Expression of ATPase Inhibitory Factor 1 in Normal and Pathological Tissues of the Female Reproductive System (Postprint)

Authors: Zhao Jun, Liu Xiao, Li Yali

Date: 2017-12-21T00:00:00+00:00

Abstract

Objective To investigate the expression of ATP synthase inhibitory factor 1 (IF1) in normal and pathological tissues of the female reproductive system, providing a basis for further exploration of its physiological functions and novel therapeutic approaches for female reproductive system diseases. Methods Normal and pathological tissues of the female reproductive system surgically resected in our hospital within the past two years were studied. Tissues were obtained from patients aged 25-69 years, including 38 cases of normal endometrium, 33 cases each of eutopic and ectopic endometrium from endometriosis, 30 cases of endometrial adenocarcinoma, 24 cases of normal myometrium, 30 cases of uterine fibroids, 18 cases of normal cervix, 30 cases of cervical squamous cell carcinoma, 11 cases of normal fallopian tube, 19 cases of fallopian tube adenocarcinoma, 15 cases of normal ovary, 21 cases of ovarian adenocarcinoma, and 30 cases each of chorionic villi and decidua. Immunohistochemistry was used to detect IF1 expression in all samples, while Western blot and RT-PCR were employed to examine IF1 and its mRNA expression in normal endometrium, eutopic endometrium from endometriosis, and ectopic endometrium. Results IF1 expression was significantly enhanced in endometrial adenocarcinoma ($P<0.01$), cervical squamous cell carcinoma ($P<0.01$), fallopian tube adenocarcinoma ($P<0.01$), and ovarian adenocarcinoma ($P<0.01$) tissues compared with corresponding normal tissues. However, no significant differences were observed between uterine fibroids and normal myometrium, or between chorionic villi and decidua. In normal endometrial tissues, IF1 expression was significantly decreased in the secretory phase compared with the proliferative phase ($P<0.01$). Furthermore, IF1 expression in ectopic endometrium from endometriosis was significantly higher than that in eutopic endometrium from endometriosis ($P<0.05$) and normal endometrium ($P<0.01$), while no significant difference was found between eutopic endometrium from endometriosis and normal endometrium. RT-PCR results showed no significant

differences in IF1 mRNA expression among normal endometrium, eutopic endometrium from endometriosis, and ectopic endometrium. Conclusion IF1 is positively expressed to varying degrees in normal and non-malignant pathological tissues of the female reproductive system, but its expression is abnormally elevated in malignant tumor tissues. These results suggest that IF1 may serve as a marker for malignant tumors of the female reproductive system and holds promise as a novel therapeutic target for these malignancies.

Full Text

Preamble

ZHAO Jun[1],[2], LIU Xiao[3], LI Yali[1]
[1]Department of Gynecology and Obstetrics, General Hospital of PLA, Beijing 100039, China;
[2]Department of Gynecology and Obstetrics, [3]Department of Pathology, Navy General Hospital, Beijing 100048, China
doi: 10.3969/j.issn.1673-4254.2016.12.07

Abstract

Objective To determine ATPase inhibitory factor 1 (IF1) expression in normal and pathological tissues of the female reproductive system to better understand the physiological role of IF1 in malignancies of the female reproductive system. **Methods** Surgical specimens of normal tissues and primary malignant tumors were obtained during the past 2 years, including 38 normal endometrium tissues, 33 endometriosis tissues, 30 endometrial adenocarcinoma tissues, 24 uterine myometrium tissues, 30 uterine fibroid tissues, 18 normal cervical tissues, 30 cervical squamous carcinoma tissues, 11 fallopian tube tissues, 19 fallopian tube adenocarcinoma tissues, 15 ovarian tissues, 21 ovarian adenocarcinoma tissues, 30 decidua tissues, and 30 villus tissues. The expressions of IF1 protein and mRNA were assessed by immunohistochemistry, Western blotting and RT-PCR. **Results** The expressions of IF1 were significantly increased in endometrial adenocarcinoma ($P<0.01$), cervical squamous carcinoma ($P<0.01$), fallopian tube adenocarcinoma ($P<0.01$) and ovarian adenocarcinoma ($P<0.01$) as compared with those in normal tissues. In normal endometrium, IF1 expression was significantly reduced in the secretory phase as compared with the proliferation phase ($P<0.01$). In patients with endometriosis, IF1 protein expression increased obviously in endometriotic tissues as compared with eutopic and normal endometrial tissues, but the expression of IF1 mRNA was similar among the three tissues. **Conclusion** IF1 expression increases abnormally in malignant tumors of the female reproductive system and may serve as a marker for malignancies of the female reproductive system as well as a promising pharmacological target for cancer treatment.

Key words: ATPase inhibitory factor 1; female reproductive system; malignant tumour; endometriosis

Introduction

It is well established that eukaryotic cells obtain ATP through two primary metabolic pathways: glycolysis and oxidative phosphorylation. Research has demonstrated that these two pathways are interrelated in tumor tissues, exhibiting a reciprocal relationship where one is suppressed as the other is enhanced [1-2]. Consequently, enhanced glycolysis coupled with suppressed oxidative phosphorylation is considered a hallmark of vigorous tumor proliferation [3], whereas enhanced oxidative phosphorylation with reduced glycolysis often indicates suppressed tumor growth and delayed progression [4-5]. ATPase inhibitory factor 1 (IF1) is an endogenous small molecular protein that inhibits ATP synthase (ATPase) function, thereby suppressing oxidative phosphorylation and promoting glycolysis [6-7]. Studies have confirmed that IF1 activation is closely associated with pH values. The substantial lactate produced by glycolysis can lower the pH of the tumor microenvironment, promoting IF1 activation and dimerization, which binds to two F1-ATPase units and consequently inhibits ATPase function [8], ultimately facilitating tumor cell proliferation, invasion, and survival [9-10]. Therefore, some scholars propose that IF1 is a potential oncogenic factor that could serve as a predictor for early tumor diagnosis, recurrence, and metastasis [11-13].

To date, no systematic studies have reported on IF1 expression in normal and pathological tissues of the female reproductive system. Therefore, this study aims to investigate IF1 expression in normal and pathological tissues of the female reproductive system to preliminarily understand the association between IF1 and hypoxic energy metabolism, cell proliferation, and malignant transformation, providing a basis for further exploration of its physiological functions and novel therapeutic approaches for female reproductive system diseases.

Methods

1.1 Sample Collection and Processing

All experimental protocols were approved by the Ethics Committee of the General Hospital of PLA, and written informed consent was obtained from all patients. Samples were collected from surgical specimens obtained at our hospital between 2013 and 2015 (Table 1). All patients had regular menstrual cycles and no history of hormone therapy within three months prior to surgery. Tumor tissues were obtained from patients undergoing primary surgery without prior radiotherapy or chemotherapy (aged 25-69 years). Other tissues were obtained from surgical patients (aged 25-49 years). Normal myometrium and uterine fibroids were collected from the same patients undergoing hysterectomy for fibroids. Normal endometrial tissues were obtained from patients undergoing surgery for fibroids. Eutopic and ectopic endometrial tissues from endometriosis patients were collected from the same individuals undergoing surgery for endometriosis. Decidua and villus tissues were obtained from patients undergoing artificial abortion at 8-10 weeks of gestation. Normal fallopian tubes were col-

lected from patients undergoing surgery for ectopic pregnancy. Normal ovaries were obtained from patients undergoing surgery for ovarian endometriosis. Samples were fixed in 4% neutral paraformaldehyde and routinely processed for paraffin embedding. Some endometrial and ectopic lesion samples were washed with saline to remove blood and immediately stored in liquid nitrogen for future use.

1.2 Immunohistochemical Detection of IF1 Expression

Paraffin sections were deparaffinized and rehydrated. Antigen retrieval was performed using EDTA buffer (pH 9.0) in a microwave. Endogenous peroxidase activity was blocked with 3% H O for 10 minutes, followed by PBS washing and blocking with 3% BSA at room temperature for 10 minutes. Sections were incubated with primary IF1 antibody (1:50, ab197806, Abcam, USA) at 4°C overnight. After PBS washing, sections were incubated with secondary antibody (K500711, Dako, Denmark) at room temperature for 1 hour. Following PBS washing, color development was performed using DAB (K500711, DAKO, Denmark) and sections were counterstained with hematoxylin, then dehydrated, cleared, and mounted. Each section was observed under 200× magnification, with three random fields photographed per slice. Images were analyzed using CMIAS software (CMIAS, Beijing University of Aeronautics and Astronautics, China) to obtain the area density of antigen expression in each field, and the mean value was calculated for each section.

1.3 Western Blotting for IF1 Protein Levels

Total protein was extracted from frozen tissues using standard methods. Protein concentration was determined by BCA assay. Samples were diluted with 4× loading buffer, denatured at 95°C for 15 minutes, and loaded at 50 g per well. Proteins were separated by 12% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% skim milk at room temperature for 1 hour, washed with TBS-T, and incubated with primary antibodies against IF1 (1:50, ab197806, Abcam, USA) and -actin (1:100, ab16039, Abcam, USA) at 4°C overnight. After TBS-T washing, membranes were incubated with HRP-conjugated secondary antibody (BS13278, Bioworld, USA) at room temperature for 1 hour. Following TBS-T washing, bands were visualized using ECL reagent (BLH01S020, Bioworld, USA). Images were captured using a gel imaging and analysis system (SCA/fluorCho, Alpha Inotech, USA) and subjected to densitometric analysis. The ratio of IF1 to -actin was calculated as the relative expression level of IF1.

1.4 RT-PCR for IF1 mRNA Expression

Frozen tissues were thoroughly ground in liquid nitrogen, and RNA was extracted using Trizol reagent (15596-026, Invitrogen, USA) according to standard protocols. Primer sequences were as follows: IF1-forward 5' -GGGCCTTCGGAAAGAGAG-3'; IF1-reverse 5'-TTCAAAGCTGCCAGTTGTTC-

3' ; -actin-forward 5' -GGAGATTACTGCCCTGGCTCCTA-3' ; -actin-reverse 5' -GACTCATCGTACTCCTGCTTGCTG-3' . For cDNA synthesis, 9 L of sample RNA was mixed with 1 L of Random primer and cooled at 70°C for 5 minutes. Using the GoScript Reverse Transcription system (Promega, USA), 10 L of Reverse Transcription mix was prepared according to the manufacturer' s instructions. Solutions A and B were combined, annealed at 25°C for 5 minutes, extended at 42°C for 1 minute, and the reverse transcriptase was inactivated at 70°C for 5 minutes to complete cDNA synthesis. RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) on a 7500 Real-Time PCR System (Applied Biosystems, USA) with 40 cycles of amplification (95°C for 15 seconds, 60°C for 1 minute). The Ct values of target and reference genes were obtained, and the relative expression level of the target gene was calculated. Each sample was run in triplicate, and the mean value was used as the expression level for that sample.

1.5 Statistical Analysis

Data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 17.0 software with one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

2.1 Strong Positive Expression of IF1 in Female Reproductive System Malignancies

Immunohistochemical results showed that IF1 was expressed at varying levels in normal tissues of the female reproductive system, with the strongest expression in proliferative endometrium (++), moderate expression in cervical epithelium, fallopian tube epithelium, and ovary (+), and the weakest expression in uterine myometrium (+/-). In contrast, villus, decidua, and malignant tumor tissues all showed strong positive expression of IF1 (+++). Image analysis revealed that compared with corresponding normal tissues, IF1 expression was significantly increased in endometrial adenocarcinoma ($P<0.01$), cervical squamous carcinoma ($P<0.01$), fallopian tube ampullary adenocarcinoma ($P<0.01$), and ovarian endometrioid adenocarcinoma ($P<0.01$). However, no significant differences were observed between villus and decidua, or between normal uterine myometrium and uterine fibroids [Figure 1: see original paper].

2.2 Higher IF1 Expression in Proliferative Phase Endometrium

Immunohistochemical results demonstrated that IF1 was positively expressed in normal proliferative endometrium (++), but was essentially absent or weakly expressed in secretory endometrium (+/-). Quantitative image analysis confirmed that IF1 expression was significantly higher in proliferative phase endometrium compared with secretory phase endometrium ($P<0.01$) [Figure 2: see original paper].

2.3 Expression of IF1 and its mRNA in Normal Endometrium, Eutopic Endometrium from EMS, and Ectopic Endometrium

Immunohistochemistry and Western blotting revealed that IF1 expression was strongest in ectopic endometrium from endometriosis (+++), while being weakly expressed in both normal endometrium and eutopic endometrium from endometriosis patients. Quantitative analysis showed that IF1 expression in ectopic endometrium was significantly higher than in normal endometrium ($P<0.01$) and eutopic endometrium from endometriosis patients (immunohistochemistry: $P<0.01$; Western blot: $P<0.05$). However, RT-PCR analysis showed no significant differences in IF1 mRNA expression among normal endometrium, eutopic endometrium from endometriosis patients, and ectopic endometrium ($P>0.05$) [Figure 3: see original paper].

Discussion

Previous studies have generally accepted that due to irreversible mitochondrial damage, oxidative phosphorylation is severely suppressed in tumor tissues, which primarily obtain energy through glycolysis. This unique metabolic pattern is known as the "Warburg effect" [14]. Although glycolysis produces less ATP, it generates energy more rapidly and produces lactate and other products that create a favorable microenvironment for tumor proliferation, making it more suitable for the rapid growth demands of tumors than oxidative phosphorylation [15-16, 33]. Currently, several glycolysis-inhibiting agents (such as 2-deoxyglucose and lonidamine) have been applied clinically to control tumor growth, though they are often effective only for rapidly growing tumors and less so for slow-growing ones [17-18].

ATPase is the core catalytic enzyme in oxidative phosphorylation. Under normal physiological conditions, ATPase phosphorylates ADP to generate ATP, providing energy for cells; under hypoxic stress, it can catalyze ATP hydrolysis in reverse [19]. Studies have found that ATPase expression is generally decreased in various tumor tissues [20], and lower ATPase expression has been associated with enhanced drug resistance and poor prognosis [21-24]. Therefore, some researchers believe that regulating ATPase expression and activity may influence tumor growth and survival [9, 25].

In 1963, Pullman and Monroy first discovered an endogenous ATPase inhibitory protein and named it ATPase inhibitory factor 1 [26]. IF1 is a small molecular protein composed of 106-109 amino acids (~10,000 Da) with highly conserved gene encoding across species, suggesting its important biological function [27]. Studies have found that IF1 is abnormally elevated in various malignant tumors including liver and lung cancers [6, 13, 28]. Our results demonstrate that IF1 expression is also significantly increased in female reproductive system malignancies such as endometrial adenocarcinoma, cervical squamous carcinoma, fallopian tube adenocarcinoma, and ovarian endometrioid adenocarcinoma compared with their corresponding normal tissues, further confirming that IF1 plays

an important role in energy metabolism of malignant tumors.

Although endometriosis (EMs) is a benign disease, it shares similar biological behaviors with malignant tumors, including invasion, recurrence, and potential for malignant transformation. Some studies have indicated that mitochondrial dysfunction is a risk factor for EMs development [29]. Our study found that IF1 expression was significantly increased in ectopic endometrium compared with both normal endometrium and eutopic endometrium from EMs patients. This suggests that the growth and metabolic pattern of ectopic endometrium in EMs is similar to tumor cells, likely obtaining energy through glycolysis. However, IF1 mRNA expression showed no significant differences among normal endometrium, eutopic endometrium from EMs patients, and ectopic endometrium, suggesting post-transcriptional regulation of IF1 gene expression.

Some studies have suggested that the metabolic shift from oxidative phosphorylation to glycolysis, accompanied by increased IF1 expression, is indicative of vigorous cell proliferation [30]. Our study found that normal endometrium also exhibited high IF1 expression, with higher levels in proliferative phase than secretory phase endometrium, while normal uterine and cervical myometrium showed very low or absent expression. Furthermore, although uterine fibroid tissues have significantly lower oxygen partial pressure (0-5 mmHg) than normal myometrium (PO , 5-20 mmHg) [31], and studies have shown that cobalt chloride-induced hypoxic injury can increase IF1 expression in cultured rat hepatocytes [32], our study found low IF1 expression in both uterine fibroids and normal myometrium with no significant difference. Meanwhile, both villus and decidua tissues, which are highly proliferative with abundant blood supply and no hypoxic state, showed high IF1 expression in our study. These results suggest that in vivo, IF1 expression is independent of tissue hypoxia status but correlates with proliferative and metabolic activity. Specifically, tissues with slow proliferation and low metabolic demand show low IF1 expression, while tissues with vigorous proliferation and relatively high metabolic demand show high IF1 expression.

Currently, no studies have reported on IF1 expression in the female reproductive system and its association with female reproductive system diseases. Our study demonstrates that IF1 is differentially expressed in normal tissues of the female reproductive system, with expression levels positively correlating with tissue proliferative and metabolic activity. IF1 expression is abnormally elevated in malignant tumors of the female reproductive system, and post-transcriptional regulation exists. IF1 may participate in the regulation of tumor energy metabolism by inhibiting ATPase function, thereby affecting tumor cell survival and growth. In summary, we propose that IF1 could serve as a diagnostic marker for malignant tumors of the female reproductive system and represents a potential new therapeutic target.

References

[1] Kinnaird A, Michelakis ED. Metabolic modulation of cancer: a new frontier with great translational potential[J]. J Mol Med (Berl), 2015, 93(2): 127-42.

[2] Smolková K, Plecitá-Hlavatá L, Bellance N, et al. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells[J]. Int J Biochem Cell Biol, 2011, 43(7): 950-68.

[3] Vander Heiden MG, Cantley LC, Thompson CB. Understanding the warburg effect: the metabolic requirements of cell proliferation[J]. Science, 2009, 324(5930): 1029-33.

[4] Bonnet S, Archer SL, Allalunis-Turner J, et al. A mitochondria-K+ Channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth[J]. Cancer Cell, 2007, 11(1): 37-51.

[5] Garcia-Cao I, Song MS, Hobbs RM, et al. Systemic elevation of PTEN induces a tumor-suppressive metabolic state[J]. Cell, 2012, 149(1): 49-62.

[6] Sanchez-Cenizo L, Formentini L, Aldea MA, et al. Up-regulation of the ATPase inhibitory factor 1 (IF1) of the mitochondrial H+-ATP synthase in human tumors mediates the metabolic shift of cancer cells to a warburg phenotype[J]. J Biol Chem, 2010, 285(33): 25308-13.

[7] Garcia-Bermudez J, Cuezva JM. The ATPase inhibitory factor 1 (IF1): A master regulator of energy metabolism and of cell survival[J]. Biochim Biophys Acta, 2016, 1857(8, SI): 1167-82.

[8] Solaini G, Harris DA. Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion[J]. Biochem J, 2005, 390(Pt 2): 377-94.

[9] Formentini L, Sánchez-Aragó M, Sánchez-Cenizo L, et al. Mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde prosurvival and proliferative response[J]. Mol Cell, 2012, 45(6): 731-42.

[10] Song R, Song H, Liang Y, et al. Reciprocal activation between ATPase inhibitory factor 1 and NF-kappaB drives hepatocellular carcinoma angiogenesis and metastasis[J]. Hepatology, 2014, 60(10): 1659-73.

[11] Gao YX, Chen L, Hu XG, et al. ATPase inhibitory factor 1 expression is an independent prognostic factor in non-small cell lung cancer[J]. Am J Cancer Res, 2016, 6(5): 1141-8.

[12] Wu JH, Shan Q, Li PD, et al. ATPase inhibitory factor 1 is a potential prognostic marker for the migration and invasion of glioma[J]. Oncol Lett, 2015, 10(4): 2075-80.

[13] Faccenda D, Tan CH, Seraphim A, et al. IF1 limits the apoptotic-signalling cascade by preventing mitochondrial remodelling[J]. Cell Death Differ, 2013, 20(5): 686-97.

[14] Mathupala SP, Ko YH, Pedersen PL. The pivotal roles of mitochondria in cancer: warburg and beyond and encouraging prospects for effective therapies[J]. Biochim Biophys Acta, 2010, 1797(6/7): 1225-30.

[15] Brooks GA. Cell-cell and intracellular lactate shuttles[J]. J Physiol, 2009, 587(23): 5591-600.

[16] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation[J]. Cell, 2011, 144(5): 646-74.

[17] Azevedo-Silva J, Queiros O, Baltazar F, et al. The anticancer agent 3-bromopyruvate: a simple but powerful molecule taken from the lab to the bedside[J]. J Bioenerg Biomembr, 2016, 48(4): 349-62.

[18] Cervantes-Madrid D, Romero Y, Duenas-Gonzalez A. Reviving lonidamine and 6-Diazo-5-oxo-L-norleucine to be used in combination for metabolic cancer therapy[J]. Biomed Res Int, 2015, 14(6): 690492.

[19] Daichi O, Ryota I, Hiroyuki N. Rotation and structure of F0F1-ATP synthase[J]. J Biochem, 2011, 149(6): 655-64.

[20] Vazquez-Martin A, Corominas-Faja B, Cufi SA, et al. The mitochondrial H+-ATP synthase and the lipogenic switch: new core components of metabolic reprogramming in induced pluripotent stem (iPS) cells[J]. Cell Cycle, 2013, 12(2): 207-18.

[21] Krasnov GS, Dmitriev AA, Snezhkina AV, et al. Deregulation of glycolysis in cancer: glyceraldehyde-3-phosphate dehydrogenase as a therapeutic target[J]. Expert Opin Ther Targets, 2013, 17(6): 681-93.

[22] Creaney J, Dick IM, Yeoman D, et al. Auto-Antibodies to beta-F1-ATPase and vimentin in malignant mesothelioma[J]. PLoS One, 2011, 6(10): 0026515.

[23] Sanchez-Arago M, Formentini L, Martinez-Reyes I, et al. Expression, regulation and clinical relevance of the ATPase inhibitory factor 1 in human cancers[J]. Oncogenesis, 2013, 24(2): e46.

[24] Yin T, Lu L, Xiong ZG, et al. ATPase inhibitory factor 1 is a prognostic marker and contributes to proliferation and invasion of human gastric cancer cells[J]. Biomed Pharmacother, 2015, 70(8): 113-20.

[25] Lin PC, Lin JK, Yang SH, et al. Expression of beta-F1-ATPase and mitochondrial transcription factor A and change in mitochondrial DNA content in colorectal cancer: clinical data analysis and evidence from an in vitro study[J]. Int J Colorectal Dis, 2008, 23(12): 1223-32.

[26] Pullman ME, Monroy GC. A naturally occurring inhibitor of mitochondrial adenosine triphosphatase[J]. J Biol Chem, 1963, 238(10): 3762-69.

[27] Bason JV, Montgomery MG, Leslie AG. Pathway of binding of the intrinsically disordered mitochondrial inhibitor protein IF1 to F1-ATPase[J]. Proc Natl Acad Sci USA, 2014, 111(31): 11305-10.

[28] Formentini L, Sánchez-Aragó M, Sánchez-Cenizo L, et al. The mitochondrial ATPase inhibitory factor 1 triggers a ROS-mediated retrograde pro-survival and proliferative response[J]. Mol Cell, 2012, 45(4): 731-42.

[29] Govatati S, Deenadayal M, Shivaji SA. Mitochondrial displacement loop alterations are associated with endometriosis[J]. Fertil Steril, 2013, 99(7): 1980.

[30] Wei SH, Fukuhara H, Kawada C, et al. Silencing of ATPase inhibitory factor 1 inhibits cell growth via cell cycle arrest in bladder cancer[J]. Pathobiology, 2015, 82(5): 224-32.

[31] Mayer A, Hoeckel M, Wree A, et al. Lack of hypoxic response in uterine leiomyomas despite severe tissue hypoxia[J]. Cancer Res, 2008, 68(12): 4719-26.

[32] Huang LJ, Chuang IC, Dong HP, et al. Hypoxia-inducible factor 1 alpha regulates the expression of the mitochondrial ATPase inhibitor protein (IF1) in rat liver[J]. Shock, 2011, 36(1): 90-6.

[33] Kroemer G. Mitochondria in cancer[J]. Oncogene, 2006, 25(34): 4633-54.

Edited by: Sun Changpeng

Note: Figure translations are in progress. See original paper for figures.