**ABSTRACT**

**Background:** Hypoxia is a common phenomenon in cancer cells. Due angiogenesis and cell proliferation the hypoxia-inducible factor family (HIFs) is the primary transcriptional factor to hypoxic stress. Cancer-testis (CT) antigens are almost expressed in male germ cells, aberrantly expressed in some malignancies as well. The CT gene, TSGA10, prevents the nuclear accumulation of HIF-α and may be involved in organ-specific regulation of hypoxic gene expression during sperm maturation. TSGA10 is supposed to regulate the HIF expression in germ cells and cancer cells. The HIF-α subunit has three isoforms, involved in oxygen transport, angiogenesis and tumor metastasis, detection of which is the subject of the current study.

**Methods:** Three cell lines, MCF7, MDA-MB-231 and HeLa were cultured, passaged and categorized into normal and synchronized groups. The cells were subjected to RNA extraction and reverse-transcribed into cDNAs. Realtime RT-PCR was performed to amplify TSGA10 and HIF-α isoforms and HPRT, as the normalizer gene, using appropriate primers. The REST and SPSS software were used for statistical analysis.

**Results:** The expression of the three isoforms of HIF-α in HeLa cell line was higher than MCF7, and MCF7 was higher than MDA-MB-231. Moreover the expression relationship between HIF-α isoforms and TSGA10 was evaluated in each three cell lines. The results were significant in all cases with \( P = 0.01 \). Before and after synchronization in each three cell lines, the isoform expressions of HIF-α and TSGA10 were evaluated, and the results were revealed their dependent expression. The relationship between HIF-α isoforms and TSGA10 expression was compared with each other. The cell lines with less TSGA10 expression had the higher expression of HIF-α isoforms and vice versa, according to the extent of TSGA10.

**Conclusion:** The significant relationship between expressions of TSGA10 and HIF-α isoforms is confirmed.

**Keywords:** TSGA10, HIF-α subunits, Cancer-Testis genes, breast cancer cell lines, MCF7, MDA-MB-231
INTRODUCTION:

Testis-specific gene A10 (TSGA10) which was identified by differential mRNA display is considered as a member of cancer-testis (CT) genes, with expression pattern in either normal testis or frequently human solid tumors such as breast cancer and acute lymphoblastic leukemia, but not in a variety of the normal tissues. Because of these features and existence of Blood-Testis barrier, TASGA10 is suggested as a candidate for immuno-therapy in such cancers. Protein-protein interaction was identified between TSGA10 and HIF-α in sperm by yeast two-hybrid screening. Immunofluorescence analysis revealed that due to the interaction between c-terminal of TASGA10 protein and HIF-α in the midpiece of spermatozoa, nuclear accumulation and transcription activity of HIF-α is extremely affected by overexpression of TASGA10 gene, which leads to reduced HIF-α transactivation activity during sperm maturation.

TASGA10 is a fibrous sheath protein of sperm and expressed in post-meiotic spermatozoa, so this mechanism may be involved in organ-specific gene regulation of HIF-α during the maturation of sperm. All cells and tissues for their biological functions require sufficient O2 concentrations, but in many different conditions, cells and tissues encounter insufficient O2 concentrations or Hypoxia conditions. In this situations the primary and major mechanism mediating adaptive response to low oxygen concentration is HIF. There are three isoforms of the HIF-α: HIF-1α, HIF-2α (also termed EPAS1, HLF, HRF, and MOP2) and HIF-3α. In human tumors, reduced O2 concentration is common and cancer cells exploit the same adaption response for their survival, but due to somatic mutation or epigenetic changes, standard feedback mechanisms are disrupted in these cells. Since increased tumor HIF-1α more likely brings about resistance to chemotherapy and radiotherapy, proliferation, metastasis and the invasive potential all of which decrease patient survival, it is vital to understand the molecular pathways which HIF-α are involved in, such as drug resistance mechanism. In addition, if TSGA10 can suppress HIF-α expression in other cells and tissues as in sperm, it is hoped that this gene can serve as a HIF-α inhibitor in cancers and with looking beyond, acts as an inhibitor of angiogenesis in cancer and its metastasis.

In this study we analyzed expression of different three isoforms of HIF-α (HIF-1α, HIF-2α, and HIF-3α) and compared it to TSGA10 expression in three cell lines HeLa, MCF7 and MDA-MB-231.

METHODS:

Cell culture and synchronization

Three cell lines were obtained from Pasteur Institute of Iran, including two breast cancers, MCF7 and MDA-MB-231 and HeLa, the cervix cell line respectively. The cells were cultured according to the manufacture’s instruction in RPMI medium, 10% FBS at 37 °c and 5% CO2. After two days, three cell lines were passaged, using 0.25% trypsin-0.02% EDTA and categorized in two groups; normal group and synchronized group. TSGA10 might play a role in cell division so to know the effect of TSGA10 on HIF-α expression in the cell cycle, the cells were synchronized. The cells in different stages of the cell cycle were brought to the same stage, so when the results are compared with normal group, the interaction between TSGA10 and HIF-α isoforms expression would be more informative. Cell synchronization was performed with chemical Blockade by serum deprivation as shown in table 1.

When the cell confluency reached approximately 2×106 cells in each flask, the cells were subjected to RNA extraction.

RNA Extraction and cDNA synthesis:

Both normal and synchronized cells were subjected to RNA extraction, using Tripure reagent (Rosch)
according to the manufacturer’s instruction and were dissolved in RNase-free water (DEPS treated water). The optical density of each RNA sample was defined by Nano Drop 1000 spectrophotometer Thermo Scientific. One microgram of total RNA was reverse-transcribed into single-strand cDNA, using TaKaRa kit (Japan), according to the manufacturer’s instructions. The cDNAs from six groups of cell lines (both normal and synchronized) were checked for their quality, using amplification of the housekeeping gene HPRT (Hypoxanthine-guanine Phosphoribosyl Transferase). The cDNAs were stored at -700c until Realtime RT-PCR.

**Realtime quantitative polymerase chain reaction:**

To determine the transcript levels of each gene, the Realtime RT-PCR was carried out by Rotor GeneTM 6000 machine (Corbett Life ScienceTM, Germany). The appropriate specific primers were amplified by a total of 200 ng/μl of each of the cDNAs and SYBR GreenI Premix (Takara, Japan) following the manufacturer’s protocol with minor modifications. To avoid of false positive results that may be caused by genomic DNA contamination, all primers were designed in different exons. Primers are listed in table 2.

The CT (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. The CT parameter and efficiency were defined by Comparative Quantitation with Rotor gene. The amount of the copy number variants of TSGA10 and HIF-α isoform transcript was quantified by measuring the CT value. The HPRT transcripts were also quantified as the endogenous RNA control. To ensure the proper interest amplification,

<table>
<thead>
<tr>
<th>Time</th>
<th>Medium</th>
<th>(%) FBS</th>
<th>* pen/ strep Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>For three days (72 hours)</td>
<td>RPMI</td>
<td>20%</td>
<td>1%</td>
</tr>
<tr>
<td>For two days (48 hours)</td>
<td>RPMI</td>
<td>10%</td>
<td>1%</td>
</tr>
<tr>
<td>For two days (48 hours)</td>
<td>RPMI</td>
<td>9%</td>
<td>1%</td>
</tr>
<tr>
<td>For two days (48 hours)</td>
<td>RPMI</td>
<td>2.5%</td>
<td>1%</td>
</tr>
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</table>

* mixed penicillin, streptomycin antibiotic.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product length</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>197</td>
<td>CCTCAGYCGMCACAGCMTGGA</td>
<td>GCATCCTGTACTGTTCCTGTGG</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>243</td>
<td>CCTCAGYCGMCACAGCMTGGA</td>
<td>ACCGTCCCTGGTCTCCAG</td>
</tr>
<tr>
<td>HIF-3α</td>
<td>146</td>
<td>CCTCAGYCGMCACAGCMTGGA</td>
<td>TGCTCTTGCTGACCGCATCGG</td>
</tr>
<tr>
<td>TSGA10</td>
<td>161</td>
<td>AAGGGAGAGGCTAAGGATTG</td>
<td>TCCACAGTGCTTATG-TTTC</td>
</tr>
<tr>
<td>HPRT</td>
<td>131</td>
<td>CCTGGCGTCGTGATTAGTGAT</td>
<td>AGACGTTCAGTCTGTCCATAA</td>
</tr>
</tbody>
</table>
the program was followed by melt curve analysis to avoid nonspecific products such as primer dimers.

**Statistical analysis:**
The REST and SPSS version 17 software were used for statistical analysis. The P-values of less than 0.05, was considered as the significant result.

**Results**

**Expression of HIF-α isoforms in HeLa, MCF7 and MDA-MB-231 cell lines:**
With comparison of HIF-1α, HIF-2α and HIF-3α isoforms, they all were up-regulated in HeLa cells than MCF7 and MDA-MB-231 cell lines which are shown in Fig 1, 2 and 3. Up-regulation was significant for HIF-2α and HIF-3α (p=0.001). Also different isoforms of HIF-α in MCF7 cell line indicated significantly up-regulated expression with p=0.001 in comparison with their expression in MDA-MB-231 cell line (although this up-regulation for HIF3-α was not significant, p= 0.171).

**Relationship between HIF-α isoforms and TSGA10 expression:**
Correlation study was conducted according to spearman method between each of the three isoforms and TSGA 10 expression. All of the three isoforms displayed significant expression correlation with TSGA10 expression in each of the three cell lines (p=0.01).

**Relationship between HIF-α isoforms and TSGA10 expression in the cell lines:**
To compare the relationship between gene expressions of HIF-α isoforms and TSGA10, first it is necessary to be sure that the three cell line HeLa, MCF7 and MDA-MB-231 are classified based on the TSGA10 level of gene expression. Assortment

![Figure 1. Expression of HIF1-α isoform in HeLa, MCF7 and MDA-MB-231 cell lines, towards each other](image-url)
Figure 2. Expression of HIF2-α isoform in HeLa, MCF7 and MDA-MB-231 cell lines, towards each other.

Figure 3. Expression of HIF3-α isoform in HeLa, MCF7 and MDA-MB-231 cell lines, towards each other.
Association of Hypoxia-inducible factor α...

of three cell lines based on TSGA10 gene expression is as follows: HeLa>MCF7>MDA-MB-231 (Fig 4). Then, by comparing expression levels between binary cell lines, the total interaction between HIF-α isoforms and TSGA10 in the different cell lines can be compared. After comparing MCF7 and MDA-MB-231 with each other, it was found that the number of HIF-α copy per each copy of TSGA10 in MCF7 cells was more than that in MDA-MB-231. In other words, MDA-MB-231 cell line that expresses TSGA10 less than HeLa and MCF7, has higher expression of HIF-α isoforms and this higher expression is significant for HIF1-α and HIF3-α isoforms (P= 0.001). In addition, when the authors assessed this expression ratio between MDA-MB-231 and HeLa cell line, this increased expression of HIF-α isoforms was also observed in the HeLa towards MDA-MB-231 cell line and the numbers of HIF-α copies, for every copy of TSGA10 in MDA-MB-231 was higher than that in HeLa cell line. But since the P-values were greater than 0.05, two types of interpretation can be done, First, it’s really not a significant increase in the expression, second, it is not significant due to small sample size and not due to lack of expression relation. Also when the MCF7 and HeLa cell lines were compared, in HeLa cell line that expressed higher levels of TSGA10 genes, all three isoforms of HIF-α showed decreased expression into the MCF7 cell line, and this reduced expression was significant in HIF1-α (P= 0.001) too.

Classification of cell lines based on TSGA10 expression after synchronization

TCell synchrony is required to study the progression of cells through the cell cycle. The Cell lines are arranged based on the expression levels of TSGA10 after synchronization as: MCF7> HeLa> MDA-MB-231 (Fig 5). The expression of TSGA10 in MDA-MB-231 cell line was less than that in two other cell lines, thus in computing HeLa and MCF7 were evaluated instead of MDA-MB-231. After

![Figure 4. Classification of three cell lines based on TSGA10 expression before synchronization](image-url)
synchronization, when the cell lines were examined again in terms of the relationship between HIF-α isoforms and TSGA10 expression almost similar results were obtained. HIF-α isoforms were overexpressed in MDA-MB-231 than MCF7 cell line, and this increased expression was significant for HIF-3α (P= 0.001). When MDA-MB-231 was compared with HeLa cell line, the number of HIF-α copies per each copy of TSGA10 in MDA-MB-231 was higher than HeLa and increased expression was significant (P= 0.001). In addition after synchronization, the expression ratio of TSGA10 had increased in MCF7. This has caused the analysis result of these two cell lines to be exactly reverse before and after the Synchronization. In other words, when MCF7 had a higher expression of TSGA10 towards HeLa cell line, expression of all three isoforms reduced and this reduced expression of HIF-2α and HIF-3α was significant (P=0.001).

**DISCUSSION:**
The authors have previously reported the overexpression of some cancer/testis genes in breast tumors and cell lines. The AURKC gene was overexpressed in breast tumors in comparison to normal breast\textsuperscript{11}. The CT gene, SYCP3, was highly overexpressed in 5 breast cancer cell lines, except for MCF7\textsuperscript{12}. RH-OXF2 and ODF4\textsuperscript{13} were upregulated in breast tumors as well as OIP5 and TAF7L\textsuperscript{14}. The CT gene, TSGA10, was overexpressed in breast tumors and breast cancer cell lines, MCF-7 and MDA-MB-23\textsuperscript{3}. Expression of TSGA10 in testis and several human primary tumors, but not in other adult tissues, makes it a valuable biomarker in cancer prognosis\textsuperscript{2,3}. During spermatogenesis process, when the cells are passing from meiotic to post-meiotic phase, Tsga10 expression showed 6.6 folds increase in mice mRNA transcripts\textsuperscript{15}. It has been shown that TSGA10 is correlated with tumor angiogenesis and metastasis, via its effects on the function of HIF-α gene\textsuperscript{8}. Human TSGA10 has four transcript variants extended in at least 22 exons. The 5'UTR region of TSGA10 has different sequences which can make
different combinations of each transcript variant in normal or unusual environmental conditions like hypoxia. The shorter 5’UTR and fewer upstream open reading frames elements are detected in breast tumors in comparison to normal testis. This finding suggests the role played by TSGA10 5’UTR in translation regulation in different conditions like hypoxia.

HIF-1α expression correlates directly with increased mortality in patients suffering from brain, cervix, breast, ovary, and non-small cell lung and head and neck tumors. Increased expression of HIF-α results in increased expression of survival factors, among which the VEGF is the most potent endothelial mitogen. VEGF is directly involved in angiogenesis, aggressive tumor growth, and ultimately metastasis. In many of the anti-cancer drugs the treatment aims are to target the HIF-1αtransactivation activity.

In the spermatozoa midpiece, TSGA10 c-terminal show a Protein-protein interaction with HIF-1α that results in reduced nuclear accumulation and HIF1-α transcription activity. TASGA10 is involved in organ-specific gene regulation of HIF-1α during the sperm maturation. HIF-1α transcription activity may be affected by TSGA10 overexpression in other types of the cells as well.

HIF-α protein belongs to the helix-loop-helix–Per-ARNT-Sim (bHLH–PAS) family and the HIF-α isoforms have high sequence similarity, therefore there could be a high possibility of similar interaction site between HIF-2α and HIF-3α isoforms with TASA10 respectively. Thus, TSGA10 overexpression in various cancers on one hand and existence of interaction site for HIF-α on the other hand, make it an optimistic candidate for HIF-α inhibitor. Previous studies have been carried out on HIF1-α and few studies on HIF-2α and HIF-3α. According to our knowledge, there is no study on the relationship between TASA10 and HIF-2α and HIF-3α.

Cancer is a multi-stage disease, and many signaling pathways are involved in its development. Thus the best treatment is the most complete treatment with the best results, in a very short time as much as possible, such as the differences in breast cancer patients’ treatment with ER/PR or HER2 positive. Up to now most of the drugs for targeting the HIF-α family have been designated to inhibit HIF-1α isoform. With further research if there is more overwhelming evidence for the effects of HIF-2α and HIF-3α expression in cancers, the anti-cancer drugs for HIF family may be evolved. According to the HIF-α isoforms or due to sequence and amino acid homology, a drug that desirably suppresses the expression of all three isoforms can be more useful. According to the results, the cancer cell lines with lower TSGA10 expression had the higher levels of HIF1-α isoforms as well as HIF-2α and HIF-3α expression. Previous results have shown that TSGA10 is overexpressed in many types of human cancers so the interaction between TSGA10 and HIF-α can make it as another candidate for HIF-α inhibitors, however, further researches are needed to show the details of the process and give more evidence.

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Conflict of Interest
The authors have no conflict of interest to declare.

References


