UNIVERSITY OF BOLTON

SCHOOL OF SPORT AND BIOLOGICAL SCIENCES

BSC (HONS) BIOLOGY

SEMESTER ONE EXAMINATION 2018/2019

CANCER BIOLOGY

MODULE NO: BIO6009

Date: Monday 14 January 2019 **Time: 10.00 am – 1.00 pm**

INSTRUCTIONS TO CANDIDATES:

Candidates are advised that the examiners attach importance to legibility of writing and clarity of expression. YOU ARE STRONGLY ADVISED TO PLAN YOUR ANSWERS

There are TWO sections in this paper.

Answer ALL QUESTIONS.

This examination is THREE hours long.

INSTRUCTIONS TO INVIGILATORS:

Please ensure all candidates are provided with a copy of the journal article (Section A), and are given access to the notes that they have prepared (Section B). Both of these should be included in the envelope.

Students must not bring in their own copy of the journal article, or their own version of the notes.

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Section A: 5 marks per question, 75 marks in total

Questions in this section relate to the following journal article:

Hamdan, FH and Zihlif, MA. (2014). Gene expression alterations in chronic hypoxic MCF7 breast cancer cell line. *Genomics*. 104 (6 Pt B), 477-481. (Supplied separately; also provided to you in advance).

If a copy of this article is not provided to you in the examination, please inform an exam invigilator. The answers to the questions below are not necessarily found in the article, but are based on topics and methodologies discussed therein. In your answers, please do **not** simply copy sections of the article text to answer the questions.

1. Discuss the rationale for this research. What are the researchers hoping to achieve by carrying out this work?

2. In addition to those used in this article, briefly discuss two other laboratory based approaches that could be used to characterise cellular properties.

3. The authors of this article have used a technique known as an MTT proliferation assay. Explain how this assay allows scientists to study changes in gene expression. What results would be predicted if cellular proliferation decreases in one sample compared to another?

4. What is meant by the term hypoxia? Why are many tumours said to be hypoxic?

5. Explain how the technique of real-time PCR (RT-PCR, otherwise known as qPCR) is used to measure changes in gene expression.

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6. Why is RT-PCR a valuable tool in cancer biology research? In your answer you should explain why some of the changes associated with cancer may result in changes in gene expression.

7. What is doxorubicin? Justify its use in this experiment.

8. Section 3.2 refers to the measurement of the IC_{50}. What is this? Also, explain in your own words what the results in Section 3.2 show.

9. Why is the reported increase in HNF4a gene expression of particular interest to the researchers?

10. What are the benefits of using a model cell-line (i.e. MCF7 cells) for a study such as this?

11. The article states that MCF7 cells are “metastatic”. What does this mean?

12. Sketch, **without** the use of graph paper, a bar chart to reflect the results shown for 19 hypoxic episodes in section 3.2.

13. What is meant by the term “biomarker”?

14. If you were to be tasked with continuing this study, what two experiments would you do first?

15. Evaluate the potential limitations of a study such as this.

Please turn the page

[SECTION A TOTAL: 75 marks]

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Section B

Over the last few months, you have been given introductory lectures in numerous aspects of cancer biology. These can be split into five main themes:

1. The prevention of cancer
2. The onset of cancer
3. The progression of cancer
4. The diagnosis of cancer
5. The treatment of cancer

This can be considered to be a timeline of how a healthy individual can initially be cancer free, but can ultimately be in need of treatment for cancer.

With reference to **each** of these aspects, synthesise a detailed narrative of some of the relevant biological aspects for a specific type of cancer of your own choosing.

In your answer, you should include elements of justification (e.g. why was something done?) and critical analysis (e.g. what were the consequences?).

Evidence of extra reading is expected, and you must show evidence of having consulted recent scientific publications in your answer. You are permitted to refer to the ONE side of A4 notes (maximum of 200 words) that you made on the topic.

[SECTION B TOTAL: 75 marks]

END OF QUESTIONS

Genomics 104 (2014) 477–481

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Gene expression alterations in chronic hypoxic MCF7 breast cancer cell line

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ARTICLE INFO

Article history:
Received 27 April 2014
Accepted 24 October 2014
Available online 31 October 2014

Keywords:
Cancer biomarkers
Hepatocyte nuclear factor 4
Doxorubicin chemoresistance
Chronic hypoxia

ABSTRACT

Hypoxia plays a significant role in tumor progression and aggressiveness and implicated in resistance to radiotherapy and chemotherapy. This study aims to characterize the changes in gene expression associated with chronic hypoxia in MCF7 breast cancer cell line and identify a possible biomarker for hypoxia in breast cancer. Breast cancer cells (MCF7) were exposed to 8-hour hypoxic episodes (<1% oxygen) three times a week for a total of 38 episodes. Gene expression changes were profiled using RT- PCR array after 19 and 38 episodes of hypoxia and compared to normoxic cells. Chemoresistance of hypoxic cells toward doxorubicin was assessed using MTT cell proliferation assay. Marked gene expression changes were indentified after 19 and 38 episodes of hypoxia. Only few changes were common in both stages with most genes rebounding at the level of 38 episodes. A notable gene (HNF4A) has been up-regulated by 2 folds after 19 hypoxic shots and further up-regulated by 6.43 folds after 38 hypoxic shots. The half maximal inhibitory concentration (IC_{50}) of doxorubicin in MCF7 cells has increased in a trend proportional to the number of hypoxic episodes then totally rebounded after incubation under normoxia for 3 weeks. This study provides evidence that exposing cells to prolonged periods of hypoxia (weeks) results in different expression changes than those induced by short-term hypoxia (less than 72 h).

1. Introduction

Hypoxia is a common trait of most solid tumors [1]. Various types of human solid tumors have been found to have a median pO2 of less than 15 mm Hg while contiguous normal cells have a median pO2 of 35 mm Hg. Examples of cancers where hypoxia has been found are carcinoma of the uterine cervix, head and neck cancers, breast cancers, and brain tumors [2–5]. Around 40% of all breast cancer and half of the locally advanced breast cancers include regions affected by hypoxia [4,6]. Hypoxia triggers an intricate series of responses involving a plethora of several molecular pathways. The hypoxia-inducible factor (HIF) pathway with its many downstream targets is the main player in the hypoxic response [7]. However, specific genetic changes involved in the adaptation of tumors to hypoxia have not yet been fully understood [8]. Accordingly, identifying the modulated genes by hypoxia in cancer is of great benefit [9].

Immense inconsistencies can be found in literature investigating the effects of chronic hypoxia *in-vitro*. Few studies included a cyclic hypoxic pattern faithful to the *in-vivo* conditions [10]. Thus, this study was designed to simulate real hypoxic conditions as much as possible with the aim of characterizing the gene expression changes that occur in breast cancer cells under chronic hypoxia. The main goal of this study is to uncover new biomarkers in tumor hypoxia that might be applied for prognosis and relapse prediction, hence helping in the clinical decision to use adjuvant chemotherapeutic agents in cancer patients. Additionally, we hope to find out whether prolonged duration of hypoxic exposures leads to different changes than those reported in most available studies which generally expose cells to a maximum of 72 h of hypoxia. Notable changes and differences may justify the need for experiments involving prolonged duration of exposure to hypoxia.

2. Materials and methods

2.1. Cell culture conditions

MCF7 breast cancer cells were obtained from the American Type Culture Collection (ATCC; Manassas, USA). The cells were grown in vented 75 cm^2 cell culture flasks (Membrane Solutions; North Bend, USA) in RPMI 1640 media (HyClone; Logan, USA). The media was supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS) (HyClone; Logan, USA), antibiotics (100U/ml penicillin and 100 μg/ml streptomycin) (HyClone; Logan, USA), 2 mM L-glutamine in addition

Abbreviations: ADORA2B, Adenosine A2b receptor; BTG1, B-cell translocation gene 1, antiproliferative; C_t, Cycle threshold; DDIT4, DNA-damage-inducible transcript 4; HIF, Hypoxia-inducible factor; HNF4A, Hepatocyte nuclear factor 4, alpha; IC_{50}, Half maximal inhibitory concentration; MCF7, Michigan cancer foundation-7; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; PCR, Polymerase chain reaction; PGF, Placental growth factor; PIM1, Pim-1 oncogene; ROS, Reactive oxygen species; RT-PCR, Real-time PCR; TXNIP, Thioredoxin interacting protein.

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http://dx.doi.org/10.1016/j.ygeno.2014.10.010
0888-7543/

to 25 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (HyClone; Logan, USA). The cells were incubated under an atmosphere containing 5% CO_2/95% air (normoxic conditions) at 37 °C in a cell culture incubator (Nuaire; Plymouth, USA).

2.2. Cell culture passaging

Passaging of cells was performed twice a week by standardizing the split of cells so that they reach 80% confluency on specific days of the week (Sunday and Wednesday). This was done to standardize the conditions where hypoxic cells get their respective shots as much as possible. Based on this standardization, the first hypoxic shot of the week was administered directly after passaging when cells are not yet attached to the surface of the cell culture flask, the second shot after 2 days of passaging and the third shot after 1 day and hence so forth. Control cells were always split alongside their hypoxic counterparts and were subjected to the same conditions except for the hypoxic episodes. Same number of cells was cultured for both control and hypoxic cells (1.2×10^6 cells on Sunday and 0.8×10^6 cells on Wednesday). Cellular viability was assessed using the Trypan blue method (Sigma-Aldrich, Irvine, UK) and the doubling time of both, control and hypoxic cells was calculated and compared on weekly basis. The cells were subcultured for 3 months, during the last of which the doubling time of cells (both hypoxic and control) slightly decreased. Consequently, the number of cultured cells was reduced to (1×10^6 cells on Sunday and 0.6×10^6 cells on Wednesday) to maintain same density and conditions.

2.3. Exposure to hypoxia

An anaerobic atmosphere generating system, AnaeroGen Compact (Oxoid; Hampshire, UK) was used to create hypoxic conditions. The system is composed of a tightly sealed bag and a gas-generating sachet. The active ingredients in the sachet (ascorbic acid and activated carbon) react promptly upon contact with air and consume oxygen thus reducing its concentration to less than 1% inside the bag. The vented culture flasks were placed inside the plastic pouches and the paper sachets opened and placed inside the bag, which was then sealed. MCF7 hypoxic cells were exposed to an 8 hour hypoxic shot using the aforementioned system three times a week. Apart from these signified shots, cells were incubated alongside their normoxic counterparts under normoxia. Over a period of three months, cells were subjected to 38 shots of hypoxia.

2.4. Cell proliferation assay

CellTiter Non-Radioactive Cell Proliferation Assay Kit® (Promega; Madison, USA) was used to determine the effects of hypoxia on drug resistance. A heavily-used drug in cytotoxic regimens in systemic treatment of breast cancer was chosen for this assay, namely doxorubicin (Ebewe; Unterach, Austria). The MTT proliferation assay was performed after the 19th and 35th hypoxic shots. Additionally, the hypoxic cells were incubated under normoxic conditions after the 38th episode without any hypoxic shots for 3 weeks and the MTT cell proliferation assay was carried out for one more time to check the reversibility of resistance.

2.5. RNA extraction

RNA extraction was achieved by using the RNeasy® Mini kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. RNA samples were marked as hypoxic cells which have been exposed to 19 episodes of hypoxia (stage 1), 38 episodes of hypoxia (stage 2) and cells which had no hypoxic shots (normoxic cells). Extraction of RNA was performed after incubation in normoxia for 48 h after last hypoxic shot.

2.6. Real-time PCR

A 96-well PCR array, RT^2 Profiler PCR array (PAHS-032Z, Human Hypoxia Signaling Pathway PCR Array, Qiagen; Valencia, USA) was used to study the effects of hypoxia on the gene expression of MCF7 breast cancer cell line according to manufacturers' instructions. The ΔΔCt method (delta delta cycle threshold) was used to calculate the folds by which the genes were up-regulated or down-regulated as recommended by the manufacturer.

3. Results

3.1. Morphology and proliferation rate

No morphological changes in the hypoxic cell line were observed at any time when compared with their normoxic counterparts. The proliferation rate of both cell lines (normoxic and hypoxic) was comparable throughout the experiment. The doubling time of hypoxic and control cell lines was 27 h in the first two months followed by a decrease to 25.33 h in the last month. However, the changes were analogous in both cell lines and numbers of nonviable cells in both cell lines were similar (4–6%).

3.2. Resistance to doxorubicin

Given the lack of discernible changes in the hypoxic cell line, resistance to a commonly used chemotherapeutic agent, doxorubicin, was checked to assure the development of the hypoxic phenotype. The half maximal inhibitory concentration (IC_{50}) was measured using the MTT cell proliferation assay. The IC_{50} was found to be increased by 1.62 fold after 19 hypoxic episodes compared to the normoxic cells, rising from 368 nM (±121) to 595 nM (±43). After 35 hypoxic episodes, the IC_{50} of the cells evidently increased by approximately 4 folds compared to the normoxic cells, increasing to an astounding 1228 nM (±232) compared to 352 nM (±15). When chemo-resistance toward doxorubicin was checked after the cells were incubated for 3 weeks under normoxia after the 38 episodes, the IC_{50} was found to be drop significantly to 369 nM (±31) which equal to the IC_{50} in normoxic cells, 370 (±2.6).

3.3. Gene expression changes after 38 hypoxic episodes

An arbitrarary cut-off point of 2 folds was chosen to indicate substantial change. Upon comparing the cells which were exposed to 38 episodes of 8-hour hypoxia to comparable passage-age normoxic cells, 7 genes were profoundly down-regulated (Table 1) and 3 were profoundly up-regulated. Hepatocyte nuclear factor 4, alpha (HNF4A) was the most conspicuously up-regulated gene by 6.43 folds. In addition two genes were up-regulated by approximately two folds, namely, thioredoxin interacting protein (TXNIP) and solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3).

3.4. Gene expression changes after 19 hypoxic episodes

To take a closer look at the hypoxic cell, the gene expression changes in MCF7 cells that were only exposed to 19 shots of 8-hour hypoxia were measured using the PCR array. Comparing cells after 19 hypoxic episodes to normoxic cells, 15 genes were notably changed including 5 genes that were also altered at the end of the experiment though not necessarily in the same trend. More often than not, genes were significantly up- or down- regulated halfway through the experiment to be normalized at the end of it. PGF which was down-regulated by 4.1 folds at the end of the experiment was even less expressed in 19 episodes hypoxic cells by 5.93 folds. Lysyl oxidase (LOX) was profoundly down-regulated by 3.97 folds while it failed to maintain substantial down-

Table 1
Genes profoundly down-regulated in hypoxic cells after 38 episodes compared to normoxic cells.

Gene symbol	Gene description	Fold regulation	Gene function (Qiagen, USA)
PGF	Placental growth factor	−4.10	Angiogenesis; regulation of cell proliferation
DDIT4	DNA-damage-inducible transcript 4	−2.58	Metabolism; regulation of apoptosis
ADORA2B	Adenosine A2b receptor	−2.34	Angiogenesis
DNAJC5	DnaJ (Hsp40) homolog, subfamily C, member 5	−2.11	Hypoxia responsive gene
HMOX1	Heme oxygenase (decycling) 1	−2.07	Angiogenesis
ODC1	Ornithine decarboxylase 1	−2.02	Regulation of cell proliferation
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	−2.00	Metabolism; transporters, channels and receptors

regulation after 38 episodes. Two genes were down-regulated by approximately two folds, namely, ADORA2B which maintained such inhibition at the end of the experiment and matrix metallopeptidase 9 (MMP9) which, on the other hand, failed to maintain such an inhibition.

Eleven genes were profoundly up-regulated after 19 episodes (Table 2) although many of them rebounded or reversed after 38 hypoxic episodes. The most notable of these genes is the TXNIP which was up-regulated by 6.05 folds and suffered substantial rebound to merely 2 fold of up-regulation after 38 episodes. Pim-1 oncogene (PIM1) was up-regulated by 3.11 folds after 19 experiments while it was not substantially down-regulated at the end of the experiment. DDIT4 was up-regulated after 19 episodes by 2.42 folds to be down-regulated by approximately 2 folds after 38 episodes. HNF4A was upregulated by 2 folds in the middle of the experiment to be highly over-expressed at the end of the experiment.

4. Discussion

With the purpose of finding a biomarker for hypoxia in cancer, we have exposed MCF7 breast cancer cells to 8-hour episodes of hypoxia three times a week for up to three months. Chemo-resistance toward doxorubicin was measured using MTT proliferation assay at different stages through the experiment. Furthermore, gene expression profiling was performed half way through the study (after 19 episodes of hypoxia/stage 1) and at the end of the experiment (after 38 episodes of hypoxia/stage 2). The resultant findings confirm that hypoxia plays a significant role in the development of a chemo-resistant phenotype in MCF7 breast cancer cell line. Most prominently, distinct differences in gene expression profiling were found in cells exposed to 38 episodes of hypoxia compared to their normoxic counterparts. In addition notable differences were measured in cells exposed to 19 episodes of hypoxia with some common features with those exposed to 38 episodes.

We have succeeded in inducing chemo-resistance toward doxorubicin after exposing MCF7 cells to 19 episodes of 8-hour hypoxia. This was demonstrated by a conspicuous increase in the concentration required to kill 50% of the cells (IC_{50}) compared to the normoxic cells by around 1.5 folds in the MTT assay. These results support the conclusions of other studies that have demonstrated that hypoxia induces chemo-resistance [11–14]. One of the speculated mechanisms by which hypoxia induces chemo-resistance is by decreasing cell proliferation rate as most chemotoxic drugs depend on the rapid proliferation rate of cancer cells [15]. Such theory does not apply to our study as the proliferation cell rate has been equal to that of comparable age normoxic cells. The lack of change in proliferation rate can be attributed to the relatively mild harshness of the thrice weekly 8-hour hypoxic episodes. This implicates that the cause of chemo-resistance in our experiment is most probably due to changes brought on by hypoxia on the genomic and proteomic level of the cell. Inadvertently, we have found that a gene implicated with chemo-resistance is profoundly up-regulated at stage 1. PIM1 was found to be up-regulated by 3.11 folds and is involved in sabotaging chemotoxic-induced apoptosis thus promoting cell survival [16]. Other genes that are associated with apoptosis and notably up-regulated in stage 1 cells are—cell translocation gene 1, antiproliferative (BTG1) and DDIT4. While BTG1 is known to be induced by hypoxia and have a promoting effect of apoptosis and thus most probably is not a player in chemo-resistance induction, DDIT4 helps the cells evade apoptosis through ROS reduction [17,18]. DDIT4 has been reported to be induced by hypoxia through hypoxia inducible factor 1α (HIF-1α) dependent and independent pathways and protects cells from apoptosis in human prostate carcinoma cells [19]. In MCF7, DDIT4 rendered cells resistant to hypoxia-induced apoptosis [18]. It has also been demonstrated that DDIT4 up-regulation protects human ovarian epithelial cell lines from apoptosis [20]. Accordingly, further studies are needed to investigate if DDIT4 plays a significant role in protecting cells from hypoxia-induced apoptosis and possibly chemotoxic-induced apoptosis.

Nevertheless, the IC_{50} of doxorubicin was more than doubled after 35 episodes of hypoxia compared to 19 episodes and nearly quadrupled when compared to normoxic cells. This suggests that chemo-resistance increases as the cells are exposed to more shots of hypoxia. However, most of the changes in gene expression that were noticed after 19 episodes of hypoxia were normalized after 38 episodes. It can be speculated that the hypoxic breast cancer cell has opted for alternative ways of response when irrevocably exposed to hypoxia (for more than two months). A plausible explanation can be the modulation of the cell on the proteomic level rather than the genomic level in agreement with other studies which demonstrated that protein levels of hypoxia responsive genes tend to be up-regulated instead of their messenger RNAs under chronic hypoxic conditions (more than 24 h but less than

Table 2
Genes profoundly up-regulated in hypoxic cells after 19 episodes compared to normoxic cells.

Gene symbol	Gene description	Fold regulation	Gene function (Qiagen, USA)
TXNIP	Thioredoxin interacting protein	6.05	Regulation of cell proliferation
PIM-1	Pim-1 oncogene	3.11	Regulation of cell proliferation; regulation of apoptosis
EDN1	Endothelin 1	2.99	Angiogenesis
BTG1	B-cell translocation gene 1, anti-proliferative	2.88	Angiogenesis; regulation of apoptosis; regulation of cell proliferation
CCNG2	Cyclin G2	2.46	Regulation of cell proliferation
DDIT4	DNA-damage-inducible transcript 4	2.42	Metabolism; regulation of apoptosis
NDRG1	N-myc downstream regulated 1	2.26	DNA damage signaling & repair
VEGFA	Vascular endothelial growth factor	2.04	Angiogenesis
EGR1	Early growth response 1	2.01	Angiogenesis; regulation of cell proliferation
EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	2.00	Hypoxia responsive gene
HNF4A	Hepatocyte nuclear factor 4, alpha	2.00	HIF1 & co-transcription factors

72 h) [8,21]. We also propose that this reversal might be due to the fact that other effectors take over when a stressful situation goes on beyond a specific point. Further studies involving protein level investigation are needed to explore the so far elusive mechanism of hypoxia-induced chemo-resistance and find a target to reverse this big hurdle in cancer management.

It must be noted that the induced resistance has totally been reversed when the cells were incubated for 3 weeks under normoxia after the 38 hypoxic episodes. We suggest that this illustrates the ability of the cell to switch off the mechanisms initiated when there is no more need for it. This is in accord with Wohlkoenig et al. [22] who succeeded in inducing reversible chemo-resistance toward cisplatin, a DNA-damaging agent, in lung cancer cells through hypoxia (exposing cells for 3 days of constitutive 1% oxygen). Further studies concerning the reversibility of hypoxia-induced chemo-resistance are needed.

The most prominent of our results is concerned with the consistent up-regulation of hepatocyte nuclear factor 4α (HNF4A). The mRNA of HNF4A has been one of the most intensely up-regulated genes in stage 2 when compared to control cells with 6.43 folds. It has also been up-regulated after 19 hypoxic shots for about 2 folds compared to control cells. Unlike every other gene in the hypoxia-signaling pathway PCR array, HNF4A was up-regulated halfway through the experiment to be even more expressed at the end of it. This marked increase that is proportional to the number of hypoxic shots in addition to chemo-resistance proposes HNF4A as a potential biomarker for hypoxia in breast cancer cells. HNF4A is a transcription factor that binds to the enhancer of the 3′ of erythropoeitin gene, a hormone that regulates erythrocyte proliferation under hypoxic conditions [23,24]. Zhang et al. [25] have suggested that HNF4 interacts with HIF-β under normoxic condition. Under hypoxia, this interaction is switched to a bond between HNF4 and HIF-1α. This interaction seems to be important in the activation of erythropoietin and its sabotage diminishes the activating effect of hypoxia on erythropoietin. The same team has suggested after a few years that this interaction may have a stabilizing effect on the HIF-1α protein [24]. We speculate that at a certain point in chronic hypoxia, and given the trend of HNF4A up-regulation, cells tend to stabilize HIF-1 not by gene expression but by stabilization of HIF-1 protein through many mechanisms that may include interaction with HNF4. HIF-1α was a member of our gene panel but was very marginally changed throughout the experiment which is not surprising given the chronic intermittent pattern in this study. Irrespective of the role of HNF4, it seems to be a promising candidate as a biomarker and further studies must be performed to investigate its role in hypoxia and validate its use as a biomarker in hypoxia in cancer.

Another prominent gene in our study is PGF which is the most markedly down-regulated gene at both stages of the experiment. Although somewhat up-regulated when comparing stage 2 with stage 1, the gene keeps being more than 4 times down-regulated compared to normoxic cells. PGF is a member of the pro-angiogenic family of the vascular endothelial growth factor [26]. PGF is believed to play a regulatory role in inflammation, tumor progression and stromal expansion [27]. The role of PGF in the tumor microenvironment is complex and dynamic [28]. Proangiogenic properties in addition to a promoting effect in cancer progression have been attributed to PGF in many studies [29–31], while many other studies have also found that over-expression of PGF inhibits angiogenesis and tumor progression [28,32,33]. Available studies do not satisfyingly explain the perplexing relationship between hypoxia and PGF expression [34]. Our results contrast with some studies that have reported the induction of PGF by hypoxia in various human tissues such as the lung and the heart [35–37]. However, it agrees with Khaliq et al. [38] and Ahmed et al. [39] that hypoxia down-regulates PGF expression. Nevertheless, our results cannot be perceived alone but as part of a big controversy concerning the role of PGF in cancer biology (reviewed in [40]).

MCF7 cell line has been chosen as a model for hypoxia in this study for many reasons. Firstly, there is a handful of studies [9,21,41–43] investigating hypoxia-induced gene expression changes in MCF7 cells which provide opportunity for comparison and extrapolation.

Many of the changes in these studies, which also included the proteomic aspect, occurred at the proteomic level but not on the genomic level. One of the limitations of this study is that it has focused on changes on the genomic level and did not extend to the equally, if not more important, proteomic aspect. Though many chronic changes would have occurred at the genetic and epigenetic levels due to the long time course of this experiment, it is also highly probable that many changes would occur too transiently without corresponding heritable changes. Secondly, hypoxia-induced doxorubicin resistance which has been chosen in this study to confirm the hypoxic phenotype of the cells has been well studied in MCF7 cell line [14,44]. Finally, as MCF7 cells are metastatic, it is heavily used to study the effects of hypoxia which is thought to encourage metastasis. Consequently, further studies should be performed on other cell lines to prove that results can be generalized in breast cancer cells.

It must be noted that the atmosphere generating system used to simulate hypoxic conditions exposed the cells in this experiment to slightly higher concentrations of carbon dioxide. This might be a two-edged sword as increased carbon dioxide may slightly affect the environment and thus the gene expression of cells, but it also faithfully simulates the change in pH that occurs under hypoxic conditions. A big limitation to this study is its inability to ensure the absence of subpopulations formed within the hypoxic cells which can be done through flow-cytometry.

5. Conclusions

In conclusion, we have been able to provide evidence that the overall view of gene expression changes in chronic hypoxia is actually clearly different than that in the available literature where cells are exposed to short-term hypoxia. In addition, we have confirmed that chronic hypoxia induces chemo-resistance toward doxorubicin in MCF7 breast cancer cell line and that this resistance increases as cells are further exposed to hypoxic conditions. Finally, we have proposed HNF4A as a potential biomarker in tumor hypoxia and a major player in MCF7 breast cancer cell response to chronic hypoxia.

Declaration of interest policy

The authors have no conflicts of interest.

Acknowledgments

We would like to thank the Deanship of Scientific Research, University of Jordan, Amman, Jordan (13/2012-2013) for funding this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2014.10.010.

References

[1] F. Bray, (2004).
[2] Y. Kim, Q. Lin, P.M. Glazer, Z. Yun, Hypoxic tumor microenvironment and cancer cell differentiation, Curr. Mol. Med. 9 (2009) 425–434.
[3] P. Vaupel, O. Thews, M. Hoeckel, Treatment resistance of solid tumors: role of hypoxia and anemia, Med. Oncol. 18 (2001) 243–259.
[4] P. Vaupel, A. Mayer, S. Briest, M. Hockel, Hypoxia in breast cancer: role of blood flow, oxygen diffusion distances, and anemia in the development of oxygen depletion, Adv. Exp. Med. Biol. 566 (2005) 333–342.
[5] A. Becker, P. Stadler, R.S. Lavey, G. Hansgen, T. Kuhnt, C. Lautenschlager, H.J. Feldmann, M. Molls, J. Dunst, Severe anemia is associated with poor tumor oxygenation in head and neck squamous cell carcinomas, Int. J. Radiat. Oncol. Biol. Phys. 46 (2000) 459–466.

[6] C. Ward, S.P. Langdon, P. Mullen, A.L. Harris, D.J. Harrison, C.T. Supuran, I.H. Kunkler, New strategies for targeting the hypoxic tumour microenvironment in breast cancer, Cancer Treat. Rev. 39 (2013) 171–179.
[7] S.E. Rademakers, P.N. Span, J.H. Kaanders, F.C. Sweep, A.J. van der Kogel, J. Bussink, Molecular aspects of tumour hypoxia, Mol. Oncol. 2 (2008) 41–53.
[8] O. Alqawi, H.P. Wang, M. Espiritu, G. Singh, Chronic hypoxia promotes an aggressive phenotype in rat prostate cancer cells, Free Radic. Res. 41 (2007) 788–797.
[9] H. Bando, M. Toi, K. Kitada, M. Koike, Genes commonly upregulated by hypoxia in human breast cancer cells MCF-7 and MDA-MB-231, Biomed. Pharmacother. 57 (2003) 333–340.
[10] C. Bayer, P. Vaupel, Acute versus chronic hypoxia in tumors: controversial data concerning time frames and biological consequences, Strahlenther. Onkol. 188 (2012) 616–627.
[11] R. Sullivan, G.C. Pare, L.J. Frederiksen, G.L. Semenza, C.H. Graham, Hypoxia-induced resistance to anticancer drugs is associated with decreased senescence and requires hypoxia-inducible factor-1 activity, Mol. Cancer Ther. 7 (2008) 1961–1973.
[12] W.R. Wilson, M.P. Hay, Targeting hypoxia in cancer therapy, Nat. Rev. Cancer 11 (2011) 393–410.
[13] N. Rohwer, T. Cramer, Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways, Drug Resist. Updat. 14 (2011) 191–201.
[14] S. Doublier, D.C. Belisario, M. Polimeni, L. Annaratone, C. Riganti, E. Allia, D. Ghigo, A. Bosia, A. Sapino, HIF-1 activation induces doxorubicin resistance in MCF7 3-D spheroids via P-glycoprotein expression: a potential model of the chemo-resistance of invasive micropapillary carcinoma of the breast, BMC Cancer 12 (2012) 4.
[15] A. Wouters, B. Pauwels, F. Lardon, J.B. Vermorken, Review: implications of in vitro research on the effect of radiotherapy and chemotherapy under hypoxic conditions, Oncologist 12 (2007) 690–712.
[16] N. Chen, X. Chen, R. Huang, H. Zeng, J. Gong, W. Meng, Y. Lu, F. Zhao, L. Wang, Q. Zhou, BCL-xL is a target gene regulated by hypoxia-inducible factor-1{alpha}, J. Biol. Chem. 284 (2009) 10004–10012.
[17] R. Nahta, L.X. Yuan, D.J. Fiterman, L. Zhang, W.F. Symmans, N.T. Ueno, F.J. Esteva, B cell translocation gene 1 contributes to antisense Bcl-2-mediated apoptosis in breast cancer cells, Mol. Cancer Ther. 5 (2006) 1593–1601.
[18] T. Shoshani, A. Faerman, I. Mett, E. Zelin, T. Tenne, S. Gorodin, Y. Moshel, S. Elbaz, A. Budanov, A. Chajut, H. Kalinski, I. Kamer, A. Rozen, O. Mor, E. Keshet, D. Leshkowitz, P. Einat, R. Skaliter, E. Feinstein, Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis, Mol. Cell. Biol. 22 (2002) 2283–2293.
[19] R. Schwarzer, D. Tondera, W. Arnold, K. Giese, A. Klippel, J. Kaufmann, REDD1 integrates hypoxia-mediated survival signaling downstream of phosphatidylinositol 3-kinase, Oncogene 24 (2005) 1138–1149.
[20] B. Chang, G. Liu, G. Yang, I. Mercado-Uribe, M. Huang, J. Liu, REDD1 is required for RAS-mediated transformation of human ovarian epithelial cells, Cell Cycle 8 (2009) 780–786.
[21] S. Rodriguez-Enriquez, L. Carreno-Fuentes, J.C. Gallardo-Perez, E. Saavedra, H. Quezada, A. Vega, A. Marin-Hernandez, V. Olin-Sandoval, M.E. Torres-Marquez, R. Moreno-Sanchez, Oxidative phosphorylation is impaired by prolonged hypoxia in breast and possibly in cervix carcinoma, Int. J. Biochem. Cell Biol. 42 (2010) 1744–1751.
[22] C. Wohlkoenig, K. Leithner, A. Deutsch, A. Hrzenjak, A. Olschewski, H. Olschewski, Hypoxia-induced cisplatin resistance is reversible and growth rate independent in lung cancer cells, Cancer Lett. 308 (2011) 134–143.
[23] Q. Ke, M. Costa, Hypoxia-inducible factor-1 (HIF-1), Mol. Pharmacol. 70 (2006) 1469–1480.
[24] T. Tsuchiya, Y. Kominato, M. Ueda, Human hypoxic signal transduction through a signature motif in hepatocyte nuclear factor 4, J. Biochem. 132 (2002) 37–44.
[25] W. Zhang, T. Tsuchiya, Y. Yasukochi, Transitional change in interaction between HIF-1 and HNF-4 in response to hypoxia, J. Hum. Genet. 44 (1999) 293–299.
[26] D. Maglione, V. Guerriero, G. Viglietto, P. Delli-Bovi, M.G. Persico, Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9267–9271.
[27] C. Fischer, M. Mazzone, B. Jonckx, P. Carmeliet, FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? Nat. Rev. Cancer 8 (2008) 942–956.
[28] E.M. Hedlund, K. Hosaka, Z. Zhong, R. Cao, Y. Cao, Malignant cell-derived PlGF promotes normalization and remodeling of the tumor vasculature, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 17505–17510.
[29] T. Odorisio, C. Schietroma, M.L. Zaccaria, F. Cianfarani, C. Tiveron, L. Tatangelo, C.M. Failla, G. Zambruno, Mice overexpressing placenta growth factor exhibit increased vascularization and vessel permeability, J. Cell Sci. 115 (2002) 2559–2567.
[30] M. Marcellini, N. De Luca, T. Riccioni, A. Ciucci, A. Orecchia, P.M. Lacal, F. Ruffini, M. Pesce, F. Cianfarani, G. Zambruno, A. Orlandi, C.M. Failla, Increased melanoma growth and metastasis spreading in mice overexpressing placenta growth factor, Am. J. Pathol. 169 (2006) 643–654.
[31] S. Van de Veire, I. Stalmans, F. Heindryckx, H. Oura, A. Tijeras-Raballand, T. Schmidt, S. Loges, I. Albrecht, B. Jonckx, S. Vinckier, C. Van Steenkiste, S. Tugues, C. Rolny, M. De Mol, D. Dettori, P. Hainaud, L. Coenegrachts, J.O. Contreres, T. Van Bergen, H. Cuervo, W.H. Xiao, C. Le Henaff, I. Buysschaert, B. Kharabi Masouleh, A. Geerts, T. Schomber, P. Bonnin, V. Lambert, J. Haustraete, S. Zacchigna, J.M. Rakic, W. Jimenez, A. Noel, M. Giacca, I. Colle, J.M. Foidart, G. Tobelem, M. Morales-Ruiz, J. Vilar, P. Maxwell, S.A. Vinores, G. Carmeliet, M. Dewerchin, L. Claesson-Welsh, E. Dupuy, H. Van Vlierberghe, G. Christofori, M. Mazzone, M. Detmar, D. Collen, P. Carmeliet, Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease, Cell 141 (2010) 178–190.
[32] T. Schomber, L. Kopfstein, V. Djonov, I. Albrecht, V. Baeriswyl, K. Strittmatter, G. Christofori, Placental growth factor-1 attenuates vascular endothelial growth factor-A-dependent tumor angiogenesis during beta cell carcinogenesis, Cancer Res. 67 (2007) 10840–10848.
[33] M. Bjorndahl, R. Cao, A. Eriksson, Y. Cao, Blockage of VEGF-induced angiogenesis by preventing VEGF secretion, Circ. Res. 94 (2004) 1443–1450.
[34] S. De Falco, The discovery of placenta growth factor and its biological activity, Exp. Mol. Med. 44 (2012) 1–9.
[35] C.J. Green, P. Lichtlen, N.T. Huynh, M. Yanovsky, K.R. Laderoute, W. Schaffner, B.J. Murphy, Placenta growth factor gene expression is induced by hypoxia in fibroblasts: a central role for metal transcription factor-1, Cancer Res. 61 (2001) 2696–2703.
[36] R.J. Torry, R.J. Tomanek, W. Zheng, S.J. Miller, C.A. Labarrere, D.S. Torry, Hypoxia increases placenta growth factor expression in human myocardium and cultured neonatal rat cardiomyocytes, J. Heart Lung Transplant. 28 (2009) 183–190.
[37] M. Sands, K. Howell, C.M. Costello, P. McLoughlin, Placenta growth factor and vascular endothelial growth factor B expression in the hypoxic lung, Respir. Res. 12 (2011) 17.
[38] A. Khaliq, C. Dunk, J. Jiang, M. Shams, X.F. Li, C. Acevedo, H. Weich, M. Whittle, A. Ahmed, Hypoxia down-regulates placenta growth factor, whereas fetal growth restriction up-regulates placenta growth factor expression: molecular evidence for "placental hyperoxia" in intrauterine growth restriction, Lab. Invest. 79 (1999) 151–170.
[39] A. Ahmed, C. Dunk, S. Ahmad, A. Khaliq, Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) and soluble Flt-1 by oxygen—a review, Placenta 21 (Suppl. A) (2000) S16–S24.
[40] K.J. Kim, C.S. Cho, W.U. Kim, Role of placenta growth factor in cancer and inflammation, Exp. Mol. Med. 44 (2012) 10–19.
[41] A.M. Scherbakov, Y.S. Lobanova, V.A. Shatskaya, M.A. Krasil'nikov, The breast cancer cells response to chronic hypoxia involves the opposite regulation of NF-kB and estrogen receptor signaling, Steroids 74 (2009) 535–542.
[42] C.L. Chen, J.S. Chu, W.C. Su, S.C. Huang, W.Y. Lee, Hypoxia and metabolic phenotypes during breast carcinogenesis: expression of HIF-1alpha, GLUT1, and CAIX, Virchows Arch. 457 (2010) 53–61.
[43] M. Larsen, S. Tazzyman, E.L. Lund, N. Junker, C.E. Lewis, P.E. Kristjansen, C. Murdoch, Hypoxia-induced secretion of macrophage migration-inhibitory factor from MCF-7 breast cancer cells is regulated in a hypoxia-inducible factor-independent manner, Cancer Lett. 265 (2008) 239–249.
[44] A.E. Greijer, M.C. de Jong, G.L. Scheffer, A. Shvarts, P.J. van Diest, E. van der Wall, Hypoxia-induced acidification causes mitoxantrone resistance not mediated by drug transporters in human breast cancer cells, Cell. Oncol. 27 (2005) 43–49.