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YAP mediate hypoxia-induced pancreatic cancer cells invasion and migration via promoting survivin expression

Xiaopeng Yang[1,a], Shanglong Liu[2,a], Jianfei Xu[1], Shougen Cao[2], Yanbing Zhou[1,2,*]

[a]Equal contributors

[1]Department of Clinical Medicine, Medical College, Qingdao University, Qingdao, 266027 China

[2]Department of General Surgery, Affiliated Hospital of Qingdao University, Qingdao, 266027 China

Abstract: **Hypoxia microenvironment is considered to be a contributing factor to tumor invasion and metastasis. However, the molecular mechanism whereby hypoxia contributes to these events has not been completely elucidated. Here we showed that two pancreatic cancer (PC) cells, Panc-1 and SW1990, display different invasive and migratory abilities under hypoxia conditions (1％O2). We also showed that YAP activation was required as an important role to switch on hypoxia-induced epithelial-mesenchymal transition (EMT) and invasion of PC cells upon hypoxia. Additionally, we found that YAP activation induced survivin expression; specific inhibition of survivin could reduce EMT and suppress the invasion and migration of PC cells. In conclusion, hypoxia promotes PC cells (Panc-1 and SW1990) invasion and migration via YAP/survivin pathway, which may be a new therapeutic target of pancreatic cancer.**

Keywords: Hypoxia; Pancreatic cancer; YAP; Survivin; Epithelial- Mesenchymal Transition

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*Corresponding Author: Yanbing Zhou, zhouyanbing99@aliyun.com

1. Introduction

Pancreatic cancer (PC), which is known as "the king of cancer", is one of the most malignant diseases in the world. The median survival rate, presently remaining at the low level of around 5% after diagnosis, has not improved over recent decades [1]. The poor prognosis of PC is attributable to its tendency for aggressive local invasion and early metastasis [1-2]. Therefore, understanding the biological behaviors of tumor and molecular mechanism underlying the extraordinarily high invasive characteristics of pancreatic cancer may increase insights into the development of novel therapeutic and diagnostic strategies [2]. Existing research has confirmed that pancreatic cancer contains regions of extremely low PO_2, suggesting that it is poorly perused and poorly vascularized [3,4]. The generation of this hypoxia microenvironment is due to the increased oxygen consumption by rapid tumor growth and the compression to the blood vessel by the rapid proliferation of stromal cells [3-5]. A number of studies have confirmed that intratumoral hypoxia is associated with a significantly increased risk of invasion and metastasis in many human cancers including pancreatic cancer [5,6]. However, the molecular mechanism whereby hypoxia contributes to PC cells invasion and migration have not been completely elucidated.

Yes-associated protein (YAP), a downstream effector of Hippo signaling pathway, has been found to play an important role in tumorigenesis, development and metastasis [7]. In mammals, the Hippo pathway is composed of upstream kinase cassette including MST1/2, LAST1/2 and two primary downstream effectors, YAP and TAZ. When hippo pathway is activated, the kinase MST1 and MST2 phosphorylate and activate the LAST1/2 kinases, which in turn phosphorylate YAP and TAZ, leading to the cytoplasmic retention of downstream effectors [8]. However, when the Hippo pathway is blocked, the YAP is in low phosphate condition and access into the nucleus, binds to the TEA domain family of transcription factors (TEAO), then promotes the transcription of target genes and results in cell proliferation, invasion and metastasis [8-10]. Prior studies have shown that YAP is hyperactive in pancreatic cancer patient tumor samples [11], suggesting that YAP may be a contributing factor to pancreatic cancer development. However, the underlying mechanism of YAP in pancreatic cancer cells invasion and migration is still unclear. A recent study have shown that YAP can be activated and translocate to the nucleus under hypoxia conditions [12]. This may provide a reasonable explanation for the enhanced invasion and metastasis of pancreatic cancer under hypoxia conditions.

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has multiple functions in the progression of cancer [13,14]. It is also expressed in PC tissues and is regarded as a prognostic maker in PC patients [15]. Previous studies focused on the role of survivin in the inhibition of apoptosis of cells [16,17]. However, the functional role of survivin in the tumor invasion and metastasis has been recognized gradually [18]. Existing research have confirmed that hypoxia-inducible factor-1α (HIF-1α)

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promotes PC cells invasion via promoting survivin expression, suggesting that survivin may be a contributing factor to the tumor invasion upon hypoxia [19].

In this study, we found that hypoxia increased PC cells invasion and migration through the activation of YAP. YAP was de-phosphorylated and accessed to the nucleus under hypoxia condition. Additionally, our data showed that YAP-induced PC cells invasion and migration under hypoxia was mediated by survivin.

2. Materials and Methods

2.1. Cell culture and reagents

The human PC cell lines (Panc-1 and SW1990) were obtained as a gift from The Second Military Medical University (Shanghai, china) and maintained in DMEM/F12 (1:1, HyClone Logan, Utah, USA) containing 10% fetal bovine serum (FBS, BIOIND, Kibbutz Beit Haemek, Israel), 2% streptomycin and ampicillin (Beyotime, Shanghai, China) in a cell culture incubator with 5% CO2. All cells were routinely screened using the Mycoplasma Detection Kit (Genloci, Jiangsu, China). The pharmacological reagent cyclopamine was provided by Selleck Chemicals (Houston, TX, USA), and siRNA-YAP and siRNA-survivin was purchased from Qiagen (Shanghai, China). Protein antigens were purchased from different resources: anti-E-cadherin, anti-Vimentin and anti-YAP were purchased from Abcam (Headquarters, Cambridge, UK); anti-GAPDH and anti-p-YAP was purchased from Sigma (St. Louis, MO, USA); and anti-survivin were purchased from CST (Danvers, Massachusetts, USA).

2.2. Hypoxia conditions

Pancreatic cancer cells were grown in normal conditions with serum-containing medium until 70% confluence. Then a fresh medium containing 1% FBS which had been treated by $CoCL_2$ was added and incubated in a hypoxic incubator (BioSpherix, Redfield, USA) with 1% O_2, balanced by CO_2 and nitrogen. At 12h, 24h and 48h after incubation, cells were harvested for subsequently experiments.

2.3. Real-time-PCR assay

Total RNA extraction from tumor cells was carried out with Trizol Reagent (Ambion, Austin, TX, USA). RNA was converted to cDNA using a transcription first strand cDNA synthesis kit (Roche, Mannheim, Germany). The amplification reaction was carried out on a LightCycler® 96 system adapted to a 20 μL system (8 μL RNase-free water, 1 μL forward and reverse specific primers, 1 μL cDNA, 10 μL SYBR green). The reaction consisted of the following steps: 1 cycle of denaturing at 95 ℃ for 10min, followed by 45 cycles of 94 ℃ for 10 s, 60 ℃ for 20s and 72 ℃ for 10 s. Each experiment was repeated at least three times and representative data are shown.

2.4. Western blot analyses

The extracts of whole cell protein were prepared directly in cell extraction buffer supplemented with a protease inhibitor cocktail (Beyotime). After 30 min incubation on ice, the cellular debris was pelleted from the cell lysis solution by centrifugation at 14,000 x g for 10min. All samples were normalized for protein concentration measured using a Quantipro BCA Assay kit (Boster, Wuhan, Hubei, China). Equal amounts of protein (20μg/lane) were electrophoresed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 1x protein loading buffer, and then transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 5% BSA-TBS buffer solution and incubated with a primary antibody target protein (E-cadherin; Vimentin; YAP; p-YAP; Survivin) overnight at 4 ℃.Subsequently, the membranes were exposed to the secondary antibodies for 1 h at room temperature. GAPDH was measured as a control. The blots were then detected using the West Pico ECL kit (Fluorescence, Beijing, China) and exposed to X-ray film.

2.5. Invasion and migration experiments for PC cells

The invasiveness of Panc-1 and SW1990 cells was examined by utilizing a Transwell® chamber assay (Corning Costar, Corning, NY, USA; 24-well format, pore size 8.0 μm). The basal surface was coated with 5 μL gelatin (2 mg/mL) while the upper surface was coated with 10 μL basement membrane matrixes (extracellular matrix). The tumor cells (5 x 10^3) in serum-free medium were plated into the upper chamber. PSC-CM containing 1% FBS was added to the lower chambers as a chemotactic factor while DMEM culture medium supplemented with 1% FBS was placed in the control plates. After 24 h incubation at 37 ℃, a cotton swab was used to gently wipe away the cells remaining on the upper surface. The invasive cells, adhering to the bottom surface of the membrane, were fixed with 4% paraformaldehyde for 20 min, then stained with crystal violet, counted and photographed under a light microscope by randomly selecting 10 fields. The migration assay of PC was performed using a Transwell® migration chamber (8.0mm, without matrigel™ or gelatin). The remaining steps were performed as described before. Each experiment was repeated at least three times.

2.6. Statistical analysis

Experimental cartography was made by

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GraphPad.Prism.v5.0 (La Jolla, CA, USA), and shown as mean ± standard deviation. The differences between the groups were analyzed by t-test. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Hypoxia induced epithelial mesenchymal transition of PC cells (Panc-1 and SW1990)

EMT was considered as a necessary step in tumor invasion and metastasis [20,21]. So we evaluated the different expression of EMT makers (E-cadherin and Vimentin) in PC cells when treated with hypoxia. After treated with hypoxia for 24h, the morphology of Panc-1 cells transformed into an elongated shape was not observed in SW1990 cells (Figure 1A). Then we evaluated the association between the expression of the E-cadherin and Vimentin in these two cell lines by RT-PCR and western blot. After 24h of hypoxia treatment, the mRNA and protein expression of the epithelial marker, namely E-cadherin, got degraded obviously while the expression of the Vimentin was significantly induced (Figure 1B).

3.2. Hypoxia enhanced the invasive and migratory abilities of Panc-1 cells, but not SW1990

To determine the invasive and migration capacities of the two PC cell lines, we performed Transwell assays as described previously. We found that the invasion and migration of Panc-1 cells (upon hypoxia exposure) were enhanced compared to those of the control group ($P<0.01$). In contrast, there was no statistical difference between the hypoxia treatment group and the control group in SW1990 cells (Figure 1C).

3.3. Hypoxia promoted the YAP expression and enhanced its nuclear translocation

Convincing evidence indicates that hypoxia induces YAP activation in several cancer cells and that YAP can act as a carcinogenic gene to accelerate the development of carcinoma [22]. Thus we explored the effect of hypoxia on the expression of YAP. We found that the expression of total YAP was induced, concomitant with a significant reduction in p-YAP levels (Figure 2A). To evaluate the effect of hypoxia on nuclear translocation of YAP, we evaluated the expression of YAP in nucleus and cytoplasm respectively. Interestingly, the results showed that hypoxia treatment reduced YAP expression in cytoplasm, while more nucleus YAP was observed (Figure 2B). These data indicated that hypoxia could increase YAP expression and triggered its activation.

3.4. YAP mediated hypoxia-induced PC cells EMT and invasion

Having established that YAP was activated by hypoxia, we tried to determine whether YAP is a contributing factor to cancer cells invasion under hypoxia conditions. We used a small interfering RNA (siRNA) to reduce the expression of YAP, and then evaluated the invasive and migratory abilities of panc-1 cells when treated with hypoxia. Our data showed that the invasive and migratory abilities of panc-1 cells were significantly weaken [Figure 2C]. It indicated that the activation of YAP was responsible for Panc-1 cells invasion and migration under hypoxia.

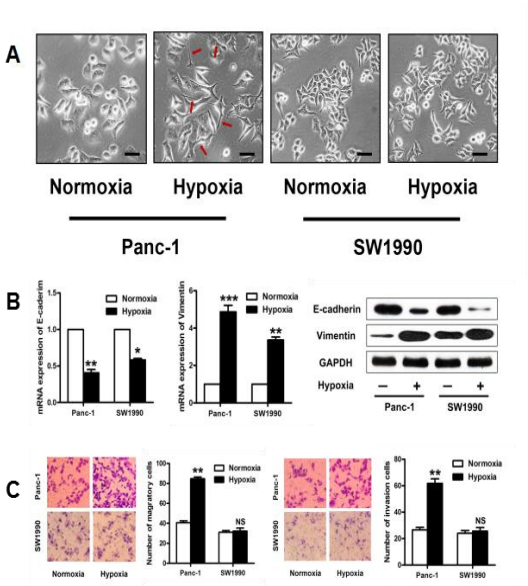


Figure 1. Hypoxia induced epithelial mesenchymal transition of PC cells, and enhanced the invasive and migratory abilities of Panc-1 cells, but not SW1990.

3.5. YAP-increased Panc-1 cells invasion under hypoxia was mediated by survivin

Existing research has noted that hypoxia induces the expression of survivin which contributes to tumor invasion and metastasis [23]. However, the mechanism in the up-regulation of survivin by hypoxia is not clear. In this study, we evaluated the effect of hypoxia on survivin expression in PC cells. We found that survivin expression was increased when incubated with hypoxia [Figure 3A]. To define the relation between YAP activation and survivin, we used a small interfering RNA (si-RNA) to reduce the expression of YAP, then evaluated the expression of survivin. We found that the expression of survivin was suppressed [Figure 3B]. Then we evaluated the effect of survivin on the PC cells invasion and migration upon hypoxia. We added an effective inhibitor of survivin, YM115, into the cell culture

medium of Panc-1 cells, to target the YAP/survivin pathway. We found that the migration of Panc-1 cells was significantly decreased [Figure 3C]. This indicated that YAP promoted PC cells invasion and migration via up-regulating survivin expression.

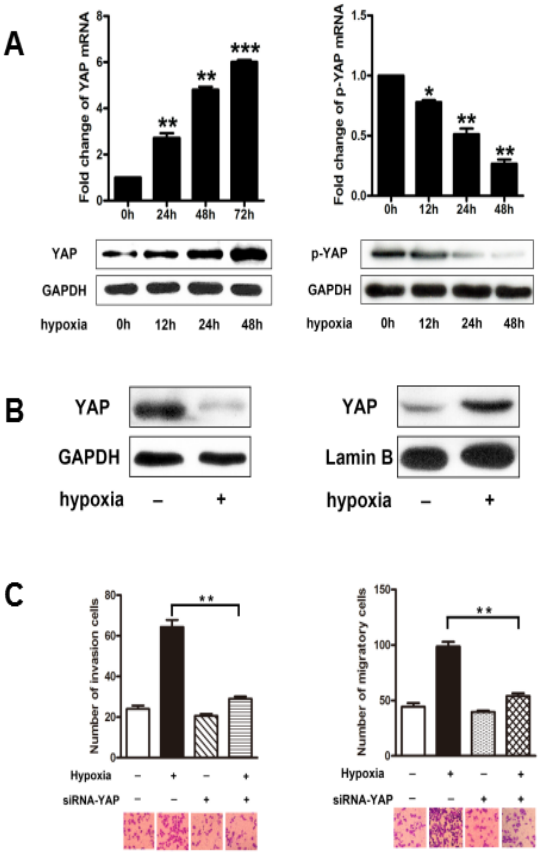


Figure 2. Hypoxia increased the YAP expression and enhanced its nuclear translocation, which mediated hypoxia-induced PC cells EMT and invasion.

4. Discussion

Hypoxia microenvironment has been demonstrated to confer in tumor occurrence and development [24]. Although hypoxia presents a particularly hostile environment for cell growth, cancer cells are able to survive and adapt via inducing the expression of genes responsible for formation of new blood vessels, metastasis, cell survival and anaerobic metabolism [25]. A number of researches have confirmed that pancreatic tumors showed high levels of hypoxia, suggesting that hypoxia may be an accomplice in PC development [26]. For instance, hypoxia stimulates angiogenesis in pancreatic cancer and increases resistance of PC cells to apoptosis induced by Gemcitabine [26,27]. In our work, we showed that hypoxia induced the EMT of PC cells (Panc-1 and SW1990) and increased the invasion and migration of Panc-1 cells. Additionally, although many studies have confirmed that EMT was positively correlated with tumor invasion, migration [28,29], some recent data have shown that EMT is not required for tumor cells metastasis [30,31]. Our data showed that the invasion and migration of SW1990 cells were not enhanced despite the fact that the EMT occurred after treatment with hypoxia, suggesting that EMT may not be a necessary condition for the invasion and migration of these cells.

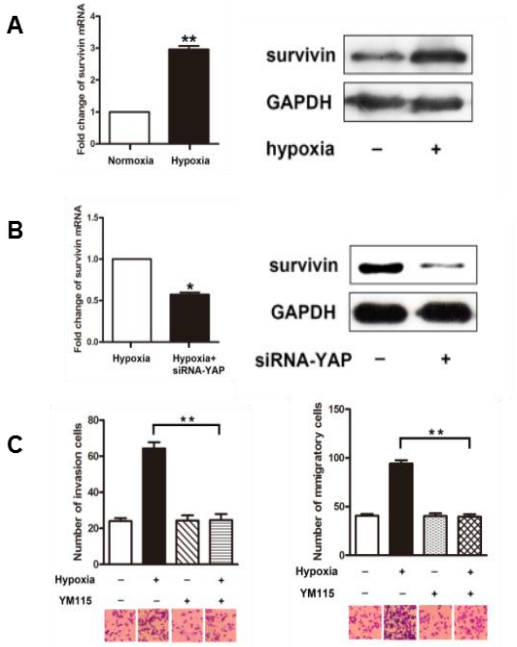


Figure 3. YAP-increased Panc-1 cells invasion under hypoxia was mediated by the up-regulation of surviving.

Multiple factors involved in tumor progression are regulated by hypoxia micro-environment [32]. For example, hypoxia-inducible factor-1α is a key player in hypoxia conditions and regulates various signaling pathway including Hippo [33]. There is increasing evidence that Hippo signaling pathway plays a critical role in cancer progression including proliferation, invasion, and metastasis and chemotherapy resistance [34]. Indeed, the manifold functions of Hippo are mediated through the activation of downstream effectors (YAP and TAZ), which promote the expression of downstream target genes like CTGF (Connective tissue growth factor), Cyclin E, AXL (AXL receptor tyrosine kinase) and so on [35]. In this study, we measured the expression of YAP in Panc-1 cells under hypoxia condition. We found that the total YAP expression was increased while the P-YAP expression was reduced. Considering the function of YAP was mediated by accessing into the nucleus and transcription target genes, we evaluated the expression of YAP in nucleus and cytoplasm of Panc-1 cells under hypoxia respectively. We found that hypoxia increased the expression of nuclear YAP, while less cytoplasm YAP

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was observed, suggesting that hypoxia was a contributing factor to YAP activation. Next, we evaluated the effect of activated YAP on the invasion and migration of Panc-1 cells. We found that the invasive and migratory abilities of Panc-1 cells were significantly decreased when treated with siRNA-YAP. These results indicated that hypoxia induced PC cells invasion and migration through activating YAP.

Survivin, an important member of the IAP family, is widely expressed in a variety of human cancers, including pancreatic cancer [36]. In tumors, a pool of survivin localizes to the mitochondria, where it influences organelle bioenergetics and promotes resistance to apoptosis, thus acting as a potential cancer driver [37]. Recently, an association between survivin expression and the invasion of PC has been reported [38]. We evaluated the expression of survivin in Panc-1 cells and found that it was increased under hypoxia condition. Then we used YM115 to reduce survivin expression to identify whether it promoted the invasion and migration of Panc-1 cells. We found that the survivin expression was suppressed, as well as the invasion and migration of Panc-1 cells. Having established that hypoxia could also trigger the activation of YAP, it is possible that survivin over expression is associated with YAP. We confirm this hypothesis by showing that knock down of YAP using siRNA leads to the decreased survivin expression in Panc-1 cells. These results showed that YAP mediated hypoxia-induced Panc-1 cells invasion and migration through up-regulating survivin expression.

5. Conclusion

Hypoxia induces pancreatic cancer cells invasion and migration. Activation of YAP participates in this process. YAP promotes PC cells invasion and migration through the up-regulation of survivin, which may be a novel target of pancreatic cancer.

Acknowledgement

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References

[1] Hidalgo M. Pancreatic cancer[J]. New England Journal of Medicine, 2010, 362(17):1605-1617.

[2] Michaud DS. Epidemiology of pancreatic cancer[J]. Seminars in Surgical Oncology, 2004, 59(2):2297-303.

[3] Koong A C, Mehta V K, Le Q T, et al. Pancreatic tumors show high levels of hypoxia[J]. International Journal of Radiation Oncologybiologyphysics, 2000, 48(4):919-922.

[4] Ide T, Kitajima Y, Miyoshi A, et al. Tumor-stromal cell interaction under hypoxia increases the invasiveness of pancreatic cancer cells through the hepatocyte growth factor/c-Met pathway[J]. International Journal of Cancer, 2006, 119(12):2750-2759.

[5] Masamune A, Kikuta K, Watanabe T, et al. Hypoxia stimulates pancreatic stellate cells to induce fibrosis and angiogenesis in pancreatic cancer[J]. Ajp Gastrointestinal & Liver Physiology, 2008, 295(4):709-717.

[6] Brahimi-Horn C, Pouysségur J. The role of the hypoxia-inducible factor in tumor metabolism growth and invasion[J]. Bulletin Du Cancer, 2006, 93(8):73-80.

[7] Zhang L, Yang S, Chen X, et al. The Hippo Pathway Effector YAP Regulates Motility, Invasion, and Castration-Resistant Growth of Prostate Cancer Cells[J]. Molecular & Cellular Biology, 2015, 35(8):1350-1362.

[8] Harvey K F, Zhang X, Thomas D M. The Hippo pathway and human cancer[J]. Nature Reviews Cancer, 2013, 13(4):246-257.

[9] Wellstein A, Sharif G, Schmidt M O, et al. The Hippo pathway controls cancer cell vascular invasion and metastasis by modulating cytokine signalling[J]. Clinical & Experimental Metastasis, 2015, 32(3):246-246.

[10] Meng Z, Moroishi T, Guan K L. Mechanisms of Hippo pathway regulation[J]. Genes & Development, 2016, 30(1):1-17.

[11] Xie D, Cui J, Xia T, et al. Hippo transducer TAZ promotes epithelial mesenchymal transition and supports pancreatic cancer progression[J]. Oncotarget, 2015, 6(34): 35949-35963.

[12] Zhou T Y, Zhuang L H, Hu Y, et al. Inactivation of hypoxia-induced YAP by statins overcomes hypoxic resistance tosorafenib in hepatocellular carcinoma cells[J]. Scientific Reports, 2016, 6.

[13] Altieri D C. Validating survivin as a cancer therapeutic target[J]. Nature Reviews Cancer, 2003, 3(1):46-54.

[14] Ryan B M, O'Donovan N, Duffy M J. Survivin: a new target for anti-cancer therapy[J]. Cancer Treatment Reviews, 2009, 35(7):553-562.

[15] Kami K, Doi R, Koizumi M, et al. Survivin expression is a prognostic marker in pancreatic cancer patients[J]. Surgery, 2004, 136(2):443-448.

[16] Okamoto K, Okamoto I, Okamoto W, et al. Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung cancers positive for EGFR mutations[J]. Cancer

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Research, 2010, 70(24):10402-10410.
[17] Pavlyukov M S, Antipova N V, Balashova M V, et al. Survivin monomer plays an essential role in apoptosis regulation[J]. Journal of Biological Chemistry, 2011, 286(26):23296-23307.
[18] Chu X Y, Chen L B, Wang J H, et al. Overexpression of survivin is correlated with increased invasion and metastasis of colorectal cancer[J]. Journal of Surgical Oncology, 2012, 105(6):520–528.
[19] Bai H, Ge S, Lu J, et al. Hypoxia inducible factor-1α-mediated activation of survivin in cervical cancer cells[J]. Journal of Obstetrics and Gynaecology Research, 2013, 39(2):555-563.
[20] Zuo J, Wen J, Lei M, et al. Hypoxia promotes the invasion and metastasis of laryngeal cancer cells via EMT[J]. Medical Oncology, 2016, 33(2):1-9.
[21] Yang J, Weinberg R A. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis[J]. Developmental Cell, 2008, 14(6):818-829.
[22] Dai XY, Zhuang L H, Wang D D, et al. Nuclear translocation and activation of YAP by hypoxia contributes to the chemoresistance of SN38 in hepatocellular carcinoma cells[J]. Oncotarget, 2016, 7(6):273-282.
[23] Fan LF, Dong WG, Jiang CQ, et al. Role of Hypoxia-inducible factor-1 alpha and Survivin in colorectal carcinoma progression[J]. International Journal of Colorectal Disease, 2008, 23(11):1057-1064.
[24] Wilson W R, Hay M P. Targeting hypoxia in cancer therapy[J]. Nature Reviews Cancer, 2011, 11(6):393-410.
[25] Kunz M, Ibrahim S M. Molecular responses to hypoxia in tumor cells[J]. Molecular Cancer, 2003, 2(1):1-13.
[26] Yokoi K, Fidler I J. Hypoxia increases resistance of human pancreatic cancer cells to apoptosis induced by gemcitabine[J]. Clinical Cancer Research, 2004, 10(7):2299-2306.
[27] Bao B, Ali S, Banerjee S, et al. Hypoxia-induced aggressiveness of pancreatic cancer cells is due to increased expression of VEGF, IL-6 and miR-21, which can be attenuated by CDF treatment[J]. Plos One, 2012, 7(12):e50165.
[28] Micalizzi DS, Ford H L. Epithelial-mesenchymal transition in development and cancer[J]. Future Oncology, 2009, 5(8):1129-1143.
[29] Guarino M. Epithelial-mesenchymal transition and tumour invasion[J]. International Journal of Biochemistry & Cell Biology, 2007, 39(12):2153-2160.
[30] Fischer K R, Durrans A, Lee S, et al. EMT is not required for lung metastasis but contributes to chemoresistance[J]. Nature, 2015, 527(7579):472-476.
[31] Zheng X, Carstens J L, Jiha K, et al. EMT Program is Dispensable for Metastasis but Induces Chemoresistance in Pancreatic Cancer[J]. Nature, 2015, 527(7579):525-530.
[32] Harris A L. Hypoxia--a key regulatory factor in tumour growth[J]. Nature Reviews Cancer, 2002, 2(1):38-47.
[33] Ma B, Chen Y, Chen L, et al. Hypoxia regulates Hippo signalling through the SIAH2 ubiquitin E3 ligase. Nature Cell Biology, 2015, 17(1):95-103.
[34] Pan D. The hippo signaling pathway in development and cancer[J]. Developmental Cell, 2010, 19(4):491-505.
[35] Di B A, Mottolese M, Sperati F, et al. The Hippo transducers TAZ/YAP and their target CTGF in male breast cancer[J]. Oncotarget, 2016.
[36] Konopka K, Spain C, Yen A, et al. Correlation between the levels of survivin and survivin promoter-driven gene expression in cancer and non-cancer cells[J]. Cellular and Molecular Biology Letters, 2014, 14(1):70-89.
[37] Altieri DC. Survivin, versatile modulation of cell division and apoptosis in cancer[J]. Oncogene, 2003, 22(53):8581-8589.
[38] Bhanot U, Heydrich R, Möller P, et al. Survivin expression in pancreatic intraepithelial neoplasia (PanIN): steady increase along the developmental stages of pancreatic ductal adenocarcinoma[J]. American Journal of Surgical Pathology, 2006, 30(6):754-759.

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