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PI3K/Akt signaling mediated Hexokinase-2 expression inhibits cell apoptosis and promotes tumor growth in pediatric osteosarcoma

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ABSTRACT

Accumulating evidence has shown that PI3K/Akt pathway is frequently hyperactivated in osteosarcoma (OS) and contributes to tumor initiation and progression. Altered phenotype of glucose metabolism is a key hallmark of cancer cells including OS. However, the relationship between PI3K/Akt pathway and glucose metabolism in OS remains largely unexplored. In this study, we showed that elevated Hexokinase-2 (HK2) expression, which catalyzes the first essential step of glucose metabolism by conversion of glucose into glucose-6-phosphate, was induced by activated PI3K/Akt signaling. Immunohistochemical analysis showed that HK2 was overexpressed in 83.3% (25/30) specimens detected and was closely correlated with Ki67, a cell proliferation index. Silencing of endogenous HK2 resulted in decreased aerobic glycolysis as demonstrated by reduced glucose consumption and lactate production. Inhibition of PI3K/Akt signaling also suppressed aerobic glycolysis and this effect can be reversed by reintroduction of HK2. Furthermore, knockdown of HK2 led to increased cell apoptosis and reduced ability of colony formation; meanwhile, these effects were blocked by 2-Deoxy-D-glucose (2-DG), a glycolysis inhibitor through its actions on hexokinase, indicating that HK2 functions in cell apoptosis and growth were mediated by altered aerobic glycolysis. Taken together, our study reveals a novel relationship between PI3K/Akt signaling and aerobic glycolysis and indicates that PI3K/Akt/HK2 might be potential therapeutic approaches for OS.

1. Introduction

Osteosarcoma (OS) is the most primary common bone malignancy and a leading cause of cancer-related death in children and adolescents [1]. Despite great advances were achieved in chemotherapy, radiation therapy and surgical resection, there are still a large number of patients who respond unfavorably to preoperative chemotherapy and have a high risk of local relapse or distant metastasis, which ultimately leads to a poor prognosis [2]. Given there is limited clinical significance to optimize conventional therapeutic approaches, more endeavors should be put in developing novel approaches for treating OS. Therefore, it is necessary to clarify the fundamental molecular mechanisms underlying the pathogenesis and progression of this deadly disease, such as histological heterogeneity, drug resistance, and distant metastasis, in order to identify novel therapeutic targets that can complement current treatment and further improve the OS patients' prognosis [3–5].

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is turned to be a key oncogenic pathway in multiple human cancers including OS [6]. The PI3K/Akt pathway is a cascade of events, whose dysregulation in OS plays a critical role in a large number of pathological processes, such as tumorigenesis [7], cell proliferation [8], cell cycle progression [9], inhibition of apoptosis [10], cell invasion [11], metastasis [12], angiogenesis [13] and chemoresistance [14]. After activation, the PI3K/Akt pathway can modulate many downstream targets to regulate various cellular functions. For example, activated Akt leads to activation of mTORC1, which mediates the phosphorylation of S6K and 4E-BP1and ultimately results in enhanced protein synthesis [15]; reduced FOXO1 induced by Akt decreases p27 and p21 expression and in turn increases CDK

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expression, which ultimately promotes cell cycle progression [16]. Tumor cells exhibits an altered metabolic phenotype known as Warburg effect, which favors tumor growth and metastasis through multiple different mechanisms [17]. Many glycolytic enzymes including Glut1, HK2 and LDHA are amplified in the pathogenesis of cancer [18–20]. These also include alterations that increase flux through glycolysis induced by PFKFB3 and PGAM1 as well as PHGDH-mediated diversion of glycolytic intermediates to biosynthetic pathways [21]. However, little is known about the relationship between the PI3K/Akt pathway and Warburg effect in OS. The aim of the present study was to investigate whether activated PI3K/Akt signaling affects the expression glycolytic enzymes, which further promotes tumor progression via enhancing Warburg effect.

2. Materials and methods

2.1. Clinical tissue samples

This study was approved by the Research Ethics Committee of the Children's Hospital of Xuzhou and Affiliated Hospital of Xuzhou Medical College. Written informed consent was obtained from all of the patients. Thirty OS patients young than 21 years was enrolled in this study between January 1, 2014 and December 1, 2014 at the Children's Hospital of Xuzhou. Tumor tissue was obtained at the time of the initial biopsy prior to treatment. All specimens were collected in full accordance with the ethical and legal standards.

2.2. Cell culture and transfection

Human OS cells including KRIB, MG63, HOS, KHOS, Saos2 and U2OS were all obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 according to ATCC protocols. All the culture media was supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% antibiotics. 2-DG, phen and LY294002 were purchased from Sigma. MK-2206 was purchased from Selleck. All reagents were diluted to preferable concentrations in each cell culture medium before use. Specific siRNA targeting HK2 as well as a negative control were obtained from GenePharma, Shanghai, China. An HK2 expression plasmid and vector were obtained from GeneCopoeia™. The transfection was performed according to the manufacturer's protocol.

2.3. Quantitative real-time PCR

Total RNA from OS cells was extracted with Trizol reagent (Invitrogen) and reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Life technologies). The cDNA products were used for real-time PCR analysis. Gene expression was monitored using ABI Prism 7300 Sequence Detection System with SYBR Green (Applied Biosystems, Life Technology). The primer sequences for genes were defined as follows: Glut1 forward 5′-ATTGGCTCCGGTATCGTCAAC-3′, reverse 5′-GCTCAGATAGGACATCCAGGGTA-3′; HK2 forward: 5′-TTGACCAGGAGATTGACATGGG-3′, reverse 5′-CAACCGCATCAGGACCTCA-3′; PFKL forward: 5′-GCTGGGCGGCACTATCATT-3′, reverse 5′-TCAGGTGCGAGTAGGTCCG-3′; PGK1 forward 5′-TGGACGTTAAAGGGAAGCGG-3′, reverse 5′-GCTCATAAGGACTACCGACTTGG-3′; PKM forward 5′-ATAACGCCTACATGGAAAAGTGT-3′, reverse 5′-TAAGCCCATCATCCACGTAGA-3′; LDHA forward 5′-ATGGCAACTCTAAAGGATCAGC-3′, reverse 5′-CCAACCCCAACAACTGTAATCT-3′; PDK1 forward 5′-ATGGCAACTCTAAAGGATCAGC-3′, reverse 5′-CCAACCCCAACAACTGTAATCT-3′; β-actin forward 5′-CATGTACGTTGCTATCCAGGC-3′, reverse 5′-CTCCTTAATGTCACGCACGAT-3′. The ΔΔCt method was used to calculate the relative fold change in mRNA expression. All samples were normalized to β-actin expression levels.

2.4. Western blotting

Cells lysates were separated by 8–10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membrane. The membrane was then blocked by 5% non-fat dry milk in Tris-buffered saline (TBS) at room temperature for 1 h to block non-specific binding. The membranes were incubated with the primary antibodies against HK2 (1:1000; Proteintech, USA). Antibody against β-actin (1:1000; Sigma, USA) was used as an internal control for protein loading. Membranes were washed three times for 5 min and incubated with HRP-conjugated secondary antibodies for 60 min. Blots were washed with TBS three times and developed with the enhanced chemiluminescence system (GE Healthcare, USA).

2.5. Immunohistochemistry

Immunohistochemistry was performed according to the immunohistochemistry-paraffin (IHC–P) staining protocol of Abcam (Cambridge, UK) (http://www.abcam.com/index.html?pageconfig=resource&rid=11384). Visualization was performed by 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained by hematoxylin. Scoring was conducted by the area of positive staining on a scale of 0–3: 0–10% scored 0; 10%–50% scored 1; 51%–75% scored 2; more than 70% scored 3. And scored at 0 and 1 was defined as low expression, while 2 and 3 was defined as high expression. Immunostaining results were independently evaluated by two clinical pathologists with no information of the clinicopathological features.

2.6. Cell apoptosis assay

Cells were seeded in 6-well plates at 4×10^5 cells per well under serum deprivation for 48 h. Adherent cells were detached with 0.25% trypsin without EDTA in 1 × PBS and harvested in complete DMEM medium and centrifuged at 1000 rpm for 5 min. Cells in each group were washed with 1 × PBS and stained with propidium iodide (PI) and Annexin V-FITC (Dojindo, Japan) following the manufacturer's instructions. Then the apoptotic cells were measured by flow cytometry.

2.7. Cell plate colony formation assay

Equal number of control cells and HK2-slienced cells were seeded in 6-well plates and cultured in medium supplemented with 10% FBS for 7 days. The media was changed every other day. Cell growth was terminated after 9 days incubation and fixed with methanol and stained with Giemsa. After staining, the number of fixed colonies in each group were recorded and counted.

2.8. Measurement of glucose uptake and lactate production

5×10^5 MG63 or U2OS cells were seeded in fresh phenol red-free medium and the culture medium was collected in the first 24 h after HK2-siRNA, LY294002 and MK-2206 treatment. The glucose and lactate levels were detected by glucose assay kits (Life technologies) or lactate assay kits (Biovision) according to manufacturer's instructions. All experiments were performed at least three times.

2.9. Statistical analysis

Data were presented as the means ± SD. Statistical analyses and graphical representations were performed with SPSS 16.0 (SPSS Inc.; Chicago, USA) and GraphPad Prism 5 (San Diego, CA) software. The student's t-test was used to determine the statistically significant differences among indicated experimental results. A P value < 0.05 was considered as statistically significant.

3. Results

3.1. Elevated expression of HK2 induced by activated PI3K/Akt signaling

To determine the possible correlation between PI3K/Akt signaling and Warburg effect, we first detected the alternations in expression of glycolytic enzymes after treatment with phen, which selectively inhibits PTEN activity and ultimately results in activation of PI3K/Akt signaling. As shown in Fig. 1A, mRNA expression of HK2 was significantly promoted by phen treatment in both MG63 and U2OS cells, while other glycolytic enzymes including Glut1, PFKL, Pgk1, PKM, LDHA and Pdk1 remain unchanged. To further confirm this observation, we treated OS cells with LY294002 and MK-2206, which are inhibitors for PI3K and Akt, respectively. Expectedly, treatment with LY294002 markedly reduced the protein expression of HK2 in MG63 and U2OS cells (Fig. 1B).Similar result was found in MK-2206-mediated suppression of Akt (Fig. 1C). Taken together, these data above indicate that PI3K/Akt signaling might affect Warburg effect through up-regulation of HK2 expression.

3.2. Expression pattern of HK2 in clinical specimens and cell lines

To observe the expression pattern of HK2 in pediatric OS, we performed immunohistochemical analysis in 30 clinical pediatric patient derived specimens. The clinical characteristics are shown in Table 1. The result showed that HK2 was overexpressed in 83.3% (25/30) OS specimens detected (Fig. 2A), while more weakly or moderately positive staining was observed in normal bone tissues (Fig. 2B). Importantly, HK2 immunoreactivity was not only found in cytoplasm of OS cells, but also in the nucleus. Meanwhile, HK2 expression closely correlated with the proliferation index as assessed by Ki67 staining ($p < 0.01$, Fig. 2C). Furthermore, HK2 expression was detected in six OS cell lines. As shown in Fig. 2D, HK2 was commonly overexpressed in OS cell lines except for KRIB. These data strongly support that HK2 expression is deregulated in OS and might contribute to tumor growth.

Table 1
Clinical characteristics of the study population.

Variable	Characteristic	Cases (n = 30)
Gender	Male	21
	Female	9
Age (years)	<14	11
	14–21	19
Enneking's stage	IIA	10
	IIB	20
Tumor location	Femur	19
	Tibia	9
	Humerus	1
	Others	1
Local recurrence	Yes	12
	No	18
Lung metastases	Yes	9
	No	21

3.3. HK2 inhibits cell apoptosis and promotes tumor growth through enhanced Warburg effect

To investigate the cellular functions of HK2, we silenced endogenous HK2 expression by small interference RNA (siRNA). As shown in Fig. 3A, both two siRNAs (si-1 and si-2) significantly reduced HK2 expression in MG63 and U2OS cells. And expectedly, suppression of HK2 led to decreased Warburg effect as demonstrated by reduced glucose consumption and lactate production ($p < 0.05$, Fig. 3B). Besides, whether treatment with LY294002 or MK-2206, the Warburg effect was significantly inhibited and this type of inhibition can be reversed by reintroduction of HK2 ($p < 0.05$, Fig. 3C). This result further confirmed the relationship between PI3K/Akt signaling and Warburg effect. Given its crucial roles of in the Warburg effect, we hypothesized whether HK2 affects the biological functions of OS cells. To test this hypothesis, we performed cell apoptosis assay and plate colony formation assay. The result showed that knockdown of HK2 remarkably promoted cell apoptosis ($p < 0.05$, Fig. 3D) and inhibited tumor cell growth

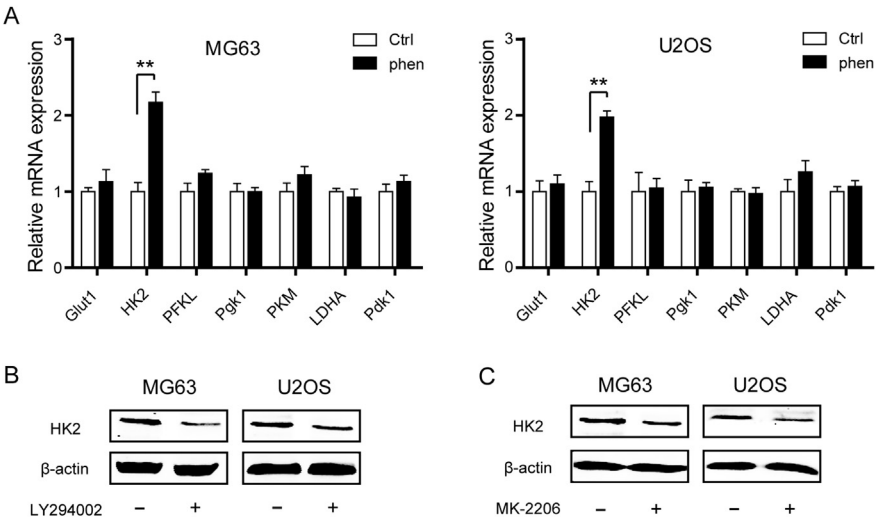

Fig. 1. Elevated expression of HK2 induced by activated PI3K/Akt signaling. (A) The alternations in glycolytic enzymes induced by phen treatment. (B) HK2 protein level was detected after treatment with LY294002. (C) HK2 protein level was detected after treatment with MK-2206.

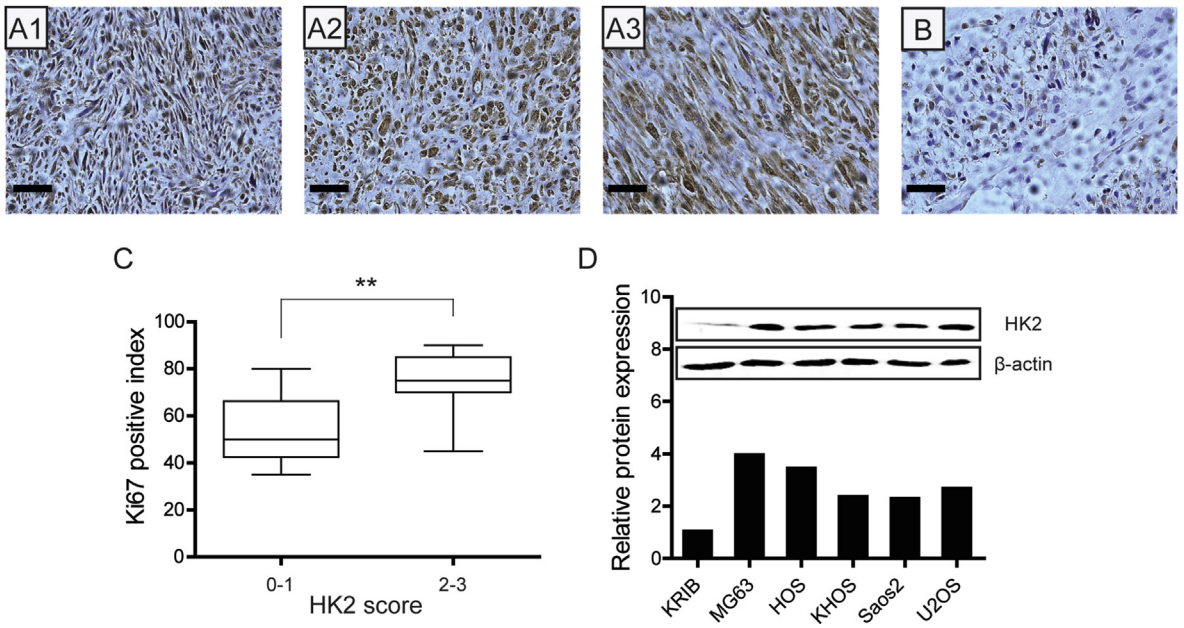

Fig. 2. Up-regulated HK2 expression in OS clinical specimens and cell lines. (A1–3) Representative images of HK2 positive staining in OS tissues. (B) Representative images of HK2 expression in normal bone tissues. (C) Correlation between HK2 expression and ki67 positive staining percentage. (D) HK2 protein expression in six OS cell lines. $^{**}P < 0.01$.

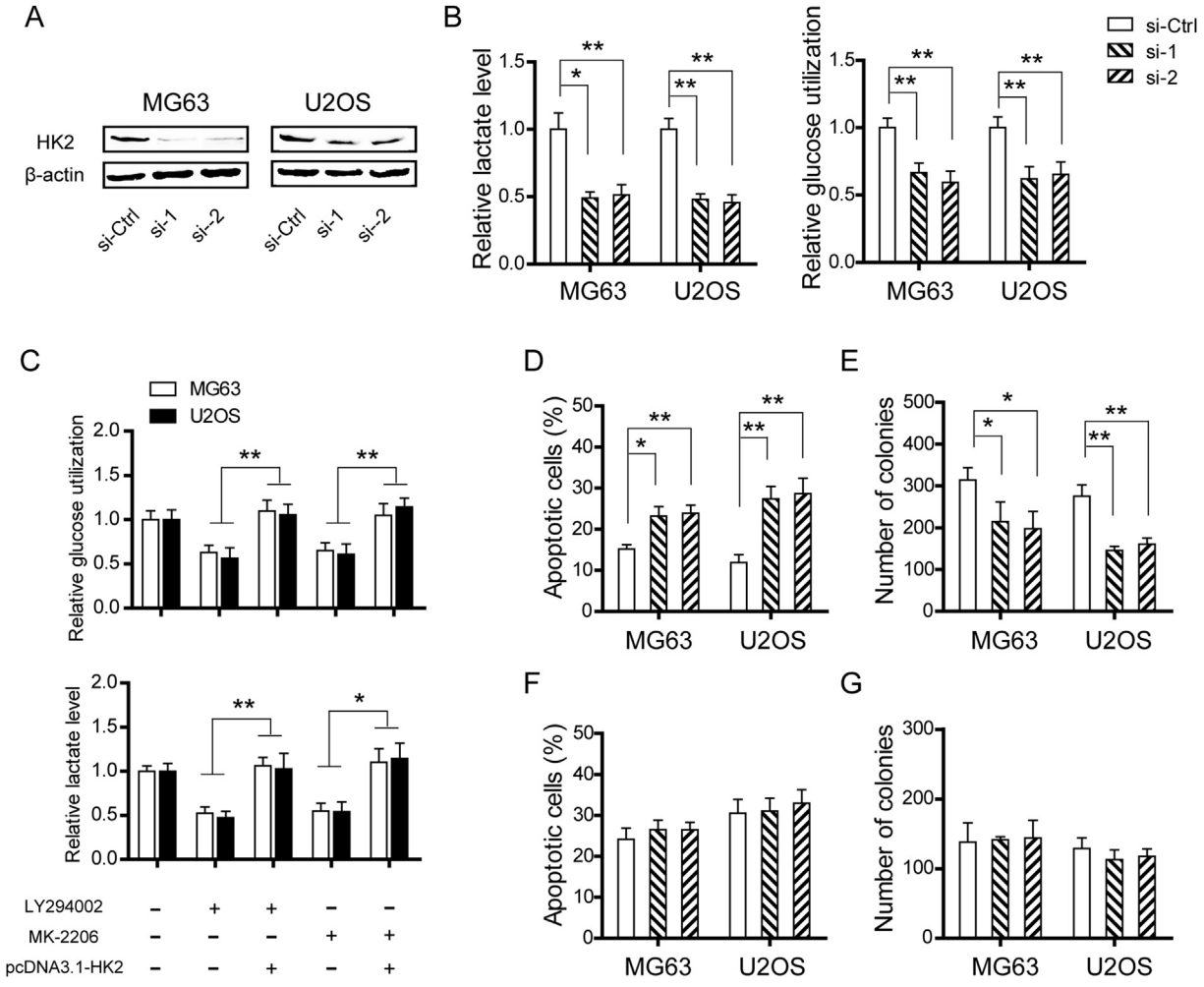

Fig. 3. HK2 inhibits cell apoptosis and promotes tumor growth through enhanced Warburg effect. (A) Interfere efficiency of HK2 in MG63 and U2OS cells was detected by Western blotting. (B) Lactate production and glucose consumption were measured after silencing of endogenous HK2. (C) Lactate production and glucose consumption were measured after treatment of LY294002, MK-2206 and reintroduction of HK2. In the absence (D) or presence (E) of 2-DG, cell apoptosis ratio was measured after silencing of endogenous HK2. In the absence (F) or presence (G) of 2-DG, colony formation assay was performed after HK2 was knocked down. $^{*}P < 0.05$; $^{**}P < 0.01$.

($p < 0.05$, Fig. 3E). In the presence of 2-DG, a glycolysis inhibitor through its actions on hexokinase (HK), however, no significant difference was observed in apoptosis ratio (Fig. 3F) or number of colonies (Fig. 3G) between HK2-slilenced cells and the negative cells, indicating that the oncogenic activity of HK2 in OS cells was dependent on enhanced Warburg effect.

4. Discussion

The study reveals a novel phenomenon that PI3K/Akt pathway mediated HK2 expression up-regulates Warburg effect and further facilitates tumor growth. We demonstrated that activation of PI3K/Akt pathway leads to elevated expression of the glycolytic enzyme HK2, thereby promoting Warburg effect. Targeting the endogenous HK2 expression promoted tumor cell apoptosis and reduced cell growth *in vitro*.

The selective advantage of aerobic glycolysis (Warburg effect) has been documented in multiple types of tumor cells and in non-neoplastic thymotes [22]. The first step of aerobic glycolysis is catalyzed by HK. HK1 is expressed in normal tissues, while its expression is replaced by HK2 during several malignant transformations [23]. Consistent with previous reports in renal cell carcinoma [24], glioblastoma [25] and laryngeal cancer [26], our observations confirmed the elevated HK2 expression in pediatric OS. However, we also observed intense staining in the nucleus of OS cells, indicating an underlying role of HK2 in nucleus distribution-dependent manner. There are many mechanisms involved in regulation of HK2 expression. The most important regulator of HK2 expression is c-Myc, a transcription factor that plays crucial roles in cell growth, apoptosis, differentiation and metabolism [27]. In this study, we found that activated PI3K/Akt pathway also can up-regulate HK2 expression but not Glut1, PFKL, Pgk1, PKM, LDHA and Pdk1 in OS cells. We aslo noted that silencing of HK2 expression exerted profound effects on aerobic glycolysis, indicating HK2 is required for maximal glycolysis. Consistent with this, HK2 is well known to the location of outer mitochondrial membrane and this sub-location is critical to its pro-glycolytic functions [28].

The roles of HK2 in tumorigenesis have been demonstrated in several different studies by pharmaceutical or genetic inhibition. Consistent with these reports that inhibition of HK2 exhibits a tumor-suppressive role in thyroid cancer [29], hepatocellular carcinoma [30] and colon cancer [31], we found that silencing of HK2 expression results in increased cell apoptosis and reduced colony formation ability. However, the exact mechanism involved in HK2-mediated glucose metabolism and tumor growth remain largely unexplored. Previous reports have shown that mitochondria-bound HK2 is closely associated with cell apoptosis and mitochondrial cell death [32,33]. Different from this type of mechanism, we showed the anti-apoptotic and pro-growth roles of HK2 are dependent on enhanced Warburg effect. Aerobic glycolysis can generate ATP at a higher rate than oxidative phosphorylation and confers a biosynthetic advantage for tumor rapid proliferation [34]. Increased expression of HK2 in cancer cells drives nucleotide and lipid biosynthesis as well as glutamine-dependent anaplerosis [35]. Accordingly, it is not surprising that the oncogenic activity of HK2 in OS was mediated by enhanced Warburg effect. Because of low specificity, current agents targeting HK2, such as 3-bromopyruvate and 2-DG, show limited clinical potential [35]. One promising strategy is to target HK2 binding to VDAC, thereby blocking its mitochondrial association and cellular functions.

In conclusion, our study identified that elevated HK2 in OS induced by activated PI3K/Akt signaling play an anti-apoptotic and pro-proliferation role through regulating Warburg effect. Importantly, our results highlight that PI3K/Akt/HK2 axis acts as a potential therapeutic approaches for treatment of OS.

Conflict of interest

The authors declare that there is no conflict of interests.

Acknowledgment

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Transparency document

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References

[1] M.L. Broadhead, J.C. Clark, D.E. Myers, C.R. Dass, P.F. Choong, The molecular pathogenesis of osteosarcoma: a review, Sarcoma 2011 (2011) 959248.
[2] R. Gorlick, Current concepts on the molecular biology of osteosarcoma, Cancer Treat. Res. 152 (2009) 467–478.
[3] J. PosthumaDeBoer, M.A. Witlox, G.J. Kaspers, B.J. van Royen, Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature, Clin. Exp. Metastasis 28 (2011) 493–503.
[4] J.P. He, Y. Hao, X.L. Wang, X.J. Yang, J.F. Shao, F.J. Guo, J.X. Feng, Review of the molecular pathogenesis of osteosarcoma, Asian Pac J. Cancer Prev. 15 (2014) 5967–5976.
[5] C.L. Haddox, G. Han, L. Anijar, O. Binitie, G.D. Letson, M.M. Bui, D.R. Reed, Osteosarcoma in pediatric patients and young adults: a single institution retrospective review of presentation, therapy, and outcome, Sarcoma 2014 (2014) 402509.
[6] J. Zhang, X.H. Yu, Y.G. Yan, C. Wang, W.J. Wang, PI3K/Akt signaling in osteosarcoma, Clin. Chim. Acta 444 (2015) 182–192.
[7] F. Pompetti, P. Rizzo, R.M. Simon, B. Freidlin, D.J. Mew, H.I. Pass, P. Picci, A.S. Levine, M. Carbone, Oncogene alterations in primary, recurrent, and metastatic human bone tumors, J. Cell. Biochem. 63 (1996) 37–50.
[8] S. Takagi, A. Takemoto, M. Takami, T. Oh-Hara, N. Fujita, Platelets promote osteosarcoma cell growth through activation of the platelet-derived growth factor receptor-Akt signaling axis, Cancer Sci. 105 (2014) 983–988.
[9] P. D'Arcy, W. Maruwge, B. Wolahan, L. Ma, B. Brodin, Oncogenic functions of the cancer-testis antigen SSX on the proliferation, survival, and signaling pathways of cancer cells, PLoS One 9 (2014) e95136.
[10] L. Ding, S. He, X. Sun, HSP70 desensitizes osteosarcoma cells to baicalein and protects cells from undergoing apoptosis, Apoptosis 19 (2014) 1269–1280.
[11] A. Nakanishi, Y. Wada, Y. Kitagishi, S. Matsuda, Link between PI3K/AKT/PTEN pathway and NOX proteinin diseases, Aging Dis. 5 (2014) 203–211.
[12] Y.S. Guo, R. Zhao, J. Ma, W. Cui, Z. Sun, B. Gao, S. He, Y.H. Han, J. Fan, L. Yang, J. Tang, Z.J. Luo, betaig-h3 promotes human osteosarcoma cells metastasis by interacting with integrin alpha2beta1 and activating PI3K signaling pathway, PLoS One 9 (2014) e90220.
[13] A. Klotzsche-von Ameln, I. Prade, M. Grosser, A. Kettelhake, M. Rezaei, T. Chavakis, I. Flamme, B. Wielockx, G. Breier, PHD4 stimulates tumor angiogenesis in osteosarcoma cells via TGF-alpha, Mol. Cancer Res. 11 (2013) 1337–1348.
[14] G. Zhao, C. Cai, T. Yang, X. Qiu, B. Liao, W. Li, Z. Ji, J. Zhao, H. Zhao, M. Guo, Q. Ma, C. Xiao, Q. Fan, B. Ma, MicroRNA-221 induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma, PLoS One 8 (2013) e53906.
[15] K. Inoki, Y. Li, T. Zhu, J. Wu, K.L. Guan, TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling, Nat. Cell. Biol. 4 (2002) 648–657.
[16] S.R. Boreddy, K.C. Pramanik, S.K. Srivastava, Pancreatic tumor suppression by benzyl isothiocyanate is associated with inhibition of PI3K/AKT/FOXO pathway, Clin. Cancer Res. 17 (2011) 1784–1795.
[17] B. Faubert, G. Boily, S. Izreig, T. Griss, B. Samborska, Z. Dong, F. Dupuy, C. Chambers, B.J. Fuerth, B. Viollet, O.A. Mamer, D. Avizonis, R.J. DeBerardinis, P.M. Siegel, R.G. Jones, AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo, Cell. Metab. 17 (2013) 113–124.
[18] K. Shibuya, M. Okada, S. Suzuki, M. Seino, S. Seino, H. Takeda, C. Kitanaka, Targeting the facilitative glucose transporter GLUT1 inhibits the self-renewal and tumor-initiating capacity of cancer stem cells, Oncotarget 6 (2015) 651–661.
[19] A.T. Ooi, B.N. Gomperts, Molecular pathways: targeting cellular energy metabolism in cancer via inhibition of SLC2A1 and LDHA, Clin. Cancer Res. 21 (11) (2015 Jun 1) 2440–2444.
[20] M. Shi, J. Cui, J. Du, D. Wei, Z. Jia, J. Zhang, Z. Zhu, Y. Gao, K. Xie, A novel KLF4/LDHA signaling pathway regulates aerobic glycolysis in and progression of pancreatic cancer, Clin. Cancer Res. 20 (2014) 4370–4380.
[21] L.W. Finley, J. Zhang, J. Ye, P.S. Ward, C.B. Thompson, SnapShot: cancer metabolism pathways, Cell. Metab. 17 (2013) 466, 466 e462.
[22] K. Brand, S. Aichinger, S. Forster, S. Kupper, B. Neumann, W. Nurnberg, G. Ohrisch, Cell-cycle-related metabolic and enzymatic events in proliferating rat thymocytes, Eur. J. Biochem. 172 (1988) 695–702.

[23] P.L. Pedersen, S. Mathupala, A. Rempel, J.F. Geschwind, Y.H. Ko, Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention, Biochim. Biophys. Acta 1555 (2002) 14–20.
[24] H. Yoshino, H. Enokida, T. Itesako, S. Kojima, T. Kinoshita, S. Tatarano, T. Chiyomaru, M. Nakagawa, N. Seki, Tumor-suppressive microRNA-143/145 cluster targets hexokinase-2 in renal cell carcinoma, Cancer Sci. 104 (2013) 1567–1574.
[25] A. Wolf, S. Agnihotri, J. Micallef, J. Mukherjee, N. Sabha, R. Cairns, C. Hawkins, A. Guha, Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme, J. Exp. Med. 208 (2011) 313–326.
[26] J. Chen, S. Zhang, Y. Li, Z. Tang, W. Kong, Hexokinase 2 overexpression promotes the proliferation and survival of laryngeal squamous cell carcinoma, Tumour Biol. 35 (2014) 3743–3753.
[27] C.V. Dang, c-Myc target genes involved in cell growth, apoptosis, and metabolism, Mol. Cell. Biol. 19 (1999) 1–11.
[28] S.P. Mathupala, Y.H. Ko, P.L. Pedersen, Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg Effect" and a pivotal target for effective therapy, Semin. Cancer Biol. 19 (2009) 17–24.
[29] J.E. Kim, B.C. Ahn, M.H. Hwang, Y.H. Jeon, S.Y. Jeong, S.W. Lee, J. Lee, Combined RNA interference of hexokinase II and (131)I-sodium iodide symporter gene therapy for anaplastic thyroid carcinoma, J. Nucl. Med. 52 (2011) 1756–1763.
[30] K.J. Ahn, H.S. Hwang, J.H. Park, S.H. Bang, W.J. Kang, M. Yun, J.D. Lee, Evaluation of the role of hexokinase type II in cellular proliferation and apoptosis using human hepatocellular carcinoma cell lines, J. Nucl. Med. 50 (2009) 1525–1532.
[31] Q. Peng, Q. Zhou, J. Zhou, D. Zhong, F. Pan, H. Liang, Stable RNA interference of hexokinase II gene inhibits human colon cancer LoVo cell growth in vitro and in vivo, Cancer Biol. Ther. 7 (2008) 1128–1135.
[32] E. Bustamante, H.P. Morris, P.L. Pedersen, Energy metabolism of tumor cells. Requirement for a form of hexokinase with a propensity for mitochondrial binding, J. Biol. Chem. 256 (1981) 8699–8704.
[33] L. Sun, S. Shukair, T.J. Naik, F. Moazed, H. Ardehali, Glucose phosphorylation and mitochondrial binding are required for the protective effects of hexokinases I and II, Mol. Cell. Biol. 28 (2008) 1007–1017.
[34] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, Nat. Rev. Cancer 11 (2011) 85–95.
[35] S. Ros, A. Schulze, Glycolysis back in the limelight: systemic targeting of HK2 blocks tumor growth, Cancer Discov. 3 (2013) 1105–1107.