Int J Clin Exp Pathol 2016;9(2):1014-1021
www.ijcep.com /ISSN:1936-2625/IJCEP0018588

Original Article

Knockdown of KIF3a inhibits hypoxia-induced epithelial-to-mesenchymal transition via suppression of the Wnt/β-catenin pathway in thyroid cancer

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Received October 27, 2015; Accepted January 21, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: Hypoxia induced epithelial-to-mesenchymal transition (EMT) to facilitate the tumor biology. KIF3a, a member of the kinesin family of motor proteins, mediates the tumor mobility, invasion, and metastasis. The definite function of KIF3a in regulating the EMT of thyroid cancer, however, is still unclear. Here, we examined the functional role of KIF3a and the underlying molecular mechanisms in hypoxia-induced EMT in thyroid cancer cells. Our results showed that KIF3a mRNA and protein expression was markedly increased in the human thyroid cancer tissues and cell lines. Knockdown of KIF3a significantly inhibited hypoxia-induced migration and invasion, and EMT process. Furthermore, knockdown of KIF3a prevented hypoxia-induced the expression of β-catenin, cyclin D1 and c-myc in FTC133 cells. In summary, the study reported here provided evidence that knockdown of KIF3a inhibited hypoxia-induced EMT of thyroid cancer cells via suppressing the Wnt/β-catenin signaling pathway. Our study uncovered a novel role for KIF3a in thyroid cancer progression, which might support the potential for KIF3a targeting in thyroid cancer therapy.

Keywords: KIF3a, hypoxia, thyroid cancer, epithelial-to-mesenchymal transition (EMT)

Introduction

Thyroid cancer is one of the most common endocrine malignancies, and the incidence of thyroid cancer has increased rapidly across the world in recent decades [1]. Despite the advancements in therapeutic methods, metastatic thyroid cancer remains incurable and results in poor patient outcomes [2]. Therefore, dissecting the molecular mechanisms that regulate thyroid cancer invasion may facilitate the advancement of clinical treatment.

Epithelial-mesenchymal transition (EMT) is one of the major molecular mechanisms involved during oncogenesis to promote cancer progress [3]. During EMT procedure, the actin cytoskeleton is reorganized and cells acquire increased cell-matrix contacts, leading to dissociation from surrounding cells and enhanced migration and invasion [4]. Accumulating evidence has shown that hypoxia, a critical microenvironmental factor, can trigger the EMT program in cancer [5-7].

The pathogenesis of thyroid cancer is believed to be a multistep process that involves multiple genetic changes, including loss of tumor suppressor genes and activation of oncogenes. KIF3a is a member of the kinesin family of motor proteins. It has been shown to regulate bone formationand ciliogenesis [8-10]. For example, Qiu*et al*. reported that selective deletion of KIF3a in osteoblasts disrupts primary cilia formation and/or function and impairs osteoblast-mediated bone formation [11]. Furthermore, KIF3a is emerging as a crucial regulator of cancer initiation and progression. Exogenous expression of KIF3a promoted cell growth in the benign prostate cells, in contrast, silencing KIF3a decreased cell proliferation, anchorage-independent cell growth, and cell migration/invasionin cancer cells [12]. The definite function of KIF3a in regulating the EMT of thyroid cancer, however, is still unclear. Here, we examined the functional role of KIF3a and the underlying molecular mechanisms in hypoxia-induced thyroid cancer EMT.

Materials and methods

Tissue specimens

Thyroid cancertissues were obtained from patients undergoing surgical treatment at the Department of Thyroid and Breast, Huaihe Hospital, Henan University (China), during the period from 2013 to 2014. Normal thyroid tissue samples were obtained from non-pathologic areas distant from tumors in surgical specimens. All patients had given informed consent. Dissected samples were frozen immediately after surgery and stored at -80°C until needed.

Cell culture and hypoxia treatment

Human thyroid cancer lines FTC133 and TPC1 were purchased from the American Type Culture Collection (USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM), with supplements of 10% (v/v) fetal bovine serum (FBS; Gibco, Rockville, MD) and 100 units/ml streptomycin and penicillin (Gibco, Rockville, MD) in a humidified atmosphere containing 5% CO_2 incubator at 37°C. We used cobalt chloride ($CoCl_2$) to simulate hypoxic conditions. Cells were seeded in dishes or plates at 300 cells/mm^2 and grown for 24 h in complete medium. The medium was then removed, and cells were washed with PBS. The cells were treated with 100 μM $CoCl_2$ and then incubated for 24 h.

Quantitative RT-PCR

Total RNA was isolated from thyroid cancer cells using the RNA plus kit (Invitrogen, Carlsbad, CA, USA). Aliquots (5 μg) of RNA were reverse transcribed to cDNA using Superscribe First-Strand Synthesis System (Invitrogen Corporation) RT-qPCR was performed by using SYBR Premix Ex Taq (Takara, Dalian, China). The specific primers for KIF3a were sense, 5'-GCTATAGACAGGCCGTCAGC-3', and antisense, 5'-GTCTTTGGAGGTTCGTTGGA-3'; and for β-actin were sense, 5'-AAATCGTGCGTGACATCAAAGA-3' and antisense, 5'-GGCCATCTCCTGCTCGAA-3'. The PCR cycling program was 95°C for 3 min, then 30 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 15 s, and a final extension at 72°C for 5 min. The relative quantification of gene expression level was compared with the internal refereeβ-actin using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Protein in each sample from was extracted using protein extraction kit (Applygen Technologies, Beijing, China). The concentration of protein was measured by BCA kit. Samples (40 μg) were subjected to SDS-PAGE and transferred to Immobilon-P Transfer membranes (Millipore). The membranes were treated using the following procedure with shaking and blocking at room temperature with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h followed by incubation in the primary antibodies at 4°C overnight. After washing with TBST buffer (0.05 mol/l Tris, 0.15 mol/l NaCl and 0.05% Tween-20), the membranes were incubated in peroxidase-conjugated secondary antibody lgG for 1 h. Then, the immune-reactive protein bands on the membrane were visualized using an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). The primary antibodies used in the experiment were as follows: anti-KIF3a, anti-E-cadherin, anti-N-cadherin, anti-vimenin, anti-β-catenin, anti-cyclin D1, anti-c-myc and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell migration and invasion assay

The migration and invasion assays were performed as in a 24-well Boyden chamber with 8 μm pore size polycarbonate membrane (Millipore, Boston, MA, USA). For migration assay, 200 μl of serum-free medium was added to the upper compartment of the chamber, and 750 μl RPMI 1640 with 10% FBS was added into the lower compartment. After incubation at 37°C for 24 h, the tumor cells remaining inside the upper chamber were removed with cotton swabs. The cells on the lower surface of the membrane were fixed in 95% ethanol and stained with 0.1% crystal violet. Six different views were randomly chosen under a microscope, and the cell number on the bottom side of the membrane was counted. The invasion assay was done by the same procedure, except that the membrane was coated with Matrigel to form a matrix barrier. The experiments were performed in triplicate.

Silencing RNA targeting KIF3a

Small interfering RNA (siRNA) targeting KIF3a and siRNA-scramble were purchased from

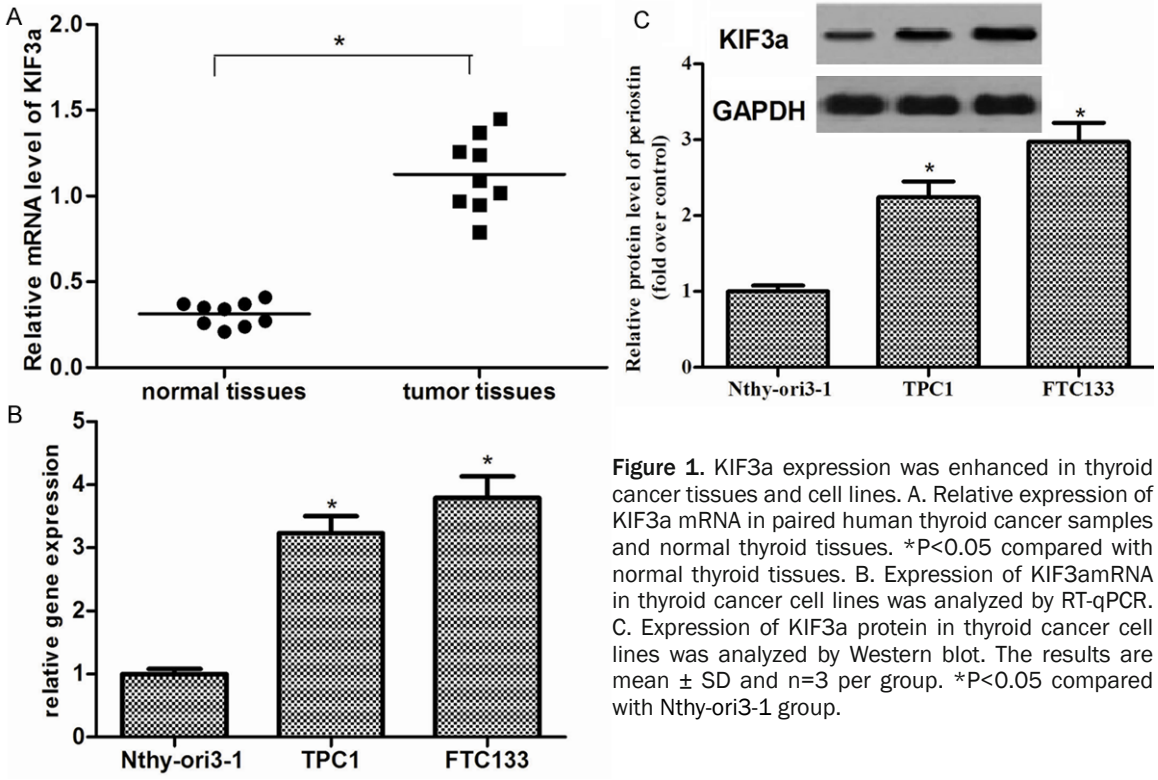

Figure 1. KIF3a expression was enhanced in thyroid cancer tissues and cell lines. A. Relative expression of KIF3a mRNA in paired human thyroid cancer samples and normal thyroid tissues. *P<0.05 compared with normal thyroid tissues. B. Expression of KIF3amRNA in thyroid cancer cell lines was analyzed by RT-qPCR. C. Expression of KIF3a protein in thyroid cancer cell lines was analyzed by Western blot. The results are mean ± SD and n=3 per group. *P<0.05 compared with Nthy-ori3-1 group.

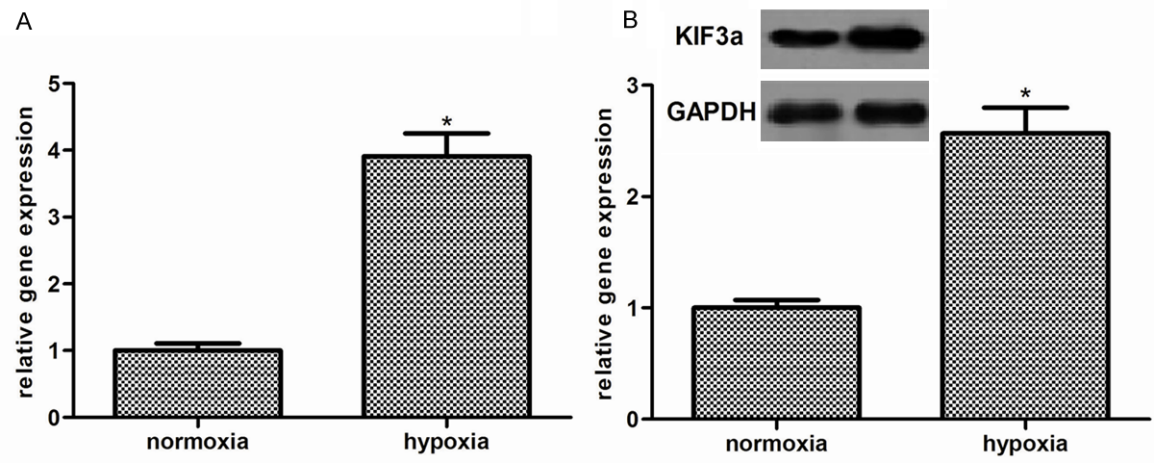

Figure 2. Hypoxia enhanced KIF3a expression in FTC133 cells. FTC133 cells were treated with 100 μM $CoCl_2$ and then incubated for 24 h. A. The mRNA expression KIF3a in FTC133 cells. B. The protein expression KIF3a in FTC133 cells. The results are mean ± SD and n=3 per group. *P<0.05 compared with normoxia group.

GenePharma Co., Ltd (Shanghai, China). For transfection, FTC133 cells were plated in 24-well culture plates at a density of 1×105 cells per well, grown for 24 h to reach 30%-50% confluence, and then incubated with a mixture of siRNA and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in 100 μl serum-free DMEM, according to the manufacturer's instructions. Then, Western blot was performed to detect KIF3a expression in transfected FTC133 cells.

Statistical analysis

Data are expressed as mean ± SD of triplicate samples. The data significance was evaluated by using Student's t-test. P<0.05 was considered a statistically significant difference.

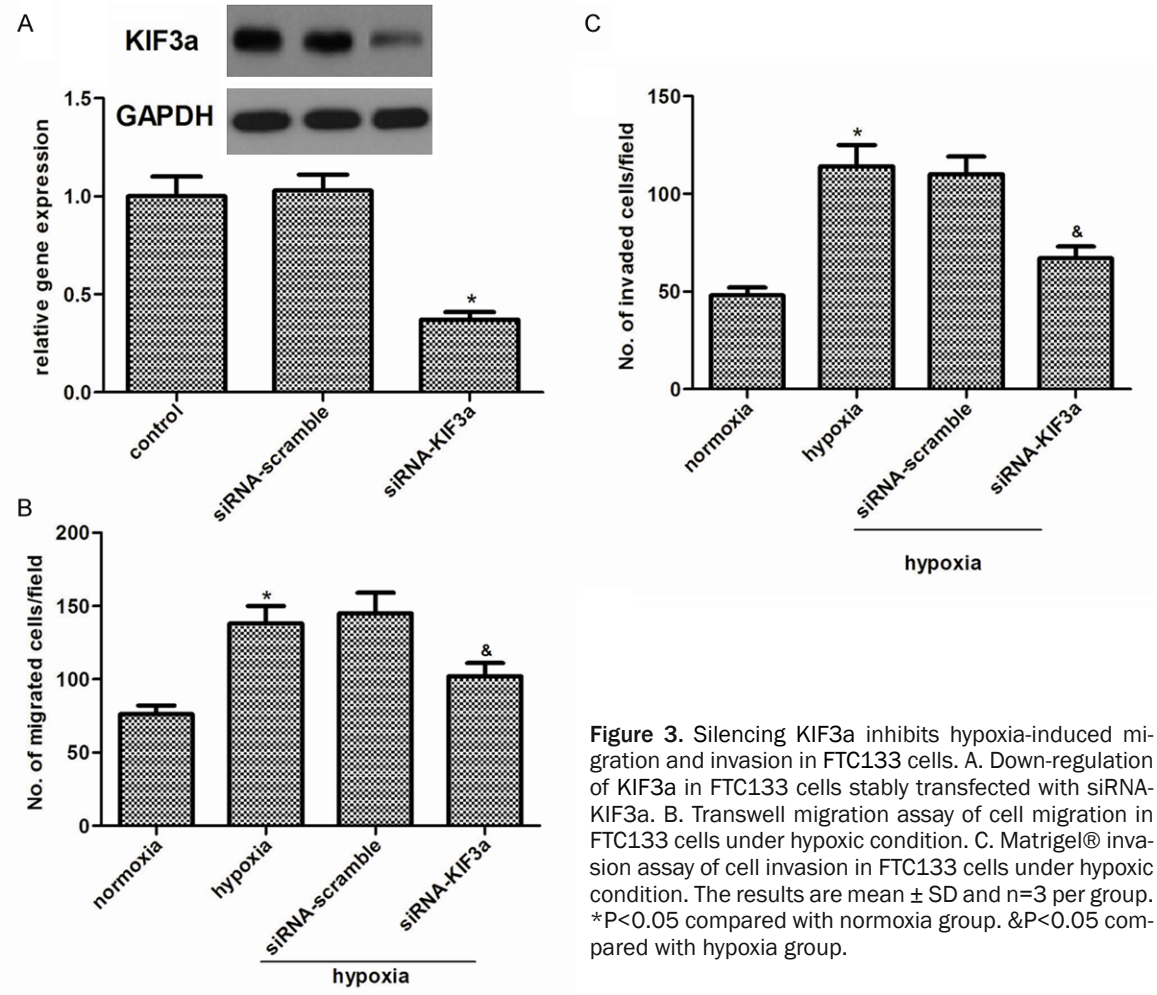

Figure 3. Silencing KIF3a inhibits hypoxia-induced migration and invasion in FTC133 cells. A. Down-regulation of KIF3a in FTC133 cells stably transfected with siRNA-KIF3a. B. Transwell migration assay of cell migration in FTC133 cells under hypoxic condition. C. Matrigel® invasion assay of cell invasion in FTC133 cells under hypoxic condition. The results are mean ± SD and n=3 per group. *P<0.05 compared with normoxia group. &P<0.05 compared with hypoxia group.

Results

KIF3a expression was enhanced in thyroid cancer tissues and cell lines

First, we detected the KIF3a mRNA levels in 9 paired thyroid cancer tissues and the corresponding adjacent normal tissues using real time RT-PCR. As indicated in **Figure 1A**, the KIF3a mRNA levels in thyroid cancer tissues were significantly higher than those in the adjacent normal thyroid tissues. Consistent with the results of KIF3a in thyroid cancer tissues, KIF3a mRNA and protein expression were also up-regulated in FTC133 and TPC1 cells (**Figure 1B** and **1C**). These results suggest that KIF3a is down-regulated in thyroid cancer.

Hypoxia enhanced KIF3a expression in FTC133 cells

Thyroid cancer cells were treated with 100 μM $CoCl_2$ and then incubated for 48 h. The mRNA expression levels of KIF3a of thyroid cancer cells under normoxia and hypoxia were assessed by RT-qPCR. The results are presented in **Figure 2A**, we found that hypoxia considerably increased the mRNA expression of KIF3a in FTC133 cells. Similarly, hypoxia also obvious increased the protein expression of KIF3a in FTC133 cells (**Figure 2B**).

Silencing KIF3a inhibits hypoxia-induced migration and invasion in FTC133 cells

To investigate whether KIF3a is involved in hypoxia-induced migration and invasion, we used siRNA to knockdown the expression of KIF3a in FTC133 cells. After transfection with siRNA-KIF3a for 24 h, a 0.65-fold decrease in KIF3a protein level was found in FTC133 cells. The transfection efficiency was approximately 60-70 % (**Figure 3A**). Then, we performed Transwell migration and Matrigel® invasion assays to investigate the effect of KIF3a on

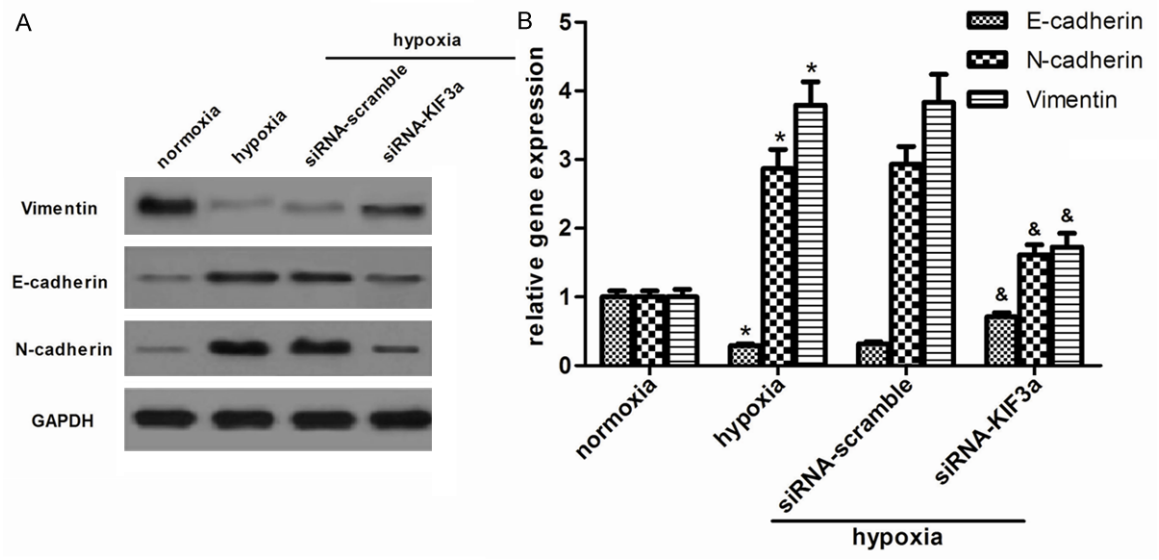

Figure 4. Silencing KIF3a suppresses hypoxia-induced EMT in FTC133 cells. FTC133 cells were treated with 100 μM $CoCl_2$ and then incubated for 24 h. The epithelial marker E-cadherin and the mesenchymal markers N-cadherin and Vimentin were determined by Western blot. The results are mean ± SD and n=3 per group. *P<0.05 compared with normoxia group. &P<0.05 compared with hypoxia group.

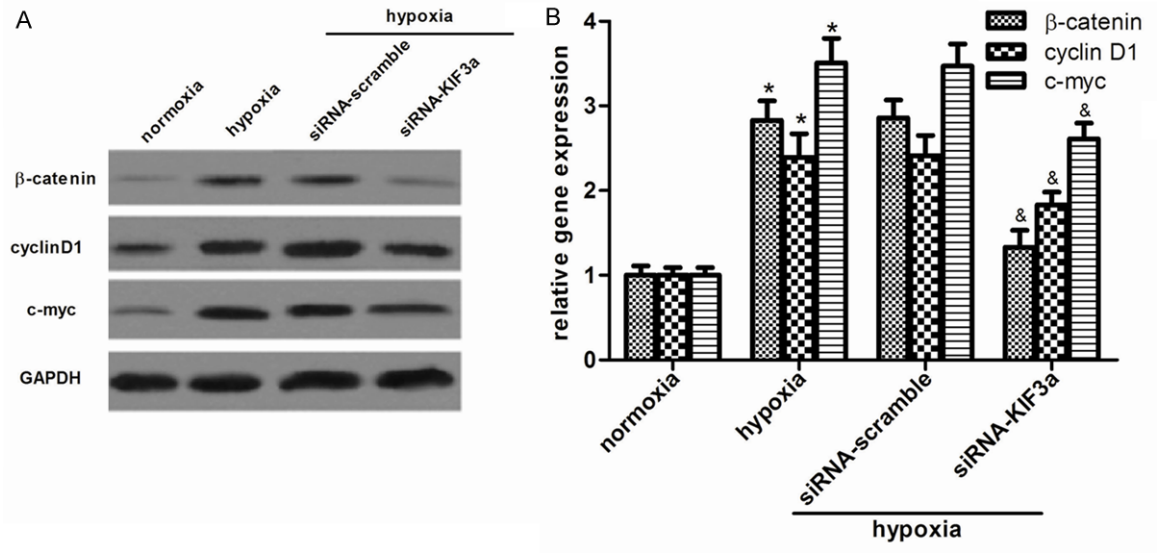

Figure 5. Silencing KIF3a suppresses hypoxia-induced Wnt pathway activation in FTC133 cells. FTC133 cells were treated with 100 μM $CoCl_2$ and then incubated for 24 h. The expression levels of β-catenin, cyclin D1 and c-myc were determined by Western blot. The results are mean ± SD and n=3 per group. *P<0.05 compared with normoxia group. &P<0.05 compared with hypoxia group.

migration and invasion of FTC133 cells under hypoxic condition. As shown in **Figure 3B** and **3C**, treatment with $CoCl_2$ significantly increased migration and invasion of FTC133 cells. The mean number of migrated and invaded cells in the mock group was 1.8-fold and 2.3-fold higher than those in the normoxia group. However, knockdown of KIF3a could prevent $CoCl_2$-induced thyroid cancer migration and invasion.

Silencing KIF3a suppresses hypoxia-induced EMT in FTC133 cells

To investigate the effect of KIF3a on the EMT process, we performed Western blotting to

evaluate the expression of EMT biomarkers: N-cadherin, N-cadherin and vimentin. As indicated in **Figure 4**, hypoxia downregulated the expression level of E-cadherin, and upregulated the expression level of N-cadherin and vimentin in FTC133 cells; however, knockdown of KIF3a increased E-cadherin expression, while decreasing the expression of N-cadherin and vimentin, indicating a blockade of the EMT.

Silencing KIF3a suppresses hypoxia-induced Wnt/β-catenin pathway activation in FTC133 cells

To explore the molecular mechanisms underlying the inhibitory effects of siRNA-KIF3a on hypoxia-induced EMT, we investigated the effects of KIF3a on the key components of the Wnt signaling pathway in FTC133 cells under normoxic and hypoxic conditions. As shown in **Figure 5**, as compared to the normoxia group, hypoxia significantly increased protein levels of β-catenin, cyclin D1 and c-myc. However, knockdown of KIF3a inhibited the expression levels of β-catenin, cyclin D1 and c-myc under the same hypoxic condition, suggesting that silencing KIF3a prevents hypoxia-induced activation of the Wnt/β-catenin pathway in FTC133 cells.

Discussion

Hypoxia plays a pivotal role in the microenvironment. $CoCl_2$ is a hypoxia-mimicking reagent that is commonly used in hypoxia studies [13-15]. In the present study, we used $CoCl_2$ in cell cultures to simulate constant hypoxia. Our results showed that KIF3a mRNA and protein expression was markedly increased in the human thyroid cancer tissues and cell lines. Knockdown of KIF3a significantly inhibited hypoxia-induced migration and invasion, and EMT process. Furthermore, knockdown of KIF3 aprevented hypoxia-induced increase in the expression of β-catenin, cyclin D1 and c-myc in FTC133 cells.

KIF3a is a member of the kinesin family of motor proteins. It has been shown to regulate tumorigenesis. Liu et al. reported that KIF3a is up-regulated in the majority of prostate cancer cell lines and primary tumor tissues, and the expression levels of KIF3a correlate with a higher Gleason score, TNM grade, and metastatic status of prostate cancer [12]. In line with these results, in the present study, we found that KIF3a mRNA and protein expression was markedly increased in the human thyroid cancer tissues and cell lines. The data suggested that KIF3a might play an important role in the development of thyroid cancer.

The EMT is a crucial step in the process of cancer. During EMT progress, tumor cells lose epithelial (E)-cadherin expression and cellular adhesion, and get the ability to invade and metastasize. In addition, hypoxia is one of the most important environmental factors that induce cancer metastasis [16, 17]. A growing body of evidence indicates that hypoxia promoted the EMT process in thyroid cancer [18, 19]. In the present study, we found that hypoxic conditioning induced thyroid cancer cell migration/invasion, and promoted the EMT program, resulting in down-regulation of E-cadherin and up-regulation of N-cadherin and vimentin. Intriguingly, the transition process was dampened by siRNA-KIF3a. These evidences suggested that the increase in metastatic property inhibited by siRNA-KIF3a in thyroid cancer cells is linked to EMT-related markers such as E-adherin, N-cadherin and Vimentin.

Several signaling pathways that are important for cancer development, such asWnt, Notch, PI3K/AKT, and MAPK signaling pathways, have been involved in regulating EMT [20-23]. In the development and progression of cancer, tumor hypoxia plays an important role in activating the Wnt pathway [24-26]. For example, hypoxia activates β-catenin in hepatocellular carcinoma cells to induce EMT and enhance their metastatic potential [27]. Furthermore, it was demonstrated that KIF3a can constrain β-catenin-dependent Wnt signaling through dual ciliary and non-ciliary mechanisms [28]. Most recently, one study reported that KIF3a increases CK1-dependent DVL2 phosphorylation and β-catenin activation in prostate cancer cells, leading to transactivation of the Wnt signaling target genes such as cyclin D1, HEF1, and MMP9 [12]. Similarly, in the present study, we found that knockdown of KIF3a inhibited the expression levels of β-catenin, cyclin D1 and c-myc under the hypoxic condition. These findings suggest that the inhibitory effects of siRNA-KIF3a on hypoxia-induced EMT process are most likely mediated by suppression of the Wnt/β-catenin signaling pathway.

In summary, the study reported here provided evidence that knockdown of KIF3a inhibited hypoxia-induced EMT of thyroid cancer cells via suppressing the Wnt/β-catenin signaling pathway. Our study uncovered a novel role for KIF3a in thyroid cancer progression, which might support the potential for KIF3a targeting in thyroid cancer therapy.

Acknowledgements

This research was supported by the Bureau of Public Health of Henan Province, China (grant number: 201003091).

Disclosure of conflict of interest

None.

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References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Vasko VV, Saji M. Molecular mechanisms involved in differentiated thyroid cancer invasion and metastasis. Curr Opin Oncol 2007; 19: 11-7.

[3] Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. Nat RevMol Cell Biol 2006; 7: 131-42.

[4] Bozdogan O, Atasoy P, Bozdogan N, Erekul S, Batislam E, Yilmaz E, Basar MM. BAG-1 expression in hyperplastic and neoplastic prostate tissue: is there any relationship with BCL-related proteins and androgen receptor status? Tumori 2005; 91: 539-45.

[5] Jiang J, Tang YL, Liang XH. EMT: a new vision of hypoxia promoting cancer progression. Cancer Biol Ther 2011; 11: 714-23.

[6] Lei J, Ma J, Ma Q, Li X, Liu H, Xu Q, Duan W, Sun Q, Xu J, Wu Z. Hedgehog signaling regulates hypoxia induced epithelial to mesenchymal transition and invasion in pancreatic cancer cells via a ligand-independent manner. Mol Cancer 2013; 12: 66.

[7] Cheng ZX, Sun B, Wang SJ, Gao Y, Zhang YM, Zhou HX, Jia G, Wang YW, Kong R, Pan SH. Nuclear factor-kappaB-dependent epithelial to mesenchymal transition induced by HIF-1alpha activation in pancreatic cancer cells under hypoxic conditions. PLoS One 2011; 6: e23752.

[8] Lin F, Hiesberger T, Cordes K, Sinclair AM, Goldstein LS, Somlo S, Igarashi P. Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. Proc Natl Acad Sci U S A 2003; 100: 5286-91.

[9] Koyama E, Young B, Nagayama M, Shibukawa Y, Enomoto-Iwamoto M, Iwamoto M, Maeda Y, Lanske B, Song B, Serra R. Conditional Kif3a ablation causes abnormal hedgehog signaling topography, growth plate dysfunction, and excessive bone and cartilage formation during mouse skeletogenesis. Development 2007; 134: 2159-69.

[10] Qiu N, Cao L, David V, Quarles LD, Xiao Z. Kif3a deficiency reverses the skeletal abnormalities in Pkd1 deficient mice by restoring the balance between osteogenesis and adipogenesis. PLoS One 2010; 5: e15240.

[11] Qiu N, Xiao Z, Cao L, Buechel MM, David V, Roan E, Quarles LD. Disruption of Kif3a in osteoblasts results in defective bone formation and osteopenia. J Cell Sci 2012; 125: 1945-57.

[12] Liu Z, Rebowe RE, Wang Z, Li Y, Wang Z, DePaolo JS, Guo J, Qian C, Liu W. KIF3a promotes proliferation and invasion via Wnt signaling in advanced prostate cancer. Mol Cancer Res 2014; 12: 491-503.

[13] Bae S, Jeong HJ, Cha HJ, Kim K, Choi YM, An IS, Koh HJ, Lim DJ, Lee SJ, An S. The hypoxia-mimetic agent cobalt chloride induces cell cycle arrest and alters gene expression in U266 multiple myeloma cells. Int J Mol Med 2012; 30: 1180-6.

[14] Cho J, Kim D, Lee S, Lee Y. Cobalt chloride-induced estrogen receptor α down-regulation involves hypoxia-inducible factor-1α in MCF-7 human breast cancer cells. Mol Endocrinol 2005; 19: 1191-9.

[15] Yang WW, Shu B, Zhu Y, Yang HT. E2F6 inhibits cobalt chloride-mimetic hypoxia-induced apoptosis through E2F1. Mol Biol Cell 2008; 19: 3691-700.

[16] Brahimi-Horn MC, Chiche J, Pouysségur J. Hypoxia and cancer. J Mol Med 2007; 85: 1301-7.

[17] Rofstad E. Microenvironment-induced cancer metastasis. Int J Radiat Biol 2000; 76: 589-605.

[18] Yang YJ, Na HJ, Suh MJ, Ban MJ, Byeon HK, Kim WS, Kim JW, Choi EC, Kwon HJ, Chang JW, Koh YW. Hypoxia Induces Epithelial-Mesenchymal Transition in Follicular Thyroid Cancer: Involvement of Regulation of Twist by Hypoxia Inducible Factor-1α. Yonsei Med J 2015; 56: 1503-14.

[19] Scarpino S, Cancellario d'Alena F, Di Napoli A, Pasquini A, Marzullo A, Ruco LP. Increased expression of Met protein is associated with up-regulation of hypoxia inducible factor-1 (HIF-1) in tumour cells in papillary carcinoma of the thyroid. J Pathol 2004; 202: 352-8.
[20] Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 2005; 17: 548-58.
[21] Vincan E, Barker N. The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression. Clin Exp Metastasis 2008; 25: 657-63.
[22] Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene 2005; 24: 7443-54.
[23] Xie L, Law BK, Chytil AM, Brown KA, Aakre ME, Moses HL. Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. Neoplasia 2004; 6: 603-10.
[24] Lim JH, Chun YS, Park JW. Hypoxia-inducible factor-1α obstructs a Wnt signaling pathway by inhibiting the hARD1-mediated activation of β-catenin. Cancer Res 2008; 68: 5177-84.
[25] Jiang YG, Luo Y, He DI, Li X, Zhang LI, Peng T, Li MC, Lin YH. Role of Wnt/β-catenin signaling pathway in epithelial-mesenchymal transition of human prostate cancer induced by hypoxia-inducible factor-1α. Int J Urol 2007; 14: 1034-9.
[26] Giles R, Lolkema M, Snijckers C, Belderbos M, Van Der Groep P, Mans D, Van Beest M, Van Noort M, Goldschmeding R, Van Diest P. Interplay between VHL/HIF1α and Wnt/β-catenin pathways during colorectal tumorigenesis. Oncogene 2006; 25: 3065-70.
[27] Liu L, Zhu XD, Wang WQ, Shen Y, Qin Y, Ren ZG, Sun HC, Tang ZY. Activation of β-catenin by hypoxia in hepatocellular carcinoma contributes to enhanced metastatic potential and poor prognosis. Clin Cancer Res 2010; 16: 2740-50.
[28] Corbit KC, Shyer AE, Dowdle WE, Gaulden J, Singla V, Reiter JF. Kif3a constrains β-catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. Nat Cell Biol 2008; 10: 70-6.