Down-regulation of HSP40 gene family following OCT4B1 suppression in human tumor cell lines

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ABSTRACT

Objective(s): The OCT4B1, as one of OCT4 variants, is expressed in cancer cell lines and tissues more than other variants and plays an important role in apoptosis and stress (heat shock protein) pathways. The present study was designed to determine the effects of OCT4B1 silencing on expression profile of HSP40 gene family expression in three different human tumor cell lines.

Materials and Methods: The OCT4B1 expression was suppressed by specific siRNA transfection in AGS (gastric adenocarcinoma), 5637 (bladder tumor) and U-87MG (brain tumor) cell lines employing Lipofectamine reagent. Real-time PCR array technique was employed for RNA qualification. The fold changes were calculated using RT² Profiler PCR array data analysis software version 3.5.

Results: Our results indicated that fifteen genes (from 36 studied genes) were down-regulated and two genes (DNAJC11 and DNAJC5B) were up-regulated in all three studied tumor cell lines by approximately more than two folds. The result of other studied genes (19 genes) showed different expression patterns (up or down-expression) based on tumor cell lines.

Conclusion: According to the findings of the present study, we may suggest that there is a direct correlation between OCT4B1 expression in tumor cell lines (and tissues) and HSP40 family gene expressions to escape from apoptosis and cancer expansion.

Introduction

The heat shock proteins (HSPs) are considered as the most conserved proteins which exist in both prokaryotic and eukaryotic cells and their level of expression increases during stresses (1, 2). A wide spectrum of physiological and environmental factors including heat shock stress could alter the expression of HSPs. This family of proteins plays essential roles in correct assembling of nascent and stress-accumulated misfolded proteins, hence preventing their aggregation (3, 4). Based on the structural localization, HSPs (either intracellular or extracellular) functions as protective and/or inducible the intracellular HSPs aid cells to survive under stress conditions while extracellular or membrane receptor like HSPs interact with some of apoptosis family member proteins to regulate a programmed cell death (apoptosis) pathway (5). As mentioned above, HSPs are expressed by normal cells; however, under stress conditions (i.e. heat-shock) their expression is up-regulated.

The mammalian HSPs are divided according to their molecular size and function, into two main groups: high and small molecular weight. HSP60, HSP70 and HSP90, are members of the high molecular weight group while other HSPs are categorized as small molecular weight group. A variety of HSPs members are constitutively expressed, but, some of them are stress-induced (6). Within stressful circumstances, the HSPs could induce proteasomal-mediated degradation of some selected trigger proteins. Altogether, these properties make HSPs as mediators that are able to regulate the processes of cell death pathways (7).

The HSPs are also up-regulated in a considerable range of human cancers and several cellular functions (varying from proliferation, differentiation, to invasion and metastasis) associated with tumor genes (7). Especially HSP70, as an anti-apoptotic molecule, is reported to be up-regulated in malignant human tumors. Interestingly, HSP70 enhances the tumorigenic potency of rodent cells (8).

Investigations showed that HSP90 is an ATP-dependent chaperone molecule which is crucial for eukaryotic cells. It is essential for both activation and continues stabilization of several proteins involved
in various cellular pathways like apoptosis. Therefore, it appears that HSP90s considered as a remarkable target for cancer treatment (9).

Studies evidenced an important relation between HSPs expression and cancer (10). Accordingly, in all cancer tissues and cancer cell lines, different subtypes of HSPs are expressed. Several different hypotheses were proposed to explain the initiation of cancer. The "cancer stem cell" hypothesis is almost widely accepted by the scientists. With regard to this theory, adult stem cells (which are present in nearly all human tissues) or reprogrammed tissue somatic cells are the primary sources for triggering tumor tissue expansion (11, 12). According to this hypothesis, specific biological marker of embryonic stem cells (such as, OCT4 series) are re-expressed in tumor tissues and cancer cell lines, which are currently employed as either tumor markers for diagnosis or treatments of cancers.

OCT4 (octamer-binding transcription factor 4) is considered as the central factor of regulation of the self-renewal potency of proteins belonging to a family of transcription regulatory proteins and contains the POU DNA binding domain (13). The high level of OCT4 expression has been primarily restricted to embryonic stem cells (ESC), and in response to its suppression, the cell differentiation processes are enhanced (14). Recent investigations showed that, OCT4 is also involved in modulating survival of cancer cells (15-17). The OCT4 gene potentially encodes different variants by various capacity via alternative splicing among which three variants (A, B and B1) of OCT4 are more famous (18-20). The OCT4A is expressed in embryonic and some adult stem cells (located in nucleus) and control the pluripotency properties of cells. While the OCT4B is widely expressed in both stem and tumor cells (more located in the cytoplasm) by no effects on stemness pathways. The OCT4B1, a new variant of OCT4 that is expressed by both pluripotent normal and cancerous stem cell lines and tissues (21-24). Complementary studies showed up-regulation of OCT4B1 in gastric (8), colorectal (9), bladder (10), and germ cell tumors (11), where it functions as an anti-apoptotic factor (23, 24, 25).

Our previous investigations showed possible involvement of the OCT4B1 variant in stress pathway (10). In the present study, we aimed to suppress OCT4B1 in three human tumor cell lines and check the profile of the stress-related genes which may either over-expressed or suppressed following OCT4B1 suppression.

Materials and Methods

Cell culture

Three human tumor cell lines, namely, AGS, 5637, and U87MG were purchased from the Iranian national cell bank (Pasteur institute of Iran, Tehran) and were further cultured in RPMI-1640 (Gibco) supplemented with 10% (v/v) of heat-inactivated FBS (Fetal bovine serum), 2 mM L-glutamine (Sigma, St. Louis, USA) and penicillin-streptomycin antibiotics (Gibco) at 37 °C and 5% CO2 atmosphere.

OCT4 variants expression profile

Real-time RT-PCR was carried out to evaluate the expression status of the OCT4 variants in the studied tumor cell lines. Total RNA was extracted from cultured cells (104 cells/ml) using Trizol reagent (Invitrogen). According to the manufacturer's guidelines, the isolated RNA was then treated with TURBO DNase to avoid possible DNA contaminations. The RNA purity and fidelity were examined by evaluation of optical density (calculation of 260/280 nm ratio) and by observation of the samples on agarose gel following their electrophoresis (1% agarose gel), respectively. The first strand cDNA was synthesized at 42 °C for 60 min using 100 pmol oligo(dT) primers, 1µg of the extracted RNA and a cDNA synthesis kit (Pars Tous, Iran) according to the manufacturer's comments. Specific primers were designed for OCT4A, OCT4B, OCT4B1, and ß-actin (as a housekeeping gene) using Gene Runner (version 3.0.2) and Allele ID (version 4.0) softwares (Table 1).

Quantitative real-time PCR was performed by addition of SYBR green master mix (Parstous, Iran), 200 ng of the generated cDNA, and 2 µg/µl of each of the appropriate primer. The following program was set on a BIO-RAD CFX96 system (Bio-Rad Company, USA): one cycle of 95 °C for 15 min, 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec (for OCT4B1 61 °C for 20 sec) and 72 °C for 30 sec. Real-time PCR was carried out in triplicate and ß-actin was employed as a housekeeping gene for normalizing of the amplified signals of the target gene. The relative measures of the PCR product were determined using the 2-∆∆Ct formula. The dissociation stages, melting curves and quantitative analyses of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA). All of the PCR products were visualized following electrophoresis on agarose to check the size and quality of the PCR product.

The siRNA transfection procedure

In order to suppress OCT4B1, two specific siRNAs complementary to the unique region of OCT4B1 sequence (exon2b), and one irrelevant (scramble) siRNA (with no complementary target sequence in the human genome) were designed, using the siRNA selection program (Whitehead institute for biomedical research, http://jura.wi.mit.edu/) and were further ordered and synthesized (MWG Germany, Table 2). Tumor cell lines (1×105 cells/ml) were seeded onto the six-well plates in RPMI1640 medium lacked antibiotics in two groups (test and control). Once cultured cells achieved a confluency of 30-50%, cells were transfected with 50 nmol/ml of OCT4B1-siRNA...
OCT4B1 suppression and HSP40 gene family expression

Table 1. Sequences of designed primers of OCT4A, OCT4B, OCT4B1 and ß-actin. F=forward R=reverse

| Target genes | Designed Oligo | Relative Sequence | Fragment length |
|--------------|----------------|------------------|----------------|
| OCT4A        | F              | CGCAAGCTCTATTTCAC| 111            |
|              | R              | CATCACCTACCCAGC  |                |
| OCT4B        | F              | CAGGGAAATGGTGATGAC| 177           |
|              | R              | AGGCAGAACCTGTGAGAC|              |
| OCT4B1       | F              | GGTTCCTTTTGGGTTTCC| 128          |
|              | R              | TTCTCCCTCCCTACTCTC|              |
| ß-actin      | F              | CACACTTCTAATGAGC | 160           |
|              | R              | ATAGCACAGCCTGGATAG|              |

Table 2. Sequences and criteria of designed siRNAs

| siRNA name | Target | Sequences |
|------------|--------|-----------|
| Version I  | Target | AAGGAGTATCCCTCCCTAG |
|            | Sense  | (GGAGUAUCCCUGAACCUAG)dTdT |
|            | Anti-sense | (CUAGGUUCAGGGAUACUCC)dTdT |
| Version II | Target | AAGGAGTGTAGTCTGGATG |
|            | Sense  | (CAGUGUAAGAGCUUAGGAUC)dTdT |
|            | Anti-sense | (AAUCCAAGCUUACCCACUC)dTdT |
| Scramble   | Sense  | GCCGGAGAGGCUUAGGUGAdTdT |
|            | Anti-sense | UACACCUAAGCUCUCGUCCGAdTdT |

HSP40 gene family profiling

Following 48 hr of transfection, cells were heat-shocked at 45 °C for 1 hr. As previously described, the total RNA was extracted from cells of either control or test groups and cDNA was synthesized immediately (26). Quantitative gene expression analysis was performed using a real-time PCR array approach (SABiosciences Company, USA).

Statistical analysis

Gene expression analysis and chart drawing were carried out using CFX96 manager software (Bio-Rad, USA), and RT² Profiler PCR Array Data Analysis version 3.5, respectively.

Results

**OCT4 variants were expressed and OCT4B1 was down-regulated after siRNA transfection**

Present findings indicated that all of the three OCT4 variants (A, B and B1) were expressed in the studied cell lines (Figure 1). We found the highest expression level of OCT4B1 in U87-MG cell line. As expected, in response to siRNA transfection, OCT4B1 expression was dramatically decreased by 24, 48 and 72 hr. As it is clearly demonstrated in Figure 2, the highest level of OCT4B1 suppression was observed 48 hr post-transfection.

![Figure 1: Expression status of OCT4 variants in tumor cell lines. The real-time PCR data showed that all three OCT4 variants (A, B and B1) were expressed in the studied 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).](image-url)
Status of changes in expression of HSP40 gene family in response to OCT4B1 suppression

Our panel PCR data indicated that the expression profile of 36 studied genes in HSP40 gene family in studied tumor cell lines after OCT4B1 suppression showed approximately unique pattern of expression. Interestingly, 15 genes were down-regulated while two genes (DNAJC11 and DNAJC5B) were up-regulated in all three studied tumor cell line (Figure 2 and Table 3). Other studied genes (19 genes) were variously regulated, so that in some cases we observed up-regulation and in some cases down-regulation was seen in tumor cell lines.

Discussion

Previous reports (23, 24, 27), emphasized on the potential roles played by OCT4B1 in apoptosis and stress-related response pathways. Farashahi and colleagues reported that under stress condition (heat shock), the expression level of OCT4B1 was significantly elevated in tumor cell lines (24).

We have already reported a significant relation between OCT4B1 and expression profile of apoptosis regulated genes (25). Here, to decipher the molecular targets in stress pathway (heat shock) which are controlled by OCT4B1, we examined the status of the expression of 36 genes from HSP40 gene family following suppression of OCT4B1 in three different tumor cell lines, by employing RNAi strategy.

According to the results obtained in this investigation, the profile of gene expression of HSP40 gene family was almost similar in all of the studied cell lines. We observed that 34 of 36 studied genes were down-regulated at least in one of three studied cell lines, while 15 genes were down-regulated in all three cell lines. We also observed that 9 genes were down-regulated more than two folds in all of investigated cell lines. Interestingly, two genes (DNAJC11 and DNAJC5B) were up-regulated in all of studied tumor cell lines. Some genes revealed different framework of regulatory, DNAJA1 was down-regulated by -5.203 folds in 5637 and -8.16 folds in U87MG but -1.7 folds in AGS tumor cell lines, respectively. DNAJB2 was down-regulated -8.13 and -16.43 folds in AGS and 5637 tumor cell lines, respectively while it was also down-regulated by -1.5 in U87Mg tumor cell line (Table 3).

The co-chaperones heat shock protein 40kDa (HSP40) constitute the largest and most diverse sub-group of the heat shock protein (HSP) family. HSP40 genes members are involved in regulation of HSP70 gene family function, as well as HSP90 chaperone machine (28).

In the present study, it was revealed that following OCT4B1 suppression, the expression of fifteen genes in HSP40 family was decreased (in all three studied tumor cell lines) and other genes were down-regulated in some cell lines. Interestingly, just two genes (DNAJC11 and DNAJC5B) showed up-regulation in all three cell lines suggesting that up-regulation of OCT4B1 in cancer cells and tissues induce the expression of HSP40 genes family. Consistent with our results, Hi.H.L. et al showed over-expression of some HSP40 gene members such as DNAJC12 in rectal cancer (29) and Morita et al reported that DNAJB8 (HSP40) was over-expressed in colon cancer (30).

The expression of OCT4B1 in cancer cells and tissue (22), in accordance with anti-apoptotic effects of the variant (23, 26), as well as its association with stress signaling pathway (24), may propose that OCT4B1 up-regulation in tumor cells in turn leads to over-expression of HSP40 family members. Accordingly, it may be considered as a probable mechanism of anti-apoptotic activity of OCT4B1 in tumor cells. By other means, down-regulation of OCT4B1 could be assumed as an alternative strategy for HSP40-mediating the molecular-based cancer therapies.

Furthermore, little is known concerning the molecular mechanisms of survival functions of HSPs. Overall, regarding results presented here, in response to OCT4B1 suppression in tumor cells, expression of 34 (out of 36) members of HSP40 family was decreased at least in one of three studied tumor cell lines, while, two genes showed up-regulation in all three studied tumor cell lines. Therefore, our data may probably demonstrate a general direct association between OCT4B1 suppression and HSP40 genes down-expression.
### Table 3. Gene expression of HSP40 family member following OCT4B1 suppression in tumor cell lines

| Symbol     | Description of Genes                                      | AGS   | 5637  | U87MG |
|------------|----------------------------------------------------------|-------|-------|-------|
| DNAJA1     | DNAJ (HSP40) homolog, subfamily A, member 1              | -1.70 | -52.03| -8.16 |
| DNAJA2     | DNAJ (HSP40) homolog, subfamily A, member 2              | -5.29 | -7.25 | -2.54 |
| DNAJA3     | DNAJ (HSP40) homolog, subfamily A, member 3              | -1.85 | -2.53 | 1.13  |
| DNAJA4     | DNAJ (HSP40) homolog, subfamily A, member 4              | 1.60  | -4.18 | -1.47 |
| DNAJC21    | DNAJ (HSP40) homolog, subfamily C, member 21             | -2.51 | -4.35 | -15.03|
| DNAJB1     | DNAJ (HSP40) homolog, subfamily B, member 1              | -2.06 | -2.82 | 1.01  |
| DNAJB11    | DNAJ (HSP40) homolog, subfamily B, member 11             | -3.72 | -5.09 | -46.40|
| DNAJB12    | DNAJ (HSP40) homolog, subfamily B, member 12             | -6.98 | -15.54| -2.39 |
| DNAJB13    | DNAJ (HSP40) homolog, subfamily B, member 13             | 1.13  | -1.21 | 5.79  |
| DNAJB14    | DNAJ (HSP40) homolog, subfamily B, member 14             | -1.78 | 1.96  | 5.59  |
| DNAJB2     | DNAJ (HSP40) homolog, subfamily B, member 2              | -8.13 | -16.43| -1.50 |
| DNAJB5     | DNAJ (HSP40) homolog, subfamily B, member 5              | -2.54 | -3.48 | -1.22 |
| DNAJB6     | DNAJ (HSP40) homolog, subfamily B, member 6              | -1.57 | -7.88 | 1.32  |
| DNAJB7     | DNAJ (HSP40) homolog, subfamily B, member 7              | -31.94| -43.75| 2.45  |
| DNAJB8     | DNAJ (HSP40) homolog, subfamily B, member 8              | -2.49 | -3.41 | -1.19 |
| DNAJB9     | DNAJ (HSP40) homolog, subfamily B, member 9              | -22.69| -11.62| -3.47 |
| DNAJC1     | DNAJ (HSP40) homolog, subfamily C, member 1              | -2.68 | -3.68 | -1.29 |
| DNAJC10    | DNAJ (HSP40) homolog, subfamily C, member 10             | 1.20  | -1.14 | 2.50  |
| DNAJC11    | DNAJ (HSP40) homolog, subfamily C, member 11             | 12.26 | 7.42  | 6.75  |
| DNAJC12    | DNAJ (HSP40) homolog, subfamily C, member 12             | -1.58 | -2.17 | 1.31  |
| DNAJC13    | DNAJ (HSP40) homolog, subfamily C, member 13             | -4.10 | -5.77 | -2.05 |
| DNAJC14    | DNAJ (HSP40) homolog, subfamily C, member 14             | -1.23 | -1.68 | 1.70  |
| DNAJC15    | DNAJ (HSP40) homolog, subfamily C, member 15             | 67.46 | 20.28 | -1.24 |
| DNAJC16    | DNAJ (HSP40) homolog, subfamily C, member 16             | -1.31 | -1.80 | 1.58  |
| DNAJC17    | DNAJ (HSP40) homolog, subfamily C, member 17             | -9.81 | -13.44| -2.36 |
| DNAJC18    | DNAJ (HSP40) homolog, subfamily C, member 18             | -1.33 | -1.83 | 1.56  |
| DNAJC19    | DNAJ (HSP40) homolog, subfamily C, member 19             | -    | -     | -     |
| DNAJC3     | DNAJ (HSP40) homolog, subfamily C, member 3              | -1.33 | 1.35  | 3.85  |
| DNAJC4     | DNAJ (HSP40) homolog, subfamily C, member 4              | -1.17 | -1.60 | 1.78  |
| DNAJC5     | DNAJ (HSP40) homolog, subfamily C, member 5              | 24.75 | -2.20 | 3.03  |
| DNAJC5B    | DNAJ (HSP40) homolog, subfamily C, member 5 beta         | 2.37  | 1.73  | 4.94  |
| DNAJC5G    | DNAJ (HSP40) homolog, subfamily C, member 5 gamma       | -1.35 | -1.40 | -2.38 |
| DNAJC6     | DNAJ (HSP40) homolog, subfamily C, member 6              | -1.44 | -1.66 | -2.70 |
| DNAJC7     | DNAJ (HSP40) homolog, subfamily C, member 7              | 1.08  | -1.27 | 2.24  |
| DNAJC8     | DNAJ (HSP40) homolog, subfamily C, member 8              | -12.50| -19.27| -2.65 |
| DNAJC9     | DNAJ (HSP40) homolog, subfamily C, member 9              | -8.84 | -4.49 | -2.84 |
| SERPINH1   | Serpin peptidase inhibitor, clade H 1                    | -4.01 | -8.27 | 3.82  |
Figure 3-1. HSP40 gene expression, 48 hr after siRNA transfection in studied tumor cell lines. Y axis showed fold gene regulation and X axis showed 36 genes of HSP40 gene family.

Figure 3-2. HSP40 gene expression, 48 hr after siRNA transfection in studied tumor cell lines. Y axis showed fold gene regulation and X axis showed 36 genes of HSP40 gene family.

Figure 3-3. HSP40 gene expression, 48 hr after siRNA transfection in studied tumor cell lines. Y axis showed fold gene regulation and X axis showed 36 genes of HSP40 gene family.
Conclusion

The data presented here may support the theory of possible direct association between the expression of OCT4B1 and HSP40 gene family. Accordingly, OCT4B1 suppression in different tumor cell lines lead to a significant down-regulation of the expression of the members of HSP40 family.

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Conflict of interest

None of authors of the present article declared conflict of interest.

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