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# The Relationship between the Expression of Small Heat-Shock Proteins and Suppression of OCT4B1

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## Abstract

**Background:** OCT4B1, a variant of OCT4, is expressed in both cancer cells and tissues. This variant has a main role in the regulation of both apoptotic and stress (heat-shock proteins) pathways. The aim of this study was to investigate the effects of OCT4B1 silencing on the expression of small heat-shock proteins (sHSPs) in three human tumor cell lines.

**Methods:** AGS (gastric adenocarcinoma), 5637 (bladder tumor) and U-87MG (brain tumor) cell lines were transfected with specific OCT4B1 siRNA (test group) and scramble siRNA (control group), using siRNA and Lipofectamine. Real-Time PCR Array technique was applied and the fold changes were calculated using RT$^2$ Profiler PCR Array Data Analysis version 3.5.

**Results:** It was revealed that HSPB1, HSPB6 and HSPE1 were down-regulated in all three studied tumor cell lines and HSPB2, HSPB7 and HSPB8 were down-regulated in two of three studied tumor cell lines (AGS and 5637). It was also revealed that HSPB3 was down-regulated in 5637 cell line and up-regulated in AGS and U87MG cell lines.

**Conclusion:** According to the results, it may be concluded that there is a direct relationship between OCT4B1 and sHSPs gene family expression. Thus, suppression of OCT4B1 may be considered in cancer therapy/research.

developed in the left eye. Two patients had no family history suspicious for keratoconus.



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## Introduction

OCT4 (octamer-binding transcription factor 4), belongs to a family of transcription regulatory proteins containing the POU DNA-binding domain (1), while its up-regulation has been presented in stem cells (2). Studies have shown that down-regulation or silencing of this gene causes cell differentiation (2,3). According to the newest hypotheses, defined as "cancer stem cell", the adult stem cells (present in nearly all human tissues) or reprogrammed somatic tissue cells are considered as origin of cancer (4-6). OCT4, as an important gene in embryonic stem cells, plays crucial roles in cancer cell survival (7,8). This gene potentially encodes at least three different variants via alternative splicing (A, B and B1) (9). The expression of OCT4A is exclusive to stem cells (embryonic or adult), while OCT4B is expressed by both somatic and stem cells and is not involved in stemness. Recently, Atlasi et al., (2008) has reported the presence of a novel variant of OCT4 (OCT4B1) in both cancer tissues and cell lines (10). Studies have shown the up-regulation of OCT4B1 in gastric (8), colorectal (9), bladder (10), and germ cell tumors (11), where it acts as an anti-apoptotic factor (7,8,12). Farashahi et al. (2011) reported that under stress condition (heat shock), the expression of both OCT4B1 and heat shock proteins (HSPs) was elevated in tumor cell lines (13).

Heat shock proteins (HSPs), as the most protected proteins, are expressed in either prokaryotes or eukaryote cells (14,15). A wide variety of physiological and environmental insults such as heat shock stress could alter the expression of HSPs. Interestingly, these proteins, as molecular chaperones, play fundamental roles in inducing correct folding of nascent and stress-accumulated misfolded proteins, which prevents their aggregation (16). Depending on their intracellular or extracellular location, HSPs have a dual (protective/inducing) function. For instance, intracellular HSPs allowing the cells to survive to lethal conditions, while extracellular or membrane-bound HSPs interact with various components of the regulated programmed cell death (PCD) pathways (17). HSPs are expressed by normal cells under physiological conditions, but their expressions are up-regulated when exposed to stress conditions such as sudden temperature jump and so on.

According to the size, mammalian HSPs have been classified into two groups of high and small molecular weight HSPs. HSP10, HSP25 and HSP27, belong to the second group. Some HSPs are expressed constitutively, while others are induced by stressful conditions (18). Under stress conditions, HSPs can lead to proteasome-mediated degradation of some selected proteins. Overall, these features make HSPs to be targets for regulating apoptosis pathways (19). HSPs are up-regulated in a wide range of human cancers and are involved in tumor cell's proliferation, differentiation, invasion and metastasis (19). As a family of ATP-independent chaperones, small HSPs (sHSPs) are encoded by HSPB genes and composed of 11 ubiquitous molecular chaperones. They are structurally determined by their chaperoning activity (20,21).

In addition to modulating the heat shock response, sHSPs can play many roles in various important cellular processes, such as secretion, translocation, protein degradation and regulation of transcription factors, especially refolding of the misfolded proteins (17).

This study aimed to investigate the function of newly discovered variant of OCT4 (OCT4B1) and its effects on the regulation of gene expression involved in the cell stress

pathway, therefore, this variant was suppressed in three human tumor cell lines and expression profile of 7 genes belonged to small molecular weight HSPs, including HSPB1, HSPB2, HSPB3, HSPB6, HSPB7, HSPB8 and HSPE1 gene families, was detected.

## Materials and Methods

### Cell culture and OCT4 variants expression

AGS, 5637 and U87MG tumor cell lines were purchased from the Iranian national cell bank (Pasteur Institute of Iran, Tehran). The cell lines were cultured in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) Fetal bovine serum (FBS) under standard cell culture conditions at 37°C in a humidified atmosphere containing 5% CO2 and 95% air. The profile of OCT4 was prepared to measure the expression of OCT4 variants in these tumor cell lines. Total RNA was isolated from cultured cells ($10^6$ cells per milliliter) using Trizol reagent (Invitrogen), according to the manufacturer's instructions. To remove DNA contamination, RNA was treated with TURBO DNase. The purity and validity of RNA were measured using spectrophotometry (260/280 nm ratio) and gel electrophoresis (agarose gel 1%), respectively. The first strand cDNA was synthesized at 42ºC for 60 min using 100 pmol oligo (dT) primer, 1µg of total RNA and a cDNA synthesis kit (Parstous, Iran), according to the manufacturer's instructions. Specific primers were designed using Gene Runner (version 3.02) and Allele ID (version 4.0) software (Table 1).

**Table 1.** Sequences for designed primers of OCT4A, OCT4B, OCT4B1 and ß-actin

| Target Genes | Designed Oligo | Relative Sequence | Fragment Length |
|---|---|---|---|
| **OCT4A** | F | CGCAAGCCCTCATTTCAC | 111 |
| | R | CATCACCTCCACCACCTG | |
| **OCT4B** | F | CAGGGAATGGGTGAATGAC | 177 |
| | R | AGGCAGAAGACTTGTAAGAAC | |
| **OCT4B1** | F | GGTTCTATTTGGTGGGTTCC | 128 |
| | R | TTCTCCCTCTCCCTACTCCTC | |
| **ß-actin** | F | CACACCTTCTACAATGAGC | 160 |
| | R | ATAGCACAGCCTGGATAG | |

F: forward, R: reverse

The expression level of mRNA was measured using qRT-PCR by SYBR green master mix (Parstous, Iran) and Bio-Rad CFX96 system (Bio-Rad Company, USA): one cycle of 95ºC for 15 min, 40 cycles of 95ºC for 30 s, 60ºC for 30 s (for OCT4B1 61ºC for 20 s) and 72ºC for 30 s. RT-PCR was carried out in triplicate and ß-actin was assessed as a reference gene for normalizing the amplification signals of the target gene. The relative levels of PCR product were calculated by the $2^{-\Delta\Delta Ct}$ method. The dissociation stages, melting curves and quantitative analysis of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA). All PCR products were screened by gel electrophoresis on 1% agarose containing 0.5 mg/ml ethidium bromide, to check the size of the PCR product.

### OCT4B1 siRNA and scramble siRNA transfection

In order to suppress OCT4B1, two specific siRNAs based on the specific OCT4B1 sequences (exon2b), and one irrelevant or scramble siRNA (with no complementary target sequence in the human genome) were designed, using a selection program (whitehead Institute for Biomedical Research, htt://jura.wi.mit.edu/) and synthesized by MWG (Germany) (Table 2).

**Table 2.** Sequences and criteria of designed siRNAs

| siRNA Name | Target | Sequences |
|---|---|---|
| **Version I** | Target | AAGGAGTATCCCTGAACCTAG |
| | Sense | (GGAGUAUCCCUGAACCUAG)dTdT |
| | Anti-sense | (CUAGGUUCAGGGAUACUCC)dTdT |
| **Version II** | Target | AAGAGGTGGTAAGCTTGGATC |
| | Sense | (CAGUGGUAAGCUUGGAUC)dTdT |
| | Anti-sense | (AAUCCAAGCUUACCACCUC)dTdT |
| **Scramble** | Sense | GCGGAGAGGCUUAGGUGUAdTdT |
| | Anti-sense | UACACCUAAGCCUCUCCGCdTdT |

Cells ($1\times10^5$ cells /ml) were cultured in 6-well plates in RPMI1640 medium lacked antibiotics in two groups (test and control). At confluences of 30-50%, cells were transfected with 50 nmol/ml OCT4B1-siRNA (for scramble/ control siRNA), using Lipofectamine 2000 (Invitrogen, USA) and Opti-MEM media, according to the manufacturer's instructions. Briefly, 5µl of siRNA (25µM) and 4.5 µl RNAi-MAX reagents were diluted in 250 µl Opti-MEM and incubated for 10 min at room temperature. The mixture was then added to the cells in a final volume of 2.5 ml. Cells were incubated at 37ºC in a humid atmosphere containing 5% CO2 and 95% air for 72 hr.

In order to confirm the efficiency of gene suppression, OCT4B1 expression was measured in OCT4B1-siRNA transfected (test group) and scramble-siRNA transfected (control group) cells. As previously described, total RNA was extracted from cells of both groups and cDNA was synthesized. OCT4B1 mRNA expression level was evaluated in both test and control groups following 24, 48 and 72 hr after cell transfection.

### Investigation of the apoptosis rate in transfected cells

The rate of apoptosis was evaluated by Annexin V-FLOUS commercial kit (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. Briefly, 48 hr after transfection, 200 µl of cultured cells were centrifuged and resuspended in 200 µl binding buffer. After 5 min, Annexin V-FLOUS (1µl) and propidium iodide (1µl) were added to tubes, incubated for 5 min at room temperature (in the dark) and analyzed by a Beckman-Coulter Elite flow cytometer (Partec, GmbH, Münster, Germany), then, the percentage of FITC/PI positive cells (apoptotic proportion) were calculated from flow histograms.

### Profiling of small molecular weight HSPs genes

Following 48 hr of transfection, cells were heat-shock treated at 45°C for 1 hr. As previously described, total RNA

was extracted from the test and control cells and cDNA was synthesized immediately (22). To evaluate OCT4 variants, quantitative Real-time PCR was carried out using Real-time PCR Array technology (SABiosciences, USA) in a similar condition. Statistical analysis of gene expression and chart drawing were performed using CFX96 manager software (Bio-Rad, USA), and RT$^2$ Profiler PCR Array Data Analysis version 3.5, respectively.

## Results

### The expression of OCT4B1 in all studied cell lines and its down-regulation after siRNA transfection

The results indicated that all three OCT4 variants (A, B and B1) were expressed in the studied cell lines (Figure 1). After siRNA transfection, the expression level of OCT4B1 was decreased sequentially, 24, 48 (the highest state of suppression) and 72 hr (Figure 2).

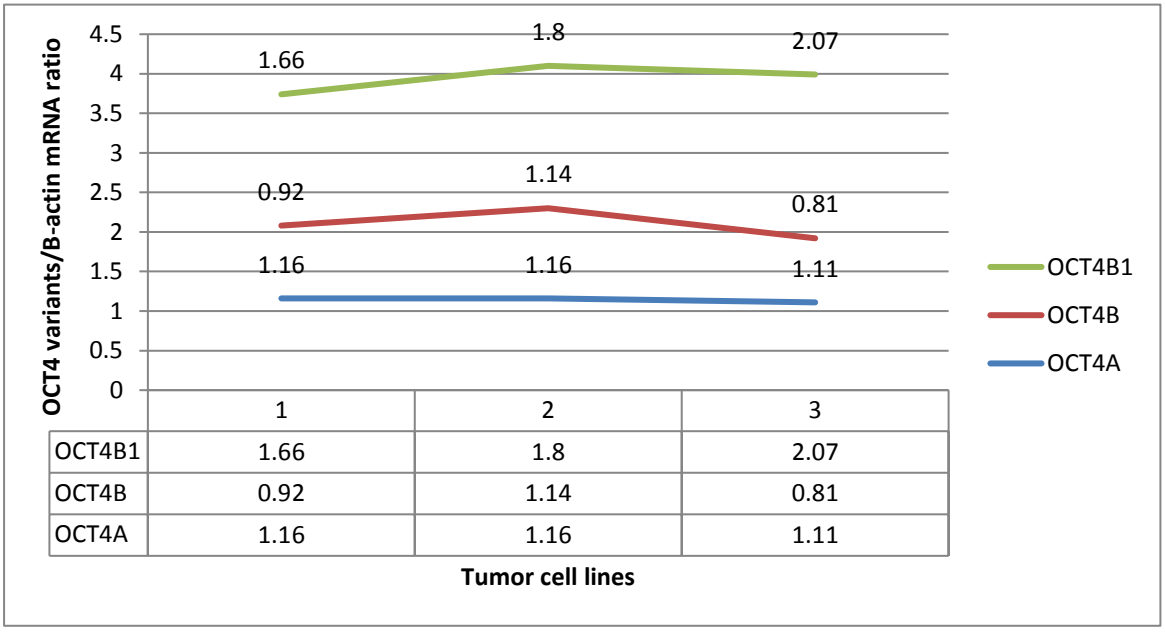


**Figure 1.** Expression of OCT4 variants in tumor cell lines. The results showed that all three OCT4 variants (A, B and B1) were expressed in the studied tumor cell lines. The Y axis shows the OCT4B1 variant mRNA expression level compared to ß-actin (as housekeeping control gene) and the X axis indicates three studied tumor cell lines (AGS, 5637 and U87MG).

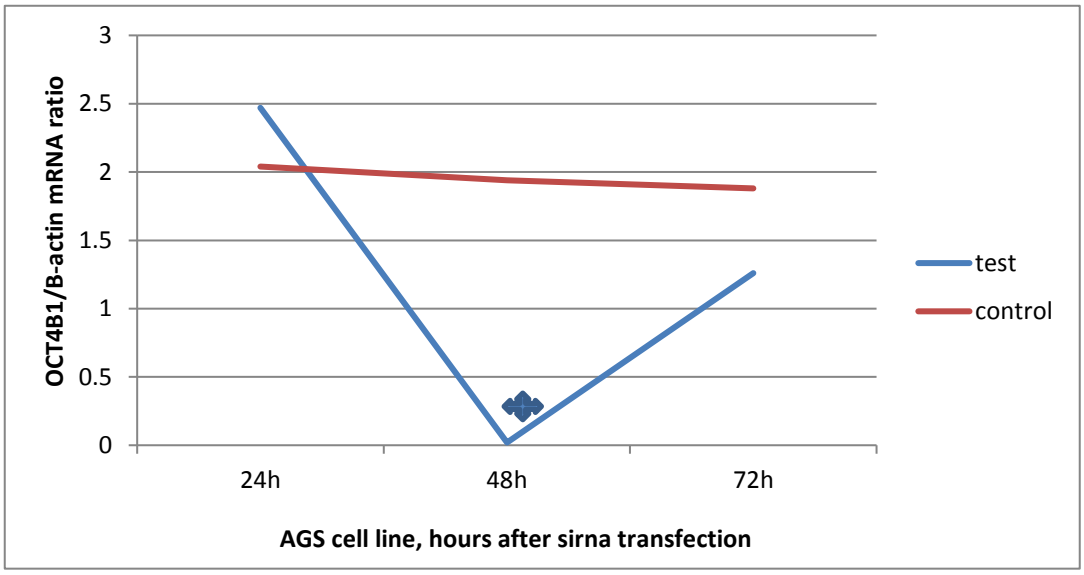


**Figure 2.** Expression status of OCT4B1 following 24, 48 and 72 hr in response to siRNA transfection.The Y axis shows OCT4B1 variance mRNA expression compared to ß-actin (housekeeping as control gene) and the X axis indicates AGS tumor cell lines. Test: transfected cells with OCT4B1 siRNA, and Control: transfected cells with scramble siRNA.

*There is a significant difference between control and test groups after 48 h ($P<0.05$).

### Elevated apoptosis in studied tumor cell lines treated with specific OCT4B1 siRNA

Flow cytometry analysis of the cells stained with Annexin V and Propidium iodide (PI) demonstrated that 48 hr after siRNA transfection, up to 29% of the test group cells (transfected by specific OCT4B1 siRNA) underwent apoptosis in comparison to the control group (transfected by scramble siRNA) (Figure 3).

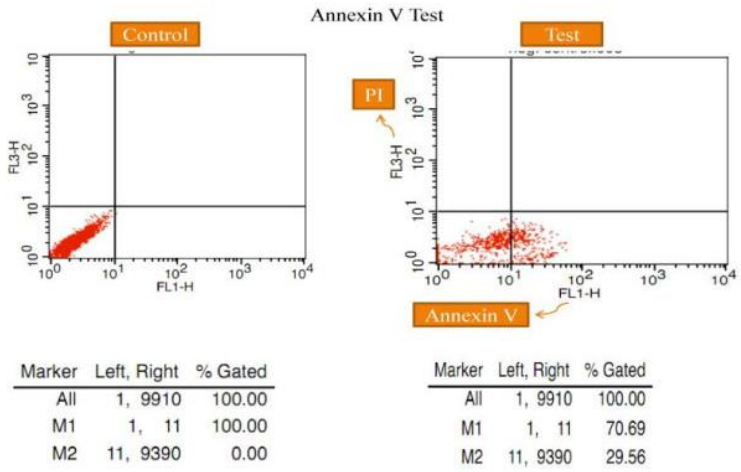


**Figure 3.** Flow cytometry analysis of OCT4B1 suppressed in AGS tumor cell line. Test: transfected cells with OCT4B1 siRNA and Control: transfected cells with scramble siRNA. The cells falling in the lower right square in scatter diagram are considered as apoptotic cells. Apoptosis was analyzed using Annexin V-FLOS assay kit, Annexin V-FLOUS binding was detected using FITC signal detector (FL1) and propidium iodide staining by the phycoerythrin emission signal detector (FL3).

### Gene expression profile of HSPBs and HSPE1 gene families

Current results show that the expression profile of the studied genes in the three studied human tumor cell lines follows approximately similar pattern of expression. The results of this study demonstrated that HSPB1, HSPB6 and HSPE1 genes family was down-regulated in all three studied tumor cell lines. Whereas, HSPB2, HSPB7 and HSPB8 were down-regulated in AGS and 5637 and up-regulated in the U87MG tumor cell lines and HSPB3 was up-regulated in AGS and U87MG and down-regulated in 5637 tumor cell lines after OCT4B1 silencing (Table 3 and Figure 4).

**Table 3.** Different pattern of genes expression of small HSP family member followed OCT4B1 suppression in tumor cell lines

| Symbol | Description of Genes | AGS | 5637 | U87MG |
|---|---|---|---|---|
| **HSPB1** | Heat shock 27kDa protein 1 | -3.19 | -4.37 | -1.53 |
| **HSPB2** | Heat shock 27kDa protein 2 | -23.65 | -32.40 | 3.42 |
| **HSPB3** | Heat shock 27kDa protein 3 | 1.22 | -1.12 | 2.54 |
| **HSPB6** | Heat shock protein, alpha-crystallin-related, B6 | -3.00 | -4.11 | -1.44 |
| **HSPB7** | Heat shock 27kDa protein family, member 7 | -2.38 | -12.33 | 2.25 |
| **HSPB8** | Heat shock 22kDa protein 8 | -1.66 | -2.28 | 1.25 |
| **HSPE1** | Heat shock 10kDa protein 1 (chaperonin 10) | -57.18 | -1.49 | -1.04 |

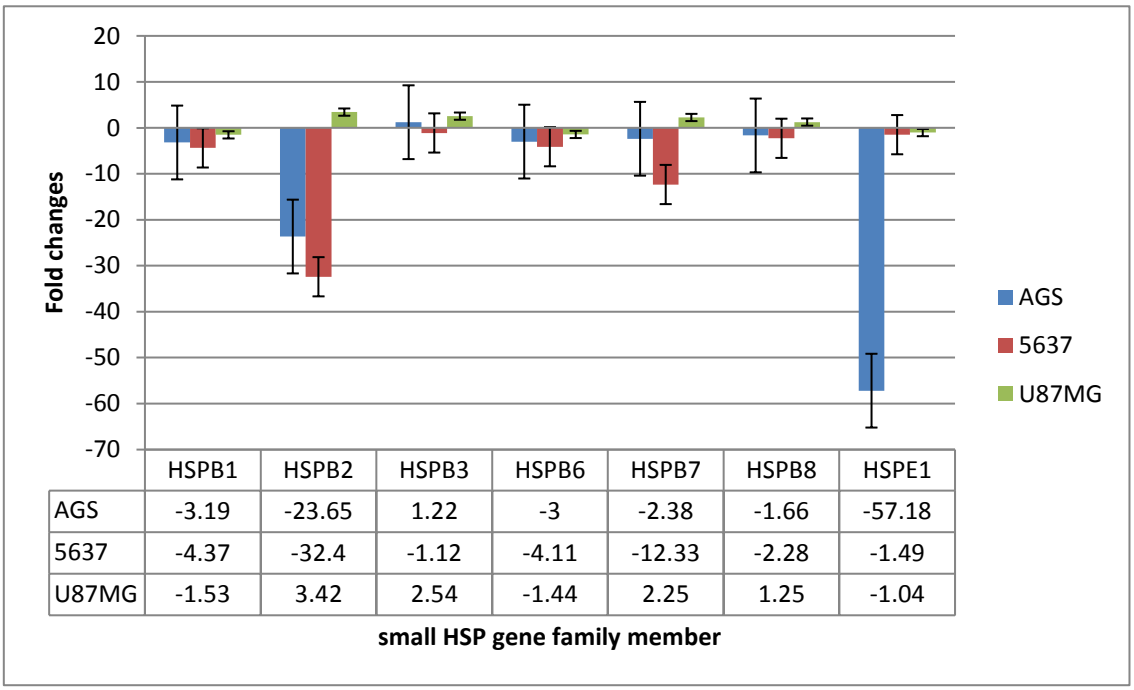


**Figure 4.** Small HSP gene expression, 48 hours after siRNA transfection in the studid tumor cell lines. Y axis shows fold gene regulation and X axis shows 7 genes of small HSP gene family.

## Discussion

To investigate the relationship between OCT4B1 and small molecular weight HSPs, this variant was suppressed by siRNA method (21 nucleotide siRNA against the 3' end of OCT4B1 mRNA) and the expression profile of the mentioned genes (including 7 genes) has been evaluated. As shown in Table 3, the gene expression profile of small molecular weight HSPs (HSPB1, HSPB2, HSPB3, HSPB6, HSPB7, HSPB8 and HSPE1) gene families in three studied tumor cell lines are approximately similar but not identical pattern. Also, three members of the HSPs (HSPB1, HSPE1 and HSPB6) were down-regulated after OCT4B1 silencing. The results also showed that HSPB2, HSPB7 and HSPB8 gene family were down-regulated in AGS and 5637, while they were up-regulated in the U87MG tumor cell lines. HSPB3 was up-regulated in AGS and U87MG and down-regulated in 5637 tumor cell lines.

It has been revealed that HSPs had two main functions. Firstly, these proteins act as molecular chaperones, thereby, they play a role in protein folding, aggregation. Secondly, they prevent cell death, therefore, dysregulated expression of these proteins can contribute to a wide range of tumor types including cancer (23). The results showed that after OCT4B1 suppression, the expression of the abovementioned genes were decreased, therefore, it is suggested that OCT4B1, which is overexpressed in cancer cell lines and tissues, acts as a positive control on the expression of sHSPs genes family. Centenera et al., (2013) have clearly demonstrated that overexpression of HSPB1 induces autophagy and HSPB1 siRNA inhibits H2O2-induced autophagy in NRK-52E cells (24). Niknejad et al., (2013) indicated that HSPB1 down-regulation by siRNA increases the podocyte apoptosis in vitro (25). Furthermore, HSPB1 regulates apoptosis in polymorph nuclear leukocytes (26). Based on the over-expression of OCT4B1 in cancer cell lines and tissues (27), anti-apoptotic effects of this variant (28) and the relationship between this variant and HSPs (13), it can be concluded that the OCT4B1 up-regulation in tumor cells leads to the over-expression of sHSPs members and it can be considered as a responsible mechanism for disruption of apoptosis in the cell lines. In other words, down-regulation of OCT4B1 can be considered as a candidate for future molecular therapies of cancers. Interestingly, several researchers approved our hypothesis that inhibitors of the HSP90 chaperone can be considered as important anticancer agents. So, it seems that OCT4B1 suppression causes decreased sHSPs expression in tumor cell lines, hence, it can be considered in future studies on cancer and cancer prevention.

Accordingly, it seems that the intracellular behavior of HSPs is complex in tumors which caused in different situations genes showed un-prospected actions.

## Conclusion

According to the results, there is a direct relationship between OCT4B1 and sHSPs gene family expression, as by OCT4B1 suppression, the expression level of HSPB1, HSPE1 and HSPB6 genes was decreased, but there was no significant relationship between OCT4B1 and HSPB2, HSPB7 and HSPB8.

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