HIF-3α Promotes Metastatic Phenotypes in Pancreatic Cancer by Transcriptional Regulation of the RhoC–ROCK1 Signaling Pathway

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Abstract

Hypoxia contributes to pancreatic cancer progression and promotes its growth and invasion. Previous research principally focused on hypoxia-inducible factor-1 alpha (HIF-1α) and HIF-2α (HIF1A and EPAS1) as the major hypoxia-associated transcription factors in pancreatic cancer. However, the role of HIF-3α (HIF3A) has not been investigated. Therefore, HIF-1α, HIF-2α, and HIF-3α expression levels were measured under normoxic and hypoxic conditions. In addition, HIF-3α expression was measured in human pancreatic cancer tissue specimens and the impact of altered HIF-3α expression on cell invasion and migration was investigated in vitro and in vivo, as well as the underlying mechanisms. Under hypoxic conditions, HIF-3α expression was stimulated in pancreatic cancer cells to a greater degree than HIF-1α and HIF-2α expression. HIF-3α protein levels were also elevated in pancreatic cancer tissues and correlated with reduced survival and greater local invasion and distant metastasis, whereas knockdown of HIF-3α, under hypoxic conditions, suppressed pancreatic cancer cell invasion and migration. Under normoxia, HIF-3α overexpression promoted pancreatic cancer cell invasion and migration and stimulated F-actin polymerization. In summary, HIF-3α promotes pancreatic cancer cell invasion and metastasis in vitro and promotes pancreatic cancer cell invasion and metastasis by transcriptionally activating the RhoC–ROCK1 signaling pathway.

Implications: HIF3α is overexpressed in pancreatic cancer, and targeting the HIF3α/RhoC–ROCK1 signaling pathway may be a novel therapeutic approach for the treatment of pancreatic cancer invasion and metastasis. Mol Cancer Res; 1–11. ©2017 AACR.

Introduction

Despite the continuing progress in combination therapy and radical surgery techniques, pancreatic cancer remains one of the deadliest tumor types, with an average 5-year survival rate of below 6% (1). Characteristics of pancreatic cancer include aggressive invasion and early metastasis, and 80%–90% of tumors are surgically unresectable at the time of diagnosis (2). Most pancreatic cancer patients who undergo surgical resection of small localized lesions later develop recurrent or metastatic tumors; these are thought to be a result of the presence of micrometastases undetectable at the initial diagnosis (3).

Pancreatic cancer is characterized by a highly hypoxic microenvironment with localized regions of tumor hypoxia (or low oxygen concentration; refs. 4, 5). Two prominent characteristics of pancreatic cancer, hypoperfusion and desmoplasia, play important roles in forming the hypoxic microenvironment (6). Hypoxia contributes to disease progression and promotes tumor growth and invasion, and clinical studies have shown that high levels of hypoxia directly correlate with a poor prognosis in pancreatic cancer (7). The observed correlation between tumor hypoxia and patient prognosis is ascribed to therapeutic failure and hypoxia-induced pro-metastatic potential, as several studies have shown that hypoxia stimulates a more invasive, metastatic phenotype via multiple mechanisms (8–11).

Hypoxia-inducible factors (HIF) regulate the cellular adaptation to hypoxia and are highly expressed in solid tumors. HIFs are a family of transcription factors comprising hypoxia-regulated α and oxygen-insensitive β subunits that regulate hypoxia-induced gene expression in normal tissues and solid tumors (12–14). Three members of the HIF family have been identified: HIF-1α, HIF-2α, and HIF-3α. HIF-3α differs from HIF-1α and HIF-2α in both its protein structure and its effect on gene expression. HIF-3α was previously believed to mainly function by opposing hypoxia-induced HIF-1α- and HIF-2α–dependent gene expression (15, 16). Under hypoxic conditions, HIF-3α competes with HIF-1α and HIF-2α for binding to the promoter region of their target genes and thus negatively regulates gene expression (14, 15, 17, 18). However, a role for HIF-3α as a positive transcriptional regulator has since been confirmed by the identification of HIF-3α target genes (14).

Hypoxia plays an extremely important role in the processes of invasion and metastasis in pancreatic cancer. The effects of HIF-1α...
and HIF-2α do not completely explain how hypoxia leads to pancreatic cancer invasion and metastasis. We therefore investigated a possible role for HIF-3α in pancreatic cancer invasion and metastasis with the aim of identifying a new therapeutic strategy for this disease.

Materials and Methods

Ethical statement

This study was approved by the Human Research Ethics Committee of Huazhong University of Science and Technology (HUST) and was conducted in accordance with the principles of the Declaration of Helsinki. All patients gave written, informed consent to participate in the study.

All animal experiments were approved by the HUST Committee on the Ethics of Animal Experiments (permit number: 2015-S117). All treatments were in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. Surgery was performed using sodium pentobarbital anesthesia.

Cell culture

BxPC-3, AsPC-1, MIA-PaCa-2, and PANC-1 cell lines were purchased from the ATCC, and were authenticated by the ATCC by short tandem repeat typing. MIA-PaCa-2 and PANC-1 cells were grown in DMEM, and BxPC-3 and AsPC-1 cells were grown in RPMI1640 Medium (Gibco). Culture media were supplemented with 10% FBS (Gibco), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Sigma) and the cells were grown at 37°C in a humidified incubator containing 5% CO₂. For hypoxia cultivation, cells were transferred to a hypoxia incubator (HeraCell TM 240i, Thermo Fisher Scientific) containing a 1:5:94 mixture of O₂:CO₂:N₂ for 24 hours.

Animals

Female 6-week-old BALB/c nude mice were purchased from HFK Bioscience. All mice were bred under specific pathogen-free conditions in the HUST Central Animal Laboratory. Female age-matched mice were used in all experiments.

Xenograft model of human pancreatic cancer

A total of 2 × 10⁴ transfected cells were subcutaneously injected into the left armpit of female 6-week-old BALB/c nude mice (n = 5 per experimental group). Mouse weight and tumor diameter were measured every week. Mice were sacrificed 6 weeks after the initiation of treatment and the tumors were evaluated both macroscopically and microscopically.

Pancreatic cancer liver metastasis model

Human pancreatic cancer cells (2 × 10⁶) were resuspended in serum-free medium and injected into the involucrum of the spleen of female 8-week-old BALB/c nude mice (n = 5 per experimental group). Splenectomy was performed 20 minutes later. Mice were weighed once a week and sacrificed when cachexia was observed or earlier if they exhibited >15% weight loss. Mouse livers were subsequently evaluated both macroscopically and microscopically.

Dual-luciferase assay

Mutant RhoC and ROCK1 promoter reporter constructs were designed to include mutation of three intermittent nucleotides complementary to the HIF-3α seed region. PANC-1 cells were cultured in 24-well plates and transfected with 400 ng of plasmid containing the wild-type, mutant pMIR/RhoC, or mutant pMIR/ROCK1 promoter controlling the firefly luciferase gene together with 100 ng of a pRL-TK vector (Promega) carrying the Renilla luciferase gene. Transfections were performed using Lipofectamine 2000 (Invitrogen). Relative luciferase activity was calculated at 48 hours post-transfection using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s protocol. For the luciferase assays, growth medium was removed from cultured cells and cells were washed in 1 × PBS. Then 20 μL/well 1 × PLB was added and cells were shaken gently for 15 minutes at room temperature. The lysate was then transferred to a luminometer tube containing 100 μL LARII and mixed gently. Firefly luciferase activity was measured directly and then 100 μL Stop & Glo Reagent was added for measuring Renilla luciferase activity.

Statistical analyses

Results for continuous variables are presented as means ± SD unless stated otherwise. Treatment groups were compared using t tests. Pairwise multiple comparisons were performed using two-sided one-way ANOVA. A P value of < 0.05 was considered statistically significant. All analyses were performed using SPSS version 17.0 (SPSS Inc.).

Results

Hypoxia induces HIF-3α expression to a greater extent than HIF-1α and HIF-2α

Pancreatic cancer cells were cultured under hypoxic or normoxic conditions for 24 hours, and then HIF-1α, HIF-2α, and HIF-3α protein and gene expression levels were assessed by Western blotting (Fig. 1A) and real-time PCR (Fig. 1B). Surprisingly, there was only a small increase in HIF-1α and HIF-2α expression levels under hypoxic conditions compared with normoxic conditions; in contrast, the level of HIF-3α expression increased considerably. Immunofluorescence staining further revealed that expression of HIF-3α protein in the cytoplasm and nucleus was higher in hypoxic conditions than that in normoxia (Fig. 1C). IHC images of serial sections of pancreatic cancer tissue showed that HIF-1α, HIF-2α, and HIF-3α show similar localization patterns and that HIF-3α showed the highest expression compared with all proteins examined (Fig. 1D; Supplementary Fig. S1A).

HIF-3α upregulation in pancreatic cancer tissues correlates with increased metastasis

IHC analysis of a tissue microarray indicated that HIF-3α was predominantly localized to the cytoplasm of pancreatic cancer cells, with much weaker nuclear staining (Fig. 2A; Supplementary Fig. S1B). We examined HIF-3α expression in 119 paired pancreatic cancer and normal pancreatic tissues samples and found significantly higher HIF-3α expression in pancreatic cancer tissues compared with adjacent normal pancreas tissues (Fig. 2B). The level of HIF-3α expression in pancreatic cancer tissues did not correlate with age, sex, tumor size, pathology grade, or tumor stage (Supplementary Table S1). However, HIF-3α expression was significantly higher in the tumors from pancreatic cancer patients with lymph node
involvement and/or distant metastasis compared with those without (Fig. 2C; Supplementary Table S1). Importantly, we found that higher intratumoral HIF-3α levels were significantly associated with a reduced overall survival rate (Fig. 2D). By comparing HIF-3α gene expression by PCR in 15 pairs of pancreatic cancer and adjacent normal pancreas tissue samples,
we also found that HIF-3α gene was upregulated in pancreatic cancer tissues (Fig. 2E).

HIF-3α promotes pancreatic cancer invasion and migration and F-actin polymerization in vitro

As our results indicated that HIF-3α expression correlated with local invasion and metastasis, we proceeded to investigate the effect of HIF-3α on the invasive capacity of pancreatic cancer cells. We used a lentiviral delivery system to generate MIA-PaCa-2 cells and PANC-1 cells stably overexpressing HIF-3α (HIF-3αU) or with stable HIF-3α knockdown (HIF-3αD). To confirm HIF-3α overexpression, we examined negative control (NC) and HIF-3αU cells in normoxic conditions by Western blotting. We also confirmed HIF-3α silencing by evaluating NC and HIF-3αD cells in hypoxic conditions by Western blotting (Supplementary Fig. S2).

Transwell and wound-healing assays revealed that HIF-3α overexpression promoted pancreatic cancer cell invasion and migration under routine culture conditions and hypoxic conditions (Fig. 3A–C; Supplementary Fig. S3A–S3D). F-actin plays an important role in cancer cell migration and invasion, and hypoxia can stimulate F-actin polymerization (19). We found that HIF-3α overexpression in normoxic conditions resulted in F-actin polymerization (Fig. 3D). However, HIF-3α knockdown under hypoxic conditions resulted in reduced F-actin polymerization.
Overexpression of HIF-3α in pancreatic cancer cells increases their invasion and metastasis ability in vivo.

To determine whether HIF-3α affects pancreatic cancer cell invasion and metastasis in vivo, we established two experimental mouse models: a subcutaneous xenograft model and a model in which intrasplenically injected human pancreatic cancer cells induce hepatic metastasis. In the xenograft model, mice were monitored and weighed weekly, and then euthanized on the sixth week. There was no significant difference of tumor growth and tumor weight observed in the NC, HIF-3αU, and HIF-3αD groups (Supplementary Fig. S4A–S4D). Hematoxylin and eosin (H&E) staining of whole xenograft serial sections indicated that mice injected with HIF-3α-overexpressing cells had more metastatic foci compared with control mice.

Figure 3.

HIF-3α overexpression promotes pancreatic cancer invasion and migration in vitro. PANC-1 cells stably overexpressing HIF-3α (HIF-3αU) and knockdown (HIF-3αD) cells in wound-healing assays (A) and transwell cell invasion and migration assays (B and C) under normoxic and hypoxic conditions (n = 3). **P < 0.01; ***P < 0.001. D, Immunofluorescence analysis of PANC-1 HIF-3αU and NC cells (top) and HIF-3αD and NC cells (bottom) under hypoxic conditions (scale bar, 10 μm).
Furthermore, HIF-3α-overexpressing cells had invaded beyond the xenograft tumor membrane and were located in the surrounding muscle and connective tissue (Fig. 4A and D). In contrast, no metastases or membrane penetration was seen in the HIF-3α knockdown group. HIF-3α expression levels were evaluated in xenograft tumor HIF-3αU group tissues by IHC staining (Fig. 4B and E).

In the hepatic metastasis model mice, H&E staining of whole liver serial sections revealed that HIF-3α overexpression resulted in an increased number of liver micrometastases compared with wild-type mice. In contrast, HIF-3α knockdown reduced the number of liver micrometastases. Further gross pathologic and survival curve analyses indicated that HIF-3α overexpression increased the number of liver metastases and
HIF-3α transcriptionally regulates RhoC–ROCK1 signaling in pancreatic cancer cells

We next used bioinformatics analysis to identify genes that are regulated by HIF-3α. The results indicated that HIF-3α may bind to the promoter regions of RhoC and ROCK1 (see Supplementary Material). This was confirmed by chromatin immuno-precipitation and dual-luciferase reporter assays (Fig. 5A and C). We further found that endogenous HIF-3α binds to the promoters of RhoC and ROCK1 under hypoxic conditions but not under normal conditions (Fig. 5B). Real-time PCR indicated that HIF-3α overexpression enhanced RhoC and ROCK1 expression (Fig. 5D).

Our results demonstrate that HIF-3α transcriptionally activates RhoC and ROCK1. To clarify the potential mechanisms and factors involved in the signaling pathway, we examined several factors in this pathway. We compared levels of RhoC, ROCK1, LIM domain kinase 1 (LIMK1), phospho-LIMK1, megalencephalic leukoencephalopathy with subcortical cysts 2 (MLC2), phospho-MLC2, coflin, and phospho-coflin in HIF-3α-transfected and negative control cells, and found increased RhoC, ROCK1, phospho-coflin, and phospho-MLC2 levels in HIF-3α-transfected cells compared with control cells (Fig. 5E). Furthermore, small-interfering RNA (siRNA) for RhoC (si-RhoC) and ROCK1 (si-ROCK1) and the RhoC–ROCK1 pathway inhibitor Y27632 inhibited the effect of increased HIF-3α activity on phospho-coflin and phospho-MLC2 levels (Fig. 5F).

HIF-3α regulates pancreatic cancer invasion and metastasis via RhoC–ROCK1 signaling

We next examined whether HIF-3α promotes pancreatic cancer cell invasion through the RhoC–ROCK1 pathway. As demonstrated above, HIF-3α overexpression promoted tumor cell invasion. We found that HIF-3α overexpression–mediated increase of invasion was ablated by si-RhoC, si-ROCK1, and the ROCK inhibitor Y27632 (Fig. 6A and B; Supplementary Fig. S5A and S5B). HIF-3α upregulation inhibited F-actin depolymerization, and si-RhoC, si-ROCK1, and Y27632 suppressed this effect, leading to reduced F-actin polymerization (Fig. 6C; Supplementary Fig. S5C).

In the Balb/c nude mouse model of human pancreatic cancer liver metastasis, while HIF-3α overexpression promoted tumor cell metastasis and reduced the overall survival time, administration of Y27632 counteracted this effect (Fig. 6D). The HIF-3α group had the shortest survival time compared with the NC and HIF-3α-D groups. However, the overall survival time did not significantly differ between the control and HIF-3α-U–1Y27632 groups. H&E staining of whole liver serial sections revealed more micrometastases in the HIF-3α-U group compared with the control group. Administration of Y27632 to HIF-3α-U–treated mice resulted in fewer micrometastases than in the control group (Fig. 6E). Together, these results suggest that HIF-3α–mediated induction of invasion and metastasis involves RhoC–ROCK1 signaling in vitro and in vivo.

Discussion

Data from a number of epidemiologic and clinical studies indicate that hypoxia and hypoxia-induced signaling correlate with a poor clinical outcome in patients with solid tumors, including pancreatic cancer (20). A correlation between hypoxia and pancreatic cancer has been recognized for a number of years (21), and a role for HIF-1α and HIF-2α in pancreatic cancer has previously been investigated (22, 23). In this study, we showed that HIF-3α expression is stimulated to a greater extent than either HIF-1α or HIF-2α under hypoxic conditions in pancreatic cancer cells. We also demonstrated that HIF-3α promotes pancreatic cancer cell invasion and metastasis by transcriptionally activating the RhoC–ROCK1 signaling pathway in vitro and in vivo.

Human HIF-3α shows high levels of similarity with HIF-1α and HIF-2α in the basic helix-loop-helix and PAS domains. Initial studies suggested that HIF-3α was a negative regulator of hypoxia-induced gene expression in the kidney (15). Li and colleagues later reported that HIF-3α is abundantly expressed in lung epithelial cells, and that the stimulation of HIF-3α–dependent transcription is important for the response to hypoxia in vitro (17). Tanaka and colleagues also showed that HIF-3α is a HIF-1α target gene, suggesting a possible role for HIF-3α in hypoxia-associated gene expression (24).

HIF-3α contains several predicted mRNA variants (at least 10) in humans from the use of different promoters, initiation sites, different transcription, and alternative splicing (25). HIF-3α has only one prolyl site and an N-terminal transactivation domain (TAD), whereas HIF-1α and HIF-2α share a homology and both have two prolyl sites and two TADs (N-terminal and C-terminal; ref. 26). HIF-3α also contains long isoforms, such as HIF-3α1 and HIF-3α2, which can upregulate a distinct set of HIF-α target genes (18). HIF-3α is reported to promote colorectal cancer cell growth through noncanonical, transcription-independent activation of the JAK–STAT3 signaling pathway (27). Ectopic overexpression of HIF-3α’s gene lacking the 3′ untranslated region might disturb the regulatory circuit by altering HIF-1α transcriptional activity (28).

We showed that HIF-3α promotes pancreatic cancer cell invasion and metastasis in vitro and in vivo due to modulation of the RhoC–ROCK1 signaling pathway. Members of the Rho subgroup of Ras GTPases, comprising RhoA, RhoB, and RhoC, induce actin cytoskeleton rearrangement and mediate a number of cellular functions (29, 30). Although RhoA and RhoB expression levels were not significantly associated with patient clinicopathologic findings, RhoC overexpression has been shown to play a role in tumor invasion and correlates with a poor patient prognosis (31). The RhoC–ROCK1 signaling pathway plays a pivotal role in actin cytoskeleton organization and is involved in many key processes, including cell adhesion, vesicular trafficking, proliferation, cell survival, cell morphology, and cell–matrix interactions (32–37). Inhibition of ROCK1 expression blocks LIMK2/cofilin phosphorylation and subsequent stress fiber formation (38, 39). MLC2 stimulates the formation and stabilization of actin stress fibers (40). In this study, we found that HIF-3α transcriptionally regulates RhoC and ROCK1 and enhances phospho-cofilin, and phospho-MLC2 levels, demonstrating activation of RhoC–ROCK1 signaling. Together, this suggests that HIF-3α may promote the invasion and metastasis of pancreatic cancer by controlling the cytoskeletal structure and stabilizing stress fibers.

In conclusion, we found that HIF-3α is upregulated in pancreatic cancer tissues and that HIF-3α overexpression...
Figure 5.
HIF-3α transcriptionally regulates the RhoC–ROCK1 signaling pathway. A, Chromatin immunoprecipitation analysis of HIF-3α recruitment to the HIF-binding site in the RhoC and ROCK1 promoters in HIF-3α-Flag-transfected and NC PANC-1 and MIA-PaCa-2 cells (n = 3); **, P < 0.01; ***, P < 0.001. B, Chromatin immunoprecipitation analysis of HIF-3α recruitment to the HIF-binding site in the RhoC and ROCK1 promoters in PANC-1 cells under hypoxic or normoxic conditions (n = 3); ***, P < 0.001. C, Dual-luciferase assay in HIF-3α U and NC cells (n = 5). ***, P < 0.001. PCR (D) and Western blotting analyses (E) of HIF-3α, HIF-3α D, and NC cells. Full-length blots are included in the Supplementary Information. F, Western blotting analysis of lysates from the indicated cells (n = 3). Full-length blots are included in the Supplementary Information.
correlated with a shorter survival time and increased local invasion and distant metastasis. We have shown that HIF-3α regulates the invasive and metastatic potential of pancreatic cancer cells in vitro and in vivo by modulating RhoC–ROCK1 signaling. These results provide insights into the molecular mechanisms underlying the enhanced invasion and metastasis of pancreatic cancer in hypoxic environments and may suggest a novel therapeutic strategy for pancreatic cancer treatment.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Authors' Contributions

Conception and design: X. Guo, C. Xie, J. Jiang
Development of methodology: X. Zhou, X. Guo, J. Jiang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zhou, X. Guo, M. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhou, X. Guo, M. Chen, J. Jiang
Writing, review, and/or revision of the manuscript: X. Guo, C. Xie, J. Jiang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Guo, J. Jiang

Study supervision: J. Jiang

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