Overexpression of *DUOX2* mediates doxorubicin resistance and predicts prognosis of pancreatic cancer

Peng-Wei Lyu[1], Xiao-Dong Xu[1], Ke Zong[2], Xin-Guang Qiu[3]

[1]Department of Breast Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; [2]Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; [3]Department of Thyroid Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Contributions: (I) Conception and design: PW Lyu, XG Qiu; (II) Administrative support: XG Qiu; (III) Provision of study materials or patients: PW Lyu, K Zong; (IV) Collection and assembly of data: K Zong; (V) Data analysis and interpretation: XD Xu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Xin-Guang Qiu. Department of Thyroid Surgery, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe East Road, Zhengzhou 450000, China. Email: zzuxinguangqiu@163.com.

Background: Dysregulation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family is frequently observed in cancers and associated with their development and progression. However, the expression, role, and clinical significance of the NOX family members in pancreatic cancer remain unexplored.

Methods: The expression levels of the 7 NOX family genes were analyzed in Gene Expression Omnibus (GEO) datasets. The messenger RNA (mRNA) expression and gene alterations were explored using The Cancer Genome Atlas (TCGA) data portal. Clinical significance analyses of the NOX family genes were conducted among pancreatic cancer patients. The expression and prognostic value of *dual oxidase 2* (*DUOX2*) were then validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC) in an independent validation cohort. The function of *DUOX2* was analyzed by gene set enrichment analysis (GSEA) and its effect on the chemosensitivity of pancreatic cancer cells was detected by Cell Counting Kit-8 (CCK-8) assay.

Results: Results showed that *NOX1*, *NOX2* (*CYBB*), *NOX4*, *DUOX1*, and *DUOX2* were upregulated, while NOX3 and NOX5 were downregulated in pancreatic cancer tissues compared with nontumor tissues. Genomic alteration analysis demonstrated that deregulation of NOX family genes was partially caused by genomic alterations. Survival analyses showed that only *DUOX2* was associated with overall survival (OS) and relapse-free survival (RFS) of pancreatic cancer patients. The *DUOX2* gene was observed to be markedly overexpressed in pancreatic cancer. In the GSEA results for pancreatic cancer patients, *DUOX2* was significantly associated with oxidoreductase activity acting on nicotinamide adenine dinucleotide hydrogen (NADH) or NADPH and uridine 5'-diphospho-glucuronosyltansferase (UDP) glycosyltransferase activity. Knockdown of *DUOX2* in pancreatic cancer cells increased their sensitivity to doxorubicin.

Conclusions: Overexpression of *DUOX2* is correlated with prognosis and recurrence in pancreatic cancer patients and acts as a good marker for pancreatic cancer course prediction; furthermore, *DUOX2* might be a therapeutic target for pancreatic cancer patients.

Keywords: Pancreatic cancer; nicotinamide adenine dinucleotide phosphate oxidase family (NOX family); prognosis; chemoresistance

Introduction

Pancreatic cancer is one of the most lethal cancers worldwide (1). Although progress in diagnosis and treatment has improved clinical outcomes, the prognosis of pancreatic cancer patients remains poor, with a 5-year survival rate of 6% (2). Therefore, it is essential to develop new therapeutic targets to improve the prognosis of pancreatic cancer patients.

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) family, which comprises 7 members NOX1–5 and *dual oxidase 1/2* (*DUOX1/2*), is involved in the generation of reactive oxygen species (ROS) (3,4). The ROS are central factors in regulating oxidative stress, and the deregulation of oxidase signaling is associated with cancer initiation and development (5-7). Studies have shown that the expression and role of NOX family members are different in cancers. The gene *NOX4* regulates the proliferation and apoptosis of gastric cancer cells by activating ROS formation through the GLI1 pathway (8); *NOX4* mediates the migration of inflammatory cells and radiation-induced senescence by producing ROS (9); *NOX1* plays a vital role in UV-associated keratinocyte responses and skin carcinogenesis (10); and *NOX1* and *NOX4* have shown different prognostic effects for liver cancer patients after hepatectomy (11). In pancreatic cancer, standard chemotherapy with gemcitabine promotes stemness and chemotherapy resistance through the NOX/ROS/NF-κB/STAT3 signaling pathway (12).

Studies have reported that NOXs play important roles in the carcinogenesis of pancreatic cancer. In pancreatic cancer, suppression of *NOX4* initiates the apoptosis of Panc-1 cells via the AKT pathway (13). The pro-inflammatory cytokine interferon (IFN)-γ activates the reactive oxygen cascade by regulation of *DUOX2* in human pancreatic cancer cells (14). In pancreatic cancer cells, *NOX4* promotes ROS signaling and contributes to the epithelial-mesenchymal transition (EMT) induced by transforming growth factor-β (TGF-β) (15). In pancreatic cancer cells, overexpression of *DUOX2* predicts poor prognosis and promotes cell invasion and growth (16). However, there is little systematic research on the expression, prognostic significance, and role of NOX family genes in pancreatic cancer. Furthermore, the effect of *DUOX2* on chemosensitivity of pancreatic cancer cells still needs to be clarified.

In this study, we explored the expression levels and clinical significance of individual NOX family members in pancreatic cancer by mining Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) datasets and confirmed that *DUOX2* might be a therapeutic target for pancreatic cancer patients. We present the following article in accordance with the MDAR reporting checklist (available at https://gs.amegroups.com/article/view/10.21037/gs-21-776/rc).

Methods

Pancreatic cancer tissues

Pancreatic cancer and adjacent nontumor tissues were collected between January 2015 and January 2016 in The First Affiliated Hospital of Zhengzhou University. A total of 44 patients were diagnosed with pancreatic ductal adenocarcinoma (PDAC) histologically. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University (No. 2018-KY-0137) and informed consent was taken from all the patients.

Cell culture and reagents

Pancreatic cancer cell lines and immortalized human pancreatic ductal epithelial cell lines (HPDE) were obtained from the Cell Repository, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, 11965092, Waltham, MA, USA) or Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco, 21870076, USA) medium containing 10% inactivated fetal bovine serum (FBS; Gibco, 10099141C, USA). All cell lines were authenticated using short tandem repeat DNA profiling within 3 months and tested for mycoplasma contamination. Antibodies used in this study were DUOX2 (ab97266, Abcam, English) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Proteintech, 160004-1-Ig, China). Control and small interfering RNA (siRNA) sequences were constructed by GeneChem (Shanghai, China) and cell transfection was performed using Lipo 3000 (Invitrogen, L3000008, Carlsbad, CA, USA) according to the manufacturer's instructions. The siRNA sequence for *DUOX2* was CCCAACGTCTTTGTGAATGAT. Doxorubicin was purchased from Sigma-Aldrich (D1515, St. Louis, MO, USA). After transfection of siRNA for 48 h, pancreatic cancer cell lines AsPC-1 and BxPC-3 were treated with increasing concentrations of doxorubicin for 24 h.

Cell viability assay

Cells (3,000 per well) were cultured in 96-well plates and cell viability was measured using Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions after treatment. The absorbance value was measured at 450 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Transwell assay

After transfection for 48 h, Panc-1 cells were washed and collected. A total of 10,000 cells were resuspended in 100 μL DMEM and cultured in the top transwell chambers (Corning, USA), which were coated with Matrigel (BD Biosciences, CA, USA). The bottom chambers contained DMEM medium with 10% fetal bovine serum. After 24 h, cells on the bottom chambers were fixed, stained and counted manually by light microscopy.

Immunohistochemistry (IHC), western blot, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

IHC staining was performed as described previously (8). Staining score was calculated based on the intensity and total area of the staining. The staining intensity was quantified as follows: 0 (none), 1 (weak), 2 (strong), or 3 (very strong). Distribution was scored as 0 (0%), 1 (1–50%), and 2 (51–100%). The IHC staining was performed as described previously (8). A TRIzol kit (Invitrogen, 15596026, USA) was used for total RNA extraction according to the manufacturer's instructions. NanoDropND-2000 spectrophotometer (NanoDrop Tech., Wilmington, DE, USA) was used for RNA quality check. Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, 28025013, USA) was used for cDNA synthesization according to the manufacturer's instructions. Messenger RNA (mRNA) expression quantification was detected by a SYBR Green PCR Kit (Takara, RR420A, Beijing, China). We used GAPDH as the housekeeping gene. The primers used in this study were: DUOX2 forward: 5'-ACGCAGCTCTGTGTCAAAGGT-3', DUOX2 reverse: 5'-TGATGAACGAGACTCGACCAGGC-3'; GAPDH forward: 5'-AGAAGGCTGGGGCTCAT TTG-3', GAPDH reverse: 5'-TGAGAGCTGTCCATT GGTAGAG-3'.

Analysis of mRNA microarray data

The gene expression profile datasets GSE16515, GSE28735, GSE62452, and GSE62165 were downloaded from GEO and analyzed utilizing the Bioconductor package Robust MultiArray Average (RMA) (R version 3.2.0; https://www.R-project.org/). The DEGs were defined by the Limma algorithm.

Statistical analyses

Overall survival (OS) and relapse-free survival (RFS) were identified as the time to occurrence of death or relapse after surgery, respectively. A two-sided Student's t-test was used to compare the expression differences of NOX family genes between the tumor group and nontumor groups. Statistical analysis was performed using the SPSS software (version 17.0; Chicago, IL, USA). Images were plotted using GraphPad Prism software (version 6.0; San Diego, CA, USA). Publicly available TCGA data were downloaded from the TCGA data portal with clinical data available. The frequency of genomic alteration was analyzed according to the cBioPortal's online instructions (https://www.cbioportal.org/). Expression levels of NOX family genes were determined by the log2[transcripts per million (TPM) +1] method. Kaplan-Meier method and the log-rank test were used for survival analysis. The independent prognostic value of *DUOX2* was evaluated by a multivariate Cox proportional hazards regression model. The GSEA software package (version 3.0; Broad Institute; Cambridge, MA, USA) was used for gene set enrichment analysis (GSEA). All experiments were performed at least 3 times. A P value <0.05 was considered statistically significant; *P<0.05, **P<0.01, ***P<0.001.

Results

Expression of NOX family genes and hierarchical clustering analysis in pancreatic cancer

We compared the expression levels of NOX family genes in pancreatic cancer tissues (T) with those in matched nontumor tissues (NT) in GSE28735 dataset (n=45). The results showed that *NOX1*, *NOX2 (CYBB)*, *NOX4*, *DUOX1*, and *DUOX2* were significantly overexpressed in pancreatic cancer, while *NOX3* and *NOX5* were significantly downregulated (*Figure 1A-1G*). Unsupervised clustering analysis of 7 NOX family members was performed in GSE28735. Notably, part of the paired PDAC tissues and
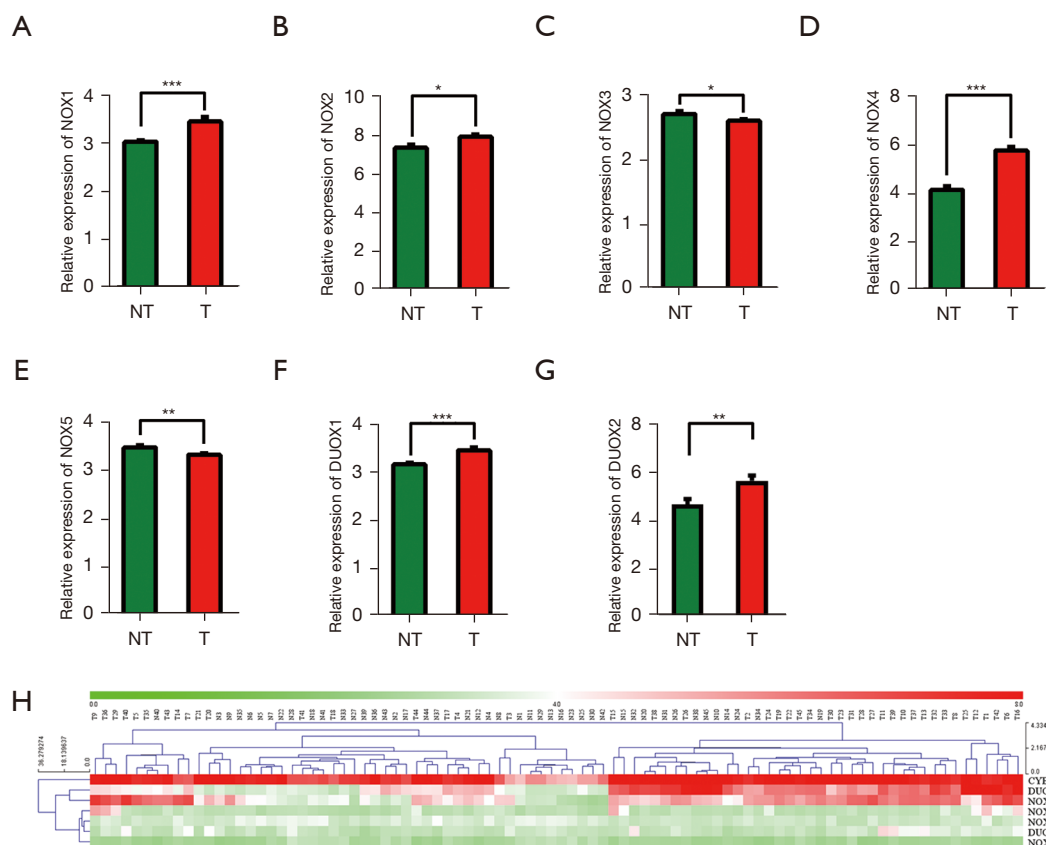
Figure 1 Expression of the NOX family genes in pancreatic cancer. (A-G) Expression of 7 NOX family genes in GSE28735 dataset. (H) Hierarchical clustering heat map of 7 NOX family genes for cancer tissues and nontumor tissues in GSE28735 dataset. Red indicates gene upregulation, green indicates downregulation. *P<0.05; **P<0.01; ***P<0.001. NOX, nicotinamide adenine dinucleotide phosphate oxidase; NT, nontumor tissues; T, pancreatic cancer tissues; DOUX, dual oxidase.

nontumor tissues could be grouped into clusters through hierarchical clustering (*Figure 1H*).

Genomic alterations of NOX family genes in pancreatic cancer

To investigate the regulation mechanism underlying the expression of NOX family genes in pancreatic cancer, genomic alterations, including copy number changes, somatic mutations, and mRNA upregulation, were analyzed using TCGA cBioPortal tool. The alterations of NOX family members occurring in 186 pancreatic cancer patients were shown (*Figure 2A*). Genetic aberrations were observed in 23.7% of 186 pancreatic cancer patients, mRNA upregulation was the most common alteration, with an alteration frequency of 18.2% (*Figure 2B*). The mRNA levels of *DUOX2* were the highest among the NOX genes

(*Figure 2C*), whereas *NOX5* and *NOX3* expression was downregulated (data not shown). Kaplan-Meier survival analysis revealed that there were no significant differences between OS and RFS in patients with NOX family genomic alterations and patients without (*Figure 2D,2E*). These findings collectively demonstrate that genomic alterations of NOX family genes are frequent and significantly contribute to their aberrant expression in pancreatic cancer.

Prognostic values of NOX family members in pancreatic cancer

To determine the survival effect of the NOX family genes in pancreatic cancer patients, Kaplan-Meier analyses were performed. The results showed that only high *DUOX2* mRNA expression was correlated with a significantly worse OS in pancreatic cancer patients (n=171; median: 17.9
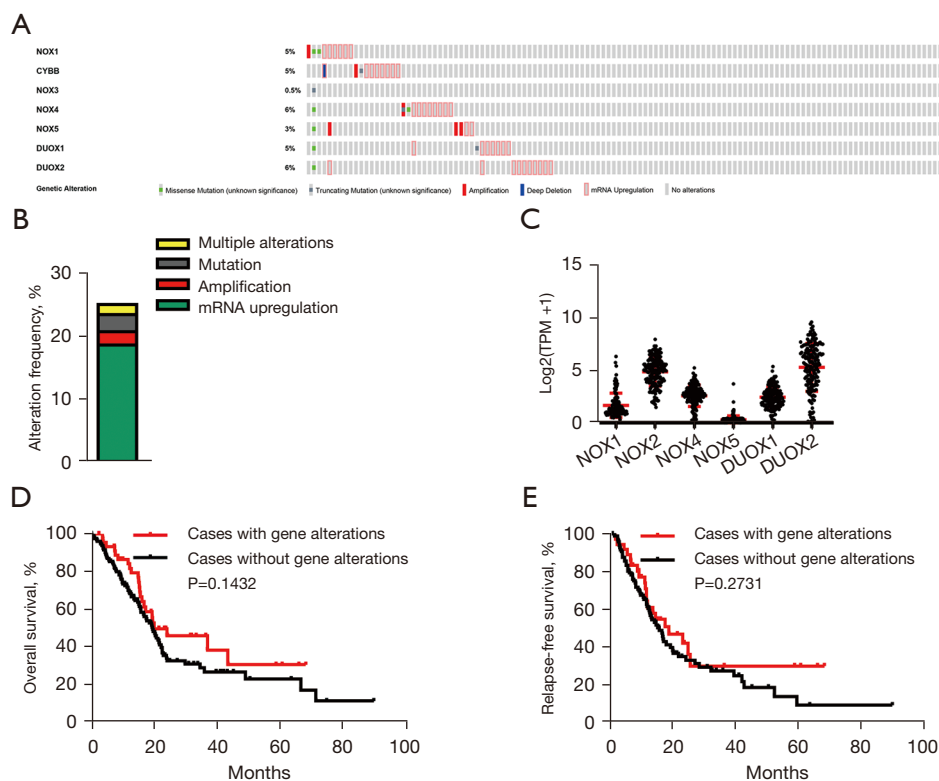
Figure 2 Genomic alterations of NOX family genes in pancreatic cancer. (A) Proportion and distribution of samples with alterations of NOX family genes. (B) Genomic alteration frequency analysis of NOX family genes in pancreatic cancer. (C) mRNA expression levels of NOX family genes in PDAC patients from the TCGA dataset (n=171). (D,E) Kaplan-Meier plots comparing OS and RFS in pancreatic cancer patients with or without NOX family genes alterations. NOX, nicotinamide adenine dinucleotide phosphate oxidase; mRNA, messenger RNA; PDAC, pancreatic ductal adenocarcinoma; TCGA, The Cancer Genome Atlas; OS, overall survival; RFS, relapse-free survival; DOUX, dual oxidase; TPM, transcripts per million.

vs. 22.47 months; log-rank test; *Figure 3A*). In addition, we found that only high *DUOX2* mRNA expression was correlated with a far shorter RFS in pancreatic cancer patients (n=132; median: 14.75 *vs.* 25.89 months; log-rank test; *Figure 3B*). To explore whether *DUOX2* is an independent prognostic factor for pancreatic cancer patients, a Cox multivariate regression analysis was performed. The results showed that *DUOX2* maintained independence in predicting both the OS [hazard ratio (HR) =1.41, 95% confidence interval (CI): 1.131 to 1.723; Table S1] and RFS (HR =1.39, 95% CI: 1.231 to 1.896; Table S2) of patients with pancreatic cancer. Collectively, *DUOX2* was revealed as an independent marker for outcome and recurrence in pancreatic cancer patients.

Expression and prognosis of DUOX2 in pancreatic cancer patients

To further explore the expression level and survival value of *DUOX2* in pancreatic cancer patients, *DUOX2* expression levels were analyzed in independent cohorts of GSE16515 (n=36) and GSE62452 (n=69) datasets. It was shown that *DUOX2* was significantly overexpressed in pancreatic cancer specimens compared with nontumor samples (*Figure 4A,4B*). Furthermore, *DUOX2* expression was detected in fresh frozen pancreatic cancer specimens from The First Affiliated Hospital of Zhengzhou University (ZZU cohort; n=44; Table S3) with clinical data available by RT-qPCR. Results showed *DUOX2* was significantly
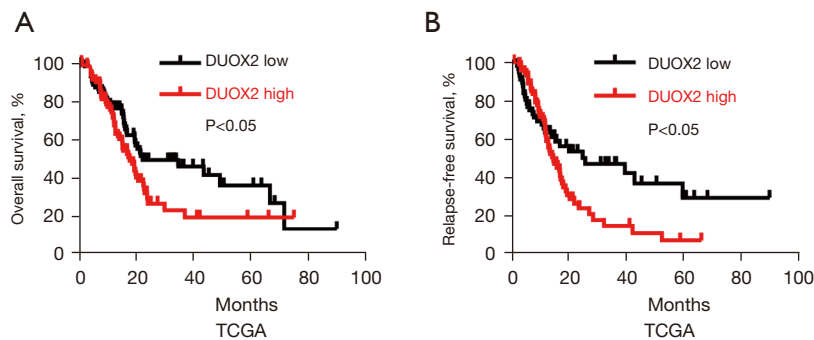
Figure 3 Prognostic values of NOX family genes in pancreatic cancer. (A) Survival analysis of OS in patients in the TCGA dataset with the mRNA expression of *DUOX2*. (B) Survival analysis of RFS in patients in the TCGA dataset with the mRNA expression of *DUOX2*. NOX, nicotinamide adenine dinucleotide phosphate oxidase; OS, overall survival; TCGA, The Cancer Genome Atlas; mRNA, messenger RNA; DOUX2, dual oxidase 2; RFS, relapse-free survival.

overexpressed in pancreatic cancer tissues compare with matched nontumor tissues (*Figure 4C*). Survival analysis showed that high *DUOX2* mRNA expression was correlated with a significantly worse OS in pancreatic cancer patients (n=44; median: 15.9 *vs.* 23.32 months; log-rank test; *Figure 4D*). These results further suggest that *DUOX2* might be a prognostic marker for pancreatic cancer patients.

Overexpression of DUOX2 is associated with a poor prognosis in pancreatic cancer

To further validate the expression level of *DUOX2* in pancreatic cancer patients, the expression of *DUOX2* was detected by IHC. It was found that *DUOX2* was significantly overexpressed in pancreatic cancer tissues compared with nontumor tissues (*Figure 5A,5B*). Late-stage pancreatic cancer patients exhibited higher expression levels of *DUOX2* compared with early-stage patients (*Figure 5C*). Patients were stratified into two groups based on the median staining score. Kaplan-Meier analysis showed that high *DUOX2* expression group participants exhibited shorter survival time compared to the *DUOX2* low expression group (median: 12.5 *vs.* 20.22 months; log-rank test; *Figure 5D*). Thus, *DUOX2* expression may serve as a valuable prognostic factor for pancreatic cancer patients.

Knockdown of DUOX2 increases chemosensitivity to doxorubicin

To understand the role of *DUOX2* in pancreatic cancer, GSEA was performed in GSE62165 dataset (n=118). In the *DUOX2* overexpression group, genes related to

uridine 5'-diphospho-glucuronosyltansferase (UDP) glycosyltransferase activity, oxidoreductase activity acting on nicotinamide adenine dinucleotide hydrogen (NADH) or NADPH and phosphotransferase activity were significantly enriched compared with the *DUOX2* downregulation group (*Figure 6A-6C*). Next, we detected *DUOX2* expression in pancreatic cancer cell lines. Compared with HPDE, *DUOX2* was upregulated in four pancreatic cancer cell lines (*Figure 6D*). We knocked down the expression of *DUOX2* by transfection of siRNA and the transfection effect was validated by RT-qPCR and western blot (*Figure 6E,6F*). The CCK-8 assays were performed to measure the cell viability after treatment with doxorubicin, which is a DNA damage-inducing first-line antineoplastic drug. The CCK-8 assays showed that inhibition of *DUOX2* suppressed the proliferation of both AsPC-1 and BxPC-3 cells compared with control group (*Figure 6G,6H*). Collectively, our results suggest that upregulation of *DUOX2* regulates doxorubicin resistance of pancreatic cancer cells.

Discussion

Recent studies have shown that NOX family genes could serve as valuable diagnostic and prognostic markers for many cancers (17). In our study, we found that the expression levels of *NOX1*, *NOX2*, *NOX4*, *DUOX1*, and *DUOX2* were increased and *NOX3* and *NOX5* were decreased in pancreatic cancer tissue samples compared with adjacent nontumor tissues, which could be partially explained by genomic alterations. Among them, *DUOX2* was a good outcome predictor and recurrence biomarker. Therefore, *DUOX2* may represent a potential biomarker
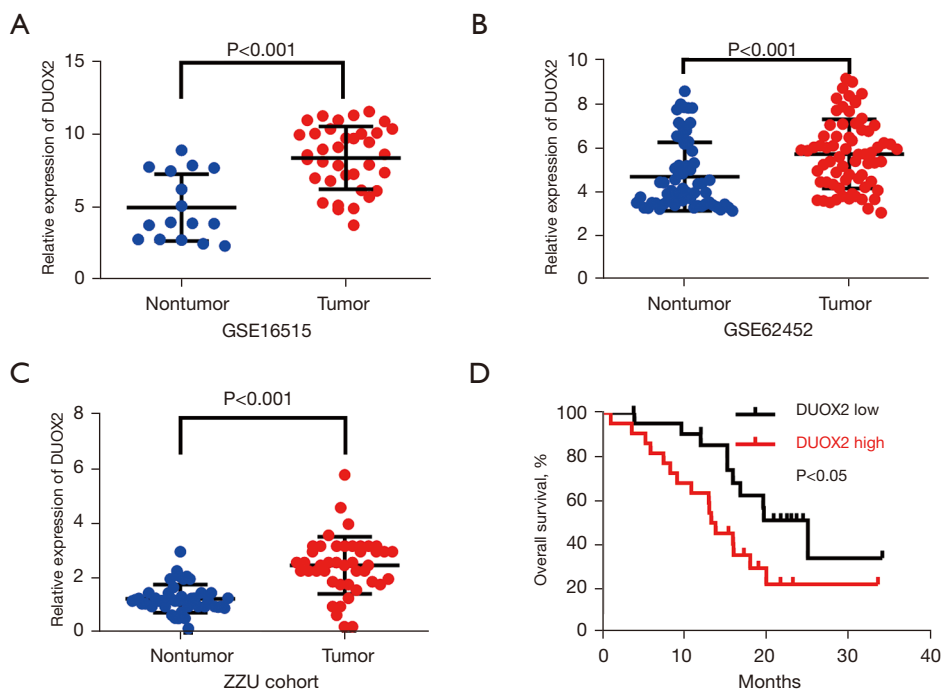
Figure 4 Expression and prognostic value of *DUOX2* in pancreatic cancer patients. (A) *DUOX2* expression in GSE16515 dataset. (B) *DUOX2* expression in GSE62452 dataset. (C) Detection of the expression of *DUOX2* by RT-qPCR. (D) Survival analysis of pancreatic cancer patients based on *DUOX2* expression. DUOX2, dual oxidase 2; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
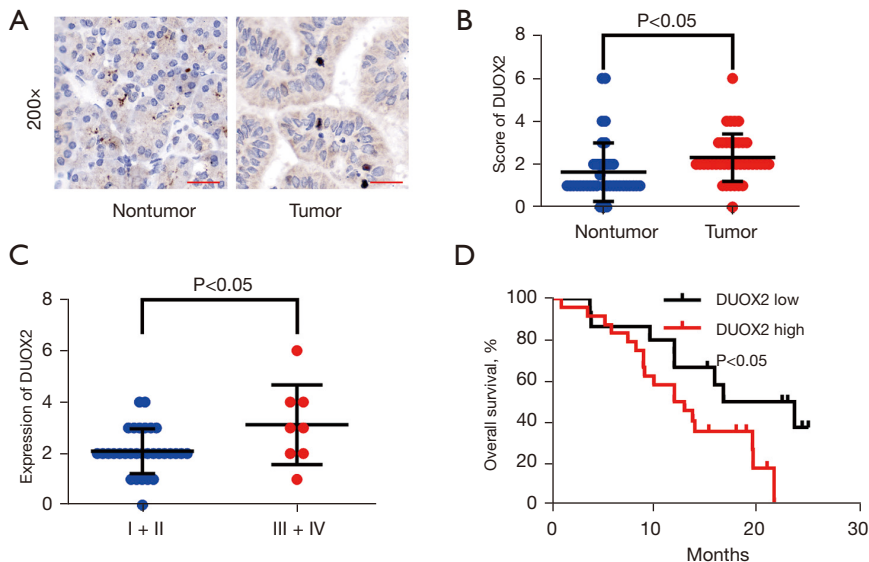

Figure 5 *DUOX2* is upregulated in pancreatic cancer. (A) Representative staining image showing *DUOX2* protein expression in pancreatic cancer tissues and nontumor tissues (IHC staining). (B) IHC scoring of *DUOX2* expression in pancreatic cancer specimens. (C) *DUOX2* expression with pathological staging stage (AJCC 7th edition) in pancreatic cancer. (D) Kaplan-Meier OS curves for patients with pancreatic cancer with low *vs.* high *DUOX2* expression. DOUX2, dual oxidase 2; IHC, immunohistochemistry; OS, overall survival.
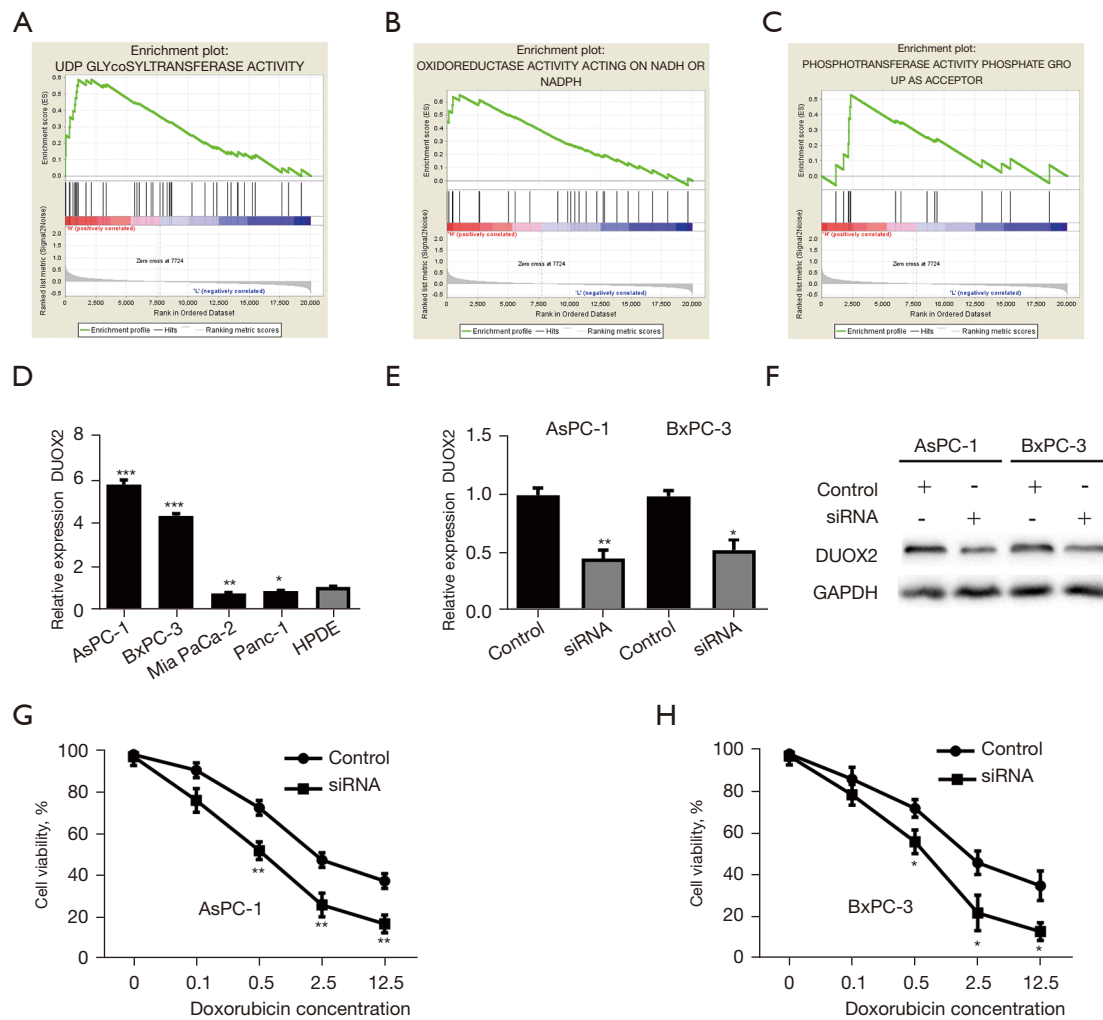
Figure 6 Knockdown of *DUOX2* increases sensitivity of pancreatic cancer cells to doxorubicin *in vitro*. (A-C) GSEA results for tumors with high DUOX2 expression in pancreatic cancer. (D) The expression of *DUOX2* in AsPC-1, BxPC-3, Mia PaCa-2, Panc-1 and HPDE cells by RT-qPCR detection. (E,F) The expression of *DUOX2* after treatment with siRNA transfection by RT-qPCR and western blot. (G,H) Cell viability was detected 72 h after transfection by CCK-8 assay. Data was reported as means ± SD for three independent experiments at least. *P<0.05; **P<0.01; ***P<0.001. DOUX2, dual oxidase 2; GSEA, gene set enrichment analysis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK-8, Cell Counting Kit-8; siRNA, small interfering RNA.

and be the subject of new therapeutic studies in the future.

Previous studies have shown that NOX family genes play vital roles in the development and progression of human cancers, although studies about the function of *NOX3* in cancer are still rare. For example, *NOX1* supports the growth of colon cancer cells by activating ROS-dependent signal transduction (18). In gallbladder cancer, *NOX1* mediates cell chemoresistance via the HIF1α/MDR1 signaling pathway (19). Natural killer cells regulate murine melanoma metastasis by NOX2-derived ROS (20).

In rectal cancer, the NOX2-ROS-HIF-1α signaling pathway plays a vital role in the growth inhibitory effect of oleanolic acid (21). It has also been shown that *NOX4* regulates drug resistance of cancer cells by functioning as a mitochondrial energetic sensor that assists cancer metabolic reprogramming (22). Additionally, *NOX4* inhibits growth of hepatocyte proliferation and progression of liver cancer (23). *In vitro*, *NOX5* protects LK-positive anaplastic large-cell lymphoma cell lines against apoptosis (24). In lung cancer, knockdown of *DUOX1* promotes invasion,

EMT, and cancer stem cell characteristics (25). Epigenetic silencing of *DUOX1* by promoter hypermethylation has been observed in human hepatocellular carcinoma (26). The DUOX2-ROS pathway promotes the elimination of the *Klebsiella pneumoniae* strain K5 from T24 cells (27). In rectal cancer, overexpression of *DUOX2* is associated with poor response to preoperative chemoradiation therapy and clinical prognosis (28). In pancreatic cancer, *DUOX2* is upregulated and associated with poor survival of pancreatic cancer patients, and regulates the invasion and proliferation of pancreatic cancer cells, which is also confirmed in our study (Figure S1). However, the relationship between chemosensitivity and dysregulation of DUOX2 was unclear.

We performed GSEA on *DUOX2* which predicted prognosis and relapse of pancreatic cancer patients. In patients with high *DUOX2* expression, the GO terms response to organic substance, actin cytoskeleton organization and biogenesis, protein transport, cell junction, Golgi apparatus, intercellular junction, cation binding, oxidoreductase activity acting on NADH or NADPH, and UDP glycosyltransferase activity were significantly enriched. Chemoresistance is a major issue for pancreatic cancer. Thus, screening biomarkers and targets to overcome resistance are significant for cancer therapy. Herein, it was our observation that increased *DUOX2* expression is associated with chemotherapy resistance and may predict the chemo-therapeutic sensitivity for pancreatic cancer.

We provided a comprehensive bioinformatic analysis of NOX family genes involved in pancreatic cancer using online databases. Importantly, overexpression of *DUOX2* was associated with worse prognosis of pancreatic cancer and contributed to chemoresistance to doxorubicin. However, further molecular biological experiments need to be conducted in the future in order to determine the vital role of *DUOX2* and the underlying molecular mechanism in pancreatic cancer.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://gs.amegroups.com/article/view/10.21037/gs-21-776/rc

Data Sharing Statement: Available at https://gs.amegroups.com/article/view/10.21037/gs-21-776/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-21-776/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of The First Affiliated Hospital of Zhengzhou University (No. 2018-KY-0137) and informed consent was taken from all the patients.

References

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
2. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016;66:115-32.
3. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004;4:181-9.
4. Diaz de Barboza G, Guizzardi S, Moine L, et al. Oxidative stress, antioxidants and intestinal calcium absorption. World J Gastroenterol 2017;23:2841-53.
5. He N, Jia JJ, Li JH, et al. Remote ischemic perconditioning prevents liver transplantation-induced ischemia/reperfusion injury in rats: role of ROS/RNS and eNOS. World J Gastroenterol 2017;23:830-41.

6. Gao X, Sun J, Huang C, et al. RNAi-mediated silencing of NOX4 inhibited the invasion of gastric cancer cells through JAK2/STAT3 signaling. Am J Transl Res 2017;9:4440-9.

7. You X, Ma M, Hou G, et al. Gene expression and prognosis of NOX family members in gastric cancer. Onco Targets Ther 2018;11:3065-74.

8. Tang CT, Lin XL, Wu S, et al. NOX4-driven ROS formation regulates proliferation and apoptosis of gastric cancer cells through the GLI1 pathway. Cell Signal 2018;46:52-63.

9. Sakai Y, Yamamori T, Yoshikawa Y, et al. NADPH oxidase 4 mediates ROS production in radiation-induced senescent cells and promotes migration of inflammatory cells. Free Radic Res 2018;52:92-102.

10. Raad H, Serrano-Sanchez M, Harfouche G, et al. NADPH oxidase-1 plays a key role in keratinocyte responses to UV radiation and UVB-induced skin carcinogenesis. J Invest Dermatol 2017;137:1311-21.

11. Zhang Z, Duan Q, Zhao H, et al. Gemcitabine treatment promotes pancreatic cancer stemness through the Nox/ROS/NF-κB/STAT3 signaling cascade. Cancer Lett 2016;382:53-63.

12. Ha SY, Paik YH, Yang JW, et al. NADPH oxidase 1 and NADPH oxidase 4 have opposite prognostic effects for patients with hepatocellular carcinoma after hepatectomy. Gut Liver 2016;10:826-35.

13. Mochizuki T, Furuta S, Mitsushita J, et al. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. Oncogene 2006;25:3699-707.

14. Wu Y, Antony S, Juhasz A, et al. Up-regulation and sustained activation of Stat1 are essential for interferon-gamma (IFN-gamma)-induced dual oxidase 2 (Duox2) and dual oxidase A2 (DuoxA2) expression in human pancreatic cancer cell lines. J Biol Chem 2011;286:12245-56.

15. Hiraga R, Kato M, Miyagawa S, et al. Nox4-derived ROS signaling contributes to TGF-β-induced epithelial-mesenchymal transition in pancreatic cancer cells. Anticancer Res 2013;33:4431-8.

16. Cao M, Zhang PB, Wu PF, et al. DUOX2 As a Potential Prognostic Marker which Promotes Cell Motility and Proliferation in Pancreatic Cancer. Biomed Res Int 2021;2021:6530298.

17. Cho SY, Kim JS, Eun HS, et al. Expression of NOX family genes and their clinical significance in colorectal cancer. Dig Dis Sci 2018;63:2332-40.

18. Juhasz A, Markel S, Gaur S, et al. NADPH oxidase 1 supports proliferation of colon cancer cells by modulating reactive oxygen species-dependent signal transduction. J Biol Chem 2017;292:7866-87.

19. Zhan M, Wang H, Chen T, et al. NOX1 mediates chemoresistance via HIF1α/MDR1 pathway in gallbladder cancer. Biochem Biophys Res Commun 2015;468:79-85.

20. Aydin E, Johansson J, Nazir FH, et al. Role of NOX2-derived reactive oxygen species in NK cell-mediated control of murine melanoma metastasis. Cancer Immunol Res 2017;5:804-11.

21. Guo Y, Han B, Luo K, et al. NOX2-ROS-HIF-1α signaling is critical for the inhibitory effect of oleanolic acid on rectal cancer cell proliferation. Biomed Pharmacother 2017;85:733-9.

22. Shanmugasundaram K, Nayak BK, Friedrichs WE, et al. NOX4 functions as a mitochondrial energetic sensor coupling cancer metabolic reprogramming to drug resistance. Nat Commun 2017;8:997.

23. Crosas-Molist E, Bertran E, Sancho P, et al. The NADPH oxidase NOX4 inhibits hepatocyte proliferation and liver cancer progression. Free Radic Biol Med 2014;69:338-47.

24. Antony S, Jiang G, Wu Y, et al. NADPH oxidase 5 (NOX5)-induced reactive oxygen signaling modulates normoxic HIF-1α and p27Kip1 expression in malignant melanoma and other human tumors. Mol Carcinog 2017;56:2643-62.

25. Little AC, Sham D, Hristova M, et al. DUOX1 silencing in lung cancer promotes EMT, cancer stem cell characteristics and invasive properties. Oncogenesis 2016;5:e261.

26. Ling Q, Shi W, Huang C, et al. Epigenetic silencing of dual oxidase 1 by promoter hypermethylation in human hepatocellular carcinoma. Am J Cancer Res 2014;4:508-17.

27. Lu H, Wu Q, Yang H. DUOX2 promotes the elimination of the Klebsiella pneumoniae strain K5 from T24 cells through the reactive oxygen species pathway. Int J Mol Med 2015;36:551-8.

28. Lin SC, Chang IW, Hsieh PL, et al. High immunoreactivity of DUOX2 is associated with poor response to preoperative chemoradiation therapy and worse prognosis in rectal cancers. J Cancer 2017;8:2756-64.
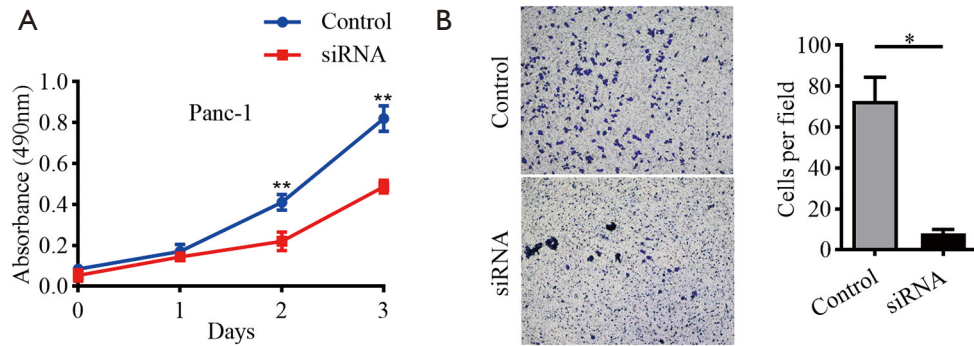
(English Language Editor: J. Jones)

Figure S1 Knockdown of *DUOX2* inhibits the growth and invasiveness of pancreatic cancer cells *in vitro*. (A) Cell viability was detected 48 h after transfection by CCK-8 assay. (B) Cell invasiveness was detected 48 h after transfection by transwell assay. Cells were stained with 0.1% crystal violet and photographed by light microscopy (400-fold). *P<0.05; **P<0.01. DOUX2, dual oxidase 2; CCK-8, Cell Counting Kit-8; siRNA, small interfering RNA.

Table S1 Multivariable Cox regression analysis of OS in pancreatic cancer patients in the TCGA dataset

Characteristics, TCGA (n=171)	Multivariable analysis		
	HR	95% CI of HR	P value
DUOX2			
High/low	1.41	1.131–1.723	<0.05
Gender			
Female/male	1.270	0.669–2.410	0.465
Age (years)			
≥65/<65	1.436	0.739–2.792	0.286
Tumor stage			
III + IV/I + II	0.931	0.115–7.502	0.946
Tumor grade			
III + IV/I + II	1.587	0.797–3.159	0.189

OS, overall survival; TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval; DOUX2, dual oxidase 2.

Table S2 Multivariable Cox regression analysis of RFS in pancreatic cancer patients in the TCGA dataset

Characteristics, TCGA (n=132)	Multivariable analysis		
	HR	95% CI of HR	P value
DUOX2			
High/low	1.39	1.231–1.896	<0.05
Gender			
Female/male	1.354	0.561–2.312	0.414
Age (years)			
≥65/<65	1.526	0.639–2.899	0.316
Tumor stage			
III + IV/I + II	0.998	0.279–7.152	0.877
Tumor grade			
III + IV/I + II	1.873	0.587–3.636	0.211

RFS, relapse-free survival; TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval; DOUX2, dual oxidase 2.

Table S3 Clinicopathologic features of 44 pancreatic cancer patients

Variables	All cases	DUOX2 expression		P value
		Low expression	High expression	
Age (years)				0.348
≥60	16	5	11	
<60	28	10	18	
Gender				0.827
Male	29	14	15	
Female	15	5	10	
Histologic grade				
Good/moderate	34	14	20	0.23
Poor	10	2	8	
Clinical stage				
I–II	34	25	9	<0.05
III–IV	10	2	8	

DOUX2, dual oxidase 2.