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A Mimic of the Tumor Microenvironment on GPR30 Gene Expression in Breast Cancer

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Introduction: The G-protein coupled receptor 30 (GPR30) gene is a member of the G-protein coupled receptor (GPCR) family; involved in breast, endometrial, and ovarian cancers. Many GPCR receptors that are implicated in several types of human cancers are correlated with increased cell proliferation and tumor progression; especially GPR30 gene.

Methods: The breast cancer MCF-7 and MDA-MB-231 cells were cultured with different concentrations of glucose (5.5, 11, and 25 mM) under normoxia/hypoxia for 24, 48, and 72 hours. Hypoxia conditions were created with Cobalt (II) chloride at a concentration of 100 μM in culture media. The scratch assay techniques were carried out to investigate the migration and finally, gene expression levels of GPR30 mRNA were investigated by quantitative Real-Time polymerase chain reaction.

Results: The MDA-MB-231 cells adaptation in hypoxic conditions is evident which enables cell survival, whereas it results in cell proliferation in the MCF-7 cells. The increased expression of GPR30 ($P \leq 0.0001$) was found to be associated with the promoted metastasis in the MDA-MB-231 cells, while an inverse relationship was seen between the GPR30 mRNA level and cellular migration in the MCF-7 cells. We found that hypoxia induces the expression of GPR30 in MDA-MB-231 cells, and MCF-7 cells; exposed to hypoxia, had a heterogeneous expression.

Conclusions: Increases /decreases in glucose concentration and hypoxia lead to changes in the expression profiles of cancer cells. The upregulation of GPR30 expression was associated with a higher risk of breast cancer metastasis; demonstrating its importance as an applicant bio-target for cancer therapy.

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INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the main cause of cancer deaths among women worldwide [1]. Breast cancer is a very heterogeneous group of diseases that differ in molecular and genetic traits, pathogenesis characteristics, clinical period, and response to treatment [2]. The disruption of pH in the region of cancer cells is an important step in the progression of the tumor to the metastasis stage. Increased abnormalities in cell proliferation, loss of cell-cell attachment, and disruption in the extracellular matrix led to a hypoxic and acidic metabolic environment as well as deprived serum, all of which are signs of pH dysregulation. Based on the Warburg effect, anaerobic glycolysis is the main energy resource even in the presence of oxygen in cancer cells [3]. Normal cells have an intracellular pH (pHi) of ~7.2 which is about 7.4 in

cancer cells. Furthermore, extracellular pH (pHe) is approximately 7.4 in normal cells, whereas it is reduced to 6.7-7.1 in cancer cells [4]. Therefore, any changes in the activity and/or expression of membrane ion pumps and proton transfers may lead to a decrease in pHe and an increase in pHi. The low pH of the external microenvironment of the tumor may increase the activity of degrading enzymes such as matrix metalloproteinases; resulting in cancer invasion and metastasis [5]. Over the years, many attempts have been made to identify the acid sensor and acid-induced factors in different cell types; however, the molecular identity of the putative acid-sensing receptor was not known [6]. Recently, it was discovered that a family of G-protein coupled receptors (GPCRs) could be activated by acidic extracellular pH [7]. Based on previous studies, ovarian cancer G-protein coupled receptor 1 (OGR1), G protein-coupled receptor 4 (GPR4), T-cell death-associated gene 8 protein (TDAG8), and G2 accumulation protein (G2A) have a role in the acid sensors [8]. Estrogen receptors are a group of proteins found in cells membrane and are activated by the estrogen hormone. There are two groups of estrogen receptors: nuclear estrogen receptors which are part of the intracellular receptor family and membrane estrogen receptors which are often coupled with G proteins. The GPCRs represent the largest and most diverse family of membrane proteins in charge of passing cellular signaling after binding with their cognate ligands to the cell interior, and they play a key role in multiple physiological activities in different tissues [9]. Several studies have shown the important role of GPCRs in tumor proliferation, invasiveness, angiogenesis, metastasis, and drug resistance. Notably, GPCRs are very attractive targets in drug design; accounting for the targets of more than 30% of all commercially available pharmaceutical drugs [10, 11]. G-Protein Coupled Receptor 30 (GPR30) is located on chromosome 7p22.3 and encodes a protein containing 375 amino acids with a molecular weight of 41 kDa. Estrogen exerts its biological effects through two different pathways. Estrogen can bind to classical estrogen receptors α (ERα) and estrogen receptors β (ERβ) to regulate target gene expression for downstream cascades [12]. Additionally, it can mediate non-genomic responses by binding to GPR30 with a high affinity. An estrogen imbalance can cause breast cancer. GPR30 is expressed in about 50% of all breast cancers. High levels of GPR30 expression in human breast cancers are often correlated with increased tumor size and metastasis [13]. GPR30 has been reported to be overexpressed in various cancers including lung, prostate, endometrial, ovarian, thyroid, and breast cancers worldwide which could be activated by diverse ligands [14, 15]. GPR30 is an important drug target that could have been a potential cancer treatment for patients diagnosed with triple-negative ERα, progesterone receptor, and epidermal growth factor receptor (EGFR). To date, several compounds are known as anti-cancer drugs; targeting GPR30 [16]. Therefore, given the importance of the estrogen receptor GPR30 in cancer and a little number of studies about it in the tumor microenvironment, the current study aimed to investigate the effects of different concentrations of glucose and hypoxia on the expression of this estrogen receptor in MCF-7 and MDA-MB-231 cell lines.

METHODS

Cell Culture

MCF-7 and MDA-MB-231 cells were acquired from the Pasteur Institute of Iran and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, England); including 10% fetal bovine serum (Gibco, South America), 1% penicillin, and streptomycin (Bioidea, Iran), in a wet cell culture incubator with 5% CO2 and at 370 C. To simulate the tumor microenvironment, different concentrations of glucose (5.5, 11, and 25 mM) and oxygen (hypoxia and normoxia) were applied in the cell culture. Hypoxia conditions were created; using Cobalt (II) Chloride (CoCl2) (Sigma Aldrich, Germany) at a concentration of 150 µM in the cell culture environment.

Wound-Healing Assay

To investigate the effect of the glucose different concentrations (5.5, 11, and 25 mM), normoxia (20% O2), hypoxia (1% O2), and tumor cell motility, a wound-healing assay was performed on MCF-7 and MDA-MB-231 cells [17]. Cells were seeded in six-well plates and grown to subconfluence. A scratch was then made in each treatment well; using a 100 uL pipette tip and the wounded monolayers were washed twice with phosphate-buffered saline (PBS) to remove cell debris and floating cells [18].

The wounds were captured at 0 to 24 h (MCF-7) and 8 h (MDA MB-231) after the scratch under an inverted microscope with a digital camera (Optika, Italy), and the distance migrated by the cells was measured at the reference points by an image-processing software (ImageJ, open-source from National Institutes of Health, Bethesda, MD, USA).

Extraction of Total RNA and Complementary DNA (cDNA) Synthesis

To extract RNA from the treated cells, the BioFACTTM Total RNA Prep Kit (Biofact Yuseong-Gu, Daejeon, K. Korea) was used. First, MCF-7 and MDA-MB-231 cells were treated with different concentrations of glucose and oxygen. Then, through the steps mentioned in the Kit protocol, RNA extractions were done (Biofact Yuseong-Gu, Daejeon, K. Korea). The integrity of the extracted RNA and the quality of 23S rRNA and 18S rRNA bands were evaluated; utilizing agarose gel electrophoresis and the purity of the RNA was measured through optical density measurement with a NonoDrop (Nano Mabna Iranian, Iran) device. The cDNA was synthesized from the total amount of extracted RNA from MCF-7 and MDA-MB-231 cells after the unification of different extracted concentrations; following a Biofactsynthesis of cDNA Kit protocol (total RNA 10 ng, 10 µL 2X RT pre-mix, 1 µL Random Hexamer Primers, and ~8 µL RNase free water) (Biofact Yuseong-Gu, Daejeon, K. Korea) with a thermal program of room temperature for 5 minutes, 50o°C for 30 minutes, and 95°C for 5 minutes.

Primer Design and Real-Time Polymerase Chain Reaction (PCR)

The primer for the GPR30 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were designed; using National Center for Biotechnology Information (NCBI) Bioinformatics and Primer 3, Oligo analyzer, and Oligo analysis v. 7 software. After selecting the sequence related to each gene on the NCBI site, the sequences were selected as exon-exon for each primer and entered into the Primer 3 software for the primer design. The designed primers were then analyzed and optimized; using Oligo analyzer, and Oligo analysis v. 7 software as well as checking in BLAST by NCBI database. The real-time PCR was performed in a total volume of 20 µL. Each well contained: 1 µL cDNA, 10 pmol/µL primer forward, 10 pmol/µL primer revers, 10 µL Master Mix SYBR Green (Biofact Yuseong-Gu, Daejeon, K. Korea), and 3 µL RNAse/DNAse free water. The thermal conditions for the thermocycler (Roche, Germany) were as follows: one cycle at 94°C for 600 seconds, 40 cycles at 94°C for 15 seconds, 63-64°C for 30 seconds (depending on the gene), 72°C for 25 seconds, and 72°C for 25 seconds. The used primers were: GPR305’-TTCCGCGAGAAGATGACCATCC-3’ (forward), 5’-TAGTACCGCTCGTGCAGGTTGA-3’ (reverse); and GAPDH 5’-AAGCTCATTTCCTGGTATGACAACG-3’ (forward), 5’-TCTTCCTCTTGTGCTCTTGCTGG-3’ (reverse). The results were analyzed: using the Pfaffl method and Cycle Threshold (CT) values resorting to the degree of GAPDH housekeeping gene expression [19]. The reactions were performed in triplicates.

Statistical Analysis

All gene expression information tests were conducted three times. Results were analyzed through one-way analysis of variance (ANOVA) to apply multiple comparisons between experimental groups. Reciprocal comparisons between experimental groups were done through GraphPad Prism v6.07.

RESULTS

Cell Morphology Remodel in MCF-7 and MDA-MB-231 Cells

The morphology of MCF-7 and MDA-MB-231 cells after treatment with hypoxia (1% O2) or normoxia (20% O2) and different concentrations of glucose (5.5, 11, and 25 mM) after 24, 48, and 72 h are shown in Figure 1. Results showed that the growth and proliferation in MCF-7 cells on the normoxia treat is dependent on high glucose concentration (25 mM, and 72 h). However, they showed better growth and differentiation in the hypoxia (Figure 1A). Moreover, the presence of normoxia (21% O2) and hypoglycemia (5.5 mM glucose) cause cell growth inhibition and leads to MDA-MB-231 cells’ death after 72 h. Likewise, adaptation to the environment is observed in the maximum physiological state in hypoxic conditions (Figure 1B).

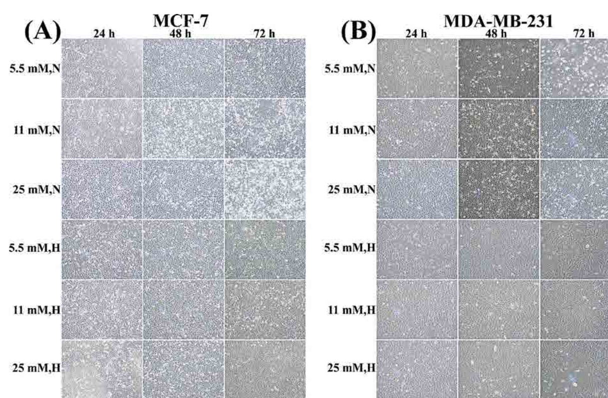

Figure 1: In Vitro Effects of Glucose Concentration and Hypoxia on Cell Morphology Against MCF-7 and MDA-MB-231

Photomicrographs of A) MCF-7 and B) MDA-MB-231 cells after 24 h, 48 h, and 72 h incubation with different glucose concentrations (5.5, 11, and 25 mM) and normoxic and hypoxic conditions.

Cell Migration

We studied the importance of glucose and oxygen concentration on the migration of the MDA-MB-231, and MCF-7 cells in vitro. For this, the cells were grown in different concentrations of glucose (5.5, 11, and 25 mM), normoxia (20% O2), and hypoxia (1% O2) and then subjected to scratch assay/wound healing assay (Figure 2). For migration assay, images were captured at 0 and 8 h for MDA-MB-231 (Figure 2A) and 0 to 24 h for MCF-7 (Figure 2B) to mark the changes in treatment conditions. Experimental data showed that hypoxia treatment leads to decrease migration in MDA-MB-231 cells after eight hours. Accordingly, migration was reduced up to 29% with 25 mM; while it was almost the same as the control for 5.5 mM 10% and 11 mM. In addition, normoxia increases the rate of migration by 25 mM, but low glucose concentration (5.5 mM)

Figure 2: Effects of Glucose Concentrations and Oxygen on the MDA-MB-231 and MCF-7 Cells Migration

A and B) The effects of different concentrations of glucose (5.5, 11, and 25 mM) under normoxic and hypoxic conditions on the A) MDA-MB-213 after 8 h; B) MCF-7 after 24 h cell migration by the scratch assay; C and D) The effects of different glucose concentrations, normoxia, and hypoxia on C) MDA-MB-231; D) MCF-7 cell migration by scratch assay [Bars correspond to mean ± SD].

causes less migration (Figure 2C). In contrast, a gradual increase in glucose concentration stimulated MCF-7 cells to promote migration in hypoxia conditions. On the contrary, high glucose (25 mM) and low glucose (5.5 mM) concentrations decreased migratory 12% and 32% in normoxia conditions, respectively (Figure 2D).

GPR30 Gene Expression in MDA-MB-231 and MCF-7 Cells

The heterogeneity of GPR30 mRNA in comparison with the untreated control MDA-MB-231 and MCF-7 cells was considered in the MDA-MB-231 and MCF-7 cells treated with different concentrations of glucose and oxygen (Figure 3). Previously, it has been shown that hypoxia leads to the over-expression of hypoxia-inducible factor 1 subunit alpha (HIF-1α)(17). Compared to untreated cells, it was observed that GPR30 mRNA expression increased in low glucose concentrations (5.5 and 11 mM) under both hypoxia and normoxia conditions in MDA-MB-231 cells; being analyzed after 24 h. However, after 48 hours, GPR30 expression was increased with 5.5 mM glucose under hypoxia, and at 72 hours, its expression level was significantly reduced in all treatment conditions. The cells treated with the high glucose concentration (25 mM) showed reduced expression after all times under both hypoxia and normoxia conditions (Figures 3 A, B, and C). To compare GPR30 mRNA gene expression in low and high metastatic breast cancer cell lines, the expression of GPR30mRNA was investigated in MCF-7 cells. The MCF-7 cells showed that GPR30mRNA expression was increased in all glucose concentrations after 24 hours, except in 5.5 mM glucose and hypoxia with further reduced expression levels (at 48 and 72 hours)(Figures 3D, E, and F). Because of the increased GPR30 mRNA expression in the MDA-MB-231 and MCF-7 cells at the primary time (24 hours), it could be concluded that this gene probably acts as an inhibitor and prognostic factor in the early stages of breast cancer.

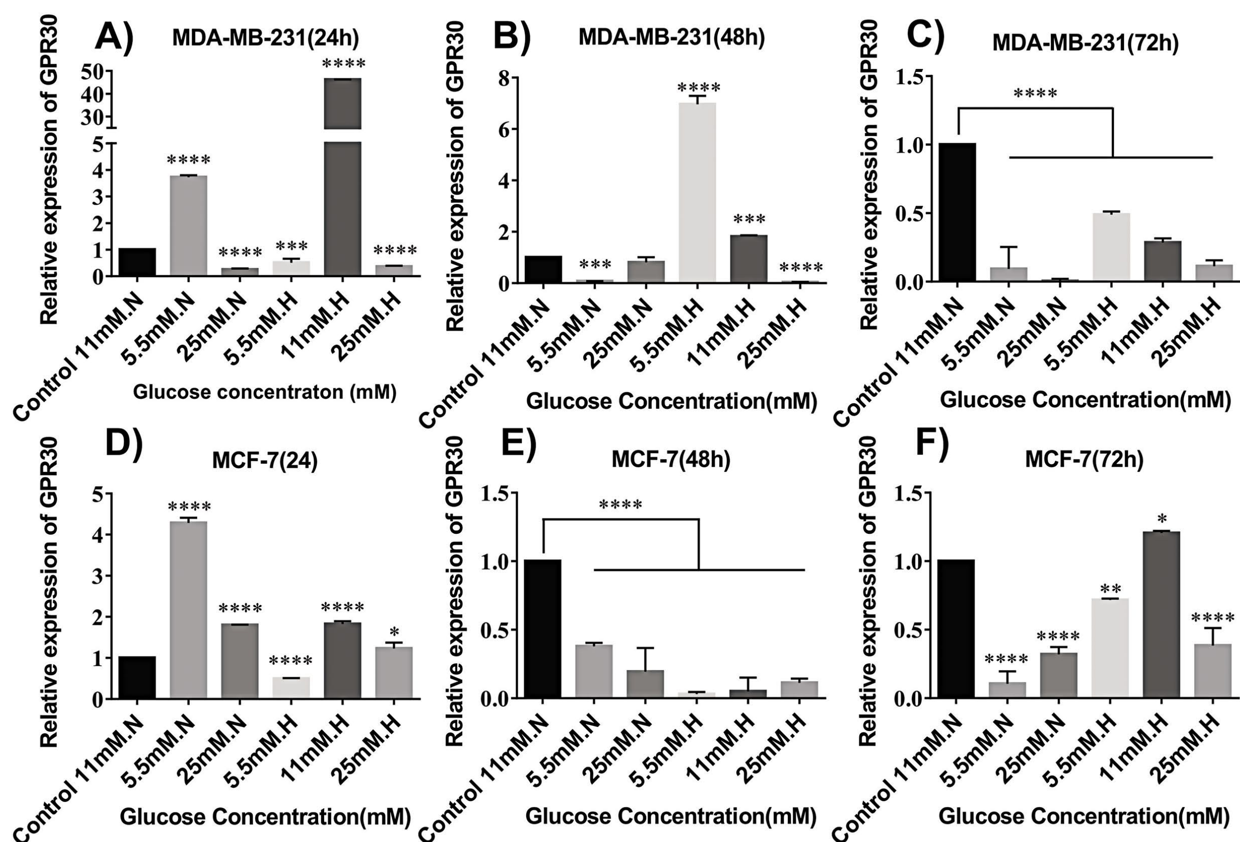

Figure 3: The GPR30 mRNA expression in the MDA-MB-231 and MCF-7 cells
Baseline GPR30 mRNA expression levels in breast cancer subtypes MDA-MB-231 and MCF-7 were evaluated by real-time PCR. Extracting total RNA and normalized to GAPDH mRNA expression levels in (A) 24 hours; (B) 48 hours; and (C) 72 hours for MDA-MB-231 and (D) 24 hours, (E) 48 hours, and (F) 72 hours for MCF-7. The data shows mean±SD. One-way ANOVA was used for statistical analyses. *P≤0.05, **P≤0.01, ***P≤0.001, and **** P≤0.0001.

DISCUSSION

Cancer cells cannot grow more than a few millimeters in the absence of sufficient oxygen and glucose [20]. We know that inadequate nutrients change the physiological conditions of cancer cells and led to pH dysregulation, activation of angiogenesis, guaranteed survival, and improper proliferation [4, 21]. Recently, more reports have shown how GPR30 affects cell signaling in breast cancer cells by motivating adenylyl cyclase, transactivating EGFRs, inducing mobilization of intracellular calcium (Ca2+) stores, activating mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) signaling pathways as well as drug resistance. Nevertheless, the reported expression profiles and functions of GPR30 in breast cancer are inconsistent and the mechanisms; leading to GPR30 expression are unclear [22, 23]. Because of the importance of the role of the GPR30 gene in cancer biology and its treatment, it is important to understand the molecular ways involved in its expression. In vivo studies in mice have shown that GPR30 expression is sensitive to D-glucose concentration and AMP-activated protein kinase (AMPK)-dependent signaling [24]. A broad spectrum of genes is induced or inhibited by D-glucose in different cell lines. It has been shown that elements responding to glucose, affect the promoter region of target genes; while high D-glucose concentration has been shown to suppress gene expression [25]. In D-glucose-deprived conditions, MAPK is activated and leads to the expression of downstream transcription factors including Forkhead Box O3A (FOXO3a), cancer risk evaluation program (CREP), tumor protein P73 (P73), and tumor protein P53 (P53) [26]. Type 2 diabetes has been shown to increase the risk of breast cancer, therefore, high blood glucose levels may suppress the expression of GPR30, thus minimizing the regulation of GPR30 signaling in breast cancer cells such as the insulin-like growth factor (IGF-1) pathway [27]. Adaptation to oxygen deficiency is one of the features of solid tumors that occurs through HIF-1α expression which binds to and activates hypoxia-responsive elements within their promoter regions [28]. To evaluate the effect of GPR30 in response to oxygen deficiency, a GPR30 expression analysis was performed. It was observed that GPR30 expression was increased in MDA-MB-231 cell lines under hypoxia. GPR30 may be considered as a new target for hypoxia-induced and HIF-1α-mediated effects. Probably because of the low expression of this gene in MFC-7 cell lines, the aggressive tumor phenotype is associated with GPR30 expression. Previous studies have shown that HIF-1a and GPR30 are located in the vascular endothelial growth factor A (VEGF) promoter region and are involved in the transcription of VEGF, and regulate the expression of the migratory factor connective tissue growth factor (CTGF) and Cyclin D in cancer-associated fibroblasts. It is noteworthy that GPR30 shows anti-apoptosis activity by estrogens in hypoxic conditions. Of note, this receptor is likely involved in the adaptation of cancer cells to hypoxia conditions [29]. In this vein, GPR30 is involved in the release of growth factors and chemokines which lead to the detachment of endothelial cells from the basement membrane and increased angiogenesis in response to the metabolism of cancer cells under conditions of oxygen deficiency [30]. GPR30 contributes to the inhibition of breast cancer cell proliferation, cell cycle arrest by M-phase, and caspase-3-mediated apoptosis in estrogen receptor-positive MCF-7 breast cancer cells; while the G2/M phase occurs in triplet negative MDA-MB-231 breast cancer cell through phosphorylation of histone H3 and apoptosis in the cell-dependent pathway so that its expression is reduced in breast cancer cells while promoter methylation needs aberrant expression and stress factors such as radiation-induced expression [31]. In some studies, the GPR30 expression pattern has been reported to be dependent on p53. In this vein, GPR30 expression is upregulated in MDA-MB-231, because of the non-functional p53 gene while it is down-regulated in MCF-7 cells that have normal wild-type p53 [32]. Results from the in vitro functional studies demonstrate that glucose concentration and oxygen play a crucial modulatory role in cancer cell migration; suggesting that glucose concentration and oxygen could be an important milieu in the drug treatment test for cancer. Taken together, GPR30 expression seems to be associated with tumor metastasis in the MDA-MB-231 cell line. But the exact mechanisms need to be clarified in further studies.

The present study suggests that increased metastasis in high glucose concentration conditions and that hypoxia plays a critical role in the breast cancer

cell microenvironment. Furthermore, cell types and oxygen concentration affect the morphology and metastasis of breast cancer cells in vitro. In addition, we found that GPR30 was a target protein in MDA-MB-231 cells metastasis. Downregulation of GPR30 occurred in glucose-deprived cells after 24 h and overexpression of this gene occurred in primary culture. So, it can manipulate key genes and predictive biomarkers of breast cancer. Further studies are required to define the role of the pathobiological and molecular pathways of different conditions such as different concentrations of glucose and oxygen. In such a heterogeneous tumor microenvironment the expression and interaction of different proteins, such as GPR30, HIF-1α, p53, etc. must be identified. Therefore, huge efforts are being made today to unravel the mystery of the regulation and function of GPR30. Indeed, the development of drugs against it could be considered innovative topic.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS APPROVAL

This is an in vitro study and doesn't have any ethical dilemma. The researchers are responsible for any possible claims..

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