Biochemical and Biophysical Research Communications 440 (2013) 137–142

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

INPP4B-mediated tumor resistance is associated with modulation of glucose metabolism via hexokinase 2 regulation in laryngeal cancer cells

Joong Won Min [a], Kwang Il Kim [b], Hyun-Ah Kim [c], Eun-Kyu Kim [c], Woo Chul Noh [c], Hong Bae Jeon [d], Dong-Hyung Cho [e], Jeong Su Oh [f], In-Chul Park [a], Sang-Gu Hwang [a], Jae-Sung Kim [a,*]

[a] Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea
[b] Molecular Imaging Research Center, Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea
[c] Department of Surgery, Korea Cancer Center Hospital, Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea
[d] Biomedical Research Institute, MEDIPOST Co., Ltd., Seoul, Republic of Korea
[e] Graduate School of East-West Medical Science, Kyung Hee University, Gyeonggi-do, Republic of Korea
[f] Department of Genetic Engineering, Sungkyunkwan University, Suwon, Republic of Korea

ARTICLE INFO

Article history:
Received 4 September 2013
Available online 17 September 2013

Keywords:
Laryngeal cancer
Glycolysis
Tumor resistance
INPP4B

ABSTRACT

Inositol polyphosphate 4-phosphatase type II (INPP4B) was recently identified as a tumor resistance factor in laryngeal cancer cells. Herein, we show that INPP4B-mediated resistance is associated with increased glycolytic phenotype. INPP4B expression was induced by hypoxia and irradiation. Intriguingly, overexpression of INPP4B enhanced aerobic glycolysis. Of the glycolysis-regulatory genes, hexokinase 2 (HK2) was mainly regulated by INPP4B and this regulation was mediated through the Akt-mTOR pathway. Notably, codepletion of INPP4B and HK2 markedly sensitized radioresistant laryngeal cancer cells to irradiation or anticancer drug. Moreover, INPP4B was significantly associated with HK2 in human laryngeal cancer tissues. Therefore, these results suggest that INPP4B modulates aerobic glycolysis via HK2 regulation in radioresistant laryngeal cancer cells.

1. Introduction

Radiotherapy in combination with surgery and/or chemotherapy is a major treatment option for eradicating tumor cells in laryngeal cancer, of which the survival rates are relatively high following radiotherapy [1,2]. Despite the effective therapeutic modality of radiotherapy for laryngeal cancer, the existence of radioresistant tumor cells contributes to locally recurrent and poor prognosis after radiotherapy. Several biological factors of the tumors such as the extent of hypoxia have been shown to contribute to resistance of these cells to radiotherapy [3]. It is well known that hypoxia as occurs in most tumors reduces the therapeutic effect by activating hypoxia-inducible factor-1α (HIF-1α) in several human cancers [3,4]. Thus, understanding these mechanisms is important for the development of strategies to overcome resistance of tumor cells to radiotherapy.

Most cancer cells prefer to metabolize glucose by glycolysis even in the presence of sufficient oxygen, known as aerobic glycolysis or "Warburg effect" [5]. Up-regulation of glycolysis in malignant tumor cells is considered as adaption to hypoxia in pre-malignant lesions. Hypoxia and genetic alteration of oncogenes or tumor suppressor genes lead to the activation of transcription factor HIF-1α and thereby reprogram the malignant tumor cells to the increased glycolytic phenotype by inducing glycolysis-regulatory genes such as glucose transporters (e.g., *GLUT1* and *2*) and hexokinases (e.g., *HK1* and *2*) [5,6]. The increased glycolysis promotes acidosis of the tumor microenvironment by producing lactic acid, end product of glycolysis [5]. Several reports have shown that enhanced aerobic glycolysis and acidosis are often linked to the resistant phenotype of cancer cells to radio- or chemotherapy. For example, overexpression of GLUT1 and HK2 in several cancers predicts poor prognosis after radio- or chemotherapy [7–10]. Thus, increased aerobic glycolysis is not only one of the essential component of the malignant phenotype but also for resistance of tumor cells to radio- or chemotherapy [5]. However,

Abbreviations: ERK-1/2, extracellular signal-regulated protein kinase-1/2; FDG, fluorodeoxyglucose; HIF-1α, hypoxia-inducible factor-1α; HK2, hexokinase 2; INPP4B, inositol polyphosphate 4-phosphatase Type II; PI3K, phosphoinositide 3-kinases; PTEN, phosphatase and tensin homolog; RR-HEp-2, radioresistant HEp-2; siRNA, small interfering RNA.

* Corresponding author. Address: Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, 215-4 Gongneung-Dong, Nowon-Ku, Seoul 139-706, Republic of Korea. Fax: +82 2 970 2417.
E-mail address: jaesung@kirams.re.kr (J.-S. Kim).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.09.041

the precise mechanisms underlying modulation of aerobic glycolysis in resistant tumor cells are still unclear.

Inositol polyphosphate 4-phosphatase type II (INPP4B) is a regulatory enzyme that selectively removes the phosphate at the fourth position of the inositol ring from phosphatidylinositol(3,4)-bisphosphate, which is involved in the phosphatidylinositol signaling pathway [11–13]. The rapid production and/or degradation of phosphoinositides participate in a wide range of cellular processes, including cell growth, differentiation, apoptosis, protein trafficking, and movement [14,15]. Recently, we identified INPP4B as a novel tumor resistance gene by systematically analyzing Unigene libraries of the human laryngeal cancer [16]. Induction of INPP4B by radiation or anticancer drugs contributes to radioresistance and chemoresistance of tumors via regulation of extracellular signal-regulated kinase (ERK)-Akt pathway [16].

In this study, we found that INPP4B expression is regulated by HIF-1α under stress conditions. Further, INPP4B was able to modulate aerobic glycolysis by inducing HK2, which consequently contributed to the resistance of tumor cells to radiation or anticancer drug. Furthermore, we provide evidence of the clinical relevance of this regulation in human laryngeal cancer tissues.

2. Materials and methods

2.1. Cell lines and treatment

A549 lung cancer cells, MCF7 breast cancer cells, and HEp-2 laryngeal cancer cells were obtained from the American Type Culture Collection. Cells were grown in DMEM (MCF7 and HEp-2) or RPMI (A549) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) and penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. Radioresistant-HEp-2 (RR-HEp-2) and HEp-2 cells overexpressing *INPP4B* were established as previously described [16,17]. Hypoxia was stimulated in a chamber with a gas mixture of 1% O_2, 5% CO_2 and 94% N_2. The cells were irradiated using a ^{137}cesium (Cs) ray source (Atomic Energy of Canada Ltd., Mississauga, Canada) at a dose rate of 3.81 Gy/min or treated with 10 μM doxorubicin (Sigma). LY294002 (10 μM; Sigma) and rapamycin (5 μM; Sigma) were used to inhibit Akt and mTOR, respectively.

2.2. RNA interference

The siRNAs were synthesized at Bioneer (Daejeon, Korea). The sequences of the siRNAs against human *INPP4B*, *HIF-1α*, and *HK2* were as follows: INPP4B; 5′-CAGAAUGUUUGAGUCACUA-3′, HIF-1α; 5′-CACCAAAGTTGAATCAGAA-3′, HK2; 5′-CACGATGAAATTGAACCTGGT-3′. Non-silencing siRNA (Bioneer) was used as a negative control. Transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.3. Western blot analysis

Western blotting was performed as described previously [17,18]. Briefly, proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and detected using specific antibodies. The following antibodies were used: rabbit polyclonal anti-phospho-Akt, anti-Akt, anti-cleaved-PARP (Asp214), and HIF-1α (Cell Signaling Technology, Beverly, MA); rabbit monoclonal anti-phospho-S6 and anti-S6 (Cell Signaling Technology); mouse monoclonal anti-HK2, anti-Myc (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-β-actin (Sigma); goat polyclonal anti-INPP4B (Santa Cruz Biotechnology Inc.). Blots were developed using peroxide-conjugated secondary antibody and enhanced chemiluminescence detection system (Amersham Life Science, Piscataway, NJ).

2.4. Quantitative real-time PCR (RT-PCR)

Quantitative RT-PCR was performed following a previously described protocol [17]. Briefly, total RNA isolated using STAT-60 (Tel-Test B, Inc., Friendswood, TX) was reverse-transcribed with ImProm-II™ reverse transcription system (Promega, Madison, WI). Quantitative RT-PCR was performed using SYBR Premix Ex Taq™ (Takara Bio, Shiga, Japan) on a chromo 4 cycler (Bio-Rad, Richmond, CA). The following PCR primers were used: carbonic anhydrase 9 (CA9), sense 5′-AAGGCTCAGAGACTCAGG-3′ and antisense 5′-CTCATCTGCACAAGGAAC-3′; HK1, sense 5′-GGTTG GACTCATTGTTGGG-3′ and antisense 5′-CACACTGTCTTGACGAGGA-TAC-3′; HK2, sense 5′-AGGAGGATGAAGGTAGAAATG-3′ and antisense 5′-CACATCCAGGTCAAACTCC-3′; INPP4B, sense 5′-AAAG AATGCAGGTACACAG-3′ and antisense 5′-CTCTGTGCTGCTCTTA GG-3′; lactate dehydrogenase A (LDHA), sense 5′-AGGGACTGATA AAGATAAGG-3′ and antisense 5′-ATGCAAGGAACACTAAGG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5′-CATCTCTGCCCCCTCTGCTGA-3′ and antisense 5′-GGATGACCTTGCC-CACAGCCT-3′; GLUT1, sense 5′-TACTCATGACCATCGCGCTAG-3′ and antisense 5′-AGCTCCTCGGGTGTCTTGTC-3′.

2.5. [^{18}F]Fluorodeoxyglucose (FDG) uptake assay

Cells were seeded at a density of 2×10^5 cells/well in 6-well plates and incubated at 37 °C. After 24 h, the cells were rinsed once with cold uptake medium (fresh DMEM with glucose (1 mg/mL) and then 2 mL of uptake medium containing 74 KBq (2 μCi) of [^{18}F]FDG was added to each well. The plates were then placed in an incubator for 30 min or 1 h to allow FDG to accumulate in the cells. The cells were then washed twice with cold PBS and lysed with 0.2% SDS. The radioactivity was immediately measured using a 1480 WIZARD gamma counter (PerkinElmer, Waltham, MA).

2.6. Measurement of lactate and pH

Cells were seeded at a density of 2×10^5 cells per 60-mm dish. Lactate concentration was measured using the Lactate assay kit (Biovision, CA, USA) according to the manufacturer's protocol. The pH of the culture medium was measured using a pH meter. The Lactate levels and pH of the culture media was normalized to cell number.

2.7. Cell death analysis

Cell death analysis was performed as previously described [17]. Briefly, cells were trypsinized, washed with PBS and then incubated with propidium iodide (5 μg/mL) for 10 min at room temperature, and analyzed with the FACScan flow cytometer (Becton Dickson, Franklin Lakes, NJ).

2.8. Immunohistochemistry

Human tissue microarrays were purchased from SuperBioChips (Cat Number: CH3; Seoul, Korea). Immunohistochemical staining was performed with anti-INPP4B rabbit polyclonal antibody (1:100 dilution; Abgent, San Diego, CA, USA) or anti-HK2 mouse monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology Inc.). Immunostaining was detected by the avidin–biotin-peroxidase method according to the manufacturer's instruction (Invitrogen). Staining intensity was scored as follows: 0 (no visible staining), 1 + (faint staining), 2 + (moderate staining) and 3 + (strong staining).

2.9. Bioinformatic analysis

Human and mouse *INPP4B* loci were aligned, and the extent of DNA sequence homology was computed with the web-based program VISTA (http://www.gsd.lbl.gov/vista). HIF-1α binding sites were predicted with rVISTA 2.0 (rvista.dcode.org) using the optimum matrix similarity. INPP4B expression or mutations in the normal and cancer tissues were analyzed by using 3 publicly available databases [Human Protein Atlas (www.proteinatlas.org), Oncomine (www.oncomine.org), or cBio Cancer Genomics Portal (www.cbioportal.org)].

2.10. Statistical analysis

The correlation between INPP4B and HK2 immunointensity was analyzed using Spearman's rank correlation test. The two-tailed Student's *t*-test was performed to analyze statistical differences between groups. $P < 0.05$ was considered as significant.

3. Results

3.1. HIF-1α-regulated INPP4B enhances glycolysis

As the functional hypoxia response elements which bound to HIF-1α and HIF-1β [19], were found on the promoter region of *INPP4B* by using VISTA analysis (Supplementary Fig. 1), we initially hypothesized that INPP4B expression might be regulated by hypoxia. Interestingly, the levels of INPP4B was induced under hypoxic condition, concomitant with increase of HIF-1α protein levels, in laryngeal cancer HEp-2 cells as assessed by Western blotting (Fig. 1A). In addition, this hypoxia-induced INPP4B expression was decreased by HIF-1α depletion with siRNA (Fig. 1B), suggesting that INPP4B expression is regulated by HIF-1α under hypoxia. Since INPP4B is induced by irradiation under normoxia [16] and irradiation activates HIF-1α [20], whether radiation-induced INPP4B levels is dependent on HIF-1α was further examined by Western blotting and RT-PCR. We found that radiation-induced INPP4B was reduced by HIF-1α depletion (Fig. 1C), indicating that hypoxia- or radiation-induced INPP4B expression is dependent on HIF-1α in laryngeal cancer HEp-2 cells.

Intriguingly, we found that stable overexpression of INPP4B promoted acidosis of HEp-2 cells, whereas INPP4B depletion in INPP4B-overexpressed cells reduced this acidosis (Fig. 1D), as judged by the pH of the medium. Since increased acidosis is tightly associated with increased glycolytic phenotype of cancer cells [5], we next examined the rate of glucose uptake and lactate production between control and INPP4B-overexpressed cells. FDG uptake assay indicated that stable overexpression of INPP4B significantly increased glucose uptake by approximately 2-fold (Fig. 1E). In addition, lactate production, the final product of glycolysis, was

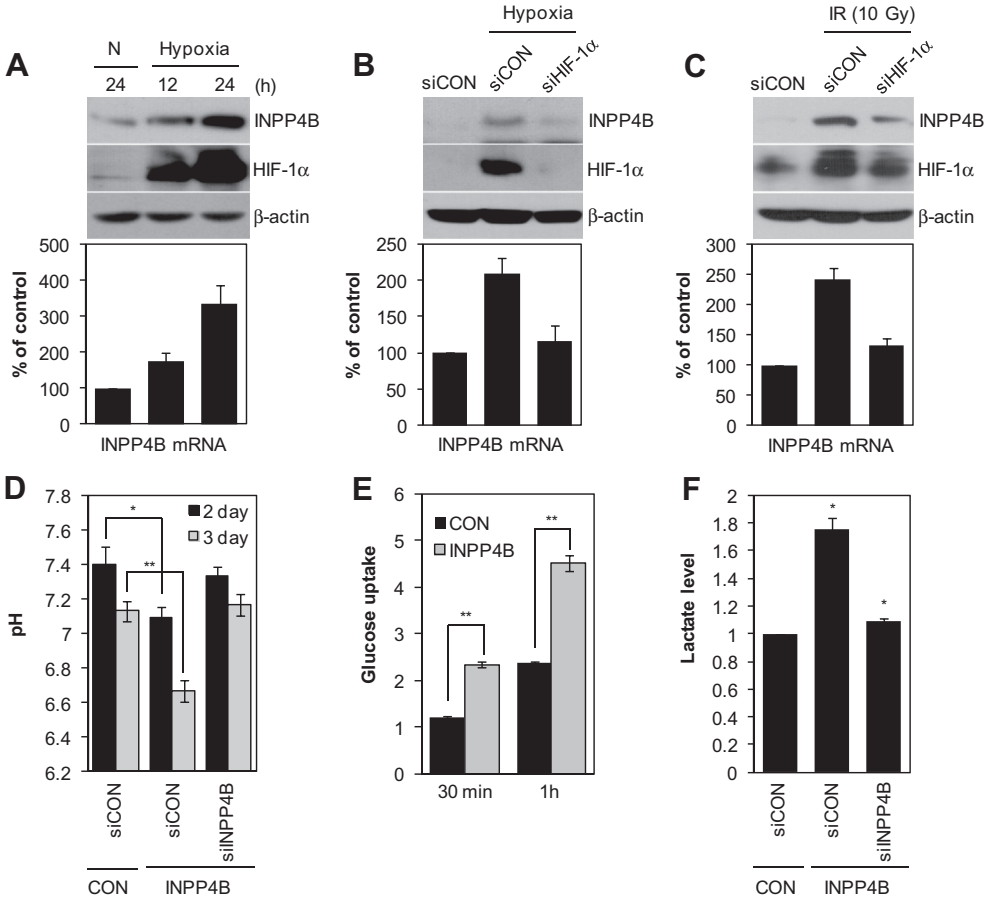


Fig. 1. Modulation of glucose metabolism by HIF-1α-mediated INPP4B. (A) HEp-2 cells were exposed to hypoxia (1% O_2) or kept in normoxia (N) for the indicated times. (B and C) HEp-2 cells were transfected with the indicated siRNAs targeting control (siCON; 50 nM) and HIF-1α (siHIF-1α; 50 nM) for 48 h and then exposed to hypoxia (1% O_2) for 24 h (B) or treated with 10 Gy radiation for 24 h (C). Protein levels of INPP4B and HIF-1α were determined by Western blotting and β-actin was used as loading control. The mRNA levels of INPP4B were measured by quantitative RT-PCR. GAPDH was used as internal control. (D and F) Control (CON) and INPP4B-overexpressing (INPP4B) HEp-2 cells were transfected with the indicated siRNAs targeting control (siCON; 100 nM) and INPP4B (siINPP4B; 100 nM) for 48 h and then the cultured media were analyzed to measure pH value (D) and lactate levels (F). (E) [^{18}F]FDG was added to control- and INPP4B-HEp-2 cells, and uptake was determined at 30 min and 1 h later. The data is presented as mean ± SD of 3 independent experiments; $P < 0.05$ (*) and $P < 0.01$ (**).

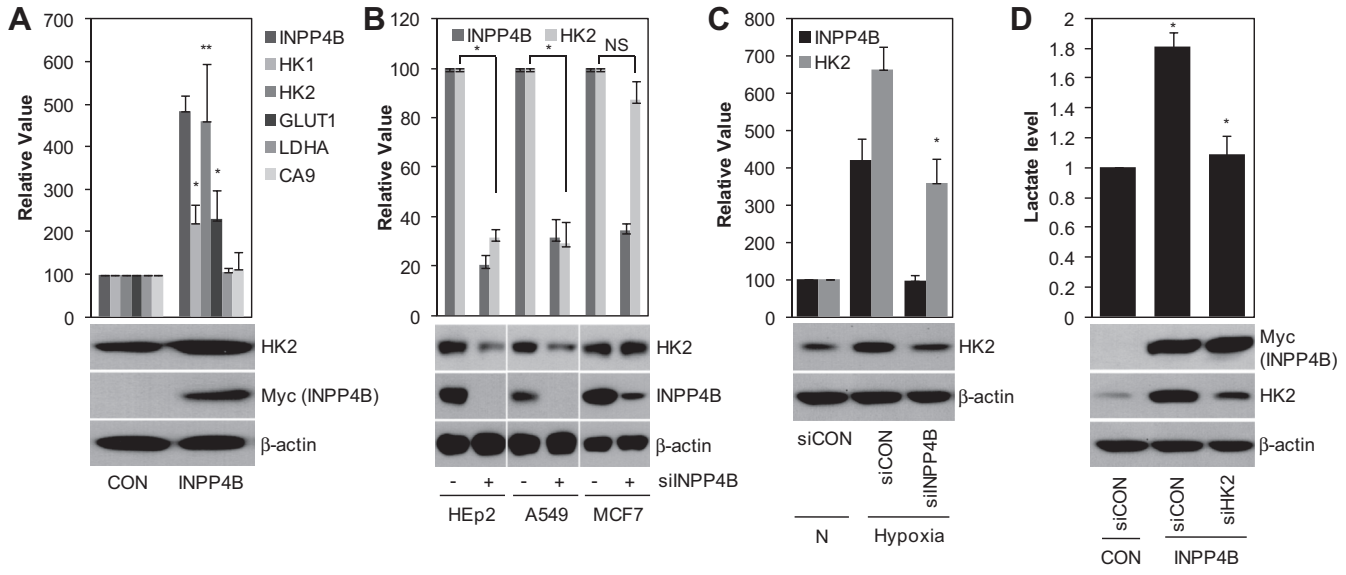


Fig. 2. Up-regulation of HK2 by INPP4B. (A) Control and INPP4B-HEp-2 cells were used to analyze the expression of the regulatory genes involved in the glycolysis pathway. (B) Three cell lines including HEp-2, A549, and MCF7 cells were transfected with 100 nM of control and INPP4B siRNA for 48 h. (C) HEp-2 cells were transfected with the indicated siRNAs targeting control (siCON; 100 nM) and INPP4B (siINPP4B; 100 nM) for 48 h and then exposed to hypoxia (1% O_2) or kept in normoxia (N) for 12 h. (D) Control- and INPP4B-HEp-2 cells were transfected with 100 nM of control and HK2 siRNA for 48 h and then the cultured media were analyzed to measure lactate levels. (A–C) Transcript or protein levels of the indicated genes were analyzed by quantitative PCR (upper panel) or Western blotting (lower panel) respectively. The data is presented as is mean ± SD from 3 independent experiments; $P < 0.05$ (*) and $P < 0.01$ (**), NS = not significant ($P > 0.05$).

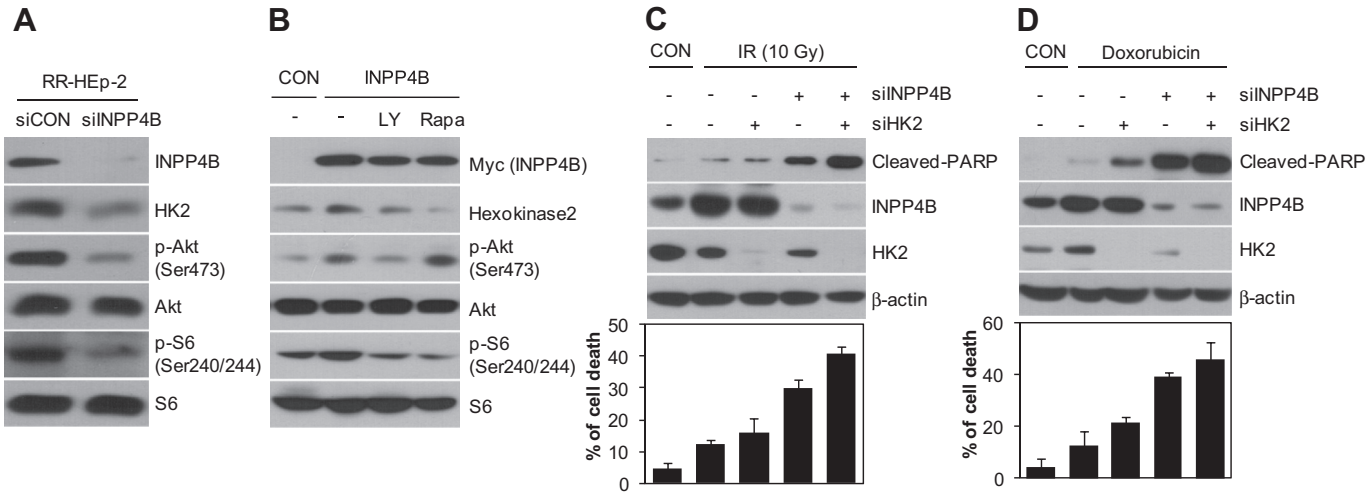
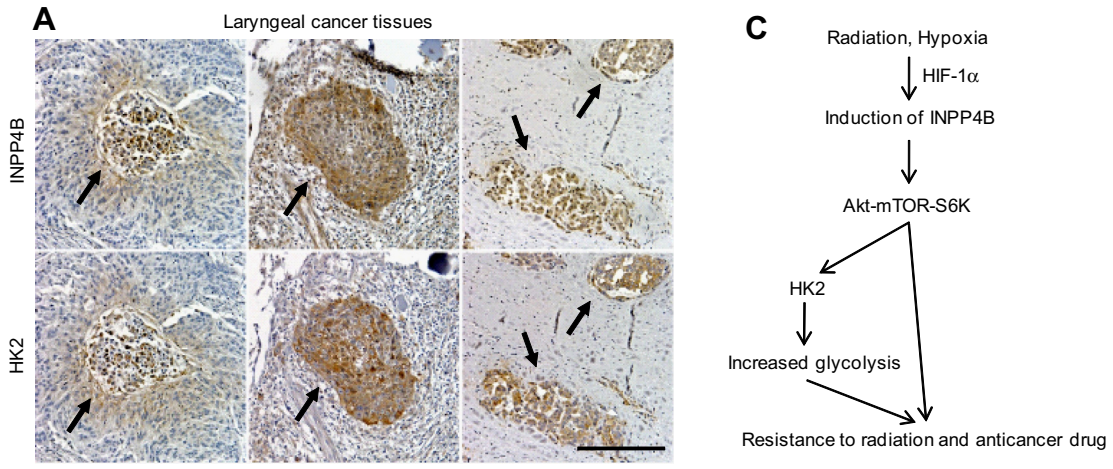


Fig. 3. Sensitization of radioresistant laryngeal cancer cells by codepletion of INPP4B and HK2 to radiation and doxorubicin via Akt-mTOR pathway. RR-HEp-2 cells were transfected with the indicated siRNAs targeting control and INPP4B for 48 h (A) or targeting control, HK2, and INPP4B for 36 h and then treated with 10 Gy radiation for an additional 48 h (C) or 1 μM doxorubicin for an additional 24 h (D). (B) Control- and INPP4B-HEp-2 cells were left untreated – or treated with 10 μM LY294002 (LY) or 5 μM rapamycin (Rapa) and then incubated for 24 h. (C and D) Cell death was determined by the levels of cleaved-PARP (upper panel) and also assessed using the FACScan flow cytometer (lower panel). The levels of the indicated proteins were analyzed by Western blotting. β-actin was used as loading control. The data presented represents a typical result of average values with standard deviations from 3 independent experiments.

increased in INPP4B-overexpressed cells compared to the control cells, and this increased lactate production was reduced by INPP4B depletion (Fig. 1F). Collectively, our results indicated that HIF-1α-regulated INPP4B has the capacity to promote aerobic glycolysis in laryngeal cancer HEp-2 cells.

3.2. INPP4B regulates aerobic glycolysis by inducing HK2

To further understand the regulatory mechanism of INPP4B-mediated glycolysis, we examined the differential expression levels of several regulatory genes related to tumor glycolysis and acidosis between the control and INPP4B-overexpressed cells. Out of the regulatory genes including HK1, HK2, GLUT1, LDHA, and CA9, HK2 was significantly up-regulated by about 4.5-fold in INPP4B-overexpressed cells (Fig. 2A). In addition, HK1 and Glut1 were also moderately up-regulated by approximately 2-fold in INPP4B-overexpressed cells (Fig. 2A, upper panel), implying that INPP4B may modulate glycolysis by regulating glycolysis-regulatory proteins. To confirm this result, INPP4B was depleted with siRNA in 3 different cell lines, HEp-2 laryngeal cancer cells, A549 lung cancer cells, and MCF7 breast cancer cells. INPP4B depletion decreased HK2 both in HEp-2 and A549 cells, but not in MCF7 cells (Fig. 2B), implying that INPP4B may regulate HK2 in cell type-dependent manner. Next, we further determined whether INPP4B regulates HK2 under hypoxic conditions. We found that hypoxia-induced HK2 was reduced by INPP4B depletion (Fig. 2C) and the



B

	Laryngeal cancer						
	INPP4B 0	INPP4B 1+	INPP4B 2+	INPP4B 3+	Total (%)	ρ	*P*
HK2 0	13	6	0	0	19 (32.2%)	0.630	< 0.0001
HK2 1+	3	10	7	4	24 (40.6%)		
HK2 2+	1	0	9	2	44 (20.3%)		
HK2 3+	2	0	0	2	4 (6.7%)		
Total	19 (32.2%)	16 (27.1%)	16 (27.1%)	8 (13.5%)	59 (100%)		

Fig. 4. Association between INPP4B and HK2 expression in laryngeal cancer tissues. (A) Representative microscopic images of laryngeal cancers sections stained with anti-INPP4B antibody (upper panel) and anti-HK2 antibody (lower panel). Arrows indicate the hypoxic regions of tumor tissues. Scale bar, 200 μm. (B) Correlation between INPP4B and HK2 immunostaining among 59 laryngeal cancer specimens. ρ indicates Spearman's correlation coefficient. Staining intensity was scored as follows: 0, no staining; +1, weak; +2, moderate; +3, strong. (C) Schematic diagram depicting the mechanism of INPP4B in radiation and anticancer drug resistance.

increased glycolysis in INPP4B-overexpressed cells was decreased by HK2 depletion (Fig. 2D). Therefore, these results indicate that INPP4B-mediated glycolysis is primarily regulated by HK2 expression.

3.3. INPP4B-mediated HK2 regulation via Akt-mTOR plays an important role in radiation and anticancer drug resistance of laryngeal cancer cells

Since it has been known that Akt-mTOR pathway is tightly associated with increased glycolysis in tumors [21] and INPP4B is involved with radioresistance of laryngeal cancer cells [16], the correlation between Akt-mTOR pathway and INPP4B-mediated HK2 regulation was further investigated in radioresistant HEp-2 (RR-HEp-2) cells. INPP4B depletion in RR-HEp-2 cells decreased the levels of phosphorylated-Akt, -S6, and HK2 (Fig. 3A), indicating that INPP4B is upstream of Akt-mTOR and HK2 regulation. To further dissect the regulatory pathway for INPP4B-mediated HK2 regulation, we used the chemical inhibitors LY294002 and rapamycin to block Akt and mTOR pathway, respectively. Treatment with these inhibitors resulted in the decrease of HK2 in RR-HEp-2 cells and this was more significant in the rapamycin-treated cells, even upon hyper-activation of Akt (Fig. 3B), indicating that INPP4B-mediated HK2 up-regulation is mediated through Akt-mTOR pathway in radioresistant laryngeal cancer cells. Next, we determined if INPP4B-mediated HK2 regulation can modulate the sensitivity of RR-HEp-2 cells to radiation and anticancer drug. As shown in Fig. 3C and D, codepletion of HK2 and INPP4B synergistically sensitized RR-HEp2 cells to the treatment of radiation or doxorubicin. However, HK2 depletion alone did not significantly sensitize RR-HEp-2 cells to radiation and doxorubicin, indicating that blockage of INPP4B-HK2 regulation can sensitize radioresistant laryngeal cancer cells to radiation and anticancer drug. These results suggest that INPP4B-mediated HK2 regulation via Akt-mTOR pathway is important for radiation and anticancer drug resistance of laryngeal cancer cells.

3.4. In vivo correlation between INPP4B and HK2 in human laryngeal cancers

To investigate the physiological relevance of INPP4B-mediated HK2 regulation in laryngeal cancers, INPP4B and HK2 expression were evaluated using tissue microarrays comprising 59 laryngeal tumor tissues. Notably, we found that INPP4B was overexpressed in 67.8% of laryngeal tumor tissues as was HK2 expression (Fig. 4B). In addition, we observed that the staining regions of these 2 proteins overlap in the hypoxic regions of serial sections from the same tissue (Fig. 4A). Moreover, INPP4B expression strongly correlated with HK2 expression in laryngeal tumor tissues based on Spearman's correlation analysis ($\rho = 0.630$; Fig. 4B). Taken together, these observations support that the INPP4B-mediated HK2 regulation in laryngeal cancers.

4. Discussion

In this study, we demonstrate that INPP4B modulates glycolytic phenotype of laryngeal cancer cells and this regulation may contribute to radioresistance and chemoresistance in tumor cells. As depicted in Fig. 4C, our data suggests that exposure of tumor cells to various stress stimuli such as hypoxia and irradiation can trigger the induction of INPP4B and is dependent on HIF-1α in radioresistant laryngeal cancer cells. Increased INPP4B has the capacity to promote glucose metabolism by increasing HK2 via Akt-mTOR pathway. The increased glycolytic phenotype by INPP4B exerts the cytoprotective capacity of radioresistant laryngeal cancer cells and thereby contributes to tumor resistance.

Our results demonstrate that INPP4B is induced by hypoxic stress, supporting that INPP4B is a stress responsive gene. This is in concordance with the earlier reports that INPP4B expression is up-regulated by stress responses such as radiation and anticancer drugs in various cancer cells [16]. Previously, we showed that radiation-induced INPP4B is dependent on ERK pathway. Since it

has been known that HIF-1α protein synthesis is regulated by activation of PI3K and ERK pathways [6], it could be possible that HIF-1α-dependent expression of INPP4B under hypoxia is mediated by ERK activation. With regards to the regulation of INPP4B by HIF-1α, the data obtained indicates that INPP4B expression is associated with increased glycotic phenotype of the laryngeal cancer cells. It is well established that hypoxic microenvironment in tumors leads to a constitutive up-regulation of aerobic glycolysis via stabilization of HIF-1α [21]. HIF-1α directly regulates the transcription of survival- and glycolysis-regulatory genes such as *IGFBPs* and hexokinases under hypoxic stress in order to overcome the stress [5,6]. Thus, our data suggests that INPP4B may be one of HIF-1α-mediated survival factors modulating glycolysis in radioresistant laryngeal cancer cells.

Acquisition of tumor glycolytic phenotype is achieved through multiple mechanisms including oncogene activation or stabilization of transcription factor HIF-1α [6]. Our data that reduction of phosphorylated Akt and S6 in INPP4B-depleted RR-HEp-2 cells indicate that INPP4B modulates oncogene activation but not by direct activation of HIF-1α. Indeed, pharmacological inhibition of Akt-mTOR pathway decreased INPP4B-induced HK2 expression (Fig. 4C), suggesting that INPP4B could regulate aerobic glycolysis via Akt-mTOR-HK2 regulation. In addition, because HK2 play a key role not only in glycolysis but also in the survival of the cancer cell by suppressing cell death [22], INPP4B-mediated HK2 regulation could contribute to not only glycolysis but also towards tumor resistance in laryngeal cancer.

Interestingly, we found that about 70% of laryngeal cancer was positively stained with both INPP4B and HK2 protein and that there is a high correlation between these 2 proteins. While HK2 is overexpressed in many cancers, it has been reported that INPP4B is decreased in aggressive basal-like breast carcinomas [23] and prostate cancer [24]. However, bioinformatic analysis using public cancer database (see Section 2 for details) clearly indicates that INPP4B is highly expressed in various cancers and the mutation rates of INPP4B are relatively low in various cancers (Supplementary Fig. 2). Therefore, this discrepancy may be due to the difference of tumor types. Although the occurrence of INPP4B mutations in laryngeal cancers was not evaluated in the present study, the similar staining pattern of INPP4B and HK2 in hypoxic regions indicates that INPP4B-mediated glycolysis via HK2 regulation might be of physiological relevance in laryngeal cancer.

In conclusion, we show that HIF-1α-dependent induction of INPP4B under hypoxia and irradiation has the capacity to modulate aerobic glycolysis via Akt-mTOR-HK2 regulation in laryngeal cancers. This could also explain the cytoprotective role of INPP4B in the development of tumor-resistance phenotype during radiotherapy.

Acknowledgments

This work was supported by Basic Science Research Program (Grant No. 2012R1A1A2002955) and the Nuclear Research and Development Program through a National Research Foundation of Korea (NRF) grant funded by the Korean government (Ministry of Education, Science and Technology).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.041.

References

[1] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Estimating the world cancer burden: Globocan 2000, Int. J. Cancer 94 (2001) 153–156 (Journal international du cancer).
[2] J.L. Lefebvre, Laryngeal preservation in head and neck cancer: multidisciplinary approach, Lancet Oncol. 7 (2006) 747–755.
[3] A.C. Begg, F.A. Stewart, C. Vens, Strategies to improve radiotherapy with targeted drugs, Nat. Rev. Cancer 11 (2011) 239–253.
[4] J.M. Brown, W.R. Wilson, Exploiting tumor hypoxia in cancer treatment, Nat. Rev. Cancer 4 (2004) 437–447.
[5] R.A. Gatenby, R.J. Gillies, Why do cancers have high aerobic glycolysis?, Nat Rev. Cancer 4 (2004) 891–899.
[6] G.L. Semenza, Targeting HIF-1 for cancer therapy, Nat. Rev. Cancer 3 (2003) 721–732.
[7] M. Kunkel, T.E. Reichert, P. Benz, H.A. Lehr, J.H. Jeong, S. Wieand, P. Bartenstein, W. Wagner, T.L. Whiteside, Overexpression of Glut-1 and increased glucose metabolism in tumors are associated with a poor prognosis in patients with oral squamous cell carcinoma, Cancer 97 (2003) 1015–1024.
[8] M. Younes, R.W. Brown, M. Stephenson, M. Gondo, P.T. Cagle, Overexpression of Glut1 and Glut3 in stage I nonsmall cell lung carcinoma is associated with poor survival, Cancer 80 (1997) 1046–1051.
[9] T.A. Smith, Mammalian hexokinases and their abnormal expression in cancer, Br. J. Biomed. Sci. 57 (2000) 170–178.
[10] D. Palmieri, D. Fitzgerald, S.M. Shreeve, E. Hua, J.L. Bronder, R.J. Weil, S. Davis, A.M. Stark, M.J. Merino, R. Kurek, H.M. Mehdorn, G. Davis, S.M. Steinberg, P.S. Meltzer, K. Aldape, P.S. Steeg, Analyses of resected human brain metastases of breast cancer reveal the association between up-regulation of hexokinase 2 and poor prognosis, Mol. Cancer Res. 7 (2009) 1438–1445.
[11] F.A. Norris, V. Auethavekiat, P.W. Majerus, The isolation and characterization of cDNA encoding human and rat brain inositol polyphosphate 4-phosphatase, J. Biol. Chem. 270 (1995) 16128–16133.
[12] F.A. Norris, R.C. Atkins, P.W. Majerus, The cDNA cloning and characterization of inositol polyphosphate 4-phosphatase type II. Evidence for conserved alternative splicing in the 4-phosphatase family, J. Biol. Chem. 272 (1997) 23859–23864.
[13] C. Gewinner, Z.C. Wang, A. Richardson, J. Teruya-Feldstein, D. Etemadmoghadam, D. Bowtell, J. Barretina, W.M. Lin, L. Rameh, L. Salmena, P.P. Pandolfi, L.C. Cantley, Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling, Cancer Cell 16 (2009) 115–125.
[14] G. Di Paolo, P. De Camilli, Phosphoinositides in cell regulation and membrane dynamics, Nature 443 (2006) 651–657.
[15] K. Kok, B. Geering, B. Vanhaesebroeck, Regulation of phosphoinositide 3-kinase expression in health and disease, Trends Biochem. Sci. 34 (2009) 115–127.
[16] J.S. Kim, H.S. Yun, H.D. Um, J.K. Park, K.H. Lee, C.M. Kang, S.J. Lee, S.G. Hwang, Identification of inositol polyphosphate 4-phosphatase type II as a novel tumor resistance biomarker in human laryngeal cancer HEp-2 cells, Cancer Biol. Ther. 13 (2012) 1307–1318.
[17] J.S. Kim, J.W. Chang, H.S. Yun, K.M. Yang, E.H. Hong, D.H. Kim, H.D. Um, K.H. Lee, S.J. Lee, S.G. Hwang, Chloride intracellular channel 1 identified using proteomic analysis plays an important role in the radiosensitivity of HEp-2 cells via reactive oxygen species production, Proteomics 10 (2010) 2589–2604.
[18] J.S. Kim, J.W. Chang, J.K. Park, S.G. Hwang, Increased aldehyde reductase expression mediates acquired radioresistance of laryngeal cancer cells via modulating p53, Cancer Biol. Ther. 13 (2012) 638–646.
[19] Q. Ke, M. Costa, Hypoxia-inducible factor-1 (HIF-1), Mol. Pharmacol. 70 (2006) 1469–1480.
[20] B.J. Moeller, Y. Cao, C.Y. Li, M.W. Dewhirst, Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules, Cancer Cell 5 (2004) 429–441.
[21] G. Kroemer, J. Pouyssegur, Tumor cell metabolism: cancer's Achilles' heel, Cancer Cell 13 (2008) 472–482.
[22] S.P. Mathupala, Y.H. Ko, P.L. Pedersen, Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria, Oncogene 25 (2006) 4777–4786.
[23] C.G. Fedele, L.M. Ooms, M. Ho, J. Vieusseux, S.A. O'Toole, E.K. Millar, E. Lopez-Knowles, A. Sriratana, R. Gurung, L. Baglietto, G.G. Giles, C.G. Bailey, J.E. Rasko, B.J. Shields, J.T. Price, P.W. Majerus, R.L. Sutherland, T. Tiganis, C.A. McLean, C.A. Mitchell, Inositol polyphosphate 4-phosphatase II regulates PI3K/Akt signaling and is lost in human basal-like breast cancers, Proc. Natl. Acad. Sci. USA 107 (2010) 22231–22236.
[24] M.C. Hodgson, L.J. Shao, A. Frolov, R. Li, L.E. Peterson, G. Ayala, M.M. Ittmann, N.L. Weigel, I.U. Agoulnik, Decreased expression and androgen regulation of the tumor suppressor gene INPP4B in prostate cancer, Cancer Res. 71 (2011) 572–582.