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ORIGINAL ARTICLE

Investigate the Inhibitory effect of Amniotic membrane proteins on HSP90 gene Expression level in PC3 Prostate cell line

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ABSTRACT
Prostate cancer is the second most common cancer among men after lung cancer, and it is the eighth leading cause of cancer death in Iran. Increasing levels of heat shock protein 90 (HSP90) gene expression is seen in this cancer. Heat shock proteins are molecular chaperones that play a fundamental role in the wrinkle of a lot of cellular proteins. The role of anticancer amniotic membrane proteins has been proven in different studies. Therefore, the present study was conducted with the aim of investigating the inhibitory effects of amniotic membrane proteins (hAMP) on heat shock protein 90 gene expression amounts. After preparing and culturing a prostate cancer cell line, the proteins in the amniotic membrane were extracted from the collected samples, and the cells with density of 1 µg/µL, were incubated for 72 hours. A decrease of 56% was seen in the growth of these cells. After incubation and RNA extraction, the amounts of HSP90 and related gene protein expressed were investigated using Real Time PCR and Western Blot, respectively. The results showed decreases of 62% and 58% in gene expression and HSP90 protein expression, respectively. It can be said that HSP90 has an important role in creating prostate cancer so that a decrease in its expression amount can lead to a considerable reduction in the growth of these cells. Therefore, using new pharmaceutical combinations to inhibit HSP90 expression can present new therapeutic opportunities in cancer inhibition.
Keywords: *Amniotic Membrane Proteins, Heat Shock Protein 90 (HSP90), PC3 Prostate Cancer Cell line*

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INTRODUCTION

Prostate cancer is the most common visceral cancer, the second most common cancer among men after lung cancer, and one of the most important causes of death all over the world [1,2]. In Iran, it is the eighth leading cause of cancer death [3-7].

Although, the causes of the cancer remains unknown, experimental and clinical observations indicate the role of whole hormonal, genetic, and environmental factors in its pathogenicity [6, 8].eat shock proteins are molecular chaperones that play a fundamental role in the wrinkle of a lot of cellular proteins. At first, these proteins were found to be intermediaries of resistance to increase body temperature [9,10]).

Heat shock protein 90 expression is higher in tumors and cancers than in normal tissue.This protein includes 4% to 6% of all existing proteins in cancer cells and 1% to 2% of all proteins in normal cells. In addition, it has been specified that heat shock protein 90 in tumors with high activity of ATPase is very complex, whereas these proteins are not complex and are in a latent state in normal cells. They help tumor cells maintain homeostasis as well as growth and survival in counter settings [11-13].

Expression of HSP90 increases in many cancers, and thus this protein plays an important role in most onco proteins involved in the growth and survival of cancer cells, like serine/threonine kinase (Akt), expressed protein from v-raf-1 murine leukemia viral oncogene homolog 1 (RAF-1), human growth factor 1 apoptosis induction, angiogenic inhibition, and cell cycle inhibition in cancer cells by amniotic impact are prevented by heat shock protein 90 [14].

The expression of heat shock protein 90 in prostate cancer cells is two- to tenfold that in natural cells [15, 5] ; therefore, if some natural combinations, including amniotic membrane proteins that are themselves

without side effects, can reduce the expression of these proteins, it can be hoped that this method could be used to produce combinations for cancer treatment by reducing angiogenesis, inducing apoptosis, and decreasing resistance to chemotherapy. Therefore, the aim of this study was to investigate the effects of amniotic membrane proteins (hAMP) on the amount of heat shock protein90 gene expression.

MATERIALS AND METHODS

Cell Culture

Cancer line PC3 of prostate cancer was bought from the cell bank of Iran's Pasteur Institute. These cells were cultured in a single layer on 2cm (Orange Scientific) 25 flasks and in RPMI-1640 culture medium (Sigma; Chemical Co., St. Louis, MO, USA)along with 10 % FBS (Gibco- Life Technologies.), streptomycin (100 µg/mL), and penicillin (Sigma) (100 units/mL), under pressure of 0.5 carbon dioxide and a temperature of 37^0 C.

Extraction of protein from amniotic membrane

Amniotic membrane was prepared based on conscious informed consent of Medical Ethics Committee. Pairs were obtained from healthy women who had undergone cesarean sections, and they were washed in a phosphate buffer containing 0.2 antibiotics/antimycotics. Human amniotic membrane protein extract (hAMPE) was obtained based on the method of Shao et al [16].

According to this method, amniotic membrane was mechanically separated from the outer membrane of the embryo, put on ice, and then subjected to homogenization and sonication. 1800 gr of amniotic membrane was centrifuged for 15 minutes and then, after collecting the supernatant, frozen at -80^0C.Protein density was measured by nano-drop (ND-1000 spectrophotometer, USA).

Treating cells with amniotic fluid proteins

The examined cells were treated with the extract of amniotic fluid proteins at a density of 1 µg/µLand incubated for 72 hours.

Cell proliferation assay

The effect of hAMPE on the PC3 cell proliferation was determined by MTT assay. The cells were seeded in 96-well tissue culture plates at a density of 15,000 cells per well and incubated at 37 °C and 5% CO2 humidified incubator. After 50% confluency, the cells were treated with1 µg/µL hAMPE. For MTT assay, 2 mg/ml of MTT solution was added to each well and incubated for 3 h at 37°C. The medium was removed and the blue formazan crystals were dissolved in 200 µl of DMSO and 25 µl Sorenson buffer. The absorbance was read in a microplate reader (Biotek, model Elx808) at 570 nm. Each experiment was repeated in triplicate format, and results were expressed as means ±SEM.

RNA extraction and c-DNA synthesis

72 hours after treatment, medium was removed from monolayer cancer cells and scrapped in 1 ml RNAX-PLUS (Cinagene, Iran). Total RNA was extracted from samples using Cinagene Kit based on the manufacturer's instruction (RNX-Plus Solution, SinaClon, Iran). Genomic DNA contaminant was removed, and then the resulting RNA was selected to DNase treatment using DNase, RNase-free (Fermentas,USA). After purification and quantification, RNA was determined by measuring optical density at 260 and 280 nm by nanodrop (NanoDrop- ND-1000). The cDNA synthesis was performing by cDNA synthase kit (Qiagene).

Real-time PCR

Real-time PCR test was used to determine the amount of the effect of the amniotic fluid protein extract on the gene expression amount of HSP90 from real-time PCR with iCycler IQ5 Multicolor Real-time PCR Detection System (Bio-Rad, USA). GAPDH was used as a housekeeping gene. The sequence of used primers is indicated in Table 1. Each experiment was repeated in triplet, and results were expressed in means ± SEM.

Table 1. Primers used for Real time- PCR

Genes	Primer sequence (5′ to 3′)
HSP90	F 5'- TGTCATGAGCCTGAGGTGAAC-3' R 5'- GTGGATCCAGACACCAACAG-3'
GAPDH	F 5′AAGCTCATTTCCTGGTATGACAACG3′ R 5′ TCTTCCTCTTGTGCTCTTGCTGG 3′

Western blot analysis

We used Western blot analysis to evaluate the HSP90 protein expression on the PC3 Prostate cell line, after treatment by hAMPE. 25µg total crude proteins were used for each well for SDS-PAGE and Western blot analysis. GAPDH gene was used as control gene in this study. Western blotting for lysed cells performed using Chemiluminescent Immunodetection kit (Western Breeze; Invitrogen). Equal crude

protein was loaded in each lane for SDS-PAGE and Western blot analysisHSP90 specific antibody (Invitrogen) were used according to the supplier's commendation. Films containing protein bands were scanned and densitometry using Image J software.

Statistics

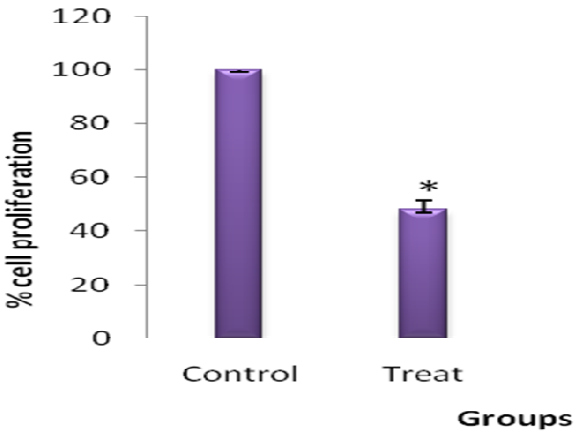
Statistical analysis was performed with SPSS version 16.0 software and student t-test was used to compare between groups. Data are represented Mean±SEM. The differences were considered significant when *P<0.05.

RESULTS

Effects of Amniotic membrane proteins on the growth of PC3 prostate cancer cell lines

In order to determine the effect hAMPE on the PC3 cell lines proliferation , MTT assay was illustrated at 24, 48, and 72 hours after incubation with hAMPE. The Prostate cell line (PC3) treated with hAMPE was compared to untreated PC3cell lines .As shown in Figure1, cell growth was inhibited considerably compared with control groups so that it can be seen in Figure 1, cell proliferation was decreased to 56% (P<0.05) after 72 hours but this reduction was not significant at 24 and 48 hours.

Figure 1. The Effects of Amniotic membrane proteins on the growth of PC3 prostate cancer cell lines

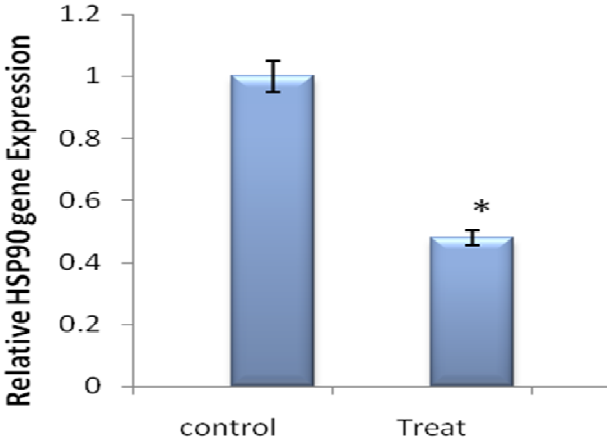


Cell proliferation (%) of PC3 human prostate cancer cell lines evaluated after 72 h of incubation with 1 lg/lL of hAMPE. Significantly different from Treated cells and controls (P<0.05)* Data are represented Mean ± SEM.

Analysis hAMPE effects on expression HSP90

The down regulation of PC3 cells were investigated by real time PCR analyzing. After 72 hours of treatment, mRNA levels of HSP90 gene were analyzed. The genes CT values were normalized against mRNA level of GAPDH as housekeeping gene and the relative expression for each group was measured. Figure 2, show that, there is significant decrease in level of HSP90 expression about %62, P<0.05) comparing with control group respectively (P<0.05).

Figure 2. The Effects of Amniotic membrane proteins on the HSP90 gene expression in PC3 prostate cancer cell lines.

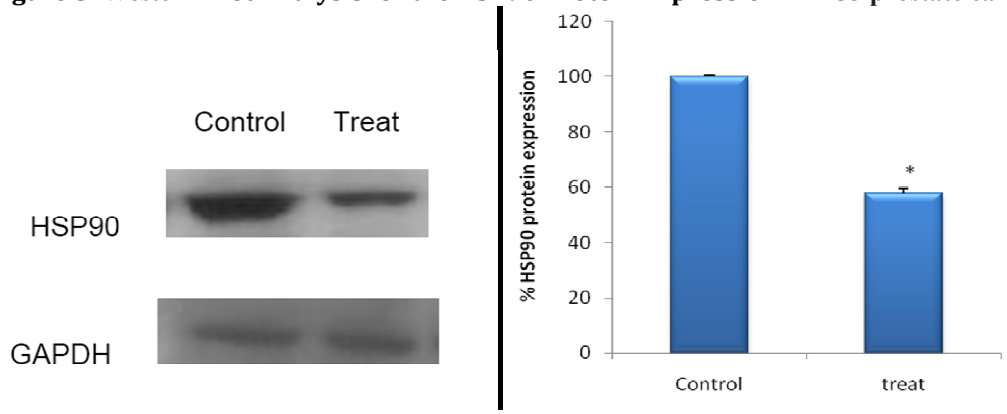


Significantly different from treated cells and controls (P<0.05)*. Data are represented Mean ± SEM. The experiments were repeated as triplicate.

Inhibitory effect hAMPE on the level of HSP90 protein expression

Western blot results show that hAMPE had inhibitory effects in PC3 Prostat cancer cells (Figure 3). The level of protein expression of this gene was significantly down-regulated. Comparing with the level of protein expression of control gene (GAPDH gene) and total protein in control group (the PC3 cell without treatment), HSP90 expression has been decreased about 58% (P<0.05).

Figure 3. Western Blot Analysis for the HSP90 Protein Expression in PC3 prostate cancer cell lines.



Significantly different from Treated cells and controls (P<0.05)*. Data are represented Mean ± SEM. The experiments were repeated as triplicate.

DISCUSSION

One of the most important causes of the creation, growth, and progression of different cancers in humans is the increased expression of one oncogene [17] .Since heat shock protein 90(HSP90) and its dependent proteins play an important role in the growth of cancer cells [18], they are used as a therapeutic target in cancer cells [19, 20]. In recent times, different studies have been conducted concerning the anticancer role of amniotic membrane proteins [20], but there is little information about the mechanism of the effect these proteins have on the growth and differentiation of cancer cells. In this study, an MTT experiment was used to investigate the effects of hAMPE on the growth of line PC3prostate cancer cells. As shown in Diagram 1, these proteins caused a nearly 73% decrease in the amount of cancer cell growth after incubation with a density of 1 µg/µL from hAMPE for 73 hours. It is necessary to say that in view of the conducted studies, the effect of these proteins (hAMPE) on cancer cells is different, so effects like stimulation, inhibition, or no response are sometimes seen [20].

Other conducted studies have specified that cells from a different line of one kind of cancer also showed different responses to treatment with these proteins, this could be attributed to the specific genetic characteristics of each cell line [21].

This study also measured the amount of HSP90 gene expression after treatment through hAMPE. Results indicated that these proteins reduce gene expression. Approximately 62% may be due to the release of other unknown factors. Much more research is needed to identify these factors and their signaling pathways. The inhibitory effect of these proteins on the proteomics level by Western blot method was also shown. This hypothesis confirms that the growth of PC3 prostate cancer cell lines occurs by increased HSP90gene expression. Its inhibition prevents the growth of this cell line considerably. Studies have indicated that the inhibition of heat shock protein 90 is the disarranging of STAT3 route so that reduced STAT3 phosphorylation is associated with decreased tumor growth. A probable explanation is that the inhibition of STAT3 phosphorylation by heat shock protein 90 leads to the inhibition of Wnt5A (a key gene in the proliferation of cancer cells) transcription [10, 11].

Although heat shock protein 90 is required by both cancer and natural cells, it seems that cancer cells have a higher sensitivity to it than the inhibition of the chaperone function of heat shock protein 90. Inhibitors of heat shock protein 90 can be used to increase or substitute chemotherapy. When heat shock protein 90 is inhibited by its inhibitors, like goldanamaysin and its derivatives, inactive proteins, and heat shock protein 90 lose their wrinkles and are destroyed by proteasomal [15]. Since many prostate cancer cells are severely dependent upon these inactive proteins, the pharmaceutical inhibition of heat shock protein 90 simultaneously inhibits numerous tumorigenic signaling pathways and can have a strong anticancer effect. It has been suggested that heat shock protein 90 most likely adjusts the motility of the PC3 cell through integrin-FAK/src signaling [15, 14].

In conclusion, it can be said that HSP90 plays an important role in creating prostate cancer along with the help of other chaperone molecules that lead to fold or open folding, transferred protein, and its decomposition. It seems that using new pharmaceutical combinations to inhibit HSP90 could present new cancer treatment opportunities.

COMPETING INTERESTS

All authors declare that they have no competing interests.

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