Original Article
Regulatory role and mechanism of NAC1 in cancer cell survival subjected to hypoxia

Yanfeng Lu[1], Lina Lei[1], Yongyong Zhang[1], Ruonan Liu[2], Xiuzhen Xue[1], Lei Li[2]

[1]Department of Obstetrics and Gynecology, The First Affiliated Hospital of Henan University of Science and Technology, Luoyang 471000, Henan, People's Republic of China; [2]Department of Gynecology, Cancer Hospital Affiliated to Zhengzhou University, Henan Cancer Hospital, Zhengzhou 450008, Henan, People's Republic of China

Abstract: In order to study the impact of nucleus accumbens-1 (NAC1) expression on survival in cancer cell and the molecular mechanism involved. NAC1 has been observed to be up-regulated in human cancer, and to play an important role in progression and recurrence of ovarian cancer. In this study, we investigated the regulatory role of NAC1 in glycolysis and the effect of the NAC1-mediated glycolysis on cancer cell survival. Cell biology, molecular biology, and pharmacology approaches and methods were used in this study. We demonstrated: 1) NAC1 is a critical regulator of cancer cell survival under hypoxic condition. Silencing of NAC1 leads to increase in the cleaved caspase-3 and cleaved PARP, and a decline in cell viability and colony formation when the cells were subjected to hypoxia; 2) NAC1 is a novel regulator of glycolysis. Depletion of NAC1 leads to an increase in the concentration of glucose in the culture medium, a decrease in the level of intercellular lactate; 3) NAC1 enhances the stability and protein level of HIF-1α. Under hypoxia, NAC1 promotes cancer cell survival and glycolysis through strengthening the stability of HIF-1α.

Keywords: Cancer cell, survival, hypoxia, glycolysis, NAC1

Introduction

Tumor tissues are always in a hypoxic environment, which is one of the most significant differences comparing to normal tissues. It is due to the high oxygen requirement to ensure the excessive proliferation, high metabolic rate, autophagy and apoptosis of tumor cells that cause tissue hypoxia. In addition to high consumption, lack of oxygen is another reason for hypoxia. For instance, the discouraged spread of oxygen from blood vessels into tumor tissue, which caused by irregular tumor cells arrangement, the anemia and decreased oxygen carrying capacity of red blood cells result in a lack of oxygen, leading to a persistent hypoxia microenvironment within the tumor tissue [1-3]. The hypoxic tumor cells can keep alive through a variety of adaptive ways, such as upregulation of some growth factors, like platelet-derived growth factor B (PDGF-B), transforming growth factor β (TGF-β) [4, 5], insulin-like growth factor 2 (IGF-2) and epidermal growth factor (EGF),

expression of tumor invasion protein likeurokinase-type plasminogen activator [6-8]. Taken together, depth investigations about the metabolic characteristics of hypoxic tumor cells and related gene expression will help us to understand the effect of abnormal metabolismon tumor biology, and also provide theoretical basis for the treatment of tumor through intervention and correction of abnormal metabolism.

Glycolysis is the most significant feature of tumor cells and widely found in various tumor tissues, which promotes proliferation and invasion of tumor cells and involves in drug resistance [9-11]. In the 50s of last century, Warburg et al. [12] proposed "Warburg effect" firstly, indicating that it was through glycolysis that tumor cells got ATP and produced large amount of lactic acid. When the diameter is larger than 1 mm, growth of tumor is restricted by limited blood vessels. Then transport of oxygen and nutrients are discouraged, resulting in a hypox-

ic microenvironment of chronic diffusion disorder. At the same time, the energy metabolism of tumor cells transforms from oxidative phosphorylation to glycolysis under effects of hypoxia, oncogene activation and tumor suppressor gene inactivation, promoting survival and proliferation of tumor cells in a unique microenvironment [13, 14].

Nucleus accumbens-associated protein 1 (NAC1) is a nuclear transcription factor encoded by NACC1. K. Nakayama et al. [15] demonstrated the correlation between NAC1 and tumor at first. They found that NAC1 was a family member of BTB/POZ, and the highly homologous domain with BTB/POZ is essential for confirmation of NAC1 dimeric. At present, many studies elaborated the mechanisms of tumor formation and development promoted by NAC1. (1) NAC1 benefits to the survival of tumor cells by suppression of GADD45 transcription, which is related to the resistance to paclitaxel treatment [16]. (2) NAC1 increases drug resistance and regulates fatty acid metabolism through upregulating fatty acid synthase (FASN) [17]. (3) NAC1 inhibits the transcription of MIZ1 target gene, cell cycle-related gene p21Cip1, by binding with MIZI [18]. (4) NAC1 involves in regulation of HMGB1-mediated autophagy and Δ Np63 mediated cellular aging escape [19, 20]. However, it is still unclear about the relationship between NAC1 and tumor cell survival subject to hypoxic. Since NAC1 is widely involved in tumor proliferation, malignant transformation, and drug resistance, we suspect that it can also facilitate tumor formation and development by regulating tumor cell survival under hypoxic condition. To verify our hypothesis, we will explore the following aspects in this study. (1) The effect of NAC1 on tumor cell survival under hypoxic condition. (2) Whether the pro-survival effect of NAC1 is related to glycolysis activation and the potential regulatory mechanism.

Materials and methods

Materials Human breast cancer cell line MCF-7 was purchased from ATCC (USA), RPMI 1640 medium with high glucose and fetal bovine serum (FBS) were obtained from GiBco (USA); Opti-MEM culture medium optimization and Lipofectamine 2000 liposomal transfection reagents were purchased from Invitrogen (USA); BCA Protein Assay Kit was from Thermo (USA);

Crystal violet solution came from Beyotime Institute of Biotechnology; Cleaved PARP antibody and Cleaved Caspase-3 antibody were purchased from Cell Signaling (USA); β-actin antibodies were obtained from Santa Cruz (USA); NAC1 antibody was from Novus (USA); NAC1 siRNA and Negative control were purchased from Shanghai GenePharma Co.,Ltd; Western Blot related reagents were from American Bio-Rad (USA); Glucose Assay Kit & L-Lactate Assay Kit came from Bioassay Systems, Cycloheximide was purchased from Sigma (USA).

Cell viability assay

Cell viability was measured by MTT assay. Briefly, cells were plated in 96-well plates at a density of 5×10^3 cells per well for different treatments and incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air. When the treatment was finished, culture medium in each well was aspirated, 100 μL fresh culture medium and 30 μL MTT reagent were added. After 4 h-incubation at 37°C, MTT residual was removed and 100 μL DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm to determine the cell viability.

Cell colony formation

Under normoxic or hypoxic environment, cells subjected to different treatments were prepared into suspension. Cell suspension was diluted by times. 2×10^3 cells were inoculated in a 35 mm diameter petri dish and placed in 5% CO_2 incubator at 37°C. After 14 days-incubation, cells were removed, the supernatant was discarded, and cells were washed gently 3 times with PBS, and then stained for 10 min with crystal violet, washed 3 times with PBS. After inversion dry, it can be photographed.

Establishment of hypoxia model

When cells in culture flasks were cultured to a certain density, culture solution was removed and cells were washed with sterile PBS again, and then added to fresh 1640 medium, the flasks were placed in a closed hypoxic culture device, Inward into gas mixture containing 95% of nitrogen (N_2)/5% carbon dioxide (CO_2) for about 7 minutes to reach the state of 1% O_2, and then the hypoxic culture device was placed in the incubator at 37°C.
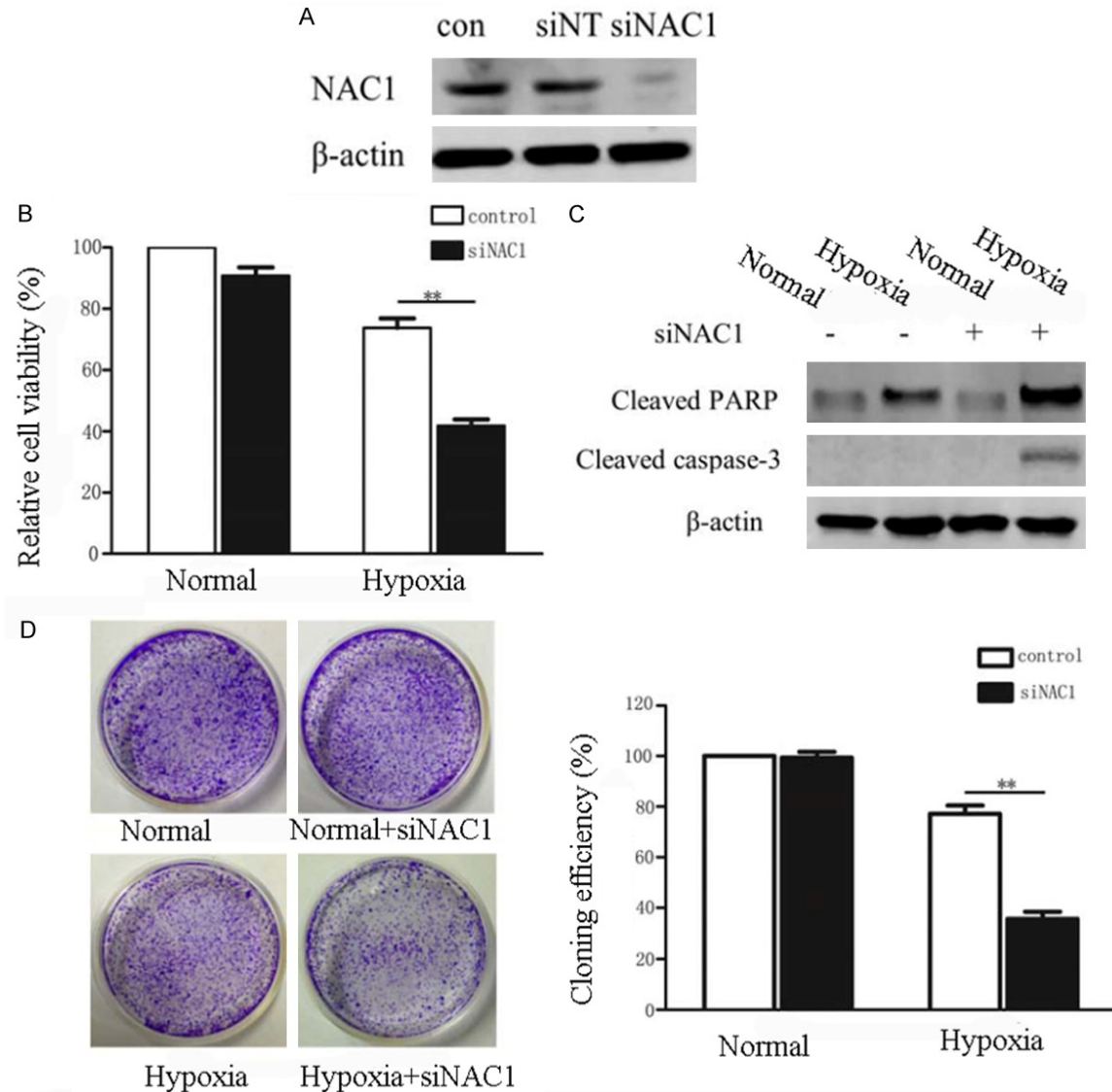
Figure 1. NAC1 promotes cancer cell survival subjected to hypoxia. A. MCF-7 cells were transfected with siNT and siNAC1, respectively, and then the expression of NAC1 in MCF-7 cells was detected. B. After 12 hours of cell culture under normoxic or hypoxic conditions, the viability of MCF-7 cells transfected with siNT or siNAC1 was detected using the MTT methods. C. After 12 hours of cell culture under normoxic or hypoxic conditions, the expressions of apoptotic proteins Cleave PARP and Cleaved capase-3 in MCF-7 cells transfected with siNT or siNAC1 were detected by Western blot. D. After 12 hours of cell culture under normoxic or hypoxic conditions, the colony formation ability of MCF-7 cells transfected with siNT or siNAC1 was detected by crystal violet staining assay. **P<0.01.

Transfection

SiRNA targeting NAC1 and scrambled control siRNA were designed and synthesized by GenePharma (GenePharma, Shanhai, China). The day before transfection, count and plate 1 × 10^5 cells per well. Cells were transfected with siRNA using Oliofectamine according to manufacturer's instruction. Medium was refreshed 4-6 hours after transfection. The cells were continued to incubate for the downstream experiments. The primer sequence was as follows: Sense: 5-GAGGAAGAACUCGGUGCCCUU-CUCCAU-3'.

Western blot analysis

To determine the expression levels of cleaved-caspase-3 and PARP in MCF-7, western blot analyses were carried out as previously described. The cell sample was ruptured with M-PER mammalian protein extraction reagent

(Pierce), centrifuged for 10 min at 12000 × g and 4°C, and the supernatants were collected. Protein concentration was determined by Bradford assay. Before electrophoresis, 30-50 μg protein was fully denatured in boiled water. Then the cell sample supernatants containing equal amounts of denatured protein were resolved by 8% SDS-polyacrylamide gel electrophoresis. Then, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. These membranes were blocked with 3% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and probed overnight at 4°C with the appropriate primary antibodies, Then, the membranes were rinsed with TBST before a 1 h incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were washed in TBST solution with 0.5% Tween-20 before chemiluminescence (ECL) detection.

Lactate and glucose assay in MCF-7 cells

Intracellular was assayed by EnzyChrom™ Lactate Aaasy Kit (BioAssay Systems) according to manufacturer's instruction. 10^6 cells were prepared in each group. Lactate in cell specifically reacts with an enzyme mix to generate a product, which interacts with lactate probe to produce color, and detected at 565 nm. Glucose levels in culture medium were determined using a glucose assay kit (BioAssay Systems). Glucose oxidized by glucose oxidase, while reacted with coloring agent. And reaction product was detected at 565 nm.

Statistical analysis

The data is analyzed by SPSS19.0 Statistical values are presented as means ± standard error of the mean. For calculation of significance in differences, Student's t-test or, if possible, a paired t-test was used. In the figures shown, a significance level of $P<0.05$ is marked with * and $P<0.01$ with **.

Results

The effect of NAC1 on viability of MCF-7 under hypoxia

To investigate the effects of NAC1 on viability of MCF-7 cell under hypoxia, the expression of NAC1 in MCF-7 was silenced by specific RNA sequence. The data showed that the expression of NAC1 was depressed after RNA interference (**Figure 1A**). We further studied the capacity of proliferation of MCF-7 cell under normal condition and hypoxic environment. The results of MTT assay indicated that the proliferation of MCF-7 cell under hypoxic environment was decreased significantly in the group that NAC1 gene was silenced (NAC1 siRNA group) compared to control group (siNT group), while under normal condition, there was no significant difference between NAC1 siRNA group and siNT group (**Figure 1B**). Clonegenic assay result also showed that MCF-7 cell of NAC1 siRNA group showed decreased clone formation ability. Expression of cleaved-caspase-3 and PARP were determined by western blot that showed a remarkable increase in NAC1 siRNA group under hypoxic environment (**Figure 1C**, **1D**). Altogether, we concluded from these results that NAC1 contributes to cell viability under hypoxia.

The influence of NAC1 on glycolysis of tumor cells under hypoxic environment

The above results suggested that NAC1 could promote the proliferation and survival of tumor cells under hypoxic conditions. However, the growth and proliferation of tumor cells require adequate energy and nutrient supply, and glycolysis is the main process to obtain energy for tumor cells under hypoxic conditions. Therefore, we proposed the hypothesis that whether NAC1 could promote the glycolysis of tumor cells to exert the survival-promoting effects under hypoxic conditions. To test this hypothesis, firstly, we analyzed and compared the glucose consumption and lactic acid production in tumor cell culture medium between NAC1 expression group and NAC1 silence group. We found that after 24 hours of MCF-7 cell culture under hypoxic conditions (1% O_2), in comparison with the control group, the glucose consumption and lactic acid production in the NAC1 silence group were significantly reduced (**Figure 2A** and **2B**).

The influence of NAC1 on the expression and stability of tumor cell HIF-1α protein under hypoxic conditions

The above results indicated that NAC1 could promote the glycolysis of tumor cells under hypoxic conditions. According to the reports of prior researches, HIF-1α played a critical role in the regulation of tumor cell's glycolysis [21].
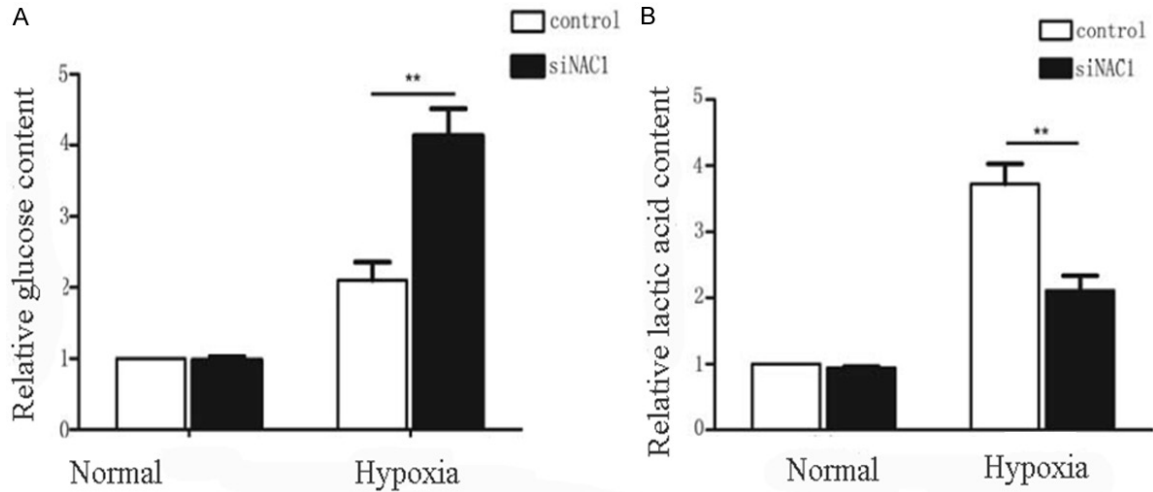
Figure 2. NAC1 promotes the glycolysis of tumor cells under hypoxic conditions. After 24 hours of cell culture under normoxic or hypoxic conditions, the amount of glucose consumption and lactic acid production were analyzed for MCF-7 cells transfected with siNT or siNAC1, respectively. **P<0.01.
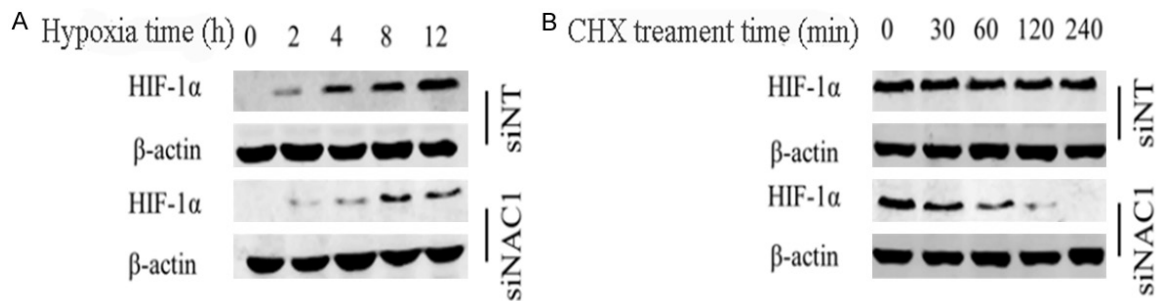

Figure 3. NAC1 stabilize the protein level of HIF-1α. A. MCF-7 cells were transfected with siNT or siNAC1 and placed under hypoxic conditions for 0, 2, 4, 8, 12 hours, respectively. Then the expression of HIF-1α protein was analyzed by Western blot. B. MCF-7 cells transfected with siNT or siNAC1 were placed under hypoxic conditions for 6 hours and then treated with CHX (20 µg/ml). Then the expression of HIF-1α protein was analyzed by Western blot.

Therefore, we proposed that NAC1 might promote tumor cell's glycolysis through HIF-1α protein. To test the hypothesis, firstly we examined the influence of NAC1 on HIF-1α protein expression under hypoxic conditions. Our results showed that the expression of HIF-1α proteins was reduced markedly in NAC1 silence group compared with control group (**Figure 3A**), and the down-regulation of HIF-1α protein expression might be attributable to the decrease of HIF-1α gene transcription or the increase of its degradation. We then applied actidione (CHX) treatment to MCF-7 cells in the control group and NAC1 silence group, respectively, and found that the stability of HIF-1α protein in the NAC1 silence group was decreased significantly (**Figure 3B**). The above findings suggested that NAC1 promoted the tumor cell glycolysis

under hypoxic conditions by enhancing the stability of HIF-1α proteins.

Discussion

As a key regulator of glycolysis, on one hand, HIF-α could reprogram the energy metabolism pathways of hypoxic tumor cells to increase tumor cells' adaptability to hypoxic environment. These pathways include promoting the phosphorylation of PDK1 to inhibit PDH activity and subsequently inhibit TCA cycle. On the other hand, HIF-α activates the transcription of relevant oncogenes and growth factors (such as VEGF) [25]. Therefore, the HIF-1α-induced glycolysis is an important mechanism that promotes the development and progression of cancer. In this study we found that silence of NAC1 could inhibit the ability of tumor cells to

survive under hypoxic conditions, while there was no obvious effect under normoxic conditions. This might be attributable to the differences of HIF-1α expression between hypoxic and normoxic conditions.

In summary, our current study found a new tumor glycolysis regulator NAC1, and we found that NAC1 could maintain tumor cells adaptation to hypoxic environment by promoting the expression of HIF-1α and enhancing its stability, which has a critical role in inhibiting stress-induced apoptosis of tumor cells. Our findings had important clinical relevance, which may help to further understand the molecular mechanisms of nuclear transcription factor NAC1 in the tumor cell's adaptive survival under hypoxic conditions, and provide a potential target of cancer treatment and prevention in clinical settings.

The specific energy metabolism patterns of tumor cells were considered closely related to the development and progression of cancers. Yet until now, the exact molecular mechanism and signal pathways regulating energy metabolism of tumor cells are not fully understood. In this study, we firstly revealed a new glycolysis regulator of tumor cells-NAC1, and found NAC1 mediated glycolysis activation was closely related to the survival of tumor cells under hypoxic conditions. Our study suggested that NAC1 could facilitate the adaptive survival of tumor cells under hypoxic environment.

As a potential carcinogen, prior studies have proved that NAC1 was highly expressed in ovarian cancer, cervical cancer, breast cancer and liver cancer, and the high expression of NAC1 was closely related to the malignant progression, recurrence and prognosis of ovarian cancer and cervical cancer [15, 22-24]. Yi Zhang et al. also found that NAC1 could reduce the sensitivity of tumor cells to cisplatin treatment by regulating HMGB mediated autophagy, but the relationship between NAC1 and tumor energy metabolism was not made clear in their study [19]. Therefore, out study was the first one that found NAC1 could promote the tumor cell's glycolysis and adaptive survival ability.

As a key regulator of glycolysis, on one hand, HIF-1α could reprogram the energy metabolism pathways of hypoxic tumor cells to increase tumor cells' adaptability to hypoxic environ-

ment. These pathways include promoting the phosphorylation of PDK1 to inhibit PDH activity and subsequently inhibit TCA cycle. On the other hand, HIF-1α activates the transcription of relevant oncogenes and growth factors (such as VEGF) [25]. Therefore, the HIF-1α-induced glycolysis is an important mechanism that promotes the development and progression of cancer. In this study we found that silence of NAC1 could inhibit the ability of tumor cells to survive under hypoxic conditions, while there was no obvious effect under normoxic conditions. This might be attributable to the differences of HIF-1α expression between hypoxic and normoxic conditions.

In summary, our current study found a new tumor glycolysis regulator NAC1, and we found that NAC1 could maintain tumor cell's adaptation to hypoxic environment by promoting the expression of HIF-1α and enhancing its stability, which has a critical role in inhibiting stress-induced apoptosis of tumor cells. Our findings had important clinical relevance, which may help to further understand the molecular mechanisms of nuclear transcription factor NAC1 in the tumor cell's adaptive survival under hypoxic conditions, and provide a potential target of cancer treatment and prevention in clinical settings.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanfeng Lu, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Henan University of Science and Technology, Luoyang 471000, Henan, People's Republic of China. E-mail: yanfenglu221@126.com

References

[1] Vaupel P, Mayer A, Hockel M. Tumor hypoxia and malignant progression. Methods Enzymol 2004; 381: 335-354.

[2] Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001; 93: 266-276.

[3] Vaupel P, Thews O, Hoeckel M. Treatment resistance of solid tumors: role of hypoxia and anemia. Med Oncol 2001; 18: 243-259.

[4] Vaupel P, Harrison L. Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. Oncologist 2004; 9 Suppl 5: 4-9.

[5] Zhang SX, Gozal D, Sachleben LJ Jr, Rane M, Klein JB, Gozal E. Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. FASEB J 2003; 17: 1709-1711.

[6] Conti E, Musumeci MB, De Giusti M, Dito E, Mastromarino V, Autore C, Volpe M. IGF-1 and atherothrombosis: relevance to pathophysiology and therapy. Clin Sci (Lond) 2011; 120: 377-402.

[7] Rofstad EK, Rasmussen H, Galappathi K, Mathiesen B, Nilsen K, Graff BA. Hypoxia promotes lymph node metastasis in human melanoma xenografts by up-regulating the urokinase-type plasminogen activator receptor. Cancer Res 2002; 62: 1847-1853.

[8] Kothari S, Cizeau J, Mcmillan-Ward E, Israels SJ, Bailes M, Ens K, Kirshenbaum LA, Gibson SB. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene 2003; 22: 4734-4744.

[9] Vander HM, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324: 1029-1033.

[10] Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L, Bhalla KN, Keating MJ, Huang P. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia. Cancer Res 2005; 65: 613-621.

[11] Deberardinis RJ. Is cancer a disease of abnormal cellular metabolism? New angles on an old idea. Genet Med 2008; 10: 767-777.

[12] Warburg O. On the origin of cancer cells. Science 1956; 123: 309-314.

[13] Bartrons R, Caro J. Hypoxia, glucose metabolism and the Warburg's effect. J Bioenerget Biomembr 2007; 39: 223-229.

[14] Airley RE, Mobasheri A. Hypoxic regulation of glucose transport, anaerobic metabolism and angiogenesis in cancer: Novel pathways and targets for anticancer therapeutics. Chemotherapy 2007; 53: 233-256.

[15] Nakayama K, Nakayama N, Davidson B, Sheu JJ, Jinawath N, Santillan A, Salani R, Bristow RE, Morin PJ, Kurman RJ, Wang TL, Shih IeM. A BTB/POZ protein, NAC-1, is related to tumor recurrence and is essential for tumor growth and survival. Proc Natl Acad Sci U S A 2006; 103: 18739-18744.

[16] Jinawath N, Vasoontara C, Yap KL, Thiaville MM, Nakayama K, Wang TL, Shih IM. NAC-1, a potential stem cell pluripotency factor, contributes to paclitaxel resistance in ovarian cancer through inactivating Gadd45 pathway. Oncogene 2009; 28: 1941-1948.

[17] Rahman MT, Nakayama K, Rahman M, Katagiri H, Katagiri A, Ishibashi T, Ishikawa M, Iida K, Nakayama N, Otsuki Y, Nakayama S, Miyazaki K. Fatty acid synthase expression associated with NAC1 is a potential therapeutic target in ovarian clear cell carcinomas. Br J Cancer 2012; 107: 300-307.

[18] Stead MA, Wright SC. Nac1 interacts with the POZ-domain transcription factor, Miz1. Biosci Rep 2014; 34.

[19] Zhang Y, Cheng Y, Ren X, Zhang L, Yap KL, Wu H, Patel R, Liu D, Qin ZH, Shih IM, Yang JM. NAC1 modulates sensitivity of ovarian cancer cells to cisplatin by altering the HMGB1-mediated autophagic response. Oncogene 2012; 31: 1055-1064.

[20] Zhang Y, Cheng Y, Ren X, Hori T, Huber-Keener KJ, Zhang L, Yap KL, Liu D, Shantz L, Qin ZH, Zhang S, Wang J, Wang HG, Shih IeM, Yang JM. Dysfunction of nucleus accumbens-1 activates cellular senescence and inhibits tumor cell proliferation and oncogenesis. Cancer Res 2012; 72: 4262-4275.

[21] Prigione A, Rohwer N, Hoffmann S, Mlody B, Drews K, Bukowiecki R, Blümlein K, Wanker EE, Ralser M, Cramer T, Adjaye J. HIF1alpha modulates cell fate reprogramming through early glycolytic shift and upregulation of PDK1-3 and PKM2. Stem Cells 2014; 32: 364-376.

[22] Nakayama K, Nakayama N, Wang TL, Shih IeM. NAC-1 controls cell growth and survival by repressing transcription of Gadd45GIP1, a candidate tumor suppressor. Cancer Res 2007; 67: 8058-8064.

[23] Nakayama K, Nakayama N, Davidson B, Katabuchi H, Kurman RJ, Velculescu VE, Shih IeM, Wang TL. Homozygous deletion of MKK4 in ovarian serous carcinoma. Cancer Biol Ther 2006; 5: 630-634.

[24] Yeasmin S, Nakayama K, Ishibashi M, Katagiri A, Iida K, Purwana IN, Nakayama N, Miyazaki K. Expression of the bric-a-brac tramtrack broad complex protein NAC-1 in cervical carcinomas seems to correlate with poorer prognosis. Clin Cancer Res 2008; 14: 1686-1691.

[25] Giatromanolaki A, Sivridis E, Kouskoukis C, Gatter KC, Harris AL, Koukourakis MI. Hypoxia-inducible factors 1alpha and 2alpha are related to vascular endothelial growth factor expression and a poorer prognosis in nodular malignant melanomas of the skin. Melanoma Res 2003; 13: 493-501.