TSGA10 Expression Status in Breast Cancer: The Role of Microenvironmental Changes

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Abstract

Background: Testis-specific gene antigen (TSGA10) mainly involves in spermatogenesis and embryogenesis. In the new defined roles, being a tumor suppressor agent or a cancer/testis antigen (CTA) is still unclear for this protein. The current study aimed to examine exact role of TSGA10 as a tumor suppressor or CTA in breast cancer and evaluate the role of microenvironment on its expression.

Methods: This study evaluated the expression of *TSGA10* and *hypoxia-inducible factor 1α (HIF-1α)* in two different breast cancer cell lines (MCF-7 and MDA-MB23) as well as their control (MCF10A) using real-time PCR. Moreover, expression of the mentioned genes evaluated in samples obtained from tumoral tissues with two types of controls: paired (tumor-free margin) and unpaired (healthy individuals). Also, in order to asses TSGA10 levels in the tumoral tissues, western blotting was performed. Furthermore, to evaluate the role epigenetic changes on *TSGA10* expression, breast cancer cell lines were treated with a histone deacetylase inhibitor (HDACI) as well as H_2O_2 for oxidative stress induction.

Results: The current study evaluated 36 patients diagnosed with breast cancer as well as 10 healthy controls. According to the results, it was shown that 35 (97.7%) and 1 (2.8%) of patients were diagnosed with ductal and lobular carcinomas respectively. The *TSGA10* levels in the tumoral samples showed 1.38±0.014-fold decrease and 1.41±0.127-fold increase compared with their paired ($P<0.001$) and unpaired ($P<0.001$) controls respectively. Moreover, results of blotting in tumoral tissues expressed significant decrease in TSGA10 levels in comparison to the paired controls ($P<0.01$). Among the cell lines, *TSGA10* expression in MCF-7 and MDA-MB23 cells had 4.9±0.283 and 4.21±0.163 folds of decrease in normoxic and 4.7±.0.283 and 7.1±0.141 folds of expression reduction in hypoxic condition respectively (all $P<0.0001$). Furthermore, the results showed that *HIF-1α* expression was up-regulated in both normoxic ($P<0.01$) and hypoxic ($P<0.01$) conditions. Also, *TSGA10* expression increased up to 7.39±0.156 folds in MCF-7 cells after HDACI treatment (all $P<0.01$). However, MDA-MB23 cells firstly experienced a decrease and then a notable increase in *TSGA10* expression (all $P<0.01$).

Conclusion: Results of current study showed that TSGA10 seems to be tumor suppressor, however, further studies are necessary.

Background

Testis-specific gene antigen (TSGA10) is a role player factor during embryogenesis and spermatogenesis. This gene which is located on chromosome 2 (2q11.2) encodes a 3 Kb mRNA that translates to a polypeptide with a molecular weight of 82 kDa [1-3]. After post-translational modifications, the protein converts to the two segments including 27 (N-terminus fragment) and 55 kDa (C-terminus fragment) polypeptides. With an eye on the functional role, C-terminus places in the midpiece and the N-terminus fragment presents in the fibrous sheath of sperm tail [4]. Although this gene expresses in the physiological situations, it is also involved in some pathological conditions such as tumors and autoimmune diseases. As it has been reported, over-expression of *TSGA10* has been seen in some

malignancies, and thus, it has been categorized as cancer/testis antigen (CTA) [5, 6]. However, new studies in this field show different results. The lower expression levels of *TSGA10* in cancer cells [7], as well as its ability to decrease cell motility and invasion [6], is totally against its CTA role. These results provide a debatable issue on the exact role of *TSGA10* in cancer as CTA or tumor suppressor agent.

According to the data, the C-terminus fragment of TSGA10 is co-located with hypoxia-inducible factor 1α (HIF1-α) and could prevent its nuclear localization [8]. HIF1-α is rapid response cytokine to the low oxygen levels in cellular microenvironment (such as a tumoral tissue) which could be expressed from a variety of cells. This cytokine has been considered as a pro-angiogenic factor and one of the most important beginners of angiogenic cascade. The increased levels of HIF-1α following hypoxia induces over-expression of different pro-angiogenic factors such as *vascular endothelial growth factor (VEGF), epidermal growth factor (EGF),* and *erythropoietin* which lead to the angiogenesis [9-11]. Thus, expression of *HIF1-α* finally leads to activation of angiogenesis cascade and the newly formed micro-vessels provide enough oxygen and nutrient supply for cellular growth. Taken together, data prove crucial of HIF-1α in tumor growth, invasion, and metastasis [12].

In the current study, it was tried to examine the accurate function of *TSGA10* in breast cancer and its possible association with *HIF-1α* expression status in different micro-environmental conditions.

Methods

Ethical considerations

This study was approved by the Medical Ethics Committees of the affiliated Research Center and Hospital. All the patients participated in the current study, signed a consent form freely after explanation of aims and methods according to their knowledge. Also, all of the authors adhered to the 1975 Declaration of Helsinki and its next revisions.

Patients and samples

Inclusion criteria for sample selection considered as any patients previously diagnosed with breast cancer through histopathological evaluations performed on tissues obtained from core/fine needle biopsies. Also, a positive history of chemotherapy, autoimmune diseases, radiotherapy, and any chronic diseases were defined as exclusion criteria. For the included patients, all of the histopathological evaluations were performed by two well-experienced pathologists on core/fine needle biopsy samples as well as provided tissues following the surgeries. Also, the control sample for each patient obtained from the histopathological approved tumor-free margin (paired control) and samples obtained from the reduction mammoplasty (unpaired controls). All the samples stored at -80º C in liquid nitrogen for the investigations.

Cell culture

In order to compare *TSGA10* expression levels, MCF-7 [13] and MDA-MB231 [14] as breast cancer and MCF10A [15] as a normal cell line were cultured (all purchased from Pasture Institute, Tehran, Iran). The cells were cultured in DMEM/F12 medium supplemented with 10% bovine calf serum (BCS) containing 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in a humidified incubator with 5% CO2.

Induction of hypoxia

In order to induce hypoxic situation, cells were treated with cobalt (II) chloride hexahydrate (CoCl2.6(H_2O), MW=237.92 g/mol. MCF-7, MDA-MB23, and MCF10A cells were counted in equal numbers using hemocytometer and planted on a 6 well plate in two replications. They have cultured in the DMEM/F12 medium supplemented with 10% BCS containing 100 U/ml penicillin and 100 µg/ml streptomycin and synchronal treated with 100 µM cobalt (II) chloride hexahydrate for 24 hours [16]. After the mentioned time period RNA extraction was performed.

Inhibition of histone deacetylase

In this study, sodium valproate was used as a histone deacetylase inhibitor (HDACI) in order to evaluate the role of epigenetic changes in the expression of *TSGA10* [17]. The malignant cells were treated with different concentrations (0.1, 0.2 and 0.4 mM) of this HDACI in the same situation as previous sections. After 24 hours of treatment with sodium valproate, the BCS free medium was added and RNA extraction performed 18 hours later.

Induction of oxidative stress

The cells were counted in equal numbers using hemocytometer and planted on a 6 well plate in two replications in DMEM/F12 medium supplemented with 10% BCS containing 100 U/ml penicillin and 100 µg/ml streptomycin for 24 hours. Then, the same BCS-free medium containing different concentrations of H_2O_2 (10, 100, and 200 µM) was added for induction oxidative-stress. The RNA extraction was performed separately in 30 minutes and 24 hours after the treatment.

Lactate assay

In order to investigate cellular proliferation lactate levels were evaluated in each of the three cell lines (MCF-7, MDA-MB231, and MCF-10A). Cells were cultured as same as the previous section but under both hypoxic and normoxic situations. In each of the mentioned situations, all the cultured cells were treated with low and normal amount of glucose. After 24 hours the supernatant was removed and the same media but without BCS was added. Following another 24-hour interval, lactate levels were evaluated according to the manufacture's instruction (Greiner lactate colorimetric LOD-PAP-Test, Monoreagent, Germany).

Real-time PCR

All of the obtained tissues from surgeries (tumor tissue and controls), as well as cultured cells (with and without oxidative-stress, hypoxic induction, and low-glucose condition), were washed with sterile phosphate-buffered saline (PBS) and homogenized using liquid nitrogen. Total RNA extraction was performed using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction.

Sense and antisense primers for *TSGA10* and *HIF-1α* were designed by robust primer design software as shown in ***Table 1***. The real-time polymerase chain reaction (PCR) was performed using Takara Kit by Rotor Gene 6000 system (Corbett Research, Australia) on the collected samples and the mentioned 3 cell lines. This method was performed as we have previously discussed in a same research [6]. After data gathering, the collected real-time PCR results from both normal and cancerous cells were compared and evaluated as normalized ratio.

Immunoblotting

For the evaluation of protein levels, western blotting protein analysis was used. For this aim, 40 µg of whole-cell total protein of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition in 8–10% gradient gel. Then, the proteins were transferred to a polyvinylidene fluoride membrane and incubated for 2 hours at 24ºC with blocking buffer (5% non-fat milk dissolved in PBS). For the detection of TSGA10, goat anti-TSGA10 antibody (Santa Cruz, USA) was used and the membranes were incubated for 24 hours at 4°C. After two times of membranes washing with PBS (5 minutes), membranes have been incubated with the secondary antibody (Santa Cruz, USA) for 2 hours at room temperature. Then after, they were re-washed and using horseradish peroxidase-conjugated reaction the TSGA10 bond visualized. The quantitation of TSGA10 band intensity was performed by TotalLab Life Science Image Analysis Software, Version 2.0 (TotalLab Ltd, Newcastle upon Tyne, UK) through normalization with β-actin protein.

Statistical analysis

Data analyses were performed by GraphPad Prism software 5.0 (GraphPad Software, San Diego, CA). Analyzed data reported as mean±standard deviation (SD) and evaluated by the t-test, one way or two-way analysis of variance (ANOVA) followed by the Tukey test. Any P-value less than 0.05 was considered to be statistically significant. In the figures showing the significance levels were followed by *P < 0.05, ** P < 0.01, ***P < 0.001.

The specificity and sensitivity index was obtained using the Receiver operating characteristic (ROC) curve by X software version Y (Company, Country). ROC curve data were interpreted by calculation of area under the curve (AUC) through the Youden index with following criteria: [1-0.9): excellent, (0.9-0.8]: good, (0.8-0.7]: fair, (0.7-0.6]: poor and 6< were considered as fail.

Result

Among the 85 eligible cases according to the inclusion criteria, 49 of them were excluded after meeting the exclusion criteria and a final number of 36 patients were enrolled in the study. The mean age of the patients (also as the paired control group) was 49.89±9.94 (ranged between 40-76) years which that 35 (97.2%) and 1 (2.8%) of them were diagnosed with ductal and lobular carcinomas respectively. For the unpaired controls (N=10), the mean age was calculated as 44.34±6.34 (ranged between 31 and 57) which had no statistically significant difference with breast cancer group (P>0.05).

As the results showed, *TSGA10* expression in the surgically obtained tumoral tissue samples had 1.38± 0.014 folds of decrease compared with their paired controls (P<0.001). However, *TSGA10* expression in the tumoral tissues compared with unpaired controls showed a 1.41±0.127 fold of increase (P<0.001).

In the *in-vitro* evaluations, cell lines were challenged with both normoxic and hypoxic situations. It was shown that in the normoxic situation the expression of *TSGA10* in MCF-7 and MDA-MB23 cell lines were 4.9±0.283 (P<0.0001) and 4.21±0.163 (P<0.0001) folds lower (respectively) than the control (MCF-10A). Also, it was revealed that in the hypoxic situation the expression of *TSGA10* had 4.7±.0.283 (P<0.0001) and 7.1±0.141 (P<0.0001) folds of decrease in MCF-7 and MDA-MB23 cells (respectively) in comparison to the control (***Figure 1***).

Moreover, according to the results of western blotting (***Figure 2***), surgically obtained breast cancer tissue had approximately 1.1±0.177 (P<0.01) folds of decrease in the TSGA10 protein level relative to the paired control.

As well as *TSGA10*, the expression of *HIF-1α* was evaluated in the *in-vitro* situation. MCF-7 and MDA-MB231 cell lines showed approximately 1.51±0.049 (P<0.0001) and 1.7±0.085 (P<0.0001) folds of increase in *HIF-1α* expression in the hypoxic situation compared to the control cell line (MCF-10A). As well, in the normoxic situation MCF-7 and MDA-MB231 cell lines experienced 1.32± 0.078 (P<0.0001) and 1.51± 0.061 (P<0.0001) folds of increase in *HIF-1α* expression compared to the control.

According to the ***Figure 3A***, it was shown that lactate concentrations (as an indicator of cellular proliferation) was statistically higher in MCF-7 and MDA-MB231 cells in comparison to the normal control (MCF-10A) in the all of the already mentioned situations including high and low glucose concentration in both hypoxic and normoxic situations (details have been shown in ***Figure 3A***).

Furthermore, the treatment of breast cancer cell lines with different concentrations of sodium valproate as a HDACI, expressed the effect of epigenetic changes on *TSGA10* expression. It was shown that in MCF-7 cell line of *TSGA10* expression levels increased up to 7.39±0.156 folds (P<0.0001) after the treatment. However, in the MDA-MB231 cells, the increased levels of *TSGA10* were only observed in 0.4mM while the other concentrations led to decrease in expression. All the changes were statistically significant in comparison to non-treated situations (details have been shown in ***Figure 3B***).

According to the results, treating cancer cell line (MCF-7 which according to the ***Figure 3B*** showed more sensitivity to the environmental changes) was the next aim of this study. It was shown that induction of

oxidative stress significantly increased *TSGA10* expression (details in ***Figure 3C***).

As mentioned, the tumoral tissues showed a significant decrease in *TSGA10* expression in comparison to the paired controls. Furthermore, the ROC curve analysis of *TSGA10* expression in tumoral and their paired controls reached an AUC of 0.889 (95% CI, 0.780-0.998; P<0.001). Also, the optimal cut off value for expression *TSGA10* was defined as 1.604 with the sensitivity and specificity of 86.7 and 100% respectively (***Figure 4***).

Discussion

Breast cancer is known as the most prevalent malignancy among women. According to the data, a higher rate of incidence of this cancer has been expected in the next years [18]. As well as other malignancies, better understanding of any factor affecting the disease could help to improve diagnostic tools, treatments, and survival predictors. The results of current study showed a significant decrease in *TSGA10* expression in the cell lines as well as obtained tumoral tissues in comparison to their already defined controls. However, for tumoral tissues, an increase of expression in *TSGA10* was observed when compared to the unpaired controls (normal healthy individuals). The most possible hypothetical cause of this paradoxical result seems to the genetic differences between the controls. Considering this hypothesis comparing the results only with the paired controls seems to be more trustable than the unpaired ones. Moreover, there is another hypothesis which considers *TSGA10* as a tumor resistance factor. During the tumoral situation, the expression of *TSGA10* as a tumor suppressive agent would be increased through the changes in the microenvironment, however, this resistance may be less in the tumoral tissue than their neighbors but higher in comparison to the healthy individuals. This theory is congruent with the previously reported anti-tumoral and anti-angiogenic activity of *TSGA10* [6, 7] and the current obtained results. Furthermore, results of *in-vitro* evaluation on two breast cancer cell lines compared to normal healthy control showed a statistically significant decrease in *TSGA10* expression indicates the more probability of the second hypothesis. Taken together, the paired samples were chosen as the tumoral tissue controls for further evaluations. In the next step, the protein levels of TSGA10 were assessed. The immunoblotting results showed a significant decrease in TSGA10 protein level in tumoral tissue compared with paired control which was along with the gene expression status.

In a similar study by Dianatpour et al. [19], it was shown that *TSGA10* is up-regulated in patients with breast cancer in comparison to the adjacent noncancerous tissue. Their results showed expression of *TSGA10* in 70% (N=35/50) using semi-nested PCR (only 10% positive expression in the first RT-PCR) but no expression (0/50) in the adjacent noncancerous tissue. Moreover, they mentioned the positive expression of *TSGA10* in MCF-7 and MDA-MB231 cells which no normal control was used for comparison. It seems that the cause of different results of current study with Dianatpour et al.'s results is their PCR method which was semi-nested PCR [19] while we used real-time PCR. However, lack of normal breast cell line was notable in their *in-vitro* investigations. Recent studies on *TSGA10* revealed evidence regarding tumor suppression function of this gene. These results have questioned some other reports about the CTA properties of TSGA10. As Yuan et al. showed [20], *TSGA10* down-regulation is related to

increased malignancy of esophagus squamous cell carcinoma. Also, as Mansouri et al. demonstrated, overexpression of *TSGA10* reduces cell growth and secretion of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinase 2 (MPP-2), MMP-9, and Chemokine (C-X-C motif) Receptor 4 (CXCR4). Furthermore, they have stated that TSGA10 could be involved in tumor angiogenesis and metastasis but not as an inducer and promoter [6]. In another study, Hagel et al. investigated a direct protein-protein interaction between TSGA10 and HIF-1α. According to their results, the C-terminus segment of TSGA10 prevents HIF-1α nuclear localization as well as transcriptional activity through an interaction with the N-terminal domain of HIF-1α [8].

During tumor growth, the micro-environment is not able to provide enough nutrition and oxygen for cellular survival and proliferation. As a response, HIF-1α starts the angiogenic cascade in order to overcome this situation [21]. According to the different studies, hypoxia could lead to the production of the mitochondrial ROS [22, 23] which these products could stabilize HIF-1α through inactivation of prolyl hydroxylases [22, 24]. Interestingly, ROS have been described not only required but also sufficient for stabilization of HIF-1α [25]. Thus, ROS is a key element in tumor angiogenesis and therefore growth and metastasis. In this regard, the effect of oxidative stress and hypoxia were evaluated on expression of *TSGA10* and *HIF-1α*. As results showed, both hypoxia and oxidative-stress induced *HIF-1α* and reduced *TSGA10* expression.

As it has been recently bolded, epigenetic is an important role player in the pathogenesis of breast cancer. Data have shown that acetylation and deacetylation of histones can modify the expression of tumor suppressor genes. Thus, histone deacetylase inhibitors are one of the new targets in breast cancer investigations [26, 27]. The induction of centromere hyper-acetylation by these inhibitors causes the release of heterochromatin proteins and consequently is associated with abnormal chromosome separation and the reduction of abnormal mitosis [28-30]. Herein, it was shown that treatment of malignant cells with HDACI led to increase in expression of *TSGA10* in MCF-7 in all doses. In this cell line, the *TSGA10* expression was decreased in low concentration of sodium valproate but increased in high concentration. Although a certain pattern was not found, considering the tumor suppressor function of TSGA10 the hypothesis of its function in histones acetylation/deacetylation is not fully accepted nor rejected.

Conclusion

In this study, it was showed that *TSGA10* is down-regulated in tumoral tissues obtained from patients diagnosed with breast cancer when compared to the tumor-free margins (paired controls). Also, this result was confirmed by *in-vitro* evaluation of two breast cancer cell lines which compared with normal control. Taken together, this study showed that *TSGA10* seems to be a tumor suppressor gene, not a CTA.

Abbreviations

TSGA10: Testis-specific gene antigen 10; CTA: Cancer/Testis antigen; HIF1-α: hypoxia-inducible factor 1α; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; BCS: Bovine calf serum; HDACI: Histone deacetylase inhibitor; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SD: Standard deviation; ROC: Receiver operating characteristic.

Declarations

Ethics approval and consent to participate

The study was approved by the Medical Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran.

Consent for publication

All the patients have signed a consent form freely for publication of their data.

Availability of data and material

The datasets used and/or analyzed during the current study could become available through the corresponding author on reasonable request.

Funding

The authors declare that the current study was conducted in the absence of any commercial or financial relationships.

Authors' contributions

K.M and M.M contributed to the conceptual design of the study. K.M, M.M and A.H.N designed the project. Samples were collected by M.M. Cell culture was performed by A.M, Z.H, and M.J. M.M and Z.H contributed to perform RNA extraction and real-time PCR. M.M and A.M, and A.H.N contributed to perform ELISA. M.J and A.H.N contributed to data analysis. All authors have read and approved the final manuscript.

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Competing interests

Authors declare no actual or potential competing interests related to this study.

References

1. Modarressi MH, Cameron J, Taylor KE, Wolfe J. Identification and characterisation of a novel gene, TSGA10, expressed in testis. Gene. 2001;262:249-55.
2. Depping R, Hägele S, Wagner KF, Wiesner RJ, Camenisch G, Wenger RH et al. A dominant-negative isoform of hypoxia-inducible factor-1α specifically expressed in human testis. Biol Reprod. 2004;71:331-9.
3. Theinert S, Pronest M, Peris K, Sterry W, Walden P. Identification of the testis-specific protein 10 (TSGA10) as serologically defined tumour-associated antigen in primary cutaneous T-cell lymphoma. Br J Dermatol. 2005;153:639-41.
4. Behnam B, Modarressi MH, Conti V, Taylor KE, Puliti A, Wolfe J. Expression of Tsga10 sperm tail protein in embryogenesis and neural development: from cilium to cell division. Biochem Biophys Res Commun. 2006;344:1102-10.
5. Salehipour P, Nematzadeh M, Mobasheri MB, Afsharpad M, Mansouri K, Modarressi MH. Identification of new TSGA10 transcript variants in human testis with conserved regulatory RNA elements in 5'untranslated region and distinct expression in breast cancer. Biochim Biophys Acta Gene Regul Mech. 2017;1860:973-82.
6. Mansouri K, Mostafie A, Rezazadeh D, Shahlaei M, Modarressi MH. New function of TSGA10 gene in angiogenesis and tumor metastasis: a response to a challengeable paradox. Hum Mol Genet. 2015;25:233-44.
7. Hoseinkhani Z, Rastegari-Pouyani M, Oubari F, Mozafari H, Rahimzadeh AB, Maleki A et al. Contribution and prognostic value of TSGA10 gene expression in patients with acute myeloid leukemia (AML). Pathol Res Pract. 2019;215:506-11.
8. Hägele S, Behnam B, Borter E, Wolfe J, Paasch U, Lukashev D et al. TSGA10 prevents nuclear localization of the hypoxia-inducible factor (HIF)-1α. FEBS Lett. 2006;580:3731-8.
9. Huang X, Trinh T, Aljoufi A, Broxmeyer HE. Hypoxia signaling pathway in stem cell regulation: good and evil. Curr Stem Cell Rep. 2018;4:149-57.
10. Morales-Garza LA, Puche JE, Aguirre GA, Muñoz Ú, García-Magariño M, Rocío G et al. Experimental approach to IGF-1 therapy in CCl 4-induced acute liver damage in healthy controls and mice with partial IGF-1 deficiency. J Transl Med. 2017;15:96.
11. De la Garza MM, Cumpian AM, Daliri S, Castro-Pando S, Umer M, Gong L et al. COPD-Type lung inflammation promotes K-ras mutant lung cancer through epithelial HIF-1α mediated tumor angiogenesis and proliferation. Oncotarget. 2018;9:32972.
12. Paduch R. The role of lymphangiogenesis and angiogenesis in tumor metastasis. Cell Oncol. 2016;39:397-410.
13. Levenson AS, Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res. 1997;57:3071-8.
14. Welsh J. Animal models for studying prevention and treatment of breast cancer. In: P. Michael Conn, editor. Animal models for the study of human disease. Amesterdam: Elsevier; 2013. p. 997-1018.

15. Qu Y, Han B, Yu Y, Yao W, Bose S, Karlan BY et al. Evaluation of MCF10A as a reliable model for normal human mammary epithelial cells. PLoS One. 2015;10:e0131285.
16. Wu D, Yotnda P. Induction and testing of hypoxia in cell culture. J Vis Exp. 2011:e2899.
17. Göttlicher M, Minucci S, Zhu P, Krämer OH, Schimpf A, Giavara S et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J. 2001;20:6969-78.
18. Anastasiadi Z, Lianos GD, Ignatiadou E, Harissis HV, Mitsis M. Breast cancer in young women: an overview. Updates Surg. 2017;69:313-7.
19. Dianatpour M, Mehdipour P, Nayernia K, Mobasheri M-B, Ghafouri-Fard S, Savad S et al. Expression of testis specific genes TSGA10, TEX101 and ODF3 in breast cancer. Iran Red Crescent Med J. 2012;14:722.
20. Yuan X, He J, Sun F, Gu J. Effects and interactions of MiR-577 and TSGA10 in regulating esophageal squamous cell carcinoma. Int J Clin Exp Pathol. 2013;6:2651.
21. Norooznezhad AH, Norooznezhad F, Ahmadi K. Next target of tranilast: inhibition of corneal neovascularization. Med Hypotheses. 2014;82:700-2.
22. Klimova T, Chandel N. Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ. 2008;15:660.
23. Kaelin Jr WG. ROS: really involved in oxygen sensing. Cell Metab. 2005;1:357-8.
24. Lu H, Dalgard CL, Mohyeldin A, McFate T, Tait AS, Verma A. Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem. 2005;280:41928-39.
25. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM et al. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1α during hypoxia a mechanism of O2 sensing. J Biol Chem. 2000;275:25130-8.
26. Lo P-K, Sukumar S. Epigenomics and breast cancer. Pharmacogenomics. 2008;9:1879-902.
27. Jovanovic J, Rønneberg JA, Tost J, Kristensen V. The epigenetics of breast cancer. Mol Oncol. 2010;4:242-54.
28. Illner D, Zinner R, Handtke V, Rouquette J, Strickfaden H, Lanctôt C et al. Remodeling of nuclear architecture by the thiodioxoxpiperazine metabolite chaetocin. Exp Cell Res. 2010;316:1662-80.
29. Marchion DC, Bicaku E, Daud AI, Sullivan DM, Munster PN. Valproic acid alters chromatin structure by regulation of chromatin modulation proteins. Cancer Res. 2005;65:3815-22.
30. Cowell IG, Papageorgiou N, Padget K, Watters GP, Austin CA. Histone deacetylase inhibition redistributes topoisomerase IIb from heterochromatin to euchromatin. Nucleus. 2011;2:61-71.

Table

Table 1. Used primers in the study.

Primer		Sequence (5’ to 3’)	Product size
TSGA10	Forward	TACTCAGCGACACCTTGCTAA	152 base pair
	Reverse	CCAGATCATTGAGGGTTCCAC	
HIF-1α	Forward	GAACGTCGAAAAGAAAAGTCTCG	124 base pair
	Reverse	CCTTATCAAGATGCGAACTCACA	
Beta actin	Forward	ACCTTCTACAATGAGCTGCG	148 base pair
	Reverse	CCTGGATAGCAACGTACATGG	
18S rRNA	Forward	GCTTAATTTGACTCAACACGGGA	69 base pair
	Reverse	AGCTATCAATCTGTCAATCCTGTC	

TSGA10: Testis-specific antigen 10; HIF-1α: Hypoxia inducible factor 1 α

Figures

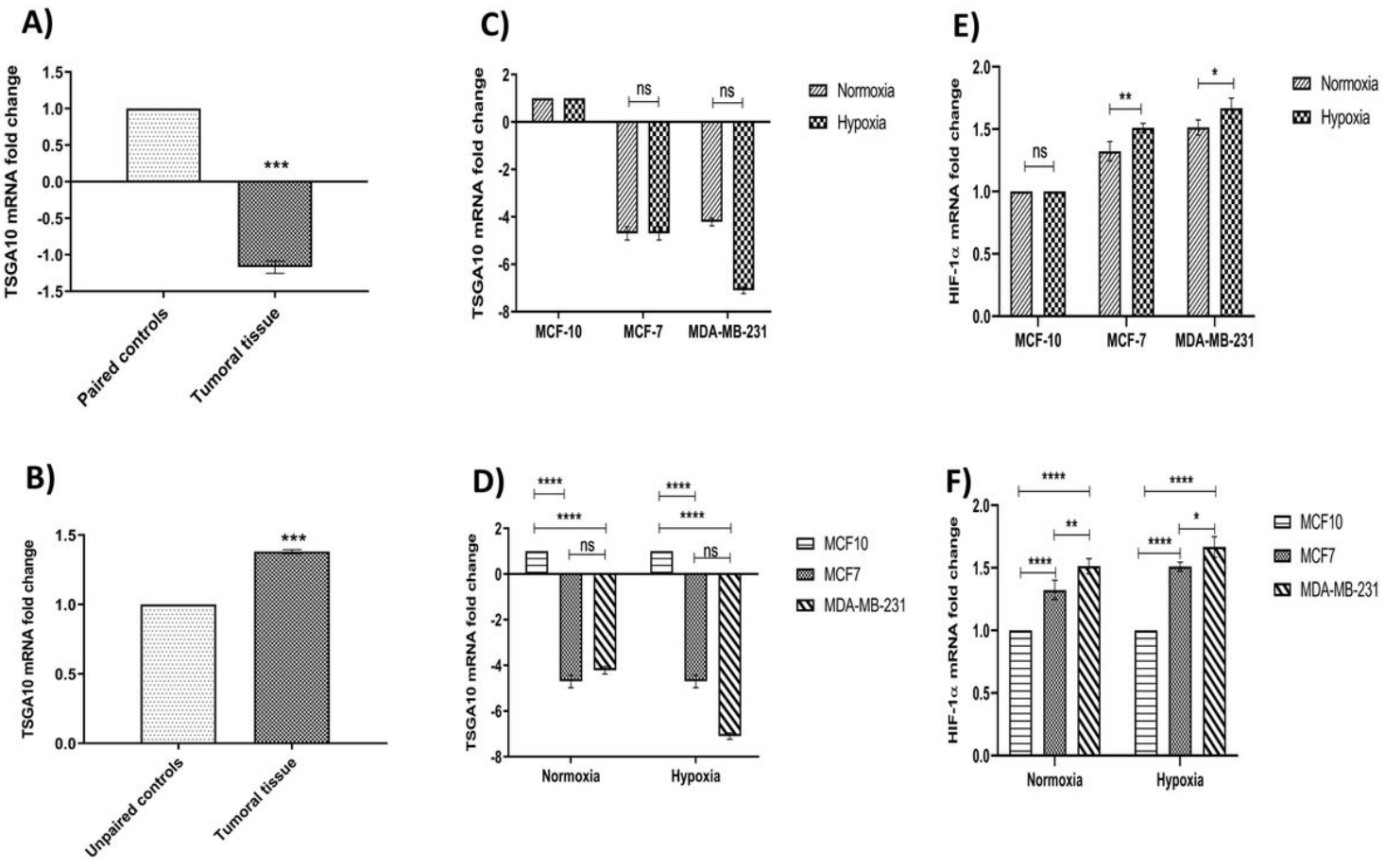

Figure 1

Evaluation of TSGA10 and HIF-1α expression. A) The cancerous tissue in paired sample shows a decrease in the expression of the TSGA10 gene. B) The cancerous tissue shows an increase in the expression of the TSGA10 gene compared with unpaired control tissues. C and D) TSGA10 gene expression in MCF-10, MCF-7, and MDA-MB231 cell line in both normoxic and hypoxic conditions (compared with each other and themselves). E and F) HIF-1α gene expression in MCF-10, MCF-7, and MDA-MB231 cell line in both normoxic and hypoxic conditions (compared with each other and themselves). ** P < 0.01, ***P < 0.001, ****P < 0.0001.

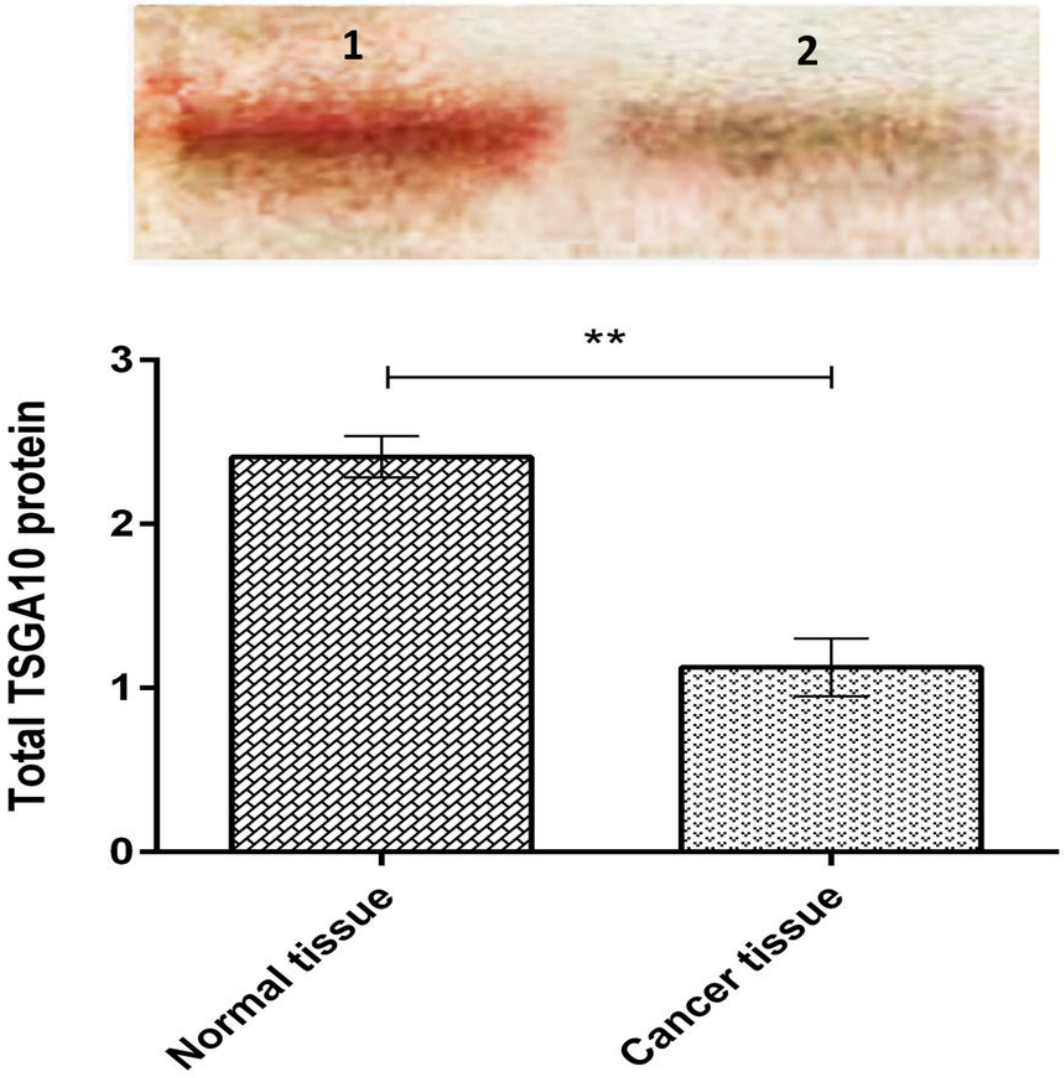

Figure 2

TSGA10 protein expression in tumoral tissue decreases -2.5 folds relative to its margin normal tissue (paired control). ** P < 0.01.

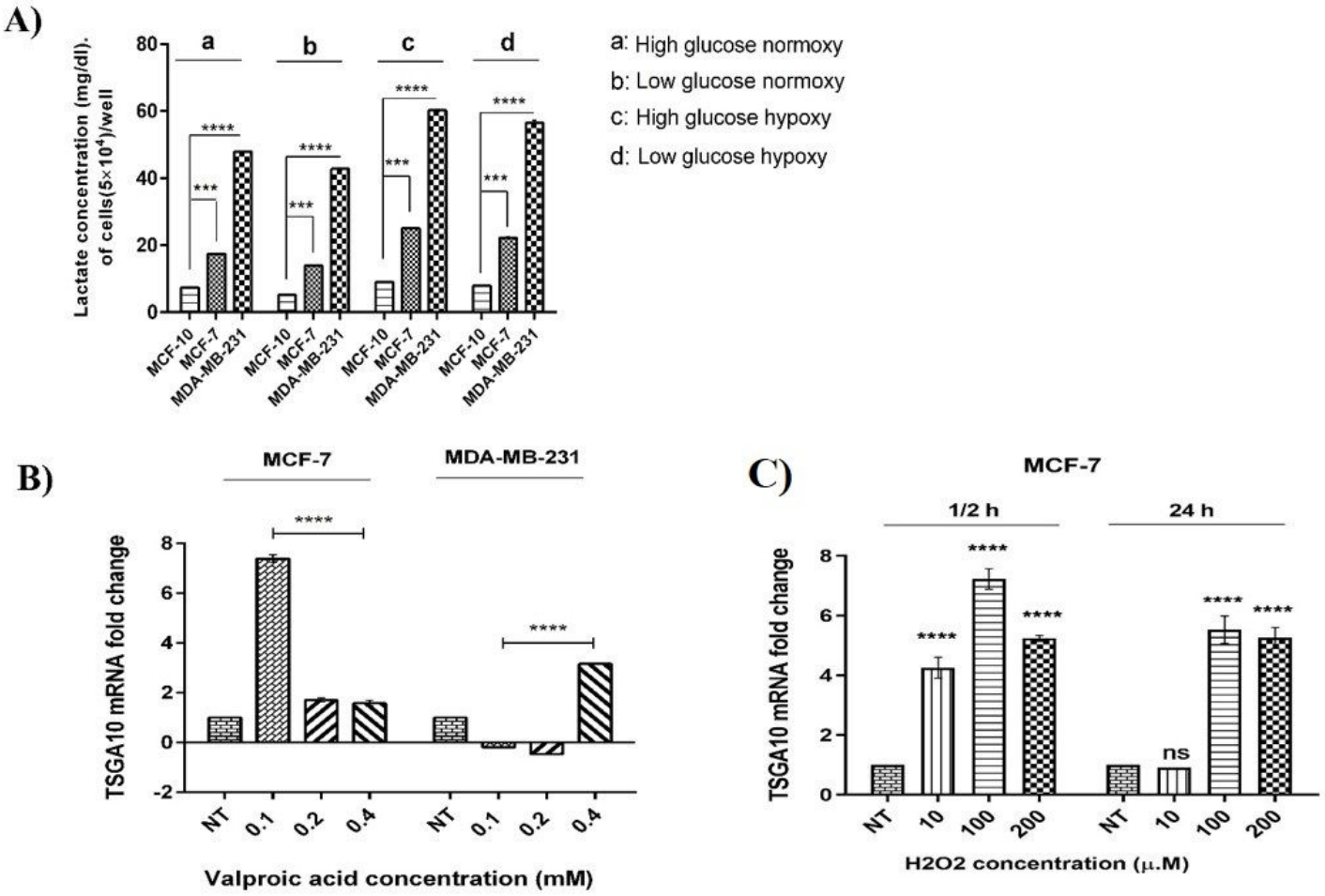

Figure 3

Role of micro environmental changes on breast cancer cell lines. A) Lactate assay in breast cancer cell lines (MCF-7, MDA-MB231) compare to normal cell line (MCF-10A) under hypoxic and normoxic conditions with high and low concentration of glaucous. B) Treatment of MCF-7, MDA-MB468 cell line with 0.1, 0.2, 0.4 mM sodium valproate as a histone deacetylase inhibitor (HDACI). C) Treatment of MCF-7 cell line with 10, 100, 200 micro liter of H2O2 at 1/2hour, 24hours. ** P < 0.01, ****P < 0.0001.

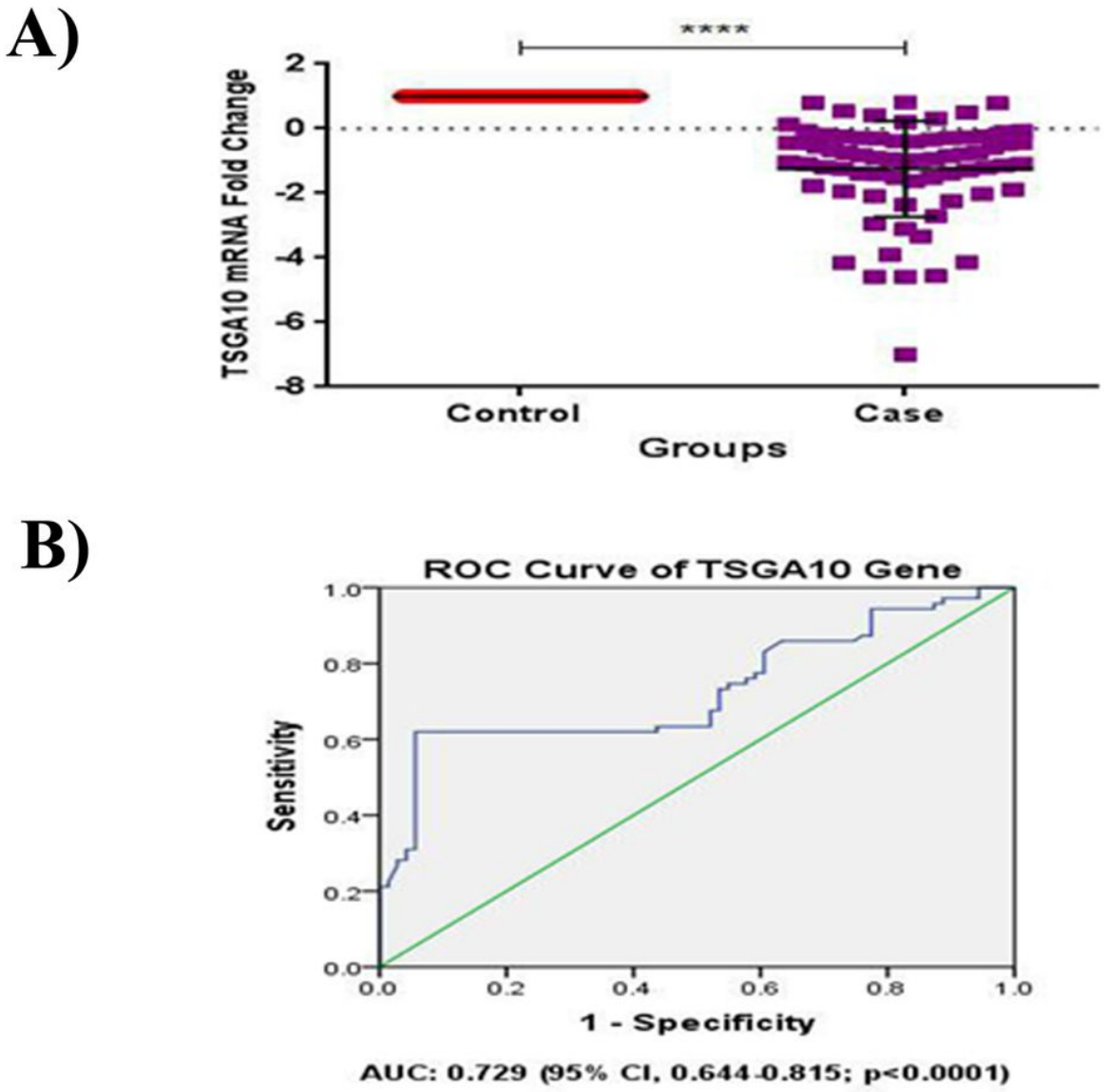

Figure 4

A) TSGA10 gene expression decrease in 36 cancer tissue relative to its marginal normal tissue in paired samples. Each data point was calculated as mean±SD independent experiments. ****P < 0.0001. B) ROC curve of TSGA10 in 36 mastectomies samples (The optimal cutoff for relative quantification that separates breast cancer patients from control was also determined. According to expression level of TSGA10 in patients and controls, the AUC was 0.889 (95% CI, 0.780-0.998; p<0.001).