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Original Research

Hepatocyte nuclear factor 4, alpha (HNF4A): A potential biomarker for chronic hypoxia in MCF7 breast cancer cell lines

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Abstract

Introduction: Hypoxia mediates cancer hallmarks and results from low oxygen levels due to irregularities in tumor vascularization or when the bulk of the tumor prevents oxygen diffusion and stimulates angiogenesis to compensate for low oxygen. Aims: This study aims to identify significant biomarkers associated with tumor hypoxia, focusing on prolonged exposure to hypoxic conditions. **Methods:** The breast cancer cells (MCF7) were exposed to 8-hour hypoxic episodes (1% oxygen) three times per week for 60 episodes and once weekly for 72 hours for a maximum of 12 episodes. After 60 and 12 episodes of hypoxia, changes in gene expression were profiled using a hypoxia RT- PCR array and compared to normoxic cells. An assay was performed after 60 and 12 episodes of hypoxia to determine the effect of hypoxia on angiogenesis migration. **Results:** The expression of the Hepatocyte Nuclear Factor 4 gene increased by 32.71-fold following exposure to 12 hypoxic injections and by an additional 14.95-fold following exposure to 60 hypoxic injections. After twelve episodes of hypoxia, the capacity for cell migration increased. **Conclusion:** This study provides empirical support for the notion that cellular exposure to prolonged durations of hypoxia, specifically three months, induces expression changes distinct from those induced by fleeting durations of hypoxia, which are less than weeks and hours. The study hypothesized that HNF4A could serve as a viable biomarker for tumor hypoxia and a major contributor to the response of MCF7 breast cancer cells to protracted hypoxic conditions.

Keywords: Hypoxia, hepatocyte, HNF4A, Breast

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INTRODUCTION

Hypoxia, which refers to oxygen deprivation, is a typical characteristic of several solid tumors[1,2]. Hypoxia is a hallmark of human malignancies, such as; head and neck, pancreatic, prostate, brain, breast, and malignant melanoma[3,4]. It has been observed that various forms of solid tumors in humans exhibit a median oxygen partial pressure (pO2) of less than 15 mmHg, which is notably lower than the median pO2 of adjacent normal cells, exceeding 35 mmHg[5,6]. Hypoxia in tumors is traditionally classified in clinical and experimental oncology into acute and chronic hypoxia[7-9].

Breast cancer is one of the most common cancers and represents the leading cause of cancer-associated deaths in women worldwide[10,11]. Additionally, the most aggressive and invasive breast cancer cells are those that were cultured in a hypoxic environment[12]. It is estimated that 40% of breast cancers have hypoxic regions[8]. Breast cancer cells that are exposed to a hypoxic environment subsequently exhibit enhanced tumor metastasis and angiogenesis as well as resistance to chemotherapy and radiation[13].

Adaptation of cells and tissues to low oxygen leads to the induction of expression of genes that participate in angiogenesis, glucose metabolism, and cell proliferation[14]. Many of the adaptive responses of cells to hypoxic conditions are mediated through the hypoxia inducible transcription factors (HIF)[15]. Hypoxia inducible transcription factors (HIF) have been proven to be a prognostic factor for aggressive tumor behavior in various types of cancer including breast cancer[16]. However,

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HIF inhibition alone failed to completely remove the tumor growth, even though it provoked a significant retardation[17]. Therefore, the quantification value of HIF as a hypoxia related gene remains questionable[18].

Many studies have focused upon hypoxia and its role in altering gene expression and signal transduction[19]. Nevertheless, the precise genetic alterations implicated in the process of tumor adaptation to hypoxia remain incompletely comprehended[20]. The main aim of this study is to identify novel biomarkers of significant factors in tumor hypoxia, specifically focusing on the effects of prolonged exposure to hypoxic conditions. This will be achieved by examining alterations in gene expression within breast cancer cells subjected to hypoxia.

MATERIALS AND METHODS

Cell Culture Conditions

The MCF-7 cells used in this study were obtained from the American Type Culture Collection (ATCC; Manassas, USA). These cells were cultured and maintained in RPMI 1640 medium (HyClone; Logan, USA) supplemented with 10% fetal bovine serum, penicillin (100U/ml), and streptomycin (100µg/ml) (HyClone; Logan, USA). Before conducting the experiments, the cells were cultured in 75cm^2 cell culture flasks (Membrane Solutions; North Bend, USA) until they reached 80% confluence. The cells were subjected to normoxic conditions by incubating them at 37°C in a cell culture incubator (Nuaire; Plymouth, USA) with an atmosphere consisting of 5% CO_2 and 95% air.

Exposure to Hypoxia

The AnaeroGen Compact system, manufactured by Oxoid in Hampshire, UK, was employed to establish a hypoxic environment by generating an anaerobic atmosphere. The system comprised a gas generation sachet enclosed within a securely sealed bag. The sachet contains active ingredients, ascorbic acid, and activated carbon, which exhibit rapid reactivity upon exposure to air. This reaction leads to the consumption of oxygen, decreasing its concentration within the bag to a level below 1%. The vented cell culture flasks with a surface area 75cm^2 were inserted into plastic pouches. The paper sachets were subsequently opened and positioned within the bag, which was later sealed. MCF7 cells were then assigned to three different tracts, each with a specific exposure schedule to a hypoxic environment. The first track (Track 1) was exposed to a hypoxic environment only once for 8 hours, representing short-term hypoxia. To study the effects of long-term hypoxia, the cells were assigned to two different tracts; Track 2a was exposed to a hypoxic environment for eight hours three times a week, which represents cells exposed to long-term chronic hypoxia, and Track 2b was exposed to a one 72-hour hypoxic shot every week which represents cells exposed to long term acute hypoxia. During three months, the cells in Track 2a were exposed to 60 intermittent episodes of hypoxia, while those in Track 2b were exposed to 12 episodes of continuous hypoxia. In addition to these designated injections, the cellular samples were subjected to identical incubation conditions alongside their normoxic control counterparts, maintaining a normoxic environment.

RNA extraction

The extraction of total RNA from MCF7 cells was performed using the RNeasy® Mini kit (Qiagen; Hilden, Germany) following the instructions provided by the manufacturer. The RNA samples were labeled as hypoxic cells undergoing 60 intermittent episodes (first track), 12 continuous episodes (second track), and normoxic cells without hypoxic exposure.

Real-time PCR

The study utilized a 96-well PCR array, specifically the RT2 Profiler PCR array (PAHS-032Z, Human Hypoxia Signaling Pathway PCR Array, Qiagen; Valencia, USA), to investigate the impact of hypoxia on gene expression in the MCF7 breast cancer cell line, following the guidelines provided by the manufacturer. The ΔΔCt method, also known as the delta cycle threshold, was employed to determine the relative changes in gene expression, specifically the up-regulation or down-regulation, per the manufacturer's guidelines.

Cell migration assay

The experimental technique employed for cell migration involved the utilization of Transwell Membranes with 8 µm pores manufactured by Corning in the United States. The migration assay was conducted in a 24-well plate configuration. A cohort comprising 40,000 cells encompassing intermittent 60 (first track), continuous 12 (second track), and normoxic cells was subjected to serum deprivation for one night. Subsequently, these cells were introduced into the upper compartment. The lower compartment was filled with a standard cell culture medium. The cells were permitted to migrate for 24 hours, following which the chambers were immobilized for a period of 10 minutes using cold 70% ethanol. The membranes were subjected to staining using a 0.5% (w/v) solution of crystal violet obtained from Sigma-Aldrich, located in St. Louis, MO, USA. This staining process lasted for a duration of 30 minutes, after which the membranes were carefully rinsed with tap water to remove any excess dye. The cells that had not undergone migration to the lower compartment were eliminated using a cotton swab. The migrated cells were quantified by solubilizing the crystal violet bound to the cells using 100% (wt/vol) methanol. The measurement of absorbance was performed at a wavelength of 600 nm.

Western blot

MCF7 breast cancer cells samples of intermittent 60, continuous 12, and normxic cells were washed with ice cold PBS and were lysed with RIPA lysis buffer supplemented with PMSF containing sodium orthovanadate solution and protease inhibitor. The protein concentration was measured using bradford assay protein standard (Bio-Rad???). 20 µg of protein from each sample were separated on a 10% bis-tris gel and then transferred to a nitrocellulose membrane. The membrane was blocked using 5% non-fat skimmed milk and then probed with antiHNF4alpha antibody (ab139420). To verify the uniform distribution of proteins, the gels were subjected to staining with Coomassie brilliant blue R-250 after transfer.

This was done to assess the successful transfer of proteins and confirm equal protein loading, as indicated by the intensities of high molecular weight proteins that remained untransferred. Densitometric analysis was conducted on the blots and the scanned images of the gels using Image Studio Digits v. 5.2, a software developed by Licor Biosciences in Lincoln, NE.

RESULTS

Gene Expression Changes after Hypoxic Episodes

Short-term hypoxic exposure showed broad and clear changes in many hypoxic-related genes. 55 of 86 genes were up or down-regulated more than two folds. Upon comparing the gene expression of the hypoxic related genes in the cells that were exposed to 60 episodes of intermittent hypoxia with such expression in the passage-age normoxic cells, 11 genes were profoundly up-regulated (Table 1) and one gene was profoundly down-regulated. Hepatocyte nuclear factor 4, alpha (HNF4A) was the most conspicuously up-regulated gene by 14.95 folds. Two additional genes were up-regulated by approximately six folds; member 1 (SLC2A1) of Solute carrier family 2 (facilitated glucose transporter), and member 3 (SLC2A3) of Solute carrier family 2 (facilitated glucose transporter).

Comparing cells after 12 hypoxic episodes to normoxic cells, the expression of 15 genes was notably altered, including six up-regulated genes and nine down-regulated genes (Table 2). The HNF4A was the most conspicuously up-regulated gene by 32.71 folds. In addition, member 3 (SLC2A3) of the Solute carrier family 2 gene (facilitated glucose transporter) was up-regulated by approximately ten folds. On the other hand, the most down-regulated gene was the Serpin peptidase inhibitor (SERPINE1), downregulated by -20.86 folds.

Migration assay results

The transwell assay was performed to illustrate the effects of hypoxia in Breast cancer MCF-7 cells on cell migration. The percentage of migrated cells was inversely proportional to the number and time intervals shots of hypoxia according to Figure 1. In MCF-7 hypoxic cells, the continuous 12 showed a higher percentage of migrated cells through the transwell (83%). In the group of cells subjected to intermittent hypoxia for 60 seconds, the rate of migrated cells was 64%. The percentage of migrated cells exhibits a positive correlation with prolonged exposure to hypoxia compared to normoxia cells (52%).

Western blots of HNF4alpha protein

Representative western blots of HNF4alpha protein in breast cancer cells are shown in Figure (2). The protein levels of the control, continuous MCF7 hypoxic cells, and intermittent MCF7 hypoxic cells were determined using the western blot and normalized using GAPDH. Quantitative assessment of HNF4alpha suggests a threefold increase in HNF4alpha protein concentration compared to the control, which confirms the results of the real-time PCR. The continuous hypoxic MCF7 protein concentration was halfway between the control and the intermittent hypoxic MCF7 cells.

On exposure to a hypoxic environment, the appearance of the bands of the continuous and intermittent cells differed. The results signify that exposing cells to a hypoxic environment alters the gene expression and causes the upregulation of the HNF4alpha gene. The density of the band of the control cells was the lowest and then increased in density by two folds for the continuous cells. The intermittent hypoxic MCF7 cells had a three-fold increase in the band density compared to the control and had the highest band density of all the samples. The results suggest that as the cells are exposed to a harsher hypoxic environment, the amount of HNF4alpha protein increases as a result of the up regulation of the HNF4alpha gene.

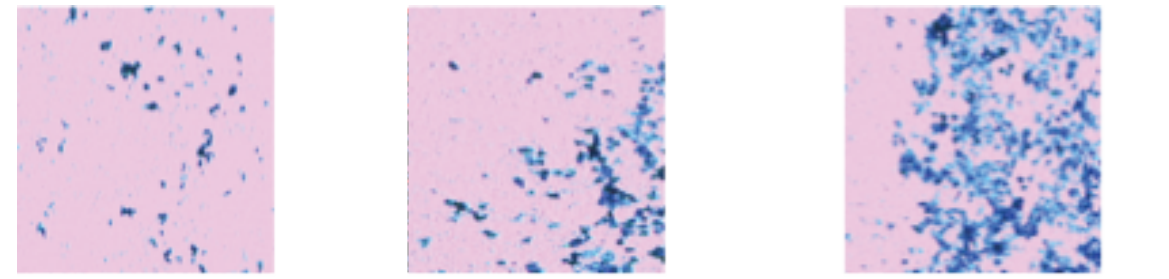
DISCUSSION

The effect of three different modalities of hypoxia on MCF7 breast cancer cell lines; short-term (track 1), chronic long-term (Track 2a), and acute long-term (Track 2b); were tested in this study. The results demonstrated different patterns of gene expression changes between short and long-term exposure to

Table 1: Up-regulated and down-regulated genes in hypoxic cells following 60 intermittent hypoxia episodes compared to normoxic cells.

Gene symbol	Gene description	Fold regulation	Gene function(Qiagen,USA)	Regulation type
DDIT4	DNA-damage-inducible transcription	2.5	Metabolism	UP-REGULATION
EDN1	endothelin 1	3.34	Angiogenesis	UP-REGULATION
EGLN2	Egl-nine-homolog 2 (C. elegans)	2.63	HIF1 Interactors	UP-REGULATION
Egr1	Early growth response 1	4.13	Angiogenesis	UP-REGULATION
EIF4EBP1	Eukaryotic translation initiations	2.72	Hypoxia Responsive Genes	UP-REGULATION
HNF4A	hepatocyte nuclear factor 4, alph	14.95	HIF1 & Co. Transcription	UP-REGULATION
IGFBP3	Insulin-like growth factor binding	2.73	Regulation of Cell Proliferation	UP-REGULATION
JMJD6	Jumonji domain containing 6	3.66	Angiogenesis	UP-REGULATION
LOX	Lysyl oxidase	-3.31	Angiogenesis	DOWN-REGULATION
SLC16A3	Solute carrior family 16, member	4.76	Coagulation	UP-REGULATION
SLC2A1	Solute carrior family 2 (facilitate	6.22	Metabolism	UP-REGULATION
SLC2A3	Solute carrier family 2 (facilitator	5.59	Metabolism	UP-REGULATION

Gene symbol	Gene description	Fold regulation	Gene function(Qiagen,USA)	Regulation type
ADORA2B	Adenosine A2b receptor	-2.53	Metabolism	DOWN- REGULATION
BLM	Bloom syndrome, RecQ helicase-like	-2.94	Angiogenesis	DOWN- REGULATION
CA9	Carbonic anhydrase IX	-3.03	HIF1 Interactors	DOWN- REGULATION
EGLN2	Egl nine homolog 2 (C. elegans)	2.82	Angiogenesis	UP-REGULATION
HNF4A	Hepatocyte nuclear factor 4, alpha	32.71	Hypoxia Responsive Genes	UP-REGULATION
IGFBP3	Insulin-like growth factor	3.51	HIF1 & Co. Transcription factors	UP-REGULATION
JMJD6	Jumonji domain containing 6	3.39	Regulation of Cell Proliferation	UP-REGULATION
LOX	Lysyl oxidase	-4.34	Angiogenesis	DOWN-REGULATION
PFKFB3	6-phosphofructo-2kinase/fructose-2,6-biphosphatase 3	-3.41	Coagulation	DOWN- REGULATION
PGK1	Phosphoglycerate kinase 1	-2.68	Metabolism	DOWN- REGULATION
PIM1	Pim-1 oncogene	-3.3	Metabolism	DOWN- REGULATION
SERPINE1	Serpin peptidase inhibitor, clade E	-20.86	Metabolism	DOWN- REGULATION
SLC16A3	Solute carrier family 16, member 3	3.42	Metabolism	UP-REGULATION
SLC2A3	Solute carrier family 2 (facilitated glucosetransporter), member 3	10.83	Metabolism	UP-REGULATION
TP53	Tumor protein p53	-2.91	Metabolism	DOWN- REGULATION



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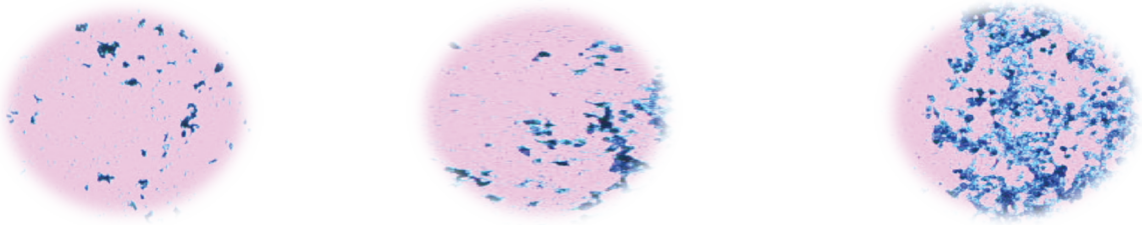

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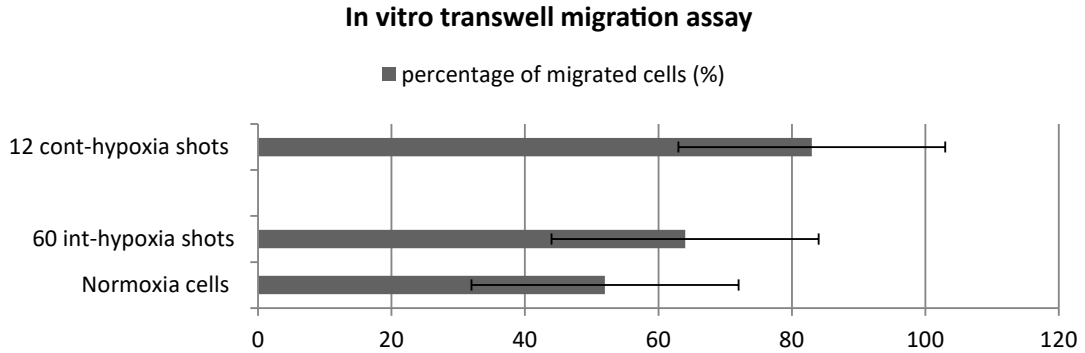

Figure 1. A In vitro transwell migration assay was conducted in MCF-7 cells with different hypoxia conditions. In vitro, transwell migration assay was performed on MCF-7 cells after being serum-starved for 24 h. Cells were allowed to migrate through an 8 µM pore with FBS as a chemo-attractant. Magnification: 10X. Data are expressed as mean ± S.E.M. ($p < 0.05$). B different hypoxia conditions in control nonhypoxic cells induced the quantification measurements of the Transmigration of MCF-7 cells. The results represent the mean±SD of three independent experiments. Statistically significant differences between the treated and control cells are shown ($p < 0.05$).

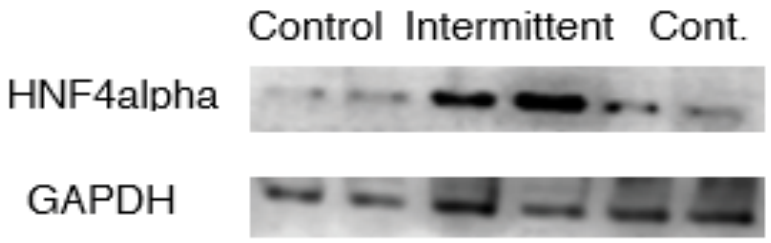

Figure 2. Western Blot for HNF4alpha

hypoxia. Cells that underwent a short-term hypoxic exposure showed broad and clear changes in many hypoxic-related genes tested, where 55 of 86 genes were up or down-regulated more than 2 folds. These changes were mostly unique to this type of exposure. The most up-regulated genes were IGFBP3 (72.4 folds), NDRG1(44.4), LOX (27.1), CA9 (26.4), ANGPTL4 (26.4), DDIT4 (21.4) and PFKFB4 (21.1). Many of those genes were upregulated by multiple research groups in cells exposed to short-term hypoxia that does not exceed 72 hours, and are considered hypoxia biomarkers that can be used as genetic targets. For example, Lal et al. (2001), who tested the transcriptional response to hypoxia in human tumors, reported that the following genes: IGFBP3, NDRG and CA9, were of the top 10 genes that a 24-hour hypoxia exposure up-regulated. CA9 was also considered a hypoxic biomarker in renal and cervical carcinoma[21].

As for the down-regulated genes, 15 were down-regulated by over 2 folds. Of those genes, BLM and OCD1 were the most downregulated, where expression of both genes decreased by 16.8 folds. ODC1 is the first rate-limiting polyamine biosynthesis enzyme associated with cell growth, proliferation, and transformation[22]. Our results support the results of Corn et al. (2005), who showed that genes regulated by c-Myc, such as ODC, were down-regulated during hypoxia[23]. The pathway analysis of the up-regulated genes in track 1 cells (short-term hypoxia), as seen in Figure 1, demonstrates clear changes in the HIF-1 signaling pathway as a response to the reduction in the oxygen supply. Within this pathway, the changes in gene expression were mainly in the genes responsible for regulating two main processes; angiogenesis and anaerobic metabolism. The VEGF and SERPINE1 genes modulate the increase in angiogenesis. On the other hand, Anaerobic metabolism was increased by multiple genes, including GLUT1, PDK and ENO1, and PFK2.

On the other hand, exposing cells to long-term hypoxia either through the cycling of 8 hours every other day (track 2a chronic long-term hypoxia) or through exposing the cells to 72 hours weekly (track 2b acute long term hypoxia) resulted in patterns of change unique to the ones seen in short term hypoxia. The extents of change were limited in the number of up or down-regulated genes and even in the extent of the fold of changes. Surprisingly, many of the highly up-regulated genes in the short term modality were down-regulated profoundly or showed no changes in the two long-term modalities. An example of such genes is the LOX and CA9 genes. LOX and CA9 were both up-regulated in the short-term (track 1) exposure by 26.4 folds and were down-regulated by 4.3 and 3 folds in track 2a (chronic long-term hypoxia) and by 3.3 and 1.2 folds in the track 2b (acute long term hypoxia), respectively.

Another relevant example is the SERPINE1 gene which is known to be induced after short-term hypoxia exposure[24]. SERPINE1 gene was up-regulated 5.1 folds in the short-term modality (track 1) and down-regulated then by 20 folds and 1.5 folds in track 2a (chronic long-term hypoxia) and track 2b (acute, long-term hypoxia), respectively. Such phenomenon is also observed in the down-regulated side, in which the most down-regulated genes (ODC1 and BLM) in the short term lose their down-regulation in the long-term hypoxic modalities. Interestingly, a new gene with the name of HNF4alpha was the gene that was up-regulated the most in the two long-term modalities by 14 folds in track 2a (chronic long-term hypoxia) and 32 folds in the track 2b (acute long term hypoxia). This up-regulation is in agreement with our previous publication[25], which demonstrated an increase in the expression of the HNF4alpha by 2 folds after 19 shots of 8 hours of hypoxia and by 6 folds after 38 shots of 8 hours of hypoxia. Furthermore, the up-regulation in the HNF4alpha gene observed in this study was confirmed on the protein level using western blotting.

The pathway analysis for the up-regulated genes in both long term hypoxia modalities (track 2a and track 2b) was similar to the short term modality concerning the HIF-1 signaling pathway which was shown to be the most up-regulated pathway in both the short-term and long term hypoxia modalities. However, in long term hypoxia (track 2a, 2b) the genes responsible for this up regulation is limited to only three genes namely: egl-9 family hypoxia-inducible factor 2(EGLN2) , eukaryotic translation initiation factor 4E binding protein 1(EIF4EBP1) , endothelin 1(EDN1) . In comparison with the short-term pathway analysis may indicate that the cells now are limiting its changing in the HIF-1 signaling pathway to the minimal and is giving the rise for an alternative pathway lead by the HNF4 alpha gene. "HNF4A is known to interact with HIF-1α to exert transcription activity. HNF4A is a nuclear transcription factor that encoded protein that with invasion, metastasis and epithelial-to-mesenchymal transition[26].

The similarities of the up-regulated genes in both long term modalities (Tracks 2a, 2b) were evident as 9 genes were commonly up-regulated more than 2 folds in both conditions.

The common up-regulated genes include SLC2A3 (10.6 in track

and 5.8 in track 2b and SLC16A3 (10.6 chronic long term hypoxia and 5.8 in acute long term hypoxia). Both SLC2A3 and SLC16A3 express monocarboxylate transporters that are known to play a major role in the adaptation to the hypoxic environment. The over-expression of monocarboxylate transporters is seen in tumor tissue and supports an apparent need for lactate shuttling to either fuel tumor growth or permit survival under stress conditions[27]. Ord et al. (2005)[28] found SLC16A3 to be up-regulated by hypoxia in bladder cancer[29]. Solute carrier family 2 member 3 (SLC2A3) was found to be up-regulated by[30] who studied the effects of chronic hypoxia on the gene expression of solute carrier (SLC) transporters in BT474 breast cancer cell line[28]. Cells were incubated at 1% oxygen for up to 64-88 days followed by gene expression profiling using drug transporter PCR array. Another important gene that has been up-regulated in the two long term modalities is the EGLN1 that encodes the Prolyl hydrase 2 (PHD2) enzyme. PHD2 enzyme is considered the key oxygen sensor-regulating hypoxia-inducible factor (HIF)[30]. This up-regulation may justify the down regulation or the stability in the hypoxia-inducible factor1 alpha gene expression that is considered a dominant player in the response of cells to hypoxia.

Another interesting feature of hypoxia that was tested in this study is the invasiveness of the MCF7 breast cancer cells. Many studies have demonstrated an increase in the aggressiveness and invasiveness of the hypoxic cells[31, 32]. The results show that the invasiveness increase in the three modalities. However, such increase varied according to the modality. The highest increase was shown in the short term modality followed by the chronic long term hypoxic modality and then by the acute long term hypoxic modality. Perhaps, the many invasive genes that have been up-regulated in the short term modality would drive such high invasiveness. For example, the LOX gene that was up-regulated 26.4 folds is known to mediate bone marrow-derived cell recruitment that forms a "pre-metastatic niche" for future metastasis and the inhibition of the LOX gene expression was reported to result in a reduction in hypoxia-induced recruitment and metastasis in the breast cancer mouse model[33].

Another invasive gene is the IGFBP3 gene that was up-regulated 72 folds. IGFBP3 mRNA was reported to markedly increase in the endothelium of human corpus luteum during early phase of luteal development which is accompanied by extensive angiogenesis and specifically in tumor endothelial cells of a murine breast cancer model[34]. ANGPTL4 was also highly up-regulated and is known to disrupt endothelial cell junctions by directly interacting with integrin, VE-cadherin and claudin-5 in a sequential manner to facilitate metastasis[35].

In the chronic long term hypoxia and acute long term hypoxia modalities the genes that may facilitate such an increase in invasiveness ability are HNF4A, SLC2A3, IGFBP3, SLC16A3, Egr-1, JMJD6. In both cases those genes were up-regulated however to different extent. Interestingly, the HNF4A gene has been up-regulated 32.7 folds in the chronic long term hypoxic modality and 15 folds in the acute long term hypoxic modality. The relatively high up-regulation in HNF4A gene may explain at least in part the increased invasiveness seen in the cells exposed to the chronic long term hypoxia modality. The gene HNF4A has been demonstrated to have an important role in cancer invasiveness. Post and colleagues showed that HNF4A is a highly useful marker for discriminating primary and metastatic breast carcinomas[36,27] showed that Knockdown of HNF4α suppressed the invasion, metastasis and angiogenesis of NB cells in vitro and in vivo and showed that ectopic expression of HNF4α promoted the invasion, metastasis and angiogenesis of NB cells. Perhaps, other genes that not included in the Hypoxic PCR array may also have a role in the high invasiveness ability, which is a limitation of our study[26].

The study focuses on mRNA and protein expression levels but does not examine post-translational modifications that could significantly influence protein function under hypoxic conditions.

CONCLUSION

We have successfully presented evidence indicating that the overall pattern of gene expression alterations in the two pathways of hypoxia differs significantly from what is currently documented in the literature, which primarily focuses on cells exposed to brief periods of hypoxia. In our study, we have suggested HNF4A as a potential biomarker for tumor hypoxia and a significant contributor to the response of MCF7 breast cancer cells under prolonged hypoxic conditions.

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest

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