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Reduced Expression Levels of the MST1 gene in the Peripheral Blood of Patients with Prostate Cancer

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ABSTRACT

Background: Prostate cancer (PC) is the second most common malignancy among men, accounting for 12.5% of all cancers. The development of molecular studies (such as RNA expression analysis) aids the characterization of this cancer, the development of new targets for therapy, and the introduction of novel prognostic and diagnostic biomarkers. Recent studies have confirmed Mammalian Sterile 20-Like kinase (MST1) as a tumor suppressor gene, which has been introduced as a biomarker for some specific cancers. In this study, we focus on MST1 expression levels in the WBC of PC patients, due to the inheritance pattern of PC.

Methods: This case-control study was conducted in two groups (20 patients with PC and 20 healthy individuals). After RNA extraction and cDNA synthesis, quantitative Real-Time PCR was done in order to determine the MST1 expression level. GAPDH was selected as an internal control gene. Statistical analysis was performed using "Rotor-Gene Q series software 2.3.1" and "Rest 2.0.13 software".

Results: This study, carried out on 20 PC patients aged 50-70 and 20 healthy individuals shows that MST1 expression level in the WBC samples of PC patients is approximately 62% lower compared to normal individuals ($P<0.01$).

Conclusion: Introducing the reduced expression level of MST1 as a prostate cancer biomarker requires complementary research. However, in this study, biomarker validation and potential of MST1 has been approved.

Keywords: Prostate Cancer, MST1, STK4, Hippo signaling, Biomarker

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INTRODUCTION:

Prostate cancer (PC) is the second most common malignancy among men (and the most prevalent urogenital malignancy), accounting for 12.5% of all cancers[1]. In the past, PC studies have focused on androgens. Today, with the development of the field of cancer genetics, it is focused on tumor suppressor genes and proto-oncogenes, approaching mutated genes in PC patients with a positive familial history, identification of recurrent chromosomal alterations, determination of gene expression profile via RNA analysis and epigenetic changes in the pathogenesis of cancer[2].

Mammalian sterile 20-like kinase (MST), also known as Serine/Threonine Kinase 4 (STK4), has been identified as a tumor suppressor gene in colon cancer[3], soft tissue sarcoma[4], and hepatocellular carcinoma[5]. MST1/2 protein kinases are a part of the Hippo signaling pathway that controls organ size and tissue homeostasis by regulating cell proliferation and apoptosis. At the core of the Hippo pathway, MST1/2 and WW45 phosphorylate Mob1 and Lats1/2 after their activation. Activated Lats1/2 leads to Yes-associated Protein (YAP) phosphorylation and inactivation. YAP is an oncogene that induces the transcription of proliferation-associated genes with the participation of TEA domain transcription factor 1 (TEAD). It should be noted that the inactivation of YAP in the Hippo kinase cascade suppresses the proliferation of cells and induces apoptosis[6]. Other results of this serine/threonine kinase activity are: central neural system development and neural channel formation, protein stabilization, MAPK pathway activation, regulation of increased protein binding, induction of apoptosis, amino acid autophosphorylation, negative regulation on cell proliferation and organ growth, differentiation of keratinocytes, regulation of cell morphogenesis and interfering with the signaling pathways of stress-activated kinases[7,8]. MST1 and MST2 can be activated via various stimuli of stress and apoptosis, i.e. sterisporin, UV-rays, hydrogen peroxide, TNF-α, retinoic acid, okadaic acid, and other anti-proliferative factors. This activation is accomplished through various mechanisms such as autophosphorylation, homo- and heterodimerization and cleavage by caspases[6].

MST1 is activated by both caspase-mediated cleavage and phosphorylation in response to apoptotic stimuli, i.e. Fas ligation[9]. It is also a direct inhibitor of Akt1[10]. Due to the effects of the MST1/LATS signaling pathway on cell proliferation, these kinases are likely to play a critical role in cancer. There are many reports that an increase in YAP/TAZ activity is associated with cell proliferation in cancer cells and stem cells. In various studies, decreasing the expression level of MST1 leads to cell proliferation and cancer in various tissues such as the liver, skin, and intestines[11]. Thus, it is possible to introduceMST1 as an anticancer target. Ingrid et al. diagnosed colorectal cancer by detecting the variation of MST1 expression using phage microarray[12]. In non-small cell lung cancer cells, Mst1 regulates apoptosis by inducing mitochondrial damage (interacting with the ROCK1/F-actin pathway)[13]. Van de Laar et al. in 2014 identified MST1/MSP as a mitogenic signal and a new biomarker for lung squamous cell carcinomas[14]. MST1 has also been identified as an early detection biomarker for colorectal cancer[15]. Many other studies have introduced MST1 as a biomarker in various cancers.

Early diagnosis of a solid tumor is related to accessible and significant biomarkers. In 2018, Chunhua et al. introduced the clinical significance of peripheral blood PCA3 gene expression in the early diagnosis of PC. In this study, peripheral blood expression analysis was introduced as a valuable hallmark for PC patients[16].

Therefore, in this study, the expression level of MST1 in patients with PC has been studied. This research is based on an approach to the investigation of a novel biomarker of PC in WBCs, based on the role of inheritance patterns in PC and by separation of white blood cells (WBCs) via non-invasive methods, this research

Table 1. Forward and Reverse primer sequences of MST1 and GAPDH (internal control gene).

Primer	Forward	Reverse	Product Size
MST1	5'-AGACCTGGAGATAATCAAAGA-3'	5'-AGATACAGAACCAGCCCCACA-3'	139bp
GAPDH	5'-CACCAGGGCTGCTTTTAACTC-3'	5'-TGGAAGATGGTGATGGGATTT-3'	180bp

is based on the approach to the investigation of a novel biomarker of PC in WBCs.

METHODS:

Study Participants and Sample Collection

This case-control study was performed on 20 PC patients, aged 50-70 who referred to Labafinejad hospital in Tehran and Velayat hospital in Qazvin in the winter of 2017, after giving written consent. Prostate-Specific Antigen (PSA) test was carried out for both patients and healthy individuals. The cancer was confirmed by pathological evaluation and biochemical tests. 20 volunteers without a prior family history of cancer or autoimmune disease (with a normal PSA value) were also sampled as a control group.

RNA Extraction and cDNA synthesis

RNA extraction was performed immediately after sampling, using the GeneAll Hybrid-RTM Blood RNA kit protocol, since RNA stability in whole blood is very low. The quality and purification of the RNAs were evaluated by Nano-drop spectrophotometer (2000UV-vis, USA). The synthesis of cDNA from RNA was carried out, based on the instructions supplied with the kit (The Thermo Scientific RevertAid First Strand cDNA Synthesis Kit). In this method, 3µl of extracted RNA was mixed with 1µl of Random Hexamer and then diluted in 8µl of RNase free water (leading to a total volume of 12µl). The mixture was incubated at 65°C for 5 minutes. Then, 4µl of 5X Reaction Buffer, 1µl RiboLuck RNase Inhibitor, 2µl of 10mM dNTP Mix and 1µl of the RevertAid M-MuLV RT-enzyme were added to the mixture. Finally, the mixture volume was adjusted to 20µl and incubated for 1 hour at 42°C. Incubation was performed to inhibit enzyme activity at 70°C for 5 minutes.

Bioinformatics analysis

Specific primers were designed for the MST1 and GAPDH genes via Primer3. GADPH was used as an internal control gene. In order to confirm the specificity and precision of the designed primers, their sequences were blasted into NCBI and Gene Runner. The sequences of the primers are listed in **Table 1**.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Statistical Analysis

The expression level of the MST1 gene in the samples was measured using Real-Time PCR technique in the Rotor Gene-Q System. In this study, 10µl of 2X qPCR-BIO SyGreen Mix Separate-Rox (NGS) Master mix,

Table 2. Temperature-time table of prepared Real-time PCR

	Stage1	Stage2		Stage3
Cycle	1		35	1
Temperature	95°C	Denaturation 95°C	Annealing and Extension 60-65°C	72°C
Time	2min	5sec	20-30sec	5min

0.8µl of forwarding primer 10µM, 0.8µl of reverse primer 10µM, 100ng of synthetic cDNA and PCR grade dH2O (with a final volume of 20µl) were used in the temperature-time table (**Table 2**). Initial analyzes were carried out using the "Rotor-Gene Q Series Software 2.3.1" and secondary analyzes and preparation of final data for drawing diagrams was done using the "Rest 2.0.13 Software" (QIAGEN Company).

RESULTS:

In the present study, a total of 40 subjects (20 patients with PC and 20 healthy controls) participated, and their MST1 expression level was evaluated. According to statistical analysis with an accuracy of 0.777, ΔCt, ΔΔCt and fold change of the MST1 gene in cases and controls were calculated after normalization (Reaction Efficacy = 0.770). This study showed that MST1 gene expression in PC patients was 0.382±0.036 (std.Error) compared to control samples (62% lower compared to normal individuals) (P<0.01). (**Chart 1**, **Chart 2**, and **Figure 1**)

DISCUSSION:

Prostate-specific antigen (PSA) screening test is a routine test in the paraclinical diagnosis of prostate cancer, alongside clinical symptoms. A false-negative PSA test and differences in operator precision and diagnostic kits leads to misdiagnosis of PC. Therefore, biomarker studies need to identify more specific, accurate and sensitive tests. For this reason, the study on biomarkers has led to the introduction of genetic-based biomarkers. In recent years, various studies have been

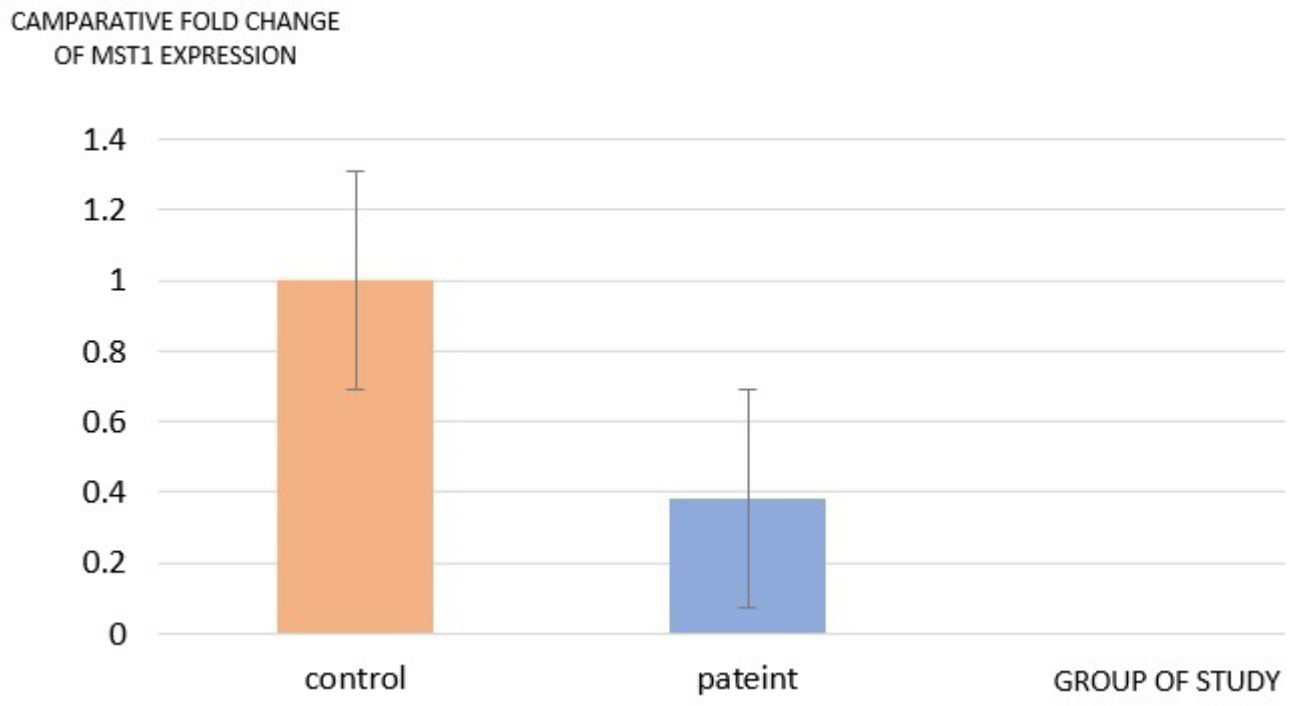

Chart 1. **Relative expression level of MST1 gene in prostate cancer patients and controls.** MST1 gene expression in samples of prostate cancer patients was 0.382 ± 0.036, compared to control samples (P<0.01).

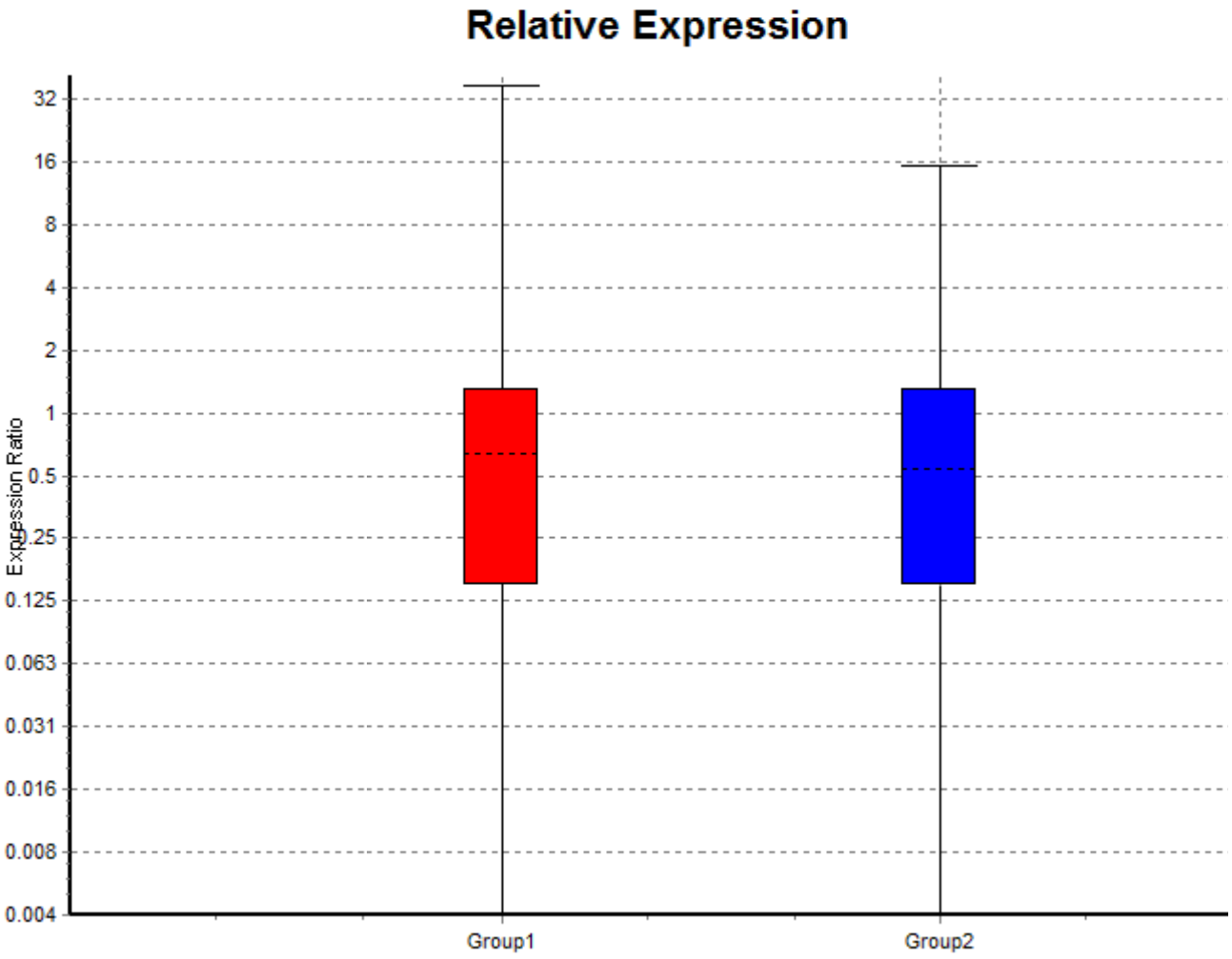

Chart 2. **Relative expression level of MST1 in prostate cancer patients compared to controls (divided into 2 groups for REST analysis)**

conducted on the diagnosis and evaluation of genetic changes involved in cancers. The various types of mutations in proto-oncogenes and tumor suppressor genes can lead to loss of control in the regulation of cell proliferation and death in malignancy. This is primarily due to changes in the regulation of cellular signaling pathways. Several pathways that have been linked to a variety of cancers include Wnt, β-catenin, TGF-β, AKT-mTOR, and MAPK signaling. Recently, the Hippo signaling pathway has also been the focus of attention[17-19]. In mammalian cells, STE20 family kinases (including MST1) are important in the regulation of cytoskeleton networks, morphological control, cellular mobility and also the regulation of cell death. YC Lee et al. showed that in colon cancer, MST1 gene expression in tumor tissues was significantly reduced compared to non-tumor sites. They showed that there is a direct ratio between the expression of MST1 gene

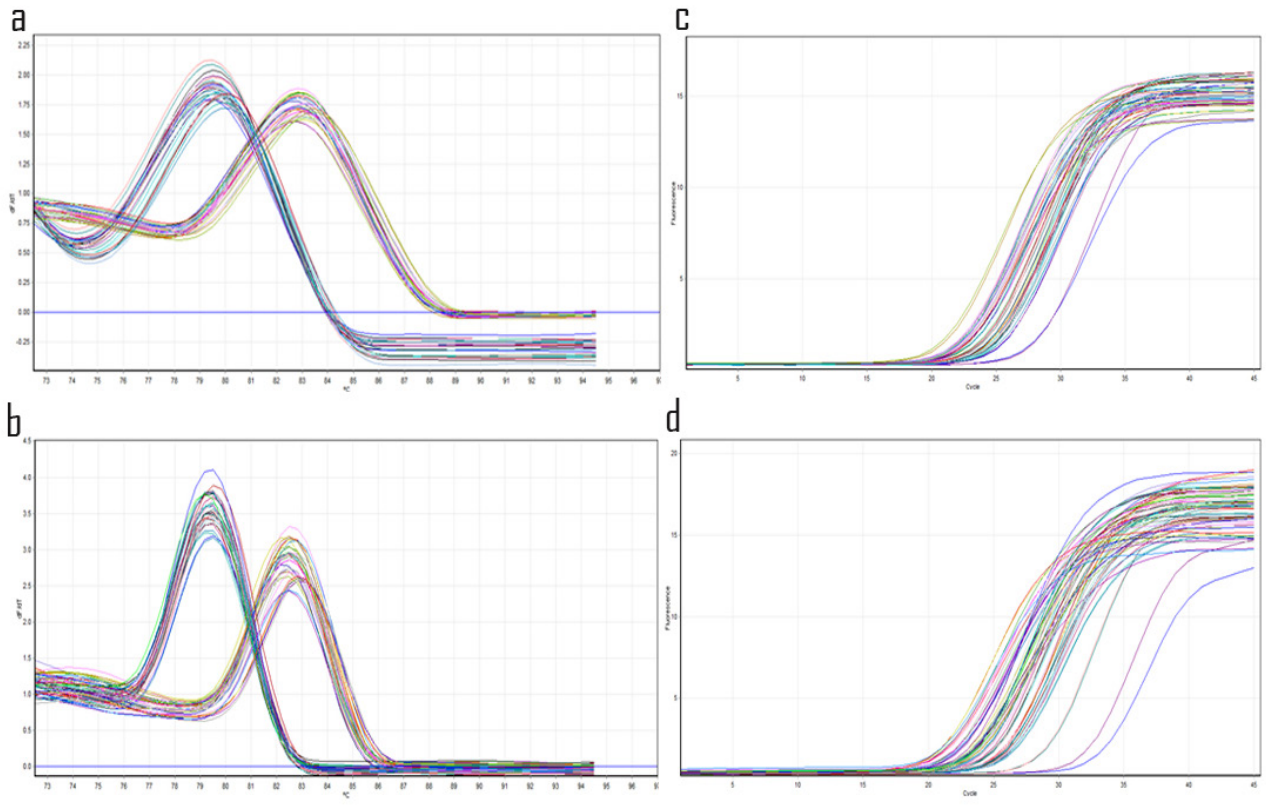

Figure 1. **Rreal-time plots and curves. a)** Melting curves of MST1 and GAPDH in control samples. **b)** Melting curves of MST1 and GAPDH in patient samples. **c)** Amplification plots of MST1 and GAPDH in control samples. d) Amplification plots of MST1 and GAPDH in patient samples.

and the prognosis of disease and the survival rate of colon cancer patients[20]. Also, in a study by Minoo et al. to investigate the role of MST1 in tumor suppression, they found that a lack of cytoplasmic expression of MST1 could be considered as a tumor marker for colorectal cancer[21]. MST1 has also been identified as one of the prognostic markers and possible targets for hepatocellular carcinoma[22]. The decreased expression level of the MST1 gene is well known in hepatocellular carcinoma, breast cancer, and lymphoma[23]. Xiao et al. in a study aiming to identify a new biomarker for the early detection of colorectal cancer introduced 3-9KD protein for this purpose and, after structural analysis, it was found to be a part of the MST1 protein[24]. So far, many studies have examined genetic issues in hereditary PC. Since many genes are involved in the disease and are characterized by variable phenotypes, PC is a complex heterogeneous disease. In this respect, the genetic study of this cancer is very important in recognizing its etiology[25]. Since there is currently no report on the biological significance of MST1 expression in PC patients, our study shows the importance of MST1 expression in the blood cells of patients with PC. It was also found that MST1 is one of the genes involved in the Hippo pathway in PC.

Our study showed that the expression level of MST1

gene is approximately 62% lower in PC patients compared to normal individuals. This suggests that the decreased expression level of MST1 further reduces the phosphorylation of Mob1 and Lats1/2 in the Hippo signaling cascade. As a result, YAP remains dephosphorylated and active and, with the aid of TEAD, stimulates the proliferation of cells. It has been suggested that further studies be carried out on the upstream and downstream cascade controller of MST1 to completely characterize the critical role of the Hippo pathway in the pathogenesis of PC. More studies are needed to introduce this gene as a PC biomarker.

Several studies have examined MST1 function in the apoptotic process. Therefore, it can be considered a tumor suppressor gene. Reduction of MST1 expression is reported in soft tissue sarcoma[4], glioblastoma[26], colorectal cancer[21,24], breast cancer, lymphoma[27], and hepatocellular carcinoma[5]. Our study on PC can also be the basis of further studies on apoptosis in malignant prostate cells.

The study by Li et al. on hepatocellular carcinoma also states that the MST1 gene is involved in regulating the TLR pathway and protecting against chronic inflammation-related hepatocellular carcinoma[23]. Therefore, based on our present results, we suggest further characterizing the role of MST1 in the regulation of inflammation in PC.

This study, based on the study by Babel et al., which was used to determine the role of an antibody against MST1 in the diagnosis of colorectal cancer, can be used a priori to study the diagnosis and prognosis of PC using a phage microarray based on MST1[28]. Since medical biotechnology and genetics are leading cancer toward gene therapy, it is possible to focus on MST1 in PC in the future. This dimension requires further study on the molecular biology of PC.

CONCLUSION:

The results of this study on the comparison of MST1 gene expression levels in WBC samples of PC patients and healthy individuals showed that expression of MST1 was lower in PC patients compared to the control group. The measurement of the expression of this gene can be helpful in identifying a novel biomarker and may be useful in genetic-based research in the future. It will help researchers in producing anti-cancer drugs against PC, and as for the Hippo signaling pathway, since numerous studies have mentioned YAP/TAZ as possible oncogenic factors, it has been proposed to use them as anticancer drug targets, too.

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CONFLICTS OF INTEREST:

There are no conflicts of interest in this article.

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