New function of TSGA10 gene in angiogenesis and tumor metastasis: a response to a challengeable paradox

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Abstract

Several studies have shown that testis-specific gene antigen (TSGA10) could be considered as a cancer testis antigen (CTA), except for one study which has identified it as a tumor suppressor gene. In order to exert its function, TSGA10 interacts closely with hypoxia inducible factor (HIF-1α) and since this interaction is still not completely defined, the exact role of TSGA10 in angiogenesis and invasion is also under question. The current study was conducted to investigate the function of TSGA10 gene and evaluate its potential effects on tumor angiogenesis and invasion. To do so, TSGA10 vector was designed for a stable transfection in HeLa cells, and then clonal selection was applied. The efficiency of transfection and the role of TSGA10 in abovementioned targets were evaluated by real-time PCR, western blot, zymography and ELISA tests in both normoxia and hypoxia. Invasion, migration and angiogenesis were assessed. Three-dimensional model of TSGA10 protein was accurately built in which TSGA10 docked to 2 domains of HIF-1α. Increased expression of TSGA10 correlated with decreased HIF-1α transcriptional activity and inhibited angiogenesis and HeLa cells invasion in normoxia as well as hypoxia. Docking analysis indicated that binding affinity of TSGA10 with TAD-C (CBP) domain of HIF-1α would be stronger than that with PAS-B domain. Our findings showed that overexpression of TSGA10 would induce disruption of HIF-1α axis and exert potent inhibitory effects on tumor angiogenesis and metastasis. Therefore, TSGA10 could be considered as a potent therapeutic candidate, prognostic factor and a cancer management tool.

Introduction

The TSGA10 gene (NC_000002.12), located on chromosome 2 band q11.2, consists of 19 exons transcribed to an RNA with a size of about 3 Kb. This RNA in turn encodes 698 amino acids in the form of an 82 kDa protein expressed in testis during developmental stages of spermatogenesis and embryogenesis (1–3). It has already been indicated that the full length TSGA10 is post transnationally modified, and subsequently processed into an N-terminus fragment of 27 kDa and a C-terminus fragment of 55 kDa. The N-terminus fragment is located in fibrous sheath of sperm tail and the C-terminus one accumulates in the midpiece of sperm, where it co-localizes with HIF-1α. The C-terminus fragment prevents nuclear localization of HIF-1α (4).

HIF-1α regulates key processes like oxygen transport, angiogenesis and glycolytic capacity (5). It plays a central role during tumor hypoxia by up-regulating expression of the genes involved...
in tumor angiogenesis, invasion and metastasis (6). Recent studies have shown that TSGA10 is widely found in normal tissues and easily tracked in auto-immune diseases, solid tumors and leukemia. In some studies it has been considered as a CTA (3,7–11). Other studies on tumoral cells have affirmed that TSGA10 expression is decreased in comparison with normal cells and characterize it as a tumor suppressor gene (12,13). The exact role of TSGA10 is unclear and still remains a challenge to researchers. Therefore, the study of TSGA10 gene function is a very appropriate area for future investigations on not only spermatogenesis but also embryogenesis, angiogenesis and tumorigenesis. Angiogenesis and metastasis are closely related to HIF-1α implying that in addition to spermatogenesis, TSGA10 is also involved in other processes such as angiogenesis and tumor metastasis. Therefore, in present study, we investigated the potential roles of TSGA10 in angiogenesis and metastasis processes.

Results
HIF-1α and TSGA10 expression
After the stable clonal selection, three clones of HeLa-pcDNA3.1-TSGA10 (cells transfected with vector carrying TSGA10) were picked out and expanded. TSGA10 mRNA and protein expression of the three mentioned clones plus those of HeLa-pcDNA3.1 (cells transfected with empty vector) and human skin fibroblast cells were assayed by real-time PCR and western blot. Overexpression of TSGA10 mRNA was quantified as follows: clone one: 1.5 ± 0.34, clone two: 3.4 ± 0.2, clone three: 5.8 ± 0.38, human fibroblast: 3.93 ± 0.45 folds increase in comparison with HeLa-pcDNA3.1. Densitometry analysis showed that protein expression levels of TSGA10 were significantly increased in clone three compared with other clones (Fig. 1A and B). It revealed that there is no direct relationship between TSGA10 mRNA and its protein levels. TSGA10 expression in fibroblast (as a representator of normal cells) is higher than HeLa-pcDNA3.1 cells (as a representator of tumoral cells). Hypoxia significantly raised HIF-1α protein expression (data not shown). Immunocytochemistry analysis showed that fluorescence signal was intensified in clone 3 which overexpressed TSGA10 compared with HeLa-pcDNA3.1 (Supplementary Material, Fig. S1).

Overexpression of TSGA10 gene suppressed cell growth and metabolite secretion
TSGA10 overexpression of more than 5 folds caused a decrease in the proliferation of TSGA10 expressing HeLa cells. To examine the correlation between TSGA10 overexpression and cell

Figure 1. Analysis of mRNA and protein levels of TSGA10 cloned in Hela cells and its overexpression effect on cell proliferation in normoxic and hypoxic conditions. (A and B) Expression of TSGA10 at mRNA and protein levels in three clones were analyzed using real-time PCR and western blot compared with HeLa-pcDNA3.1 (as control) and fibroblast cells (as normal cells). (C) Effect of TSGA10 overexpression on HeLa cells proliferation in normoxic and hypoxic conditions. (D) Analysis of TSGA10 overexpression effects on HUVEC proliferation as a result of a decreased metabolite secretion from HeLa-pcDNA3.1-TSGA10 cells challenged in normoxic and hypoxic conditions. Quantification of the protein bands in western blot analysis carried out using densitometric analysis (TotalLab software, Wales, UK). Fold change was normalized against beta-actin and compared with the control. Each data point was presented as mean ± SD from 4–5 independent experiments. **P < 0.01 and ***P < 0.001 compared with the control.
proliferation, three different clones of HeLa-pcDNA3.1-TSGA10 were selected and mRNA levels of TSGA10 were proved to be implicated in cell proliferation. The clone with TSGA10 mRNA overexpression of more than 5 folds was selected since, based on MTT results, its proliferation was significantly decreased by 22.80 ± 4.782% and 34.69 ± 2.399% in normoxia and hypoxia respectively, after 48 h compared with HeLa-pcDNA3.1 cells. Proliferation of other clones with TSGA10 mRNA overexpression of less than 5 folds was not significantly affected in both normoxia and hypoxia (Fig. 1C).

Our study showed that HeLa-pcDNA3.1 metabolites significantly increased HUVECs proliferation at certain HeLa-pcDNA3.1 conditioned-medium concentrations (25 and 50%). Under normoxic conditions, HeLa-pcDNA3.1 metabolites at conditioned-medium concentrations of 25 and 50% increased proliferation of HUVECs by 117.4 ± 3.356% and 132.87 ± 2.57% respectively. While in hypoxic conditions, these values were determined 121.2 ± 1.68% and 140 ± 4.66% respectively. There were no significant changes at conditioned-medium concentration of 10% compared with the non-challenged HUVECs group. In addition, we found that challenging endothelial cells with metabolites from HeLa-pcDNA3.1-TSGA10, slightly increased HUVECs proliferation (106.4 ± 2.6% in hypoxia condition) at HeLa-pcDNA3.1-TSGA10 conditioned-medium concentrations of 50%, but no statistically meaningful differences were observed at 25%. HeLa cells are considered as highly vascular and aggressive cervical tumor-derived cells, secreting pro-angiogenic factors such as VEGF (a key regulator of angiogenesis) and other factors causing endothelial cells proliferation. We showed that overexpression of TSGA10 inhibits metabolite secretion from HeLa cells (Fig. 1D).

**TSGA10 inhibits cell motility and invasion**

Tumor cells exposed to normoxic and hypoxic conditions have shown an increased tendency to spread to distant organs, and these cells have been characterized by these increased migratory and invasive potentials (14). Therefore, we investigated the effect of TSGA10 on the invasive potential of HeLa cells in vitro. Wound healing and Transwell migration assays were used to determine whether TSGA10 could inhibit the motility and invasion of HeLa cells. After 24 h, wounded cell monolayers of HeLa-pcDNA3.1 had completely filled in the cleared area, while HeLa-pcDNA3.1-TSGA10 had significantly hindered (Supplementary Material, Fig. S2). To investigate the effects of TSGA10 overexpression on cell invasion, Transwell invasion assay was applied to determine the ability of HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 cells to invade through matrices in vitro. As shown in Figure 2A, HeLa-pcDNA3.1-TSGA10 cell invasiveness was significantly decreased. HeLa-pcDNA3.1 cells permeated the filter in Normoxia (100% as control) and hypoxia (117±6.51) while permeation of HeLa-pcDNA3.1-TSGA10 cells was 23.3±2.56% in normoxia and 13.8±2.28 in hypoxia.

**Figure 2.** TSGA10 inhibits motility, invasion and angiogenic activity of HeLa cells. (A) Cell invasion assay. Migration of HeLa-pcDNA3.1 (I, III) and HeLa-pcDNA3.1-TSGA10 cells (II, IV), the cells were seeded onto the matrigel-coated Transwell and incubated for 24 h in normoxic and hypoxic conditions. Cell invasion was determined as described in results. (B) Effect of the TSGA10 overexpression on angiogenesis; (I, III) By adding HUVEC-attached beads to collagen gel and co-culturing with HeLa-pcDNA3.1, the HUVECs began to sprout outward off the beads and developed short, capillary like structure after 48 h in normoxic and hypoxic conditions; (II, IV) however co-culturing with HeLa-pcDNA3.1-TSGA10 decreased the number of HUVECs sprouts formed compared to co-culturing with HeLa-pcDNA3.1. (Magnification 100x). Each data point was presented as mean ± SD from 4–5 independent experiments. ***P < 0.001 compared with the control.
Vascular sprout formation in co-cultures models

Cancer metabolites increase HUVEC proliferation and angiogenic activity (15). Angiogenesis of endothelial cells co-cultured with HeLa-pcDNA3.1-TSGA10 cells through the collagen matrix was decreased in normoxia (angiogenic activity: 31.60 ± 5.11% compared with HeLa-pcDNA3.1 as control). Angiogenesis of endothelial cells co-cultured with HeLa-pcDNA3.1 (as control) through the collagen matrix in hypoxia was increased (angiogenic activity: 135.4 ± 7.78%) but angiogenesis of the endothelial cells co-cultured with HeLa-pcDNA3.1-TSGA10 cells was decreased in hypoxia (angiogenic activity: 32.40 ± 5.49%) as shown in Figure 2B.

TSGA10 decreases expression of HIF-1 target genes

HIF-1α is a master transcriptional regulator of angiogenic and metastatic genes. To analyze whether the inhibition of HIF-1α by TSGA10 would result in decreased expression of HIF-1 target genes, cells were incubated under normoxic and hypoxic conditions and after 18 h mRNA expression of HIF-1α as well as HIF-1-dependent factors such as VEGF, bFGF, PDGF, MMP2, MMP9, IGf, PLAU, CXCR4 and CXCL12 were all investigated using real-time PCR. The results illustrated that TSGA10 downregulated the expression of HIF-1-dependent factors but had no effect on HIF-1α mRNA levels but inhibited transcription of HIF-1α target genes expression playing critical roles in many aspects of tumor biology, including angiogenesis and metabolic adaptation for survival under normoxic and hypoxic conditions.

TSGA10 inhibits secretion of VEGF and the matrix metalloproteinases 2 and 9

Metastasis and invasion have been known to profoundly depend on cell matrix degradation and high levels of MMPs and VEGF in tumor tissues. In this regard, HIF-1α up-regulates the secretion of VEGF and MMPs which in turn trigger and expedite angiogenesis (16). Aside from the results showing TSGA10 inhibited cell invasion and motility; we also demonstrated anti-gelatinase activities of TSGA10 in both normoxia and hypoxia. TSGA10 inhibits secretion of MMP-2 and MMP-9 as confirmed by gelatin zymography and ELISA analysis (Fig. 3A–C). VEGF secretion was also decreased by TSGA10 overexpression in the abovementioned conditions as confirmed by ELISA analysis (Fig. 3D).

Sequence analysis, and homology modeling

To obtain a possible mechanism by which TSGA10 prevents HIF-1α transcription activity and tumor growth, TSGA10 was docked to HIF-1α. The obtained alignment using six templates was introduced into MODELLER to generate 3D models of TSGA10. Ramachandran plot and summaries of structure models were obtained from PROCHECK program. Ramachandran plot analysis showed that main-chain conformations for 90.3% of amino acid residues are within the most favored or additionally allowed regions (Fig. 4A). In general, a score close to 100% was implied a good stereochemical quality of the models (17). Therefore, these PROCHECK results suggested that the predicted models were of good quality.

Molecular docking by computer-assisted methods was used to improve our understanding of the interaction between TSGA10 and HIF-1α domains. The HADDOK was used to calculate the possible conformation of the TSGA10 that binds to the HIF-1α/CBP recognition (TAD-C) and HIF-1α/ARNT PasB (Fig. 4B). To gain a better understanding of the effects of each residue on binding affinity, a per-residue decomposition of the total energy was carried out to evaluate the energetic influences of critical residues on the binding in the two systems of TSGA10/HIF-1α/CBP recognition (TAD-C) and TSGA10/ARNT PasB. At first, using WHAT IF Web Interface web server that reads a pdb file with two or more chains to find the residues involved in the interaction of subunits in a selected amino acid chain, important residues in the interaction were determined. Fig. 4C shows output of this analysis. As it is illustrated, some regions have more important roles in the interaction. The results showed the residues involved in interaction of subunits selected confirming that the binding to HIF-1α/CBP recognition (TAD-C) involved more amino acids.

To evaluate the binding capability of TSGA10, docking energy landscapes were analyzed (Table 2A). The values of total interaction energy, van der Waals energy and electrostatic energy are reported for both complexes. Docking analysis revealed that the binding affinity of TSGA10 to HIF-1α/ARNT PasB was lower than those of TSGA10/HIF-1α/CBP recognition (TAD-C) (Table 2A).

The important residues in each interaction site were separately studied, and the van der Waals, electrostatic, and total interaction energies of these residues in complexes were determined. Energy of interaction between individual residues in these sites was calculated with the ‘Calculate Interaction Energy’ protocol encoded in Discovery Studio. The complexes were typed with CHARMM force field and the dielectric model was assigned.

Table 1. TSGA10 did not affect HIF-1α mRNA levels but inhibited transcription of HIF-1α target genes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Target gene name</th>
<th>Fold change in Hela-pcDNA3.1-TSGA10 compared with Hela-pcDNA3.1 in Normoxia</th>
<th>Fold change in Hela-pcDNA3.1-TSGA10 compared with Hela-pcDNA3.1 in Hypoxia</th>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
<td>−1.66 ± 0.2</td>
<td>−2.13 ± 0.29</td>
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<td>MMP2</td>
<td>Matrix Metalloproteinase2</td>
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<td>MMP9</td>
<td>Matrix Metalloproteinase9</td>
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<td>−1.8 ± 0.24</td>
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<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
<td>−1.8 ± 0.2</td>
<td>−2.56 ± 0.24</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
<td>−1.1 ± 0.15</td>
<td>−1.7 ± 0.28</td>
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<tr>
<td>IGf</td>
<td>Insulin-like Growth Factor</td>
<td>−0.75 ± 0.05</td>
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<tr>
<td>PLAU</td>
<td>Plasminogen Activator, Urokinase</td>
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<td>−2.44 ± 0.2</td>
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<tr>
<td>CXCR4</td>
<td>Chemokine (C-X-C motif) Receptor 4</td>
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<td>−1.63 ± 0.06</td>
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<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
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<tr>
<td>HIF-1α</td>
<td>Hypoxia Inducible factor 1 Alpha</td>
<td>0.12 ± 0.029</td>
<td>0.092 ± 0.037</td>
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</table>

Hela-pcDNA3.1 and Hela-pcDNA3.1-TSGA10 cells were incubated under normoxia and hypoxia for 18 h. mRNA expression was analyzed by real-time PCR. Each point represents the mean ± SD.
Figure 3. TSGA10 inhibits MMP-2, MMP-9 VEGF secretion. HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 cells were cultured in normoxic and hypoxic conditions and after 16 h the conditioned media were analyzed for MMP-2 and MMP-9 gelatinase activity and ELISA tests. (A) Gelatin zymograms (upper band: MMP-9, lower band: MMP-2) respectively. I: Control (purified MMP-2) II: Control (purified MMP-9) III: MMPs secretion pattern of HeLa-pcDNA3.1-TSGA10 in normoxia IV: MMPs secretion pattern of HeLa-pcDNA3.1 in normoxia V: MMPs secretion pattern of HeLa-pcDNA3.1-TSGA10 in hypoxia VI: MMPs secretion pattern of HeLa-pcDNA3.1 in hypoxia. The bands were visualized and quantified by densitometric analysis (TotalLab software, Wales, UK). As demonstrated by the graph in (A), overexpression of TSGA10 results in a decreased gelatinase activity of MMPs. (B–D) Effects of stable TSGA10 overexpression on MMP-2, MMP-9 and VEGF secretion were assayed by ELISA method. Each data point was presented as mean ± SD from 4–5 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. Ramachandran plot and summary of results generated via HADDOCK and WHAT IF Web server for TSGA10. (A) Ramachandran plot analysis showed the residues in most favored (red), additionally allowed (yellow), generously allowed (pale yellow) and disallowed regions (white color). (B) Binding between TSGA10 and HIF-1α/CBP and, HIF-1α/ARNT PASB. (C) The results of WHAT IF Web Interface web server: residues involved in interaction of subunits selected confirming that the binding to HIF-1α/ CBP recognition (TAD-Q) involved more amino acids.
The heterodimeric HIF-1 transcription factor consists of two basic helix-loop-helix (bHLH) containing subunits: HIF-1α and HIF-1β. They also share a PER-ARNT-SIM (PAS) domain with other transcription factors. HIF-1 heterodimer formation is feasible through HLH and PAS domains. Both subunits belong to bHLH-PAS family. These basic regions are sensitive to O2 drops and also responsible for DNA-binding. HLH and PAS domains (PAS-A and PAS-B) mediate protein–protein interactions. In both subunits, HLH and PAS domains together create a functional interface for subunit dimerization (23–25). The carboxyl halves of HIF-1α and HIF-1β contain one or more transactivation domains, presumed to interact directly or indirectly with the components of the transcription initiation complex which affects transcription rate of the gene to which it is bound. HIF-1α expression rises exponentially as a fast response to the befallen hypoxia, then cells are made to discontinue the phase they repeatedly go through under normoxic conditions involving a cycle of synthesizing, ubiquitinating and degrading HIF-1α. To maintain this factor, degradation is inhibited through hypoxia, so dimerization can happen which permits HIF-1 binding to HREs within target genes. Then it starts the transcription phase by recruiting P300/CBP and Ref co-activators (26). These co-activators, namely P300/CBP and Ref, playр two critical roles to stabilize the transcription initiation complex. Their recruitment is necessary for RNA polymerase to access the DNA within chromatin and subsequent transcription of DNA into RNA (26,27).

A 41 amino acid residue domain of HIF-1α is the only necessary domain to bind to CH1 domain of P300/CBP and Ref-1. This domain is HIF-1α/CBP recognition (TAD-C) which includes the amino acids 786-826 required for trans-activation process in hypoxic cells. This binding triggers HIF-1 transcriptional activity (28).

As demonstrated by HeLa-HUVEC co-culture model, TSGA10 overexpression in HeLa cells leads to the decrease of migration and angiogenic responses of HUVECs. Tumor cells are known as secretors of growth factors with paracrine effects on vascular endothelium. These growth factors play major roles in regulating tumor angiogenesis; and most of them are increased when tumor cells undergo hypoxia and HIF overexpression (5). The due results suggested that soluble factors (growth factors) production, during cancer cell development, has great roles in tumor angiogenesis.

Numerous cell-specific angiogenic growth factors and cytokines are regulated either directly or indirectly by HIF-1 (5,6). VEGF, bFGF, PDGF, IGF, PLAU, MMP2, MMP9, CXCR4, CXCL12, as HIF-1-dependent factors, were all investigated in this study. TSGA10 affects HIF-1 dependent factors, as shown by our results obtained from ELISA, real-time PCR, zymography, challenged and co-culture models. Secretion of soluble growth factors from HeLa-pcDNA3.1-TSGA10 cells was significantly decreased which resulted in inhibition of angiogenesis proving that these factors are critical in tumor angiogenesis and metastasis (Fig. 5A).

As stated in clonal selection, three clones were selected. Since proliferation and angiogenic factors secretion and activity were inhibited in only one of them, namely the clone 3, so, this clone was appropriate. Since in HeLa-pcDNA3.1 cells TSGA10 mRNA level should reach a threshold of more than 5 folds to produce enough protein in order to act as a tumor suppressor under normoxic and hypoxic conditions if its mRNA level is below this amount, TSGA10 cannot be considered as an anti-tumorigenic and anti-angiogenic factor in HeLa cells.

**Discussion**

It has perfectly been established that malignant epithelial tumors cannot grow more than several cubic millimeters if oxygen and/or glucose levels are not sufficient to meet their demands which are higher compared with physiological conditions (18,19). Thus, cancer cells will encounter a limitation in oxygen and nutrition sources leading to competition for these vital factors. Metabolite diffusion is also hindered because of high interstitial pressure. Tumor cells that undergo these conditions are induced to secrete factors which trigger neovascularization from the preexisting vasculature. This in turn guarantees tumor cell survival and abnormal proliferation (20). The due situation needs a hypoxic environment paradigm and results in altered angiogenic responses. A nuclear factor named HIF-1α, has been identified to be induced in hypoxic situations in cells. It binds to hypoxic response element (HRE) which permits transcription of genes essential for an adaptive response to hypoxia (21,22).

**Table 2.** (A) Total interaction, van der Waals and electrostatic energies for TSGA10/ARNT PasB and TSGA10/HIF-1ALPHA/CBP interactions. Details of interaction energies of individual residues involved in (B) TSGA10/HIF-1α/CBP (TAD-C) and (C) TSGA10/HIF-1α/ARNT PasB.

<table>
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<tr>
<th>Residue</th>
<th>van der Waals energy (Kcal/mol)</th>
<th>Electrostatic energy (Kcal/mol)</th>
<th>Interaction energy (Kcal/mol)</th>
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<td>TSGA10/HIF-1α-CBP</td>
<td>21.20060</td>
<td>-534.10064</td>
<td>-512.90004</td>
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<td>TSGA10/ARNT PasB</td>
<td>77.04334</td>
<td>-468.77197</td>
<td>-391.72863</td>
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(A) Total interaction, van der Waals and electrostatic energies for TSGA10/ARNT PasB and TSGA10/HIF-1ALPHA/CBP interactions.
The main question is how TSGA10 regulates the expression of genes involved in tumor growth, proliferation and specially angiogenic activity of tumor cells.

S. Hđgele et al. showed in spermatozoa TSGA10 co-localized with HIF-1α and consequently prevented its nuclear localization and transcriptional activity. According to their study, the C-terminal of TSGA10 interacted with the first 400 amino acids of HIF-1α (the N-terminal domain). Since the full length cloned HIF-1α auto-activates itself, S. Hđgele et al. faced a limitation in terms of cloning the full length protein. So they just cloned the first 400 amino acids of HIF-1α (which interacted with TSGA10) by yeast two hybridization and pull down methods. In their study, the role of HIF-1α C-terminal interaction with TSGA10 as well as whether TSGA10 acts in favor of tumor or not, remained unclear (4).

As stated before, there are two significant domains in HIF-1α, named PASB (N-terminus) and TAD-C (C-terminus). PASB is involved in HIF-1 dimerization and contributes to HIF-1 activity while TAD-C binds to P-300 and contributes to co-activation of P300/CBP. Our bioinformatics studies showed that TSGA10 can dock to both these domains while its binding to TAD-C is much stronger than PASB and more amino acids are involved in this binding. TSGA10 inhibits HIF-1 dimerization by docking to PASB, and forms a complex with TAD-C leading to inactivation of HIF-1α by preventing P300 recruitment and consequently represses HIF-1 target gene transcription which results in impaired cell growth, metastasis and angiogenesis. Therefore TSGA10 blocks HIF-1α binding to co-activators (Fig. 5B). However, TSGA10 affected neither HIF-1α mRNA and protein levels nor β-actin levels in HeLa-pcDNA3.1 cells. So these data could lessened the possibility of nonspecific effects of TSGA10 on transcription or/and translation.

According to Behnam’s and Miryounesi’s studies, TSGA10 is expressed in undifferentiated embryonic stem cells at a low level (3,29). Its expression increases during transition from proliferation phase to differentiation phase. Since cell cycle is almost inactive in differentiation phase and most cells are non-growing and non-proliferating at G0 phase, TSGA10 overexpression could be associated with cell cycle inactivation. Accordingly, TSGA10 overexpression is considered as one of the factors controlling cell cycle. Due to high HIF-1α expression and drastically increased expression of TSGA10 in tissues like testis, retina, brain and kidney (3,30,31), TSGA10 could be considered as a controlling factor for HIF-1α activity.

As mentioned, HIF-1α rises in majority of tumors. In order to control HIF-1α, TSGA10 gene must be expressed to a certain threshold to produce enough protein leading to HIF inhibition. It has been found that both stimulators and inhibitors of angiogenesis are produced in the microenvironment of tumors. Stimulators are more elevated than inhibitors. Such disruption of angiogenesis balance in favor of neovascularization is a key stage in the progression of tumor growth and metastasis (32). This phenomenon is likely confirmed in the case of tumors whose TSGA10 expression is less than a certain threshold able to inhibit HIF-1α. So, TSGA10 could be considered as both tumor suppressor and tumor marker.

As shown by our results, TSGA10 overexpression can inhibit HeLa cell growth, motility and invasion. One potential mechanism mediating this phenomenon could be TSGA10 effects on
the factors like MMPs, CXCR4 and CXCL12 which are the most important factors in tumor metastasis and invasion. Expression of vimentin in cancers correlates with tumor invasion and growth (33). Also in the study of Roghanian et al. (34) it was shown that TSGA10 interacts with vimentin. Therefore TSGA10 interaction with vimentin could be considered as another possible mechanism by which TSGA10 exerts its antitumor effects.

In addition to a few tumors, TSGA10 is also broadly distributed in normal tissues (3,9,10,35). Accordingly it cannot be considered as a CTA. CTAs are a big family of tumor associated antigens that are expressed in human tumors of different histological origin, while in normal tissues they are not expressed except for testis and placenta. Their biological roles in both germline tissues and tumors have remained unclear. Their expression could be involved in tumor genesis, as indicated by increasing evidence (36).

In TSGA10 research, a major paradox still remains unsolved. In previous studies, elevated levels of TSGA10 RNA in malignancy status were considered as a predictive index of malignant stages. Our results are in line with data obtained from two previous studies in which TSGA10 expression was decreased in tumor cells compared with normal cells. Also in one of those studies TSGA10 has been stated as a tumor suppressor gene (12,13). In comparison with those studies, we showed HIF-1-dependent antiangiogenic and antimetastatic effects of TSGA10 on the condition that its mRNA level reaches a suitable threshold. Hence in our study, both the mechanisms of TSGA10 effects and its roles are different.

As stated before, mRNA level cannot be regarded as an index of protein level. Therefore, to clarify gene functions and disease mechanisms, a combination of genomic and proteomic tests is expected to result in better perceptions than either approaches alone (37–39).

In conclusion, the obtained results from in vitro studies indicate that proliferation, migration, invasion and angiogenic activity of a tumor are significantly affected by TSGA10 overexpression. These new findings provide the first evidence consistent with the hypothesis that TSGA10 has a significant role in tumor angiogenesis and metastasis. The due results could be considered as a good prognosis in tumors with overexpression of TSGA10 in terms of protein levels. In primary cancers, it is suggested that developing methods to elevate TSGA10 level to a certain threshold in which it exerts its described biological effects, inhibition of tumor growth and neovascularization would be feasible as a result. Finally, the more precise our knowledge of TSGA10 functions, the more potential we could provide for cancer therapeutic interventions.

**Materials and Methods**

Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Calf Serum (FCS), and trypsin–EDTA were purchased from Invitrogen (Life Technologies, Gaithersburg, MD, USA). Tissue culture flasks were from Orange Scientific (Braine l’Alleud, Belgium) and disposable plastic ware was purchased from Greiner Bio-One (Frickenhausen, Germany). Phenyl methylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and Nonidet P-40 were from Roche (Mannheim, Germany). Polyvinylidene difluoride membrane was from Millipore (Schwalbach, Germany). ECL reagents were from Amersham Pharmacia Corp. (Piscataway, NJ, USA). T4 ligase was purchased from Takara Bio, Inc. (Dalian, China). PcDNA3.1 eukaryotic expression vectors and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All other reagents were from Sigma-Aldrich (Taufkirchen, Germany) and Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Construction of recombinant expression vectors**

The RT-PCR amplification mixture contained the following primers: 5′-AGGATCCGCCACCATGATGCCAAGTGGTCT-3′ (up-stream) carrying a BamHI site, underlined and 5′-AGGAATTCCT CAGAAATCTCTGTAGC-3′ (downstream) carrying an EcoRI site, underlined, were designed for the TSGA10 gene’s CDS (sequence coding for amino acids in protein, Accession NO.AF254756.1) domain. This sequence was amplified by quantitative reverse transcription-polymerase chain reaction (RT-PCR) with primer T1 and T2 from 10 g of human testis tissue. The research protocol was approved by the Research Ethics Committee on Human Experimentation of Kermanshah University, Faculty of Medicine. Quantitative RT-PCR conditions were as follows: denaturing at 94°C for 1 min, annealing at 60°C for 90 s, and extension at 72°C for 1 min. The PCR was run for 35 cycles. Final extension was performed at 72°C for 10 min. The fragment carrying both the BamHI and EcoRI site was acquired by quantitative RT-PCR. This PCR produced a product of 2115 bp. The product was subjected to a double digestion with BamHI and EcoRI enzymes, and the digested DNA product was ligated into a 5.4-kb fragment of pcDNA3.1 (Invitrogen, USA) that was digested with the same enzymes. The ligated product was transformed into DH5α cells. Restriction endonuclease analysis, quantitative RT-PCR and plasmid sequencing were performed to validate the recombinant plasmid reading frame.

**Cell culture and transfection**

In order to compare TSGA10 expression level in normal and tumor cells, human skin fibroblast cells were isolated in a procedure described previously (40) and considered as normal cells, whereas HeLa cells (purchased from the Pasteur Institute of Iran) were considered as tumor cells. The cells were cultured in DMEM/F12 medium supplemented with 10% bovine calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO2. Twelve hours before transfection, cells were seeded onto a 24-well plates to the amount of 1–2 × 105 cells per well. Cells were transfected when plate confluence was approximately 85–90%. The cells were transfected with 1.25 µg/well of pcDNA3.1-TSGA10 vector using 2 µl Lipofectamine 2000 (Invitrogen). HeLa transfectants were selected by cultivating in medium containing 1 mg/ml of G418 (Alexis Biochemicals) for 4 weeks. The TSGA10 transfected cells and empty vector-transfected cells were separately cultured. These cell lines were designated as HeLa-pcDNA3.1-TSGA10 and HeLa cells were transfected with pcDNA3.1 plasmid (empty vector), the stable cell line designated as HeLa-pcDNA3.1 as control. After selection using 1 mg/ml G418 for 4 weeks, the positive cell clones were picked out and expanded into clonal cell strain. When stably transfected cells were obtained, the cells were continuously maintained in 200 µg/ml of G418. G418-resistant cells were detected for TSGA10 mRNA, protein by real-time PCR, western blot and immunocytochemistry analysis.

**Real-time PCR**

HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 cell cultures were incubated for 18 h at 37°C, and 90% humidity in normoxia and hypoxia conditions. At the end of the incubation time, cells were harvested and used for total RNA extraction. Total RNA was extracted from the cells using Trizol reagent (Invitrogen), as recommended by the supplier. Sense and antisense primers of target genes were designed by robust primer design software real-time PCR was performed on RNA samples using Takara Kit.
by Rotor Gene 6000 system (Corbett Research, Australia). Cycling conditions were 50°C for 2 min and 95°C for 5 min. In addition, 40 cycles of 95°C for 15 s and 60°C for 1 min at the optimized conditions were also run (for PCR step). Finally, melting was carried out at 60–95°C (0.5°C increments) for 5 s for each step. Relative quantification analysis was carried out with the Relative expression software tool (REST). The analysis used the sample’s crossing point, the efficiency of the reaction, and the number of cycles completed and other values to compare the samples and generate the ratios. The results were expressed as a normalized ratio.

TSGA10 and HIF-1α detection by western blot

For western blotting protein analysis, 40 µg of total protein was separated by 8–10% gel SDS-PAGE under reducing conditions. Proteins were subsequently transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was incubated with blocking buffer (PBS containing 5% non-fat milk) for 2 h at room temperature. Primary antibodies were goat anti-TSGA10 and rabbit anti-HIF 1α (Santa Cruz, USA). Membranes were incubated overnight at 4°C with gentle shaking. The membranes were then washed twice with PBS for 5 min and incubated with secondary antibodies (Santa Cruz, USA) for 2 h at room temperature. After washing, TSGA10 and HIF-1α were detected using a horseradish peroxidase-conjugated reaction. Protein levels were normalized to β-actin protein. The results were analyzed with TotalLab2.0 software.

Immunocytochemistry for detection of TSGA10

Transfected cells were cultured on slides and fixed in methanol for 15 min. After blocking with normal goat serum for 10 min at room temperature, the slides were incubated with goat anti-TSGA10 polyclonal antibody (1:500) for 48 h at 4°C, and then with an rabbit anti-goat FITC for 30 min at 37°C. Cells were washed three times in PBS and visualized against controls (PBS was used instead of primary antibodies) using an optical immunofluorescence microscope (TS100; Nikon, Tokyo, Japan) with appropriate fluorescence filters, and images were captured using a Nikon camera (ELWD 0.3/OD75; Nikon).

Cell proliferation assay

Cells were seeded at 5 × 10³ cells per well in a 96-well plate and cultured with 10% fetal bovine serum for 48 h. The cell culture dishes were placed into a hypoxia chamber inside the cell culture incubator and exposed to hypoxia (1%). Hypoxia was achieved by flushing low oxygen gas (by injecting nitrogen to displace oxygen). Another set of cell culture dishes were cultured under normal conditions (normoxia). The cells were pulsed with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA) 20 µl/well (5 mg/ml in phosphate-buffered saline (PBS)) to measure cell growth. The purple-blue MTT formazan precipitate was dissolved in 200 µl of DMSO and swirled for 30 min. Absorbance (UA) was measured at 570 nm, with background subtraction of 630 using a spectrophotometer. This absorbance is also called optical density, which reflects the cell count and may be used to construct cell growth curves. Experiments were repeated six times.

ELISA assay for MMP-2, MMP-9 and VEGF

VEGF, MMP-2 and MMP-9 are of key mediators of angiogenesis and metastatic process. We assessed these factors using ELISA method. Supernatants were collected from HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 after 16 h of incubation under the indicated conditions. Total cell protein levels were measured by Bradford protein assay. Cell-free culture supernatants were collected and MMP2, MMP9 and VEGF concentrations were assessed using a quantitative ELISA kit (R&D Systems), according to the manufacturer’s instructions.

Substrate gel zymography

The matrix metalloproteinase-2 (MMP-2) and MMP-9 gelatinolytic activities of conditioned media from HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 were determined by electrophoresis (41). Conditioned media from cell cultures were centrifuged and their protein concentrations were determined by the method of Bradford (42) and then separated on 7.5% SDS polyacrylamide gels containing 2 mg/ml (0.2%) gelatin under non-reducing conditions at 4°C. PAGE gels were run at 120 V, renatured by exchanging SDS with 2.5% Triton X-100 for 1 h at room temperature, and then incubated for 16 h at 37°C in the activation buffer (50 mM Tris–HCl, pH 7.5, 10 mM CaCl2) for development of active bands. After staining with Coomassie brilliant blue R-250 (10% glacial acetic acid, 30% methanol, and 1.5% dye) for 2–3 h, the gel was destained with a solution of 10% glacial acetic acid, and 30% methanol for 1 h. The gelatinolytic activities were detected as transparent bands against the background of Coomassie-stained gelatin. Protein standards were run concurrently for determining the molecular weight of MMP bands. The results were analyzed with TotalLab2.0 software.

2D challenge model

Since in pathologic angiogenesis, most inducer cytokines are released from cancer cells, thus it was decided to use the rich angiogenic inducer supernatant of HeLa cells in this model. To investigate if TSGA10 have any effects on secreting tumor associated factors, we used 2D challenge model. This model was performed as previously described (15) as HeLa-pcDNA3.1, HeLa-pcDNA3.1-TSGA10 cells (2 × 10⁵ cells/cm²) and HUVECs (5 × 10⁴ cells/cm²) were plated onto 8-wells chamber slides in normoxic and hypoxic condition. When confluency of cell line reached approximately 60–70%, conditioned medium was extracted and used in HUVECs cell line at concentrations of 10, 25 and 50%. In HUVEC-HeLa-pcDNA3.1 and HUVEC-HeLa-pcDNA3.1-TSGA10 challenge model, MTT assays were performed.

Scratch motility (wound Healing) assay

The scratch assay is a useful and straightforward method for evaluation of cell migration in vitro. HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 at the same cell number were seeded into 6-well plates. The next step involved creating a cell free area of confluent monolayer cells by scraping the cell monolayer with a 1000 µl pipette tip at following day. After washing the detached cells, images were captured during a 24 h period of cell migration. For comparing the migration rate, scratches were photographed at five separate fields and then free cell area was analyzed by NIH Image J software. The percentage of wound closure was estimated as previously described (16) by the following equation: wound closure % = [1 – (wound area at t/wound area at t₀)] × 100%, where t₀ is the time after 24 h of wounding and t is the time immediately after wounding.
Transwell assay

Transwell filters (Costar, USA) were coated with matrigel (3.9 µg/µl, 60–80 µl) on the upper surface of the polycarbonate membrane (6.5 mm in diameter, 8 µm pore size) as described previously (43). The matrigel solidified after 30 min of incubation at 37°C, and served as the extracellular matrix for tumor cell invasion analysis. Hela-pcDNA3.1 and Hela-pcDNA3.1-TSGA10 groups (1 × 10^5) were harvested in 100 µl of serum free DMED/F12 medium and added to the upper portion of the chamber. The medium was removed from the upper chamber after 24 h of incubation at 37°C and 5% CO₂ in normoxia and hypoxia conditions, and then stained with Crystal violet Staining Solution. Noninvasive cells on the upper surface were wiped with a cotton swab. The number of cells invading the matrigel was counted from three randomly selected visual fields, each from the central and peripheral portion of the filter, using an inverted microscope at 200× magnification.

In vitro angiogenesis by co-culturing HUVEC with Hela-pcDNA3.1 and Hela-pcDNA3.1-TSGA10 in a three-dimensional co-culture model

Co-culture assays were performed as described previously (44,45). First, HUVECs were cultivated on Cytox3 3-microneribbon beads which previously prepared according to the manufacturer’s instruction. Mixed suspension was placed into humid 37°C incubator with 5% CO₂ and was beaded which previously prepared according to the manufacturer’s instruction. 23 mg/ml NaHCO₃ with the ratio of 8:1:1 respectively. The beads were mixed with collagen solution (collagen type I, 10X DMEM medium, 23 mg/ml NaHCO₃ with the ratio of 8:1:1 respectively) on ice and then 50 µl of the resultant mixture loaded into each 96-well plate and allowed to solidify for 45 min at 37°C. After DMEM/F12 media was added to the collagen gel containing HUVEC-coated beads, cells from HeLa-pcDNA3.1 and HeLa-pcDNA3.1-TSGA10 were harvested and counted so that approximately 20,000 tumor cells were layered on top of the collagen gels. The model was incubated at 37°C and 5% CO₂ in normoxia and hypoxia conditions. Only capillary-like structure composed of at least three tightly connected endothelial cells was included in the quanification process. Occasionally, single endothelial cells were found in an elongated shape. These spindle shaped structures were also not included in counting. Sprout formation was measured as the mean number of sprouts in ten beads for each well and presented as the percentage.

Modeling

Due to lack of PDB file of TSGA10, it was modeled by homology modeling. In homology modeling (HM) step, we would like to look for an experimentally determined structure of high sequence identity with the TSGA10.

The sequence of TSGA10 (Q9BZW7) was obtained from Uniprot database (http://www.uniprot.org/uniprot/Q9BZW7.fasta). In order to find out the suitable templates for HM, a sequence similarity search was performed with the query of Q9BZW7 against protein data bank on the NCBI online web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and 4IKM (CARD8), 1OB8 (PHAGE CP-1), 2IXU (Cpi-1 lysine), 3CRU (putative glutathione S-transferase), 3S4R (vimentin central α-helical domain), and 4E61 (EB1-like motif of Bim1p) were chosen as the templates for HM. HM using Multiple templates can calculate a good alignment between the templates and the target sequence, so this technique can increase the accuracy and quality of the models developed. This method is particularly suited for proteins with lower sequence identity (<40%) (46,47) which is the very situation.

To align the sequence of human TSGA10 with templates, the CLUSTALW program was employed directly from its website at http://www2.ebi.ac.uk/CLUSTALW.

MODELLER (48) version 9.8 was used to build homology models of TSGA10. 1000 models were generated by the MODELLER and the one corresponding to the lowest value of the probability density function (pdf) and fewest restraints violations was selected for further analysis. An ab initio method implemented in the MODELLER which has been demonstrated to predict the conformations of loop regions, was used to refine some of the loops of the selected model.

The overall stereochemical quality of the final developed 3D model for each TSGA10 was evaluated by the program PROCHECK (49). G-factor was calculated for the developed model using PROCHECK. Environment profile of final developed model was checked using Verify-3D (Structure Evaluation Server) (50).

Molecular docking

The calculations regarding the interaction between TSGA10 and HIF-1α structures were carried out using HADDOCK web server (51), which makes use of chemical shift perturbation data to derive the docking while allowing various degrees of flexibility. The structure of HIF-1α (ARNT PasB) and HIF-1α/CBP Recognition (C-Terminal) were obtained from the Protein Data Bank (PDB ID: 4H6J, 1L8C). The docking procedure was performed in three steps as follows: first, randomization and rigid body energy minimization; second, semi-flexible simulated annealing; and third, flexible explicit solvent refinement. TSGA10 and HIF-1α residues involved in the binding were set to be ‘active’ residues, whereas neighbors of active residues were defined as ‘passive’ residues according to HADDOCK definition. Among the structures built by HADDOCK, one structure with the lowest energy was selected for detailed analysis and display.

Statistical analysis

Data were presented as means ± SEM and evaluated by the t-test, one way or two way analysis of variance (ANOVA) followed by the Tukey test (Graph Pad Software, version 5.0, Inc., La Jolla, CA, USA). A P-value <0.05 was considered to be statistically significant. Each point or column represents the mean ± SEM (n = 4–5). *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Material

Supplementary Material is available at HMG online.

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