NADPH Oxidase 4 Promotes Hypoxia-induced Epithelial-to-mesenchymal Transition via Histone Modification in Pancreatic cancer

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Abstract

Background

Hypoxia is a characteristic of the tumor microenvironments within Pancreatic cancer (PC) which has been linked to its malignancy. Oxidative stress, characterized by NADPH oxidase (NOX) activation, and epithelial-to-mesenchymal transition (EMT) could be induced by hypoxia which involved in tumor progression and metastasis. However, the relationship between hypoxia-induced oxidative stress and EMT has not been clarified, and the regulatory mechanism of NADPH oxidase is still unknown.

Methods

A hypoxic-related gene signature and its associated pathways in PC were identified by bioinformatics method. Candidate downstream gene (NOX4), responding to hypoxia was validated by RT-PCR and western blot. In vitro and in vivo assays as well as tumor samples from our centre were preformed to explore the phenotype of NOX4 in PC. Immunofluorescence, western blot and chromatin immunoprecipitation assays were further applied to search for detailed mechanism.

Results

We established a hypoxia-related gene signature within PC which was prognostic and linked with up-regulated EMT pathway. Then we found that hypoxia could induce stable up-regulation of NOX4, which is essential for EMT activation. Elevated expression of NOX4 was observed in PC samples and positively associated with advanced tumor grade and unfavorable prognosis. In vivo and in vitro experiments demonstrated NOX4 overexpress or inhibition in pancreatic cancer cells caused changes of proliferation and invasion ability. Then we found NOX4 could increase the methylation modification of histone H3 and regulated the transcription of EMT-associated gene_ snail family transcriptional repressor 1 (SNAIL1).

Conclusions

This study highlights the prognostic role of hypoxia-related genes in PC and strong correlation with EMT pathway. Our results also creatively discovered that NOX4 was an essential mediator for hypoxia-induced histone methylation modification and EMT in PC cells.

Introduction

Hypoxia is a vital feature of the tumor microenvironment, including pancreatic cancer (PC)(1, 2). Increased desmoplasia and resulting insufficient perfusion are important causes of hypoxia in PC (3, 4).
Triggered by hypoxia, tumor cells activate a variety of molecular pathways in order to adapt to environmental change(5). Epithelial-to-mesenchymal transition (EMT) is a process of transforming tumor cells from epithelial to mesenchymal cell types which could be induced by hypoxia, contributing to tumor invasion and metastasis(6, 7). In this regard, most studies proposed that hypoxia-inducible factor 1 subunit alpha (HIF1α) is held accountable for the induction of EMT (8, 9). However, this view has been updated by the latest studies showing that the change of histone modifications under hypoxia occurred more rapidly than HIF1α induction. This rapid response in histone modification is able to induce EMT process in Hela cells(10, 11). However, the mechanism of hypoxia-induced histone methylation and its association with EMT in PC is not yet clear.

Oxidative stress is another crucial process in tumor cells after exposure to hypoxia. And NADPH oxidase (NOX), a main reactive oxygen species (ROS) producer, can be activated in response to hypoxia(12, 13). Different from the inevitable physiological "leakage" of the mitochondrial respiratory chain during normal function, the enzymes NADPH oxidase are the primary sources of non-mitochondrial ROS production, and more importantly, they generate ROS in a regulated manner(14). Hence, the NADPH oxidase has been suggested as a possible cellular oxygen sensor(15). More importantly, NADPH oxidase has also been reported to induce EMT in breast cancer cells(16). Therefore, NADPH oxidase may be involved in hypoxia-induced EMT in PC.

In this study, we took advantage of GEO and TCGA database to establish a list of hypoxia-associated genes. Further analysis uncovered a prognostic value of hypoxia-related gene sets, which is also associated with EMT-related pathways. Then, we identified NOX4, the enzyme activated by hypoxia in PC, activated EMT pathway by regulating intracellular ROS levels and by modifying chromatin methylation modification.

**Materials And Methods**

**Analysis of pancreatic cancer gene expression data from GEO and TCGA database**

The Hypoxia-related gene expression signature consisted of 200 genes and EMT-related gene expression signature consisted of 200 genes were obtained from the gene set of HALLMARK_HYPOXIA and HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION in The Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/). Microarray gene expression data from GSE15471 and GSE16515 were used to screen for genes that were highly expressed in PC tissues and then merged with 200 hypoxia-related genes. Level3 TCGA RSEM Gene expression data and clinical information for pancreatic cancer were downloaded from UCSC (https://xena.ucsc.edu/public). We got 30 highly expressed hypoxia-related genes in pancreatic cancer tissues. Then we calculated the hypoxia score of TCGA samples using the 30 genes as previously described(17). Briefly, for each gene, samples with the top 50% of expression value were given a score of +1, and samples with the bottom 50% of expression value were given a score of −1. The hypoxia score in each sample was the sum of the scores of 30 genes (Table S1). Similarly, EMT
score of TCGA samples was calculated using 200 EMT-related genes using the same method (Table S2). TCGA subtype defined by Bailey et al. (18), Collisson et al. (19), and Moffitt et al. (20) were derived from the most recent TCGA pancreatic adenocarcinoma subclassification (21).

**Functional Analysis and GSEA**

Spearman correlation analysis was used to find genes related to hypoxia scores (Table S2) and NOX4 expression. The R package “clusterProfiler” (22) was applied for the Gene Ontology (GO) analysis and KEGG analysis of genes positively related to hypoxia score. Hallmark gene sets were enriched using Metascape (23). Gene-set enrichment analysis (GSEA) was applied to enrich hallmark gene sets related to NOX4 in TCGA samples.

**Tumor samples**

50 PC Formalin-Fixed Paraffin-Embedded (FFPE) samples were obtained from the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China) between January 2007 and August 2013. Frozen specimens of 6 PC patients and 6 benign pancreatic lesions were collected from the same hospital between 2018 and 2019. The experimental study was approved by the Affiliated Drum Tower Hospital of Nanjing University Medical School, and the informed consent forms were obtained from patients enrolled in this study.

**Cell culture and treatment**

Human pancreatic cancer cell lines HPAC and Panc1 were a gift from the Technical University of Munich, Germany. ALL cell lines used in this study are considered to be identical to the reference cell line in the Cell Bank STR database, as the STR profile yields a 100% match. All cell lines were cultured in DMEM medium (Wisent Inc, Montreal, Canada) with 10% FBS (Biological Industries, Beit-Haemek, Israel) under 5% CO₂ at 37 °C. HPAC cells were treated with GLX351322 (MedChemExpress, Shanghai, China) with a concentration of 0, 1, 5, 10μM for 48 hours. The cells were then used to detect CCK8. Other interventions for cells were shown in supplementary materials.

**NOX4 knockdown and overexpress**

NOX4 short-hairpin RNAs (shRNAs) or scrambled control shRNA were designed, synthesized and packaged into lentivirus particles (Corues Biotechnology, Nanjing, China). Cells were plated into six-well plates (3×10^4 cells per well). Before transfection, the culture medium was replaced with DMEM with 10%FBS and 1 μg/mL Polybrene (GeneChem, Shanghai, China). Infectious lentivirus particles were harvested for 72 hours after transfection.
To establish NOX4 overexpressed HPAC cells, HPAC cells were transfected with NOX4 Human Tagged ORF Clone (RC208007) and the pcmv6 empty vector (Origene, MD, USA) using lipo3000 (Thermo Fisher Scientific, MA, USA). After 72 hours, cells were treated with G418 (Sigma-Aldrich Corp., MO, USA) to select stably transfected clones.

**RNA extraction and RT-PCR**

Total RNA was isolated from cells using RNAiso Plus Reagent (Takara, Kusatsu, Japan). RT reactions were performed using the PrimeScript™ RT Master Mix (Takara). Then quantification of mRNA expression was performed using SYBR® Advantage® qPCR Premix (Takara) in a total reaction volume of 20 µl according to the manufacturer's instructions. The reaction was performed using the LightCycler® 96 system (Roche Diagnostics, Basel, Switzerland). ACTB was used as an internal control. The primer sequences used were shown in the supplementary material.

**Immunoblot analysis**

Cell or tissue homogenate was used for immunoblot analysis. Specific information was described in supplementary materials.

**Immunohistochemistry**

Paraffin sections of tissues from 50 PC patients were used for immunohistochemical detection of NOX4 expression. The detailed protocol was presented in the supplementary material.

**Immunofluorescence**

Cells or frozen tissue sections (15µm thick) were fixed with paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 2% BSA in PBS and incubated with NOX4, CDH2, VIM, H3K4ME3 antibodies overnight at 4°C. Then the sections and cells were incubated with secondary antibody (1:500, supplementary material) for 2 hours, counterstained with DAPI (Beyotime) and visualized by a fluorescence microscope (Olympus, Tokyo, Japan).

**Cell Counting Kit-8 (CCK8) assay**

CCK8 assay (Dojindo, Kumamoto, Japan) was used to detect the cell viability of PC cells according to the manufacturer's instructions. HPAC cells were planted in 96-well plates (5000 cells each) and incubated with 10 µL CCK8 for 2 h at 37 °C. The absorbance was recorded at 450nm.
Migration and invasion assays

Migration and invasion assays were proceeded in transwell chamber (Corning, NY, USA) with (invasion) or without (migration) Matrigel matrix (Corning) in a 24-well plate. 5\times10^6 cells resuspended in serum-free DMEM were added in the up chamber, and DMEM medium with 20% FBS were added in the bottom chamber. Then cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Cells were imaged and counted using a 20× microscope.

Experimental mice

Four-week-old male BALB/c nude mice (weighing 16–18 g) were purchased from Changzhou Cavens Experimental Animal Co. Ltd (Changzhou, China). To establish the subcutaneous transplanted model, HPAC cells were injected subcutaneously to the flank region of nude mice of 6 weeks old. Tumors were measured using vernier callipers twice a week, and the volume of tumors was calculated using the formula (length x width^2/2). After one month, all mice were sacrificed, and tumors were collected. For lung-metastasis xenografts, 1\times10^6 HPAC cells suspended in 100 µL cold PBS were injected into the lateral tail vein (4 for each group). After one month, all mice were sacrificed, and lung tissues were fixed with 4% paraformaldehyde. H&E staining was used to evaluate the proportion of metastatic lesions. All animals were approved by the Ethics Committee of Nanjing Drum Tower Hospital. All animals used in this study were treated humanely and followed guidelines set by the Animal Care Committee. The study was approved by the Ethics Review Committee for Animal Experimentation at Nanjing Drum Tower Hospital (Nanjing, China).

CHIP-PCR

Chromatin Immunoprecipitation(CHIP)-PCR was performed to analyze the effect of NOX4 on the binding of H3K4ME3 to the SNAIL1 promoter sequence. Detailed information was presented in the supplementary material.

Statistical analysis

All bioinformatics analyses were performed using R (https://www.r-project.org/) and Rstudio software (B Corps™, DE, USA). All statistical analysis was performed using GraphPad Prism v6.0 (GraphPad Inc., La Jolla, CA, USA) software. All data were reported as the mean ± SD. The differences between two groups were analyzed using T-test and the differences among multiple groups were analyzed using one-way ANOVA or two-way ANOVA followed by Tukey’s test. Correlations between two groups were analyzed by the Person’s Rank-Order method. Kaplan-Meier curve (Log-rank tests) was used to determine any significant associations of patient outcome and hypoxia score or NOX4. P<0.05 was considered statistically significant.
Result

Evaluation of hypoxia-related gene expression in PC

First, we quantified hypoxia-related gene expression in gse15471 and ges16515 using 200 genes from hallmark HYPOXIA gene set (Molecular Signatures Database [MsigDB]). This analysis identified 30 shared hypoxia-related genes that were overexpressed in PCs, compared to normal controls in both databases (Figure 1a, b). Figure 1c showed the expression of these 30 genes across PAAD tumor samples in TCGA dataset. Then, we used these 30 genes as hypoxia-related gene signature, and a hypoxia score was calculated for each individual sample according to the expression levels of these genes (described in the method part, Table S1). Next, we explored the prognostic significance of hypoxia-related gene expression in TCGA PAAD dataset. Tumors with the top 50% of hypoxia score values had a worse overall survival (OS) and progression-free survival (PFS) than those with the bottom 50% of hypoxia score values. And also, hypoxic tumors were more likely to have a higher grade and pathologic stage (Figure 1f). To verify whether hypoxia scores are related to specific molecular subtypes, we grouped TCGA PAAD samples by the four-groups classification of Bailey et al.(18), the three-group classification of Collisson et al.(19), and the two-group classification of Moffitt et al(20). This analysis revealed that the subtypes with unfavorable prognosis (squamous in Bailey clusters, quasimesenchymal (QM) in Collisson clusters and basal-like in Moffitt clusters) were more likely to have a higher hypoxia score (Figure 1g).

Epithelial-mesenchymal transition was induced in hypoxia samples

Then, we calculated the pearson correlation coefficient between each hypoxia score and all the other genes and ranked them according to the correlation coefficient in TCGA PAAD samples, and a total of 385 genes showed a significant positive correlation with hypoxia score ($R \geq 0.5$) (Table S2). Their biological processes and pathways were analyzed using the GO terms of biological processes and KEGG pathways. The GO analysis showed that the biological processes such as extracellular structure organization and extracellular matrix organization were significantly enriched (Figure 2a), and the KEGG pathway analysis identified focal adhesion and PI3K-AKT signalling pathway as the enriched pathways (Figure 2b). Next, we used the Hallmark gene sets from MSIGDB (https://www.gsea-msigdb.org/) to analyze the enriched sets of same 385 genes, which showed EMT pathway is the most enriched one (Figure 2c). Figure 2d showed the expression of EMT markers consisting of 200 genes increases along with hypoxia score. As above mentioned, we then calculated an EMT score for each individual sample and tested if it was correlated with the hypoxia score. This analysis revealed that EMT and hypoxia score was strictly correlated with each other (Figure 2e). To experimentally validate this, we tested the expression of EMT-related genes in two PC cell lines treated with/without hypoxia (1% $O_2$). This analysis revealed that the mRNA and protein expression of vimentin (VIM), cadherin 2 (CDH2) and SNAIL1 increased in HPAC and Panc1 cell lines upon hypoxia exposure for 24 hours. Accordingly, the mRNA and protein expression of cadherin 1 (CDH1) decreased in both cell lines (Figure 2f, 2g).
EMT induced by hypoxia is dependent on NOX4 expression

Since hypoxia was described to stimulate the production of ROS, we first evaluated the correlation between hypoxia-related expression and NOX family members which are responsible for generating non-mitochondrial ROS (24). This analysis revealed a significant correlation between NOX4 expression and hypoxia score (Figure 3a). We used the median value to distinguish groups with high and low expression of NOX4 in TCGA samples and carried out a GSEA analysis. Importantly, this analysis identified EMT and hypoxia pathways were significantly (with a cutoff for FDR 5% and p value < 0.01) enriched in high expression group of NOX4 (Figure 3b).

Further, we detected the location of NOX4 in the bulk tissues of two PC patients. NOX4 was expressed in both fibroblasts and epithelial cells (Supplementary Figure 1a). To further verify the expression of NOX4 after hypoxia, we examined the mRNA and protein expression of NOX4 after hypoxia exposure for 0-24 hours. We found that NOX4 expression increases at a very early stage (Figure 3c, 3d). We also found that the level of ROS increased steadily after hypoxia exposure in HPAC cells (Supplementary Figure 1b). Since superoxide is generated from NOX4 and quickly converts to H2O2 through dismutation (25), we hypothesized that H2O2 was partially responsible for NOX4 and hypoxia-induced EMT phenotype. Indeed, H2O2 activated an EMT program in HAPC and Panc1 cells in a dose-dependent manner (Figure 3e). In line, the level of ROS increased significantly, when NOX4 was overexpressed in HAPC and Panc1 cells (Figure 3f). Correspondingly, EMT process is also activated in these NOX4-overexpressing cells (Figure 3g, Supplementary Figure 1c,e). When NOX4 was knocked down using two short-hairpin RNAs (shNOX4#1 and shNOX4#2) in HPAC cells, the hypoxia-induced EMT process was partially compromised (Figure 3h, Supplementary Figure 1d,f). Thus, the hypoxia-induced EMT in PC cells largely depends on the function of NOX4.

Hypoxia-induced NOX4 activation was independent of HIF1α, but dependent on TGFβ1

HIF1α is a vital transcription factor induced by hypoxia. We then analyzed whether NOX4 is a downstream gene of HIF1α, however, we found the upregulation of NOX4 preceded to HIF1α induction upon hypoxia exposure (Figure 3c). And also, the hypoxia-induced NOX4 expression was not affected after the knockdown of HIF1α using siRNA (Figure 3i). Given that transforming growth factor beta 1 (TGFβ1) is a NOX4 inducer, we found TGFβ1 secreted by HPAC cells increased significantly after hypoxia (Figure 3j). Then, we treated HPAC cells with TGFβ1 in time and concentration gradient, and similarly to previous studies, NOX4 is significantly activated by TGFβ1 in PC cells (Figure k). TGFβ1 neutralizing antibody treatment attenuated hypoxia-induced NOX4 activation in HPAC cells (Figure 3l). Therefore, TGFβ1 may be the upstream signal of NOX4 during hypoxia.
NOX4 is highly expressed by PC and positively correlated with the degree of hypoxia in tumor tissue

To investigate the relevance between NOX4 expression and hypoxia in PC tissues, we compared the IHC scores of NOX4 in HI1Fα-positive and negative PC tissues within a cohort of 56 PC patients from our centre and found that NOX4 was highly expressed in hypoxic PC tissues (Figure 4a). We also found that PC patients with higher NOX4 expression had worse overall survival and higher histologic grade than those with lower NOX4 expression (Figure 4b,c). We also analyzed NOX4 expression in GEO databases (GSE15471 and GSE16515) and found that NOX4 expression is up-regulated in bulk PC tissues (Figure 4d). Similar results were found in 7 PC tissues compared with 6 benign pancreatic tissues collected in our centre (Figure 4e). We also observed that PC cell lines had a higher level of NOX4, as compared to human ductal pancreatic epithelial cells (HPDE cell line, Figure 4f). By analyzing TCGA data, the expression of NOX4 was significantly correlated with tumor stage and grade (Figure 4g,4h).

NOX4 promotes the proliferation and metastasis of PC cells

To investigate the functional significance of NOX4, we first performed cell proliferation assay after the knockdown and overexpression of NOX4 in HPAC cells. After the knockdown of NOX4, the cell growth was inhibited, while the overexpression of NOX4 promoted the growth of HPAC cells (Figure 5a,b). Then we established subcutaneous transplanted model to investigate the role of NOX4 on the proliferation of HPAC cells in vivo. The volume of xenograft tumors was significantly increase after NOX4 overexpression (Figure 5c) and decrease after the knockdown of NOX4 (Figure 5d). And also, we used GLX351322 (a NOX4 inhibitor) to treat HPAC cells, the growth of cells was also inhibited (Figure 5e).

In migration and invasion assays, we found that NOX4 overexpression enhanced the migration and invasion of HPAC cells (Figure 5f). Likewise, a similar outcome was observed in HPAC cells after NOX4 knockdown (Figure 5g). Then, a lung-metastasis xenograft mouse model was established to study the potential role of NOX4 in PC metastasis in vivo. The percentage of metastatic area were significantly reduced after NOX4 knockdown (Figure 5h).

NOX4 up-regulates SNAIL1 expression by increasing histone methylation

The previous study has shown that hypoxia induced a robust increase of histone methylation markers. In particular, chromatin immunoprecipitation followed by deep sequencing (ChIP-sequencing) of H3K4me3, a methylation marker associated with active gene transcription, identified that EMT was up-regulated after hypoxia exposure. To investigate whether NOX4 significantly affected histone methylation modifications, we tested various histone methylation markers after overexpressing NOX4. We found that NOX4 induced an increase in histone methylation (Figure 6a). Then we focused on the H3K4me3, as we
verified the previous findings that the expression of H3K4me3 was indeed significantly induced by hypoxia in HPAC cells (Figure 6b). Compellingly, the knockdown of NOX4 reversed the upregulation of H3K4me3 caused by hypoxia (Figure 6c). Then, we analyzed the ChIP-sequencing results of PC cell line for H3K4ME3 in the GEO database (GSE945856), and found a peak in the promoter region of SNAIL1. Thus, we performed ChIP-PCR of H3K4me3 at ChIP-sequencing peak for SNAIL1 in HPAC cells after altering the expression of NOX4. The results revealed a marked increase in H3K4me3 at SNAIL1 after NOX4 overexpression or 24 hours of hypoxia exposure (Figure 6d,e). When NOX4 was knocked down with shRNAs, it was partially compromised (Figure 6e).

**Discussion**

Hypoxia is an adverse living condition for tumor cells; however, it leads to aggressive phenotypes in a variety of tumors(4,26,27). As we presented, hypoxia-related gene signature was prognostic and linked with up-regulated EMT pathway. NOX4-induced oxidative stress and rapid changes in chromatin modification status was a necessary processes facilitating this hypoxia-induced EMT process.

We quantified hypoxia score in the TCGA using 30 hypoxia-related genes that are highly expressed in PC and described the correlation between hypoxia score and clinical parameters in these samples. Similar to previous studies in other tumors(28,29), samples with high hypoxic score exhibited a worse prognosis. Molecular subtypes of PC have been defined by several studies and linked with prognosis and response to treatment(18–20). Here, we showed that samples with high expression of hypoxia-related genes were more concentrated in the subtype with the worst prognosis (squamous in Bailey clusters, QM in Collisson clusters and basal-like in Moffitt clusters). These results suggested that hypoxia is a driving factor that promoted the transformation of tumors to a more aggressive phenotype.

Hypoxia triggers varied molecular responses in tumor cells. To better understand the potential regulatory mechanism of PC cells under hypoxia, we used hypoxia-related gene signature to predict possible downstream pathways. Here, we found a strong positive correlation between hypoxia-related gene expression and EMT process in PC samples. EMT was induced by hypoxia in a variety of tumors including non-small cell lung cancer (NSCLC), ovarian carcinoma(30,31) and the mechanism involves the regulation of SNAIL, twist family bHLH transcription factor (TWIST) and snail family transcriptional repressor 2 (SLUG)(32,33). Stabilization of HIF-1α is a crucial transcription factor caused by intratumoral hypoxia which can induce EMT binding directly to the promoter of TWIST and SNAIL(33,34). However, we found a rapid induction of NOX4 after hypoxia in PC cells which significantly promotes the EMT process even before the activation of HIF1α.

*In vivo and in vitro* experiments demonstrated NOX4 overexpress or inhibition in pancreatic cancer cells caused changes of proliferation and invasion ability. And these were consistent with the analysis of clinical data from TCGA and our database. NOX4 has been reported to be activated to promote the anti-apoptotic ability of PC (35,36). It has also been shown that NOX4 can promote tumor metastasis in some tumors such as human colorectal cancer and non-small cell lung cancer(37,38). More importantly,
GLX351322, a NOX4 selective inhibitor, could attenuate proliferation ability of pancreatic cancer cells, which means that PC may benefit from NOX4-targeting therapy.

Changes in chromatin modification after hypoxia, especially histone methylation, are important newly discovered mechanisms in recent years \(^{10}\). This rapid oxygen sensing mechanism caused a series of critical changes of important pathways to make tumor cells respond to hypoxia. We firstly demonstrate that NOX4 can induce stable histone methylation after hypoxia which leads to the regulation of important pathways including EMT. And this process of oxygen sensing seems to be earlier than the activation of HIF1α.

In summary, we demonstrate for the first time that up-regulation of NOX4 after hypoxia can induce histone methylation through altering intracellular ROS levels. Up-regulation of histone modifications, especially activation of H3K4ME3, associated with active gene transcription of SNAIL1 which cause rapid and robust EMT process. However, the mechanism of hypoxia-induced NOX4 activation and how NOX4 affects histone methylation still need further investigation.

**Conclusions**

In summary, our study established a list of 30 hypoxia-related genes in PC which was prognostic and linked with up-regulated EMT pathway. Further analysis found NOX4 was induced by hypoxia and crucial for activation of EMT process. More specifically, NOX4 could up-regulate histone modifications to promote the transcription of SNAIL1 which cause robust EMT process. And clinical samples combined with in vivo and in vitro experiments verified the role of NOX4 promoting the proliferation and metastasis of PC.

**Abbreviations**

PC
pancreatic cancer
NOX
NADPH oxidase
EMT
epithelial-to-mesenchymal transition
SNAIL1
snail family transcriptional repressor 1
HIF1α
hypoxia-inducible factor 1 subunit alpha
ROS
reactive oxygen species
GO
Gene Ontology
Declarations

Ethics approval and consent to participate

The experimental study was approved by the Affiliated Drum Tower Hospital of Nanjing University Medical School, and the informed consent forms were obtained from patients enrolled in this study.

All animals used in this study were treated humanely and the study was approved by the Ethics Review Committee for Animal Experimentation at Nanjing Drum Tower Hospital (Nanjing, China).

Consent for publication
The content of this manuscript has not been previously published and is not under consideration for publication elsewhere.

**Competing interests**

The authors declare that they have no conflict of interest.

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**Authors' contributions**

XZ and SS conceptualized the study. HL, CZ and CP performed the experiments and interpreted the data. HL and CP performed the bioinformatics analysis. SN, XQ, ZS and MS collected the clinical samples. YL evaluated the histopathological results of PC samples. XD, SZ, BZ and XL helped with the experiments. GX, YL, LW, BK and HF revised the manuscript. All of the authors discussed the study and approved the final manuscript.

**Availability of data and materials**

Data sharing is not applicable to this article as no datasets were generated during the current study.

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**Figures**
Figure 1

Hypoxia related gene expression is enriched in a subset of pancreatic cancer samples with prognostic value. (a) The volcano diagrams showed differentially expresses mRNAs between pancreatic cancer...
tissue and normal tissue samples from GSE15471 and GSE16515. Hypoxia related genes were highlighted using gene name. (b) Venn diagram showed that 30 hypoxia-related genes were both up-regulated in tumor tissues in 2 databases. (c) Expression profiles of 30 hypoxia-related genes across pancreatic cancer samples in TCGA. (d) Overall survival and (e) progression free survival between hypoxia the high hypoxia score group and low hypoxia score group. (f-g) The associations of hypoxia score with clinical parameter (f) and molecular subtypes (g) in samples of pancreatic cancer in TCGA.
Figure 2

Hypoxia-related gene expression was positively correlated with EMT-related gene expression in pancreatic cancer specimens. (a) Hypoxia related GO biological processes, (b) KEGG terms and (c) Hallmark gene sets were enriched and visualized. p<0.05. (d) Expression profiles of 200 EMT markers were shown across pancreatic cancer samples in TCGA. (e) Person's correlation analyses between hypoxia score and EMT score. (f) The mRNA and (g) protein expression of EMT markers including SNAIL1, CDH1, CDH2, VIM after 1%O2 treatment for 24 hours were assessed by qRT-PCR. The efficiency of hypoxia was validated using HIF1α. ***p < 0.001, **p < 0.01, *p < 0.05 vs 20% O2. The data were presented as the mean ± SD.
Figure 3

EMT induced by hypoxia in pancreatic cancer cells was dependent on NOX4 expression. (a) The heat map showed person’s correlation analyses between hypoxia score and NADPH oxidase. (b) GSEA analysis of genes positively related to NOX4 expression showed enriched pathways associated with EMT and hypoxia. (c) The mRNA and (d) protein expression of NOX4 after time gradient of hypoxia treatment in HPAC and Panc1 cells. The data were presented as the mean ± SD. (e) The protein expression of CDH1, CDH2, VIM and SNAIL1 after the concentration gradient of H2 O2 treatment. (f) Intracellular ROS level after NOX4 expression in HPAC cells was measured by fluorescence assay. (g) The protein expression of CDH1, CDH2, VIM and SNAIL1 after NOX4 overexpression. (h) The protein expression of CDH1, CDH2, VIM and SNAIL1 after NOX4 knockdown and hypoxia treatment. (i) The protein expression of NOX4 and HIF1α after knockdown of HIF1α and treatment of 1% O2. (j) The secretion of TGFβ1 by HPAC cells after hypoxia were determined by ELISA. ***p < 0.001 vs 20% O2. (k) The protein expression of NOX4 after treatment with TGFβ1 in time and concentration gradient in HPAC cells. (l) The protein expression of NOX4 after treatment with TGFβ1 neutralizing antibody and/or 1%O2 in HPAC cells.
Figure 4

NOX4 was upregulated in pancreatic cancer and correlated with the clinical pathologic characteristics. (a) The representative IHC sections of NOX4 and HIF1α in PC tissues and T test was used for comparison between groups. *p < 0.05. (b) Kaplan-Meier survival analysis for NOX4 expression in 56 PC patients. (c) The representative IHC section of PC tissues for NOX4 and IHC scores of 56 PC patients for NOX4 corresponding to different tumor grades. *p < 0.05. (d) The mRNA level of NOX4 in pancreatic cancer tissues and adjacent normal tissues from GSE15471 and GSE16515. (e) The protein expression of NOX4 in human PC tissues and benign pancreatic lesions (f) The protein expression of NOX4 in human ductal pancreatic epithelial cells and human PC cells. The NOX4 expression of pancreatic cancer samples of different (g) pathologic stages, (h) histologic grades. ***p < 0.001, **p < 0.01, *p < 0.05.
Figure 5

NOX4 promotes the proliferation and metastasis of pancreatic cancer cells. Cell viability by CCK8 assays after (a) NOX4 knockdown, (b) NOX4 overexpress and (c) GLX351322 treatment. The volumes of subcutaneous tumor xenografts after (d) NOX4 knockdown and (e) overexpress. ***p < 0.001, **p < 0.01, *p < 0.05 vs control group. Migration and invasion of HPAC cells after (f) NOX4 overexpress and (g) knockdown by transwell assays. ***p < 0.001, **p < 0.01 vs control group. (h) The percent of metastatic lesions in the lung section 4 weeks after HPAC cells with NOX4 knockdown injection. ***p < 0.001, **p < 0.01, *p < 0.05 vs control group. The data were presented as the mean ± SD.
Figure 6

NOX4 up-regulates SNAIL1 expression by promoting histone methylation. (a) The protein expression of histone methylation markers after NOX4 overexpression. (b) The expression of H3K4ME3 and the quantification of signal intensities after (b) NOX4 overexpression or (c) knockdown with or without of 1%O2 treatment determined by immunofluorescence. *p < 0.05; **p < 0.01 vs control; bar=20µm. (d) ChIP-qPCR analysis of H3K4me3 for SNAIL1 in HPAC cells after overexpression and knockdown of NOX4 with or without of 1%O2 treatment. *p < 0.05; **p < 0.01 vs control.

Supplementary Files

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