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Original Article

Hypoxia induces TWIST-activated epithelial–mesenchymal transition and proliferation of pancreatic cancer cells in vitro and in nude mice

Shi Chen [a, b, c], Jiang-zhi Chen [b], Jia-qiang Zhang [a, c], Hui-xin Chen [b], Mao-lin Yan [a], Long Huang [a], Yi-feng Tian [a], Yan-lin Chen [b, **], Yao-dong Wang [a, *]

[a] Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fujian Medical University, Fuzhou, China
[b] Department of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, Fuzhou, China
[c] Department of General Surgery, Research Institute of Pancreatic Disease, Ruijin Hospital Affiliated to Shanghai Jiaotong, University School of Medicine, Shanghai, China

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ABSTRACT

The epithelial–mesenchymal transition (EMT) plays a crucial role in pancreatic ductal adenocarcinoma (PDAC) development and progression. TWIST activated by intra-tumoral hypoxia functions to promote the EMT. We hypothesized that TWIST and the downstream gene pathway could mediate PDAC progression under hypoxia. Therefore, 90 PDAC tissue specimens were immunostained for TWIST and other proteins. Pancreatic cancer cell lines were used for in vitro experiments and nude mice were used to confirm the *in vivo* data. Expression of TWIST and HIF-1α proteins was significantly upregulated, whereas expression of E-cadherin and p16 was down-regulated in PDAC tissues compared to that of non-tumor tissues and in tumor tissues obtained from patients with tumor involving splenic artery than those without splenic artery involvement. Up-regulated TWIST in tumor tissues were associated with worse prognosis in PDAC patients. The in vitro data showed that HIF-1α-induced TWIST overexpression promoted tumor cell growth and EMT under a hypoxic condition via TWIST interaction with Ring1B and EZH2. In vivo data showed that TWIST overexpression or a hypoxic condition induce xenograft growth, abdominal metastasis and low mouse survival, whereas knockdown of either Ring1B or EZH2 expression suppressed tumor xenograft growth and metastasis and prolonged survival of nude mice. TWIST was the key player in promotion of pancreatic cancer development and metastasis under a hypoxic condition through interaction with Ring1B and EZH2 to regulate expression of E-cadherin and p16 proteins in pancreatic cancer cells.

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Introduction

Pancreatic cancer is a lethal disease with a dismal 5-year survival rate [1]. Pancreatic cancer is usually diagnosed at the advanced stages of the disease, thus, curable surgical intervention is usually ineffective in pancreatic cancer patients [2]. Pancreatic cancer has a fast progression potential by invasion and metastasis to the adjacent or distant tissues or organs [2], and is generally resistant to chemo- or radiotherapy [3,4]. Moreover, pancreatic cancer localized in the body and tail of the pancreas usually shows worse prognosis than that localized at the pancreatic head [5]; however, this concept is still debatable. In routine clinical practice for resectable pancreatic cancer, tumors that involve the pancreatic body and tail and the splenic artery (SA) have the worst prognosis [6]. Thus, there is an urgent need to identify biomarkers for early detection and prediction of prognosis or treatment response in pancreatic cancer. Moreover, better mechanistic understanding of pancreatic cancer development and progression will aid in developing more effective therapeutic strategies to control and cure this aggressive disease.

Hypoxia frequently occurs in the tumor microenvironment due to the fast tumor growth and plays an important role in cancer progression, including pancreatic cancer [7–9]. For example, Chang et al. reported that hypoxia was associated with pancreatic cancer cell invasion and metastasis in mouse orthotopic tumor

* Corresponding author. Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Fujian Medical University, No. 134, Dong Jie Road, Fuzhou 350001, China.
** Corresponding author. Department of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, No. 29, Xin Quan Road, Fuzhou 350001, China.
E-mail addresses: drchenyl@126.com (Y.-l. Chen), wangyaodongch@163.com (Y.-d. Wang).

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xenograft model [10]. Dhani et al. demonstrated the presence of hypoxia in pancreatic cancer, in which intravenous pimonidazole probe was used to detect pancreatic epithelial tissues 16–20 h before pancreatectomy [11]. Tissue hypoxia could promote cell epithelial–mesenchymal transition (EMT), especially in tumor cells, and thus enhances tumor migration and invasion [12–14]. Previous studies clearly demonstrated that tissue hypoxic microenvironment could influence the prognosis of cancer patients by regulating tumor cell EMT, while HIF-1α as a biomarker for hypoxia could suppress E-cadherin expression [15,16] and regulate the functions of other key EMT regulators such as TWIST, Snail, and ZEB promoting EMT [16–18]. TWIST protein is a transcription factor and plays an essential role in cancer metastasis [19,20]; it binds to the E-box DNA sequences of the E-cadherin gene recruiting Bmi-1 to form a transcriptional complex that inhibits the transcription of E-cadherin gene [21]. In addition, TWIST could down-regulate the expression of $p16^{Ink4A}$ (p16) and induces cell cycle progression [21,22]. However, the role and expression of TWIST in pancreatic cancer remains controversial [23–27]. Thus, in this study, we investigated the role of TWIST and the downstream gene pathway in mediating pancreatic cancer progression in ex vivo, in vitro, and animal models. Our study provides insightful information regarding pancreatic cancer progression and the potential role of TWIST and the downstream genes as prognostic biomarkers and as a therapeutic target in pancreatic cancer patients.

Materials and methods

Tissue samples

Ninety pancreatic cancer patients who underwent distal pancreatectomy at Fujian provincial hospital, Fujian Union Hospital affiliated to Fujian Medical University, or Ruijin Hospital affiliated to Medical School of Shanghai Jiaotong University between September 2004 and September 2010 were retrospectively enrolled into this study. Paraffin embedded tissue blocks were collected from each patient and used to construct tissue microarrays (TMAs). The detailed clinicopathological data of all patients, including patients' age, sex, tumor size and location, pathological grade, perineural invasion, lymph node metastasis, and tumor staging were also collected and shown in Table 1. These patients were followed up after surgery up to September 2015 with a median follow-up duration of 14 months (ranged between 1 and 81 months). This study was approved by the Ethics and Research Committees of these three hospitals and conducted in accordance with the Declaration of Helsinki Principles.

Immunohistochemistry

These TMA sections were immunostained with anti-E-cadherin, p16, HIF-1α, and TWIST antibodies (all from Cell Signaling Technology, see details below) according to previous studies [28–30]. The immunostained TMA sections were then blindly reviewed and scored by two pathologists (JL and HL). A semiquantitative scoring of these TMA sections was counted for both the staining intensity (SI) and the percentage of staining (PP) in the tissue sections, i.e., SI was defined as follow: 0 (no staining), 1 (light yellow), 2 (light brown), 3 (brown), while PP was 0 (no positive cells); 1 (0–10%); 2 (11–50%); 3 (51–80%); and 4 (80–100%). Finally, immunoreactive score (IRS) system was used to summarize immunostaining data of each TMA and was calculated as follows: IRS = SI (staining intensity) × PP (percentages of positive cells). IRS scores were expressed as following: negative (−), 0–1 point; mild (+), 2–3 point; moderate (++), 4–8 point; strongly positive (+++), 9–12 point.

Cell lines and culture

Human pancreatic cancer cell lines (SW1900 and PATU-8988s) and a human embryonic kidney cell line (HEK-293T) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified chamber with 95% air and 5% CO_2. SW1900 and PATU-8988s cells were also exposed to a hypoxic culture condition, which was defined as 1% O_2, 94% N_2, and 5% CO_2.

Plasmid construction

Enhancer of zeste homolog 2 (EZH2) cDNA was amplified from the genomic DNA using PCR with primers (5′-CGCGGATCCGCGGGCCAGACTGGGAAGAAATC-3′ and 5′-CCGCTCGAG CGGTCAAGGGATTTCCATTTCTC-3′) and subcloned into pcDNA3.1-Flag vector (Invitrogen) between *BamH*I and *Xhol* sites, while E3 ubiquitin-protein ligase (RNF2 or Ring1B) cDNA was amplified from the genomic DNA template using PCR with primers (5′-CCCAAGCTTTCTCAG GCTGTGCAGACAAAC-3′ and 5′-GGAATTCTCATTTGTGCTCCTTTGTAGG-3′) and subcloned into pCMV4-Flag vector (Sigma) between *Hind*III and *Eco*RI sites. Moreover, TWIST cDNA subcloned into pCMV5-HA vector between *Eco*RV and *Xba*I sites and hypoxia-inducible factor-1α (HIF-1α) cDNA with deletion of amino acids between 401 and 603 (ODD) subcloned into pcDNA3.1 vector between *Eco*RI and *Xba*I sites have been previously described [17,31]. In addition, DNA sequences of E-cadherin and p16 promoters were amplified from the human genome DNA to obtain-361 to +45 and −869 to −1 promoter regions, respectively, and then subcloned into pGL4 vector. After DNA sequencing confirmation, these vectors were used for gene transfection or luciferase reporter assay.

Lentivirus carrying pLKO.1-shRNAs targeting TWIST, Ring1B and EZH2 were purchased from Thermoscientific and used to stably knockdown the expression of these genes in pancreatic cancer cell lines. Gene transfection was conducted by using Lipofectamine 2000 (Invitrogen) in pancreatic cancer cell lines according to the manufacturer's instruction, while lentiviruses were used to infect pancreatic cancer cell lines and selected with 2.5 μg/ml of puromycin. pGL4 vector carrying E-cadherin and p16 promoter sequences was transfected into HEK-293T cells for luciferase reporter assay according to a previous study [21].

Cell proliferation assay

SW1990 cells stably expressing TWIST or HIF-1α or SW1990 cells with EZH2 or Ring1B knockdown under a normoxic or hypoxic condition were seeded into 12-well plates at a density of 1×10^3 cells per well and cell proliferation assay was performed as described previously [28]. In brief, cells were seeded into the 12-well plates at a density of 1×10^3 per well and were then put in the incubator at 37 °C for 24 h to let the cells attach to the bottom of the cell culture dishes. The next day (corresponding to Day 0), the whole field of each well were viewed under a microscope at the indicated time points (Day 0, 2, 4, and 6), and the total numbers of cells were counted from each well. Three wells were used for each cell line and the experiment was done in triplicates.

Transwell tumor cell migration assay

SW1990 cells with stable expression of TWIST or HIF1-α (ΔODD) and SW1990 cells with EZH2 or Ring1B knockdown under a normoxic or hypoxic condition were harvested after serum-free starvation for 12 h and resuspended in DMEM and then were seeded into the upper chambers of Transwell inserts with 8-μm pores filters (Corning, Corning, NY, USA) at a density of 1×10^4 cells per well. DMEM with 20% FBS was added to and cells were cultured for 24 h. At the end of each experiment, tumor cells remained on the upper surface of the filter were removed with a cotton swab, while the cells that migrated into the lower surface of the filter were fixed and stained with 1% crystal violet solution. The numbers of cells on the membranes were counted under a microscope for randomly selected six microscopic fields (×100 magnification) per chamber. The data were summarized as means ± standard deviation and presented as a percentage of the control. The experiments were done in duplicate and were repeated three times.

Immunoprecipitation and western blot assay

To assess protein-protein interaction of TWIST with other proteins, we transiently transfected HEK-293T cells for 24 h with plasmids carrying Flag-EZH2, Flag-Ring1B, or HA-TWIST cDNA and then the nuclear/total cellular protein and histones were extracted for immunoprecipitation and Western blot assay according to a previous study [32]. Antibodies used were a mouse monoclonal anti-Flag (Cat #F7425, Sigma-Aldrich), anti-HA (Cat #MMS-101P, Covance, NJ,USA), anti-EZH2 (Cat #15217-662, Millipore, Billerica, MA, USA), anti-SUZ12 (Cat #sc-271325, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Snail (Cat #sc-28199, Santa Cruz Biotechnology), anti-BMI1 (Cat #05637, Upstate Biotechnology, Lake Placid, NY, USA), anti-TWIST (Cat #sc-15393, Santa Cruz Biotechnology), and anti-Ring1A (Cat #2820), Ring1B (Cat #5694), anti-H2A (Cat #2578), anti-H3 (Cat #9715), anti-ubiquityl-Histone H2A (Lys119) (Cat #8240), anti-E-cadherin (Cat #3195), anti-Tri-Methyl-histone H3 (Lys27) (Cat #9733) and normal IgG (Cat #2729) antibodies (all from Cell Signaling Technology, Danvers, MA, USA), while anti-β-actin antibody was from Proteintech (Rosemont, IL, USA).

Chromatin immunoprecipitation (ChIP)

Gene-transfected SW1990 cells were grown in 10-cm plates to reach 70–90% confluency and then harvested. The ChIP assay was performed with a ChIP assay kit from Millipore according to the manufacturer's protocol with antibodies specific to TWIST, Ring1B, EZH2, H2AK119Ub1 or H3K27Me3. The immunoprecipitated DNA samples were then amplified using quantitative PCR with primers flanking the known E-boxes in the E-cadherin promoter from −171 to +30 bp (5′-TAGAGGGTCACCGCGTCTAT-3′ and 5′-TCACAGGTGCTTTGCAGTTC-3′) or the p16 promoter covering −38 to −158 bp (5′-GCACTCAAACACGCC TTTGC-3′ and 5′-AGAGCCAGCGTTGGCAAGGA-3′).

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