Original Article
Hypoxia promotes proliferation of pituitary adenomas by HIF-1α/ALKBH5 signaling *in vitro*

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Abstract: Hypoxia is a common phenomenon in pituitary adenomas (PAs). The role and mechanism of hypoxia in the PAs remains elusive. This work aimed to explore the effect of hypoxia on PAs in vitro. PA cells GT1-1 were cultured and treated under hypoxic condition. Cell proliferation assay showed the proliferation of PA cells was increased significantly by hypoxia treatment, with a peak at 12 hours. qPCR and western blot indicated that the expression of HIF-1α, ALKBH5, and Nanog were elevated by hypoxia stimuli. In conclusion, our funding demonstrated that hypoxia could increase Nanog expression through HIF-1α/ALKBH5 signaling, thereby promoting the proliferation of PA cells.

Keywords: Pituitary adenoma, hypoxia, HIF-1α, ALKBH5, Nanog

Introduction

Pituitary adenoma (PA) is one of the most common intracranial benign tumors [1]. Unlike other neoplasms, most PAs are hypovascular, with a lower oxygen supply compared to adjacent normal tissues [2]. Studies have demonstrated the growth and invasion of PAs were related to hypoxia [3-6].

As the central transcriptional regulator for hypoxic response, hypoxia inducible factors 1α (HIF-1α) is involved in almost all biochemical and metabolic regulation for adapting to an oxygen-deficient environment [7]. HIF-1α is reportedly concerned with the growth and invasion of PAs [3-6].

N6-methyladenosine (m6A) modification of mRNA plays critical effect in the initiation and progression of various cancers [8]. AlkB homolog 5 (ALKBH5), a demethylating catalyzer in m6A, was confirmed to be the direct target of HIF-1α [9]. Recently, research reported it could promote the progression of breast cancer in a HIF-1α dependent manner [10].

However, the role of HIF-1α and ALKBH5 in PAs is unclear. Therefore, the current study explores the effect of hypoxia on proliferation as well as the expression of HIF-1α, ALKBH5, and Nanog in PA cells GT1-1 in vitro.

Material and methods

Cell culture

Mouse GT1-1 cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium/High Glucose (DMEM/High Glucose, Hyclone) containing 10% fetal bovine serum (FBS, Biological Industries, 04-001-1A), at 37°C, 5% CO_2. For hypoxic culture, cells in the logarithmic growth phase were cultured in a hypoxic incubator with condition of 37°C 94% N_2, 5% CO_2 and 1% O_2.
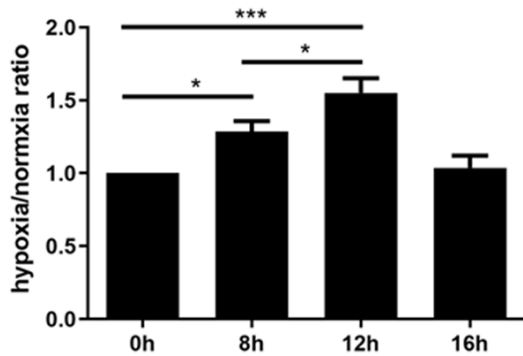
Figure 1. Effect of hypoxia on the proliferation of GT1-1 cells in vitro. *$P<0.05$, ***$P<0.001$.

Cell proliferation assay

The effect of hypoxia on cell proliferation was detected by Counting Kit-8 (CCK-8, Beyotime, C00037). After hypoxia treatment for different periods, CCK-8 reagent was added according to manufacturer's instructions, and incubation continued for another 1.5 hours. The absorbance (OD value) was read at 450 nm by enzyme labeling instrument (Spectra Max M5). After collecting data, cell proliferation rate was counted by following formula:

$$Cell\ proliferation\ rate = \frac{OD_{hypoxia} - OD_{blank}}{OD_{control} - OD_{blank}}$$

Quantitative real-time polymerase chain reaction (q-PCR)

q-PCR was carried out to explore the mechanism of hypoxia treatment on PA cells. Total RNA was extracted with Trizol RNA extraction kit (TRNzol-A⁺, TIANGEN, China). Q-PCR was performed according to the kit instructions with following cycling conditions: 60 sec at 95°C, followed by 40 cycles of 15 sec at 95°C and 15 sec at 60°C. The expression levels of HIF-α/Alkbh5/Nanog were normalized to GAPDH.

The primers were as below: HIF-1α F 5'-TAGATTTGGAGATGCTGGCTCC-3' R 5'-AACTGTGCTCATACTTGGAGGG-3' Alkbh5 F 5'-ATCGTGTCCGTGTCTTTCTTCA-3' R 5'-TTTCATCAGCAGCATACCCACT-3' Nanog F 5'-AACCAAAGGATGAAGTGCAAGC-3' R 5'-TCCAGATGCGTTCACCAGATAG-3' GAPDH F 5'-AGTGTGACGTTGACATCCGT-3' R 5'-GCAGCTCAGTAACAGTCCCC-3'.

Western blot

The expression of HIF-α, ALKBHh5, and Nanog protein were investigated by western blot. Cells were collected and total proteins were extracted in RIPA lysis buffer (Meilunbio MA0001). After quantification by BCA protein assay kit (P0001 Beytotime), proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Next, membranes were sealed, labeled with primary antibodies (overnight at 4°C), and then corresponding secondary antibodies (90 min at room temperature). Finally, proteins were detected under gel imaging system by ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using Image J software.

Primary antibodies included: anti-HIF-1α (1:2000, Abcam), anti-Alkbh5 (1:2000, Abcam), anti-Nanog (1:2000, Abcam) and anti-β-actin (1:6000, Santa Cruz).

Statistical analysis

All data were verified by at least three independent trials, and analyzed with SPSS 18.0 (Chicago, IL). Quantative data was expressed as means ± SD. Statistical analysis was performed using one-way ANOVA. The threshold value for the statistical significance was set at $P<0.05$.

Results

Hypoxia promotes the proliferation of PA cells

The proliferation of GT1-1 cells increased significantly in hypoxic circumstance as a function of time, and reached peak at 12 hours, as shown by a cell proliferation assay. It should be noted that the proliferation of GT1-1 cells decreased after hypoxic treatment more than 16 hours ($P<0.05$, **Figure 1**).

Effects of hypoxia on the expression of HIF-1α, Alkbh5, and Nanog in PA cells

As evidenced by q-PCR and western blot, the expression of HIF-1α, Alkbh5, and Nonag were increased significantly by hypoxic treatment less than 12 hours both at mRNA and protein
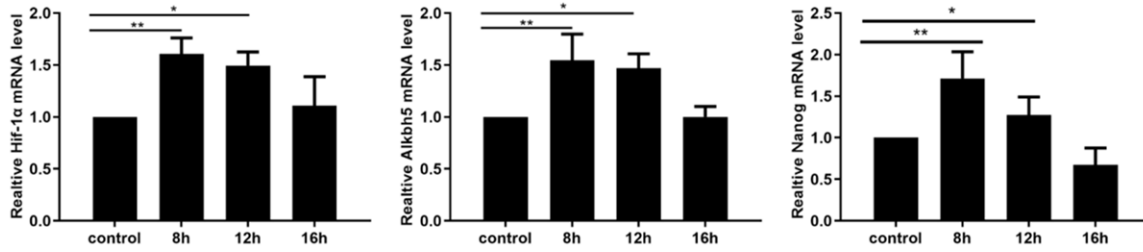
Figure 2. Effects of hypoxia on the expression of HIF-1α, ALKBH5, and Nanog mRNA in GT1-1 cells. *P<0.05, ***P<0.001.
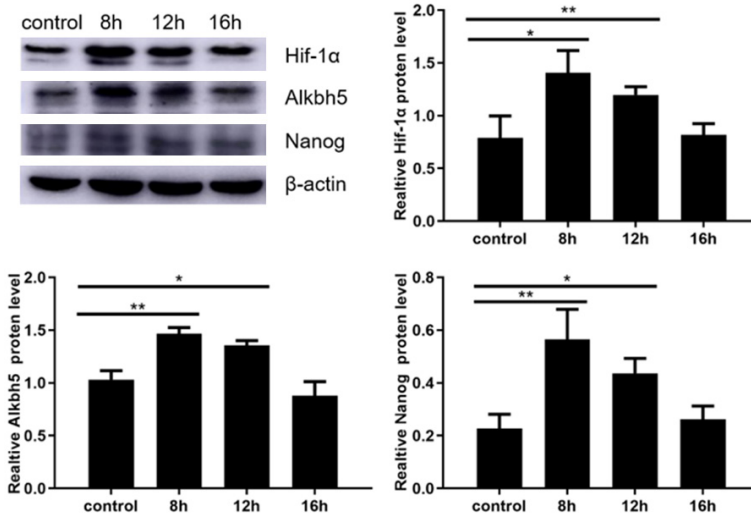

Figure 3. Effects of hypoxia on the expression of HIF-1α, ALKBH5, and Nanog protein in GT1-1 cells. *P<0.05, ***P<0.001.

levels, with a trend similar to the cell proliferation (P<0.05, **Figures 2**, **3**).

Discussion

Hypoxia is a common phenomenon in solid tumors. Under hypoxic microenvironment, tumor cells undergo a variety of complex metabolic regulations, thereby promoting cells to adapt to hypoxia, survival, or even progression [11]. Evidence demonstrated that the response to hypoxia by cells is mainly exerted through hypoxia inducible factors (HIF). Three isoforms of HIF have been identified: HIF-1, HIF-2 and HIF-3. HIF-1, the most well-known, is a heterodimer that consists of two subunits: α subunit and β subunit. HIF-1β is located within the nucleus, while HIF-1α is present in the cytoplasm and regulated by oxygen. Under hypoxic stimuli, HIF-1α is activated and translocated to the nucleus, then combined with the β subunit leading to activation of genes involved in cell metabolism, vascularization, and invasion [7, 12]. It was evidenced that the elevation of HIF-1α expression was correlated closely with the proliferation and invasion of tumors in cancers of breast [13], colorectal [14], pancreatic [15], ovarian [16], prostate [17], as well as PAs [3-6].

As a most common modification of RNA, N6-methyladenosine (m6A) plays critical roles in cancerous pathologic processes, such as cell motility, and immune response. m6As act in a dynamic and reversible manner, and are controlled by proteins known as m6A "writers" and "erasers" [18]. Interestingly, it is confirmed that the AlkB homolog 5 (ALKBH5), a m6A "eraser", is the direct target of HIF-1α [9]. In addition, a recent study showed ALKBH5 could promote the progression of breast cancer in a HIF-1α dependent manner [10]. However, the relationship between m6As and PAs remains unclear.

Nanog is an essential transcription factor regulating the pluripotency maintenance of embryonic stem cells (ESC) [19]. Recent research demonstrated that elevated Nanog is associated with initiation, progression, and invasion of various cancers, including breast [20], ovarian [21], gastric [22], colorectal [23], hepatocellular [24], and lung carcinomas [25]. The role of Nanog in PAs, however, still needed to be illuminated.

In this work, we found hypoxic conditions could promote the proliferation of PA cells in vitro. In addition, we confirmed that expressions of HIF-

1α, ALKBH5, and Nanog were elevated by hypoxic stimuli. Therefore, we speculated that hypoxia could increase Nanog expression by HIF-1α/ALKBH5 signaling, thereby promoting the progression of PAs.

In conclusion, we demonstrated that hypoxia could promote the proliferation of PAs, which might result from the elevation of HIF-1α, ALKBH5, and Naog expression induced by hypoxic environment. Further research should be carried out to clarify their exact roles in PAs.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Ostrom QT, Gittleman H, Truitt G, Boscia A, Kruchko C and Barnholtz-Sloan JS. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2011-2015. Neuro Oncol 2018; 20 Suppl 4: iv1-iv86.

[2] Kristof RA, Aliashkevich AF, Hans V, Haun D, Meyer B, Thees C and Schramm J. The regional oxygen saturation of pituitary adenomas is lower than that of the pituitary gland: microspectrophotometric study with potential clinical implications. Neurosurgery 2003; 53: 880-885.

[3] He W, Huang L, Shen X, Yang Y, Wang D, Yang Y and Zhu X. Relationship between RSUME and HIF-1α/VEGF-A with invasion of pituitary adenoma. Gene 2017; 603: 54-60.

[4] Zhang C, Qiang Q, Jiang Y, Hu L, Ding X, Lu Y and Hu G. Effects of hypoxia inducible factor-1α on apoptotic inhibition and glucocorticoid receptor downregulation by dexamethasone in AtT-20 cells. BMC Endocr Disord 2015; 15: 24.

[5] Yoshida D and Teramoto A. Elevated cell invasion is induced by hypoxia in a human pituitary adenoma. Cell Adh Migr 2017; 1: 43-51.

[6] Yoshida D, Kim K, Noha M and Teramoto A. Anti-apoptotic action by hypoxia inducible factor 1-alpha in human pituitary adenoma cell line, HP-75 in hypoxic condition. J Neurooncol 2006; 78: 217-225.

[7] Balamurugan K. HIF-1 at the crossroads of hypoxia, inflammation, and cancer. Int J Cancer 2016; 138: 1058-1066.

[8] Chen B, Li Y, Song R, Xue C and Xu F. Functions of RNA N6-methyladenosine modification in cancer progression. Mol Biol Rep 2019; 46: 2567-2575.

[9] Thalhammer A, Bencokova Z, Poole R, Loenarz C, Adam J, O'Flaherty L, Schödel J, Mole D, Giaslakiotis K, Schofield CJ, Hammond EM, Ratcliffe PJ and Pollard PJ. Human AlkB homologue 5 is a nuclear 2-oxoglutarate dependent oxygenase and a direct target of hypoxia-inducible factor 1α (HIF-1α). PLoS One 2011; 6: e16210.

[10] Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, He X and Semenza GL. Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m6A-demethylation of NANOG mRNA. Proc Natl Acad Sci U S A 2016; 113: E2047-2056.

[11] Najafi M, Farhood B, Mortezaee K, Kharazinejad E, Majidpoor J and Ahadi R. Hypoxia in solid tumors: a key promoter of cancer stem cell (CSC) resistance. J Cancer Res Clin Oncol 2020; 146: 19-31.

[12] Pezzuto A and Carico E. Role of HIF-1 in cancer progression: novel insights. A review. Curr Mol Med 2018; 18: 343-351.

[13] Kuo CY, Cheng CT, Hou P, Lin YP, Ma H, Chung Y, Chi K, Chen Y, Li W, Kung HJ and Ann DK. HIF-1-alpha links mitochondrial perturbation to the dynamic acquisition of breast cancer tumorigenicity. Oncotarget 2016; 7: 34052-34069.

[14] Li W, Zong S, Shi Q, Li H, Xu J and Hou F. Hypoxia-induced vasculogenic mimicry formation in human colorectal cancer cells: involvement of HIF-1a, Claudin-4, and E-cadherin and Vimentin. Sci Rep 2016; 6: 37534.

[15] Hao J. HIF-1 is a critical target of pancreatic cancer. Oncoimmunology 2015; 4: e1026535.

[16] Ai Z, Lu Y, Qiu S and Fan Z. Overcoming cisplatin resistance of ovarian cancer cells by targeting HIF-1-regulated cancer metabolism. Cancer Lett 2016; 373: 36-44.

[17] Lee JE, Shin SH, Shin HW, Chun YS and Park JW. Nuclear FGFR2 negatively regulates hypoxia-induced cell invasion in prostate cancer by interacting with HIF-1 and HIF-2. Sci Rep 2019; 9: 3480.

[18] He L, Li H, Wu A, Peng Y, Shu G and Yin G. Functions of N6-methyladenosine and its role in cancer. Mol Cancer 2019; 18: 176.

[19] Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M and Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 2003; 113: 631-42.

[20] Dehghan Harati M, Rodemann HP and Toulany M. Nanog signaling mediates radioresistance in ALDH-positive breast cancer cells. Int J Mol Sci 2019; 20.

[21] Mahalaxmi I, Devi SM, Kaavya J, Arul N, Balachandar V and Santhy KS. New insight into NANOG: a novel therapeutic target for ovarian cancer (OC). Eur J Pharmacol 2019; 852: 51-57.

[22] Lin T, Ding YQ and Li JM. Overexpression of Nanog protein is associated with poor prognosis in gastric adenocarcinoma. Med Oncol 2012; 29: 878-85.

[23] Xu F, Dai C, Zhang R, Zhao Y, Peng S and Jia C. Nanog: a potential biomarker for liver metastasis of colorectal cancer. Dig Dis Sci 2012; 57: 2340-6.

[24] Liang C, Zhang K, Ge H, Li W, Li G and Wu J. Prognostic and clinicopathological value of Nanog in hepatocellular carcinoma: a meta-analysis. Clin Chim Acta 2018; 477: 24-31.

[25] Chang B, Park MJ, Choi SI, In KH, Kim CH and Lee SH. NANOG as an adverse predictive marker in advanced non-small cell lung cancer treated with platinum-based chemotherapy. Onco Targets Ther 2017; 10: 4625-4633.