Expression of Hypoxia-Inducible Factor-1 alpha and 2 alpha in Advanced Breast Cancer after Neoadjuvant Therapy

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Abstract

Advanced breast cancer may aggressively progress under hypoxic conditions, leading to therapy resistance and metastasis, and the effects of neoadjuvant therapy on tumor hypoxia remain unclear.

The current study aimed to evaluate the expressions of hypoxia-inducible factor-1 alpha (HIF-1α) and hypoxia-inducible factor-2 alpha (HIF-2α) in advanced breast cancer cells following neoadjuvant treatment and their correlation with clinical characteristics.

In total, 48 breast cancer specimens were collected before and after neoadjuvant therapy (28 treated with chemotherapy, 20 with hormonal therapy). Total RNA was isolated from 50 mg specimens. The mRNA levels of HIF-1α and HIF-2α were measured using qRT-PCR. The 18S rRNA was used as housekeeping gene.

This study demonstrates that HIF-2α mRNA expression was significantly lower than those before therapy, whereas that of HIF-1α remained unchanged. We categorized the data based on clinical characteristics, and found that the increased expression of HIF-1α was associated with reduced patient survival. Moreover, high expressions of HIF-1α and HIF-2α were found in patients aged >40 years having breast cancer of low histopathological grades.

Thus, we conclude that high expressions of HIF-1α and HIF-2α may be used as biomarkers for the prediction of patient survival following neoadjuvant therapy in advanced breast cancer.

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Introduction

Breast cancer is the most common malignancy worldwide, with an estimated one million women developing breast cancer each year, and >410,000 dying from the disease.[1,2] Recently, neoadjuvant systemic (chemo- and hormonal) therapy was applied as a preoperative approach to decrease tumor volume and facilitate breast conservation in breast cancer patients.[3] Hypoxia (i.e. conditions of reduced oxygen supply to the cells) plays an important role in cancer. The physiological response to hypoxia is controlled by the hypoxia-inducible factor (*HIF*), which act as transcription factors for several genes involved in the adaptation response to hypoxia.[4] Expressions of HIF-1α and HIF-2α have been associated with poor prognosis of most types of cancer, and increased expressions of both genes are important in the development of more aggressive cancer cells.[5,6] The current study aimed to evaluate the expressions of hypoxia-inducible factor-1 alpha (HIF-1α) and hypoxia-inducible factor-2 alpha (HIF-2α) in advanced breast cancer cells following neoadjuvant therapy and their correlation with clinical characteristics.

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Methods

This was an experimental paired design study using 48 advanced breast cancer specimens (stages IIIB and IV) from patients aged 30-70 years before and after 6 months receiving either chemotherapy (28 patients) or

hormonal therapy (20 patients) to analyze their mRNA expressions of HIF-1α and HIF-2α. Histopathological grading of breast cancer specimens was determined according to Scarff-Bloom-Richardson modification.[7] The study protocol was approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Indonesia-Cipto Mangunkusumo Hospital. The samples were obtained from fine needle biopsy (pre-therapy) and mastectomy (post-therapy) at the National Cancer Hospital of Dharmais and kept in liquid nitrogen for further analysis. The mRNA expressions were measured in the Laboratory of Oxidative Stress and Hypoxia, Department of Biochemistry and Molecular Biology Faculty of Medicine, Universitas Indonesia.

About 50 mg of breast cancer tissues was homogenized and total RNA was isolated using TriPure® RNA Isolation Reagent (Roche, Merck, Germany) according to the manufacturer's protocol; the RNA concentration was quantified and its purity was analyzed using a spectrophotometer (Varioskan®, Thermo Scientific, USA) at wavelengths of 260 nm and 280 nm. We used the Bioneer Accupower® CycleScript RT Premix (dN12) kit to generate cDNA from the total RNA (100 ng) and performed the expression analysis using a two-step qRT-PCR with Bioneer Accupower® GreenStarTM qPCR master mix premix qRT-PCR Kit. The primers are 5'- GGC GCG AAC GAC AAG AAA AAG -3' (forward) and 5'- AGT GGC AAC TGA TGA GCA AG -3' (reverse) for HIF1α, 5'- ATA GCA GTG GCA AGG GGG CT -3' (forward) and 5'- TCA GGG CTA TTG GGC GTG GA -3' (reverse) for HIF2α. We used 18S rRNA for housekeeping gene; the primer is 5'- AAA CGG CTA CCA CAT CCA AG -3' (forward) and 5'- CCT CCA ATG GAT CCT CGT TA -3' (reverse). Data analysis was performed using statistical software SPSS and mean comparison between 2 group was analyzed using Student's t-test.

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Results

The expression of HIF-2α decreased in advanced breast cancer samples following neoadjuvant chemotherapy and hormone therapy; however, we found no significant difference in the expression of HIF-1α before and after neoadjuvant therapy (Figure 1). The samples were divided into three groups according to their relative mRNA expression levels:low expression (expression < 1.5-fold), intermediate expression (expression between 1.5- and 7-fold), and high expression (expression > 7-fold). HIF-1α expression was found to be low in 26 samples, intermediate in 18 samples, and high in 4 samples; HIF-2α expression was found-

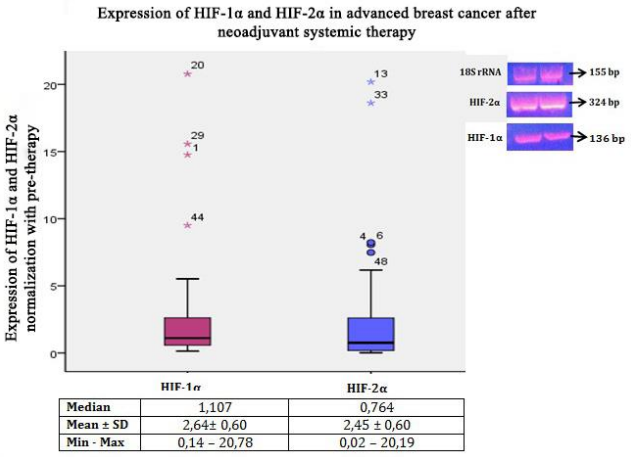


Figure 1. Expression of HIF-1α and HIF-2α in advanced breast cancer after systemic neoadjuvant therapy.

To be decreased in 28 samples, intermediate in 15 samples, and high in 5 samples (Figures 2 and 3). Based on the patient survival rates, most patients survived >2 years post-therapy (Figures 4 and 5). Based on age, the relative expressions of HIF-1α and HIF-2α were higher in patients aged >40 years than in those aged ≤40 years (Figures 6 and 7). Based on the histopathological characteristics, high expressions of HIF-1α and HIF-2α are commonly found in samples with lower histopathological grades (grades 1 and 2; Figures 8 and 9).

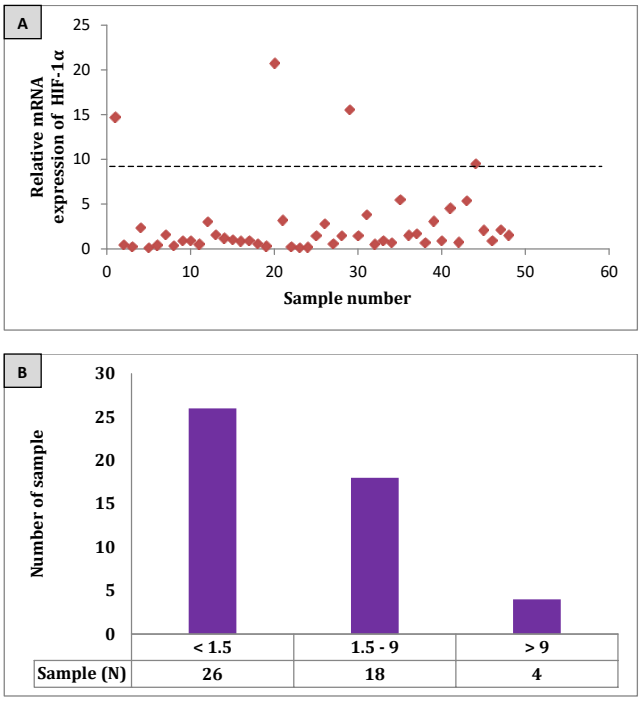
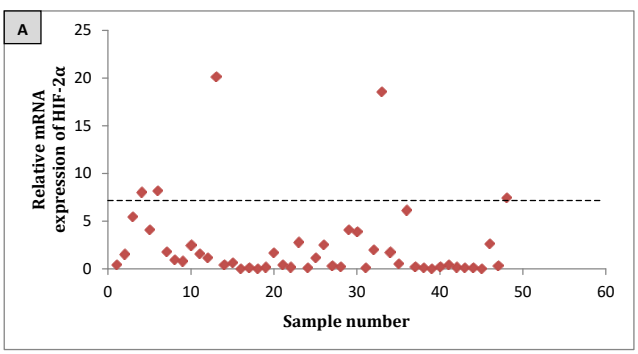


Figure 2. Distribution of expression of HIF-1α mRNA in advanced breast cancer tissue after systemic neodjuvant therapy



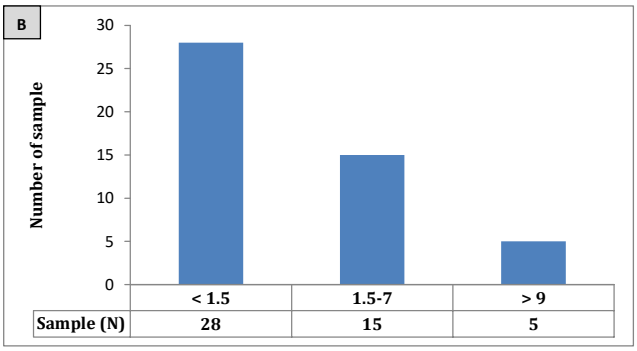


Figure 3. Distribution of expression of HIF-2α mRNA in advanced breast cancer tissue after systemic neodjuvant therapy

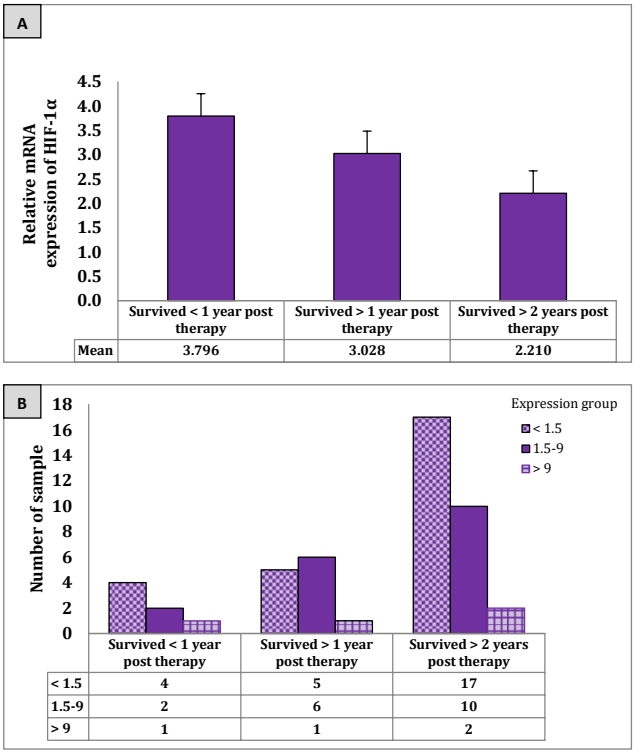


Figure 4. Expression of HIF-1α mRNA in advanced breast cancer patients after therapy based on survival (A). Distribution of HIF-1α expression in advanced breast cancer tissue after therapy based on survival (B)

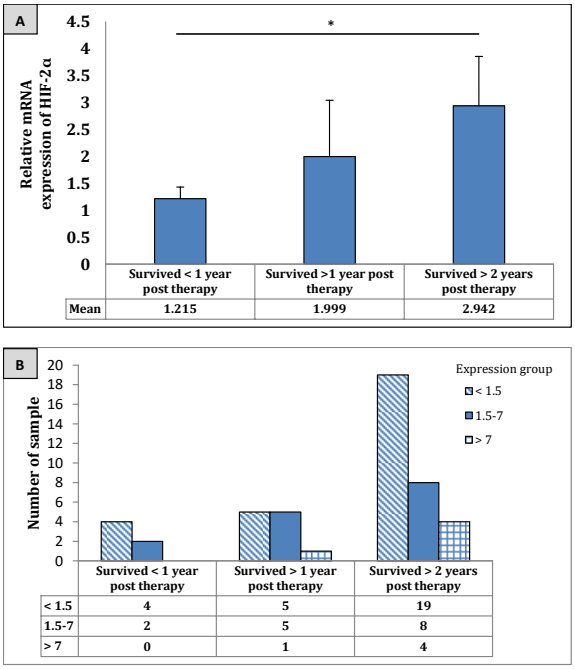


Figure 5. Expression of HIF-2α mRNA in advanced breast cancer patients after therapy based on survival (A). Distribution of HIF-2α mRNA in advanced breast cancer tissue after therapy based on survival (B)

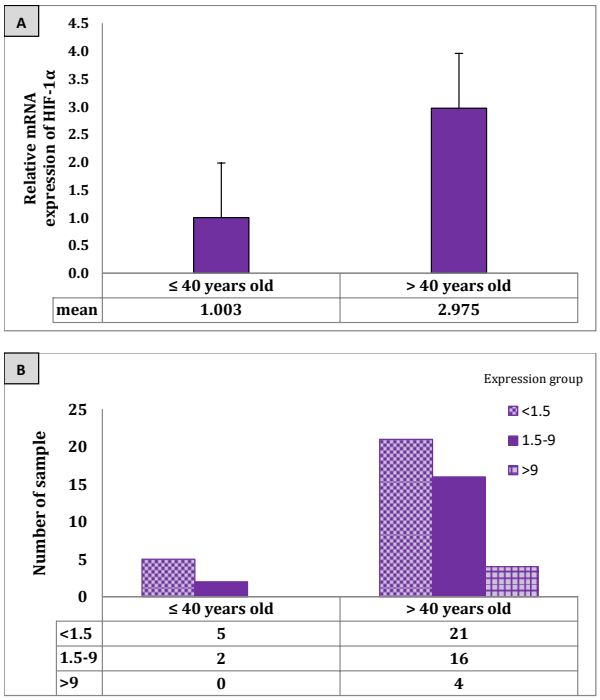


Figure 6. Expression of HIF-1α mRNA in advanced breast cancer patients after therapy based on age (A). Distribution of HIF-1α expression in advanced breast cancer tissue after therapy based on age (B)

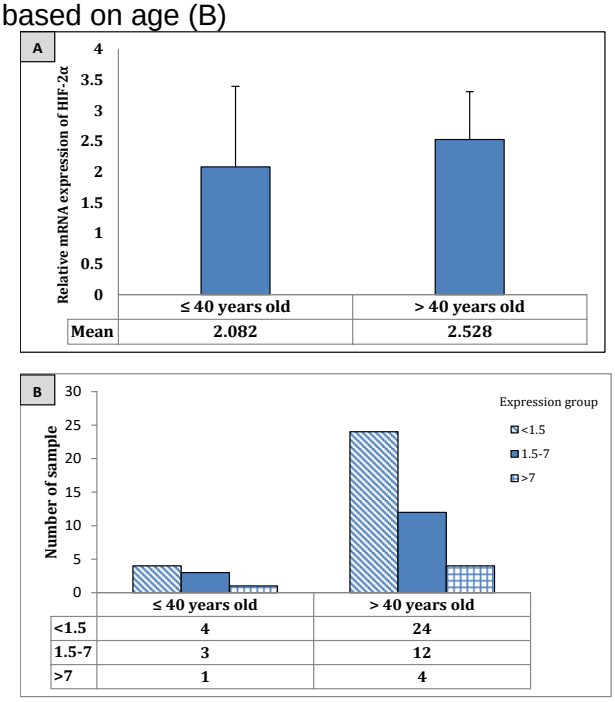


Figure 7. Expression of HIF-2α mRNA in advanced breast cancer patients after therapy based on age (A). Distribution of HIF-2α expression in advanced breast cancer tissue after therapy based on age (B)

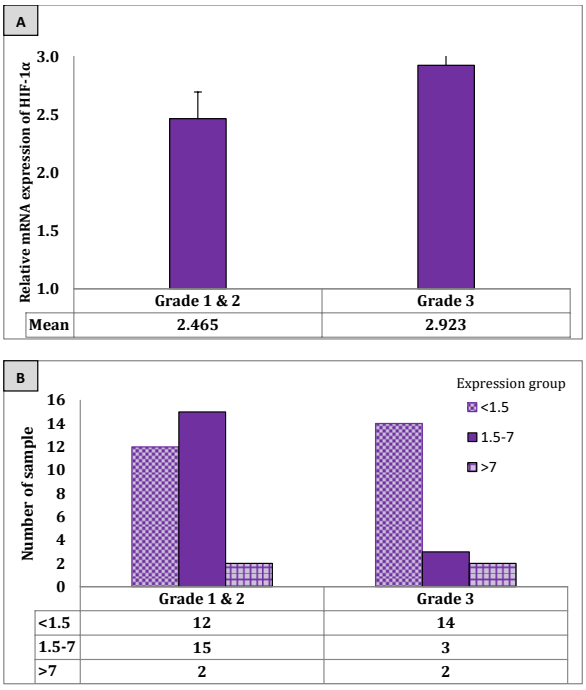


Figure 8. Expression of HIF-1α mRNA in advanced breast cancer patients after therapy

based on histopathological degree (A). Distribution of HIF-1α in advanced breast cancer tissue after therapy based on histopathological degree (B)

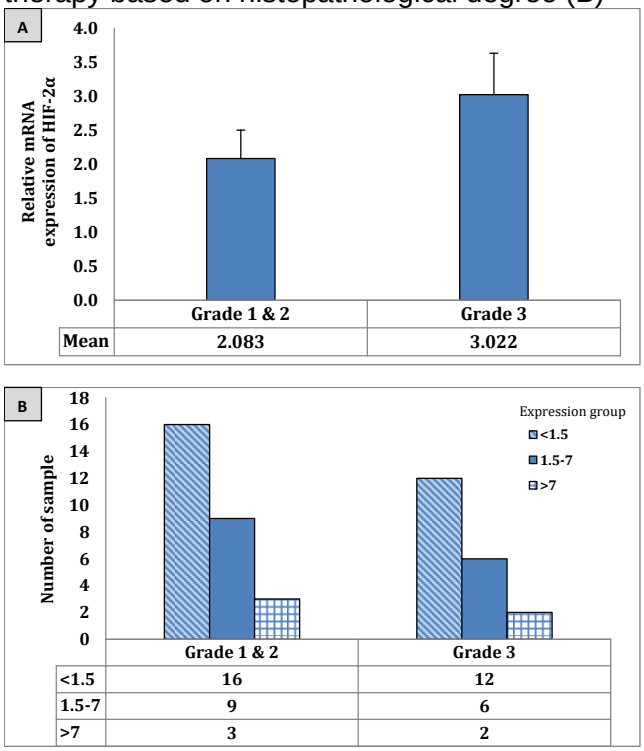


Figure 9. Expression of HIF-2α mRNA in advanced breast cancer patients after therapy based on histopathological grade (A). Distribution of HIF-2α expression in advanced breast cancer tissue after therapy based on histopathological grade (B)

Discussion

Hypoxia is one of the factors that affect the progression of cancer. Under hypoxic conditions, cancers behave more aggressively and become resistant to various treatments[8,9], such as radiation and some anticancer drug compounds, in part because hypoxic cancer cells are distant from the blood vessels that circulate the drugs to the target sites. In addition, HIF-1α and HIF-2α have been shown to mediate resistance to radiation, chemotherapy drug function, and inhibition of p53 activity.[10] The study of He et al. has shown that silencing HIF-2α with small interfering RNA (siRNA) is a potential strategy to improve the effects of doxorubicin on liver cancer.[11]

Significant differences between the expression levels of HIF-1α and HIF-2α were found between the samples before and after neoadjuvant therapy, whereas no significant difference was found between the samples treated with chemotherapy and those treated with hormonal therapy. The average relative expressions of HIF-1α and HIF-2α increased after therapy compared with those before treatment; however, no differences were observed based on the type of therapy used. More than 50% of the samples tested demonstrated low expression of HIF-1α (54.2%) and HIF-2α (58.3%) after therapy, and those with high expression likely represented resistant cases. Previous studies have demonstrated that HIF-2α expression is higher in advanced-stage cancers such as colorectal, gastric, and lung cancers.[6,12] It has been reported that the overexpression of HIF-1α is associated with poor prognosis in colorectal cancer, suggesting that HIF1α could be used as a biomarker to develop a potential target in cancer therapy.[13]

Scortegagna et al. has revealed that HIF-2α regulates the expression of certain antioxidant genes.[14] Another study comparing the ROS levels using dichlorofluorescein diacetate in control cells and HIF-2α knocked-down A498 cells demonstrated that cells with low HIF-2α levels had higher ROS levels.[15] They concluded that low concentrations of HIF-2α induce an increase in ROS. Their study has also suggested that HIF-2α inhibits p53 activation by limiting ROS levels and inhibiting the phosphorylation of p53. Moreover, the role of HIF-2α in regulating antioxidant enzymes that are involved in xenobiotic metabolic processes, such as SOD-1, SOD2, Gpx1, and catalase has been reported.[16] Bertout et al.[15] found that samples with high HIF-2α expression showed significantly increased expression of antioxidant-related genes, such as HMOX1, CP, and crystallin, and subsequent tracing demonstrated the role of HIF-2α in regulating redox status and suppressing cellular radiation responses in cancer cells.

Differences were also found between the expressions of HIF-2α and HIF-1α based on patient survival. The relative expression of HIF-2α gene was lower in patients who died within 1 year of treatment than that in patients who survived for >1 year. The tumor conditions of patients who died within 1 year aggravated to the point where hypoxia reduced because of blood vessel formation via angiogenesis, thus

supplying oxygen to the tumor environment. HIF-2α regulates high malignancy and increased pluripotency[17], whereas HIF-1α plays a role in the initial stages of hypoxia during which oxygen pressure is minimal as well as in the regulation of metabolism under acute hypoxic conditions by regulating the expression of genes such as GLUT, ALDH, and CA IX.[4,18] Unusually however, tumors of patients with stage 4 metastatic cancer demonstrated low expression of HIF-1α and HIF-2α despite their malignancy.

Substitution of roles between HIF-1α and HIF-2α occurs if oxygen pressure stabilizes at >5%.[19] In long-term hypoxia, the role of HIF-2α is more dominant than that of HIF-1α, as it enhances the regulation of a number of cancer markers, such as MMP9, Oct4, and CD 133.[20] A study by Koh suggested that prolonged hypoxic conditions activate hypoxia-associated factor (HAF), a known ubiquitin E3 ligase that plays a role in HIF-1α and HIF-2α exchange as well as proteasomal degradation.[21]

From the histopathological characteristics of the samples, we concluded that grade 3 samples had higher HIF-1a and HIF-2a expression than grades 1 or 2. HIF-2α has been detected in most chronic cancers such as those of the brain, bladder, breast, intestinal, ovarian, pancreatic, renal, and prostate cancers, but not in the normal surrounding tissues.[15,22] Clinically, high HIF-2α expression may serve as a marker for highly aggressive cancers that are not effectively treated (including breast, ovarian, cervical, oligoden-droglioma, esophageal, and oropharyngeal cancers).[11] In breast cancer, high expression of HIF-2α indicates increased therapeutic failure and mortality, even in cases with low histopathological classification.[23,24]

Conclusions

An increase in the relative expression of HIF1α and HIF-2α mRNA were found after neoadjuvant systemic therapy of advanced-stage breast cancer. Increased expressions of HIF-1α and HIF-2α after therapy were associated with low survival rates of breast cancer patients. Increased expressions of HIF-1α and HIF-2α were also found in breast cancer patients aged >40 years, and those with higher histopathological grades. Based on the results of this study, we could suggest that HIF-1α and HIF-2α expressions could be used as molecular biomarkers for the prediction of patient survival following neo-adjuvant therapy in advanced breast cancer. Further studies are required to improve breast cancer treatment outcomes. A study assessing the expressions of HIF-1α and HIF-2α in breast cancer tissues of young patients with low cancer stages is warranted.

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Declaration of Interest

The authors report no conflict of interest.

References

1. Coughlin SS, Ekwueme DU. Breast Cancer as A Global Health Concern. Cancer Epidemiol. 2009;33(5):315-8.
2. Saika K, Sobue T. Epidemiology of Breast Cancer in Japan and the US. JMAJ. 2009;52(1):39-44.
3. Thompson AM, Moulder-Thompson SL. Neoadjuvant Treatment of Breast Cancer. Ann Oncol. 2012;23(Suppl 10):x231–6.
4. Semenza GL. Regulation of Mammalian O2 Homeostasis by Hypoxia-Inducible Factor 1. Annu Rev Cell Dev Biol. 1999;15:551–78.
5. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Oberhuber G. Overexpression of Hypoxia-inducible Factor 1α Is a Marker for an Unfavorable Prognosis in Early-Stage Invasive Cervical Cancer. Cancer Res. 2000;60(17):4693-6.
6. Helczynska K, Larsson A-M, Holmquist Mengelbier L, et al. Hypoxia-Inducible Factor-2α Correlates to Distant Recurrence and Poor Outcome in Invasive Breast Cancer. Cancer Res. 2008;68(22):9212-20.
7. Howell LP, Gandour-Edwards R, O'Sullivan D. Application of the Scarff-Bloom-Richardson Tumor Grading System to Fine-Needle Aspirates of the Breast. Am J Clin Pathol. 1994;101(3):262-5.
8. Vaupel P, Harrison L. Tumor Hypoxia: Causative Factors, Compensatory Mechanisms, and Cellular Response. Oncologist. 2004;9(Suppl 5):4-9.
9. Vaupel P, Thews O, Hoeckel M. Treatment Resistance of Solid Tumors: Role of Hypoxia and Anemia. Med Oncol. 2001;18(4):243-59.
10. Harrison L, Blackwell K. Hypoxia and Anemia: Factors in Decreased Sensitivity to Radiation Therapy and Chemotherapy? Oncologist. 2004;9(Suppl 5):31-40.
11. He C, Sun X-P, Qiao H, et al. Downregulating Hypoxia-Inducible Factor-2α Improves the Efficacy of Doxorubicin in the Treatment of Hepatocellular Carcinoma. Cancer Sci. 2012;103(3):528–34.
12. Rakha EA, El-Sayed ME, Lee AH, et al. Prognostic Significance of Nottingham Histologic Grade in Invasive Breast Carcinoma. J Clin Oncol. 2008;26(19):3153–8.
13. Baba Y, Nosho K, Shima K, et al. HIF1A Overexpression is Associated with Poor Prognosis in a Cohort of 731 Colorectal Cancers. Am J Pathol. 2010;176(5):2292–301.

14. Scortegagna M, Morris MA, Oktay Y, Bennett M, Garcia J. The HIF Family Member EPAS1/HIF-2α is Required For Normal Hematopoiesis in Mice. Blood. 2003;102(5):1634-40.
15. Bertout JA, Majmundar AJ, Gordan JD, et al. HIF2α Inhibition Promotes p53 Pathway Activity, Tumor Cell Death, and Radiation Responses. Proc Natl Acad Sci U S A. 2009;106(34):14391–6.
16. Patel SA, Simon MC. Biology of Hypoxia-Inducible Factor–2α in Development and Desease. Cell Death Differ. 2008;15(4):628-34.
17. Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD. Hypoxia Inducible Factors Regulate Plorypotency and Proliferation in Human Embryonic Stem Cell Cultured at Reduced Oxygen Tension. Reproduction. 2010;139(1):85-97.
18. Wang GL, Jiang BH, Rue EA, Semenza GL. HIF-1 is A Basic Helix-Loop-Helix-PAS Hetrodimer Regulated by Cellular O2 Tension. Proc Natl Acad Sci U S A. 1995;92(12):5510-4.
19. Loboda A, Jozkowicks A, Dulak J. HIF-1 and HIF-2 Transcription Factor Smiliar But No Identical. Mol Cells. 2010;29(5):435-42.
20. Seidel S, Garvalov BK, Wirta V, et al. A Hypoxic Niche Regulates Glioblastoma Stem Cell Through HIF-2. Brain. 2010;133(Pt 4):983-95.
21. Koh MY, Darnay BG, Powis G. Hypoxia-Associated Factor, a Novel E3-Ubiquitin Ligase, Binds and Ubiquitinates Hypoxia-Inducible Factor 1α, Leading to Its Oxygen-Independent Degradation. Mol Cell Biol. 2008;28(23):7081–95.
22. Wiesener MS, Jugensen JS, Rosenberg C, et al. Widespread HIF2 Alfa in Distinct Cell Population of Different Organ. FASEB J. 2003;17(2);271-3.
23. Gilkes DM, Semenza GL. Role of Hypoxia-Inducible Factors in Breast Cancer Metastasis. Future Oncol. 2013;9(11):1623-36.
24. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W. Chemokine Receptor CXCR4 Downregulated by von Hippel–Lindau Tumour Suppressor pVHL. Nature. 2003;425(6955):307–11.