Prostate Cancer Risk Prediction Based on Complete Prostate Cancer Family History. *Journal of Diabetes*, **75**, 390-398. https://doi.org/10.1002/pros.22925

[2] Lorenzo, P.I., Arnoldussen, Y.J. and Saatcioglu, F. (2007) Molecular Mechanisms of Apoptosis in Prostate Cancer. *Critical Reviews™ in Oncogenesis*, **13**, 1-38. https://doi.org/10.1615/CritRevOncog.v13.i1.10

[3] Hubert, R.S., Vivanco, I., Chen, E., Rastegar, S., Leong, K., Mitchell, S.C., Madraswala, R., *et al.* (1999) STEAP: A Prostate-Specific Cell-Surface Antigen Highly Expressed in Human Prostate Tumors. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 14523-14528. https://doi.org/10.1073/pnas.96.25.14523

[4] Korkmaz, K.S., Elbi, C., Korkmaz, C.G., Loda, M., Hager, G.L. and Saatcioglu, F. (2002) Molecular Cloning and Characterization of STAMP1, a Highly Prostate-Specific Six Transmembrane Protein that Is Overexpressed in Prostate Cancer. *Journal of Biological Chemistry*, **277**, 36689-36696. https://doi.org/10.1074/jbc.M202414200

[5] Korkmaz, C.G., Korkmaz, K.S., Kurys, P., Elbi, C., Wang, L., Klokk, T.I., Hammarstrom, C., *et al.* (2005) Molecular Cloning and Characterization of STAMP2, an Androgen-Regulated Six Transmembrane Protein that Is Overexpressed in Prostate Cancer. *Oncogene*, **24**, 4934-4945. https://doi.org/10.1038/sj.onc.1208677

[6] Wellen, K.E., Fucho, R., Gregor, M.F., Furuhashi, M., Morgan, C., Lindstad, T., Vaillancourt, E., *et al.* (2007) Coordinated Regulation of Nutrient and Inflammatory Responses by STAMP2 Is Essential for Metabolic Homeostasis. *Cell*, **129**, 537-548. https://doi.org/10.1016/j.cell.2007.02.049

[7] Passer, B.J., Nancy-Portebois, V., Amzallag, N., Prieur, S., Cans, C., de Climens A.R., *et al.* (2003) The p53-Inducible TSAP6 Gene Product Regulates Apoptosis and the Cell Cycle and Interacts with Nix and the Myt1 Kinase. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 2284-2289. https://doi.org/10.1073/pnas.0530298100

[8] Wang, L., Jin, Y., Arnoldussen, Y.J., Jonson, I., Qu, S., Maelandsmo, G.M., Kristian, A., *et al.* (2010) STAMP1 Is Both a Proliferative and an Antiapoptotic Factor in Prostate Cancer. *Cancer Resarch*, **70**, 5818-5828. https://doi.org/10.1158/0008-5472.CAN-09-4697

[9] Galluzzi, L., Morselli, E., Kepp, O., Tajeddine, N. and Kroemer, G. (2008) Targeting p53 to Mitochondria for Cancer Therapy. *Cell Cycle*, **7**, 1949-1955. https://doi.org/10.4161/cc.7.13.6222

[10] Wadgaonkar, R. and Collins, T. (1999) Murine Double Minute (MDM2) Blocks p53-Coactivator Interaction, a New Mechanism for Inhibition of p53-Dependent Gene Expression. *Journal of Biological Chemistry*, **274**, 13760-13767. https://doi.org/10.1074/jbc.274.20.13760

[11] Gomes, I.M., Maia, C.J. and Ve Santos, C.R. (2012) STEAP Proteins: From Struc-

ture to Applications in Cancer Therapy. *Molecular Cancer Research*, **10**, 573-587. https://doi.org/10.1158/1541-7786.MCR-11-0281

[12] Steiner, M.S., Zhang, X., Wang, Y. and Lu, Y. (2000) Growth Inhibition of Prostate Cancer by an Adenovirus Expressing a Novel Tumor Suppressor Gene, pHyde. *Cancer Research*, **60**, 4419-4425.

[13] Ohgami, R.S., Campagna, D.R., McDonald, A. and Fleming, M.D. (2006) The Steap Proteins Are Metalloreductases. *Blood*, **108**, 1388-1394. https://doi.org/10.1182/blood-2006-02-003681

[14] Grunewald, T.G.P., Bach, H., Cossarizza, A. and Matsumoto, I. (2012) The STEAP Protein Family: Versatile Oxidoreductases and Targets for Cancer Immunotherapy with Overlapping and Distinct Cellular Functions. *Biology of the Cell*, **104**, 641-657. https://doi.org/10.1111/boc.201200027

Appendix

AR: Androgen Receptor

ARRE: Androgen Receptor Response Element

ChIP: Chromatine Immunoprecipitation

STAMP: Six Transmembrane Protein of Prostate

RTqPCR: Quantitative Polymerase Chain Reaction

TUBA: The Türkiye Academy of Sciences

TUBITAK: The Scientific and Technological Research Council of Türkiye