NQO1/CPT1A promotes the progression of pancreatic adenocarcinoma via fatty acid oxidation

Ran Xu (✉ 372836136@qq.com)
Yanbian University Medical College

Yanbian University Medical College

Liang Ma
Siping Central People Hospital

Yao Sun
Siping Central People Hospital

Hai Feng Liu
Siping Central People Hospital

Yang Yang
Yanbian University Medical College

Tie Feng Jin
Yanbian University Medical College

Da Wei Yang
Siping Central People Hospital

Research Article

Keywords: NQO1, PAAD, metastasis, fatty acid oxidation, CPT1A

Posted Date: July 18th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1735915/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

NQO1, a cytosolic enzyme, is closely related to progression of cancers and patients’ poor outcome. However, the molecular biological mechanism of NQO1 tumorigenicity in pancreatic adenocarcinoma (PAAD) has not been clearly reported. Therefore, we demonstrate the molecular mechanism of NQO1 PAAD in proliferation, metastasis and fatty acid oxidation (FAO). In this report, NQO1 is overexpressed in PAAD, connects with lymph node metastasis and poor overall survival. The overexpression of NQO1 improves tumor growth, metastasis and FAO in PAAD. Mechanistically, NQO1 is able to bind to carnitine palmitoyltransferase 1A (CPT1A), a key enzyme controlling FAO. And NQO1 promotes the PAAD progression via CPT1A-mediated FAO. In short, our findings identify CPT1A-depedent FAO as an essential metabolic pathway for NQO1 to promote PAAD process. Targeting NQO1/CPT1A in PAAD to attenuate proliferation and dissemination is a potential approach to promote a better antitumor effect and improve patient outcomes. Key words: NQO1; PAAD; metastasis; fatty acid oxidation; CPT1A

1 Introduction

Pancreatic adenocarcinoma (PAAD) is the third leading cause of cancer-related death with a five-year survival rate of approximately 11%. Although multiple therapeutic strategies such as immunotherapy and targeted therapies have been tried to improve the survival of PAAD patients, the effect is still limited. This may be partly due to the complexity and heterogeneity of PAAD. Therefore, the exploration of a promising biomarker, which regulates biological dynamics and behavior of PAAD, will contribute remarkably to optimize treatment.

NAD(P)H: Quinone oxidoreductase-1 (NQO1) gene, also known as DT diaphorase, is located on chromosome 16q22 and consists of six exons and five introns. It uses NADH or NADPH as a substrate to catalyze the two-electron reduction of quinine to its hydroquinone form, directly blocking quinone to hydroquinone. In addition, more reports have found that NQO1 is closely related to clinical significance and molecular mechanism in different malignant tumors. Jiang et al. found that the expression of NQO1 protein in gastric cancer tissue is higher than that in normal gastric tissue, it is closely related to the patients’ tumor stage and age. Hirose et al. found that the positive expression of NQO1 in colorectal and liver metastatic tumor cells may be an important factor for the poor prognosis after hepatectomy. Yang et al. in our team revealed that the NQO1/PKLR axis promotes lymph node (LN) metastasis and breast cancer progression by regulating glycolytic reprogramming. Meanwhile, NQO1 could promote tumor formation by regulating the process of glucose metabolism. Previous researches have revealed that the high expression of NQO1 is positively correlated with the glucose metabolism and progression of tumors. Whereas, the relationship between NQO1 and the metabolic mechanism of PAAD progression have not been reported yet.

Dysregulated metabolism, recognized as the hallmark of cancer development, such as aerobic glycolysis, fatty acid metabolism and so on. Fatty acid oxidation (FAO) is a major pathway regulating fatty acid metabolism in PAAD. Our study demonstrates the molecular mechanism of NQO1 PAAD in proliferation, metastasis and FAO. The overexpression of NQO1 improves tumor growth, metastasis and FAO in PAAD. Mechanistically, NQO1 is able to bind to carnitine palmitoyltransferase 1A (CPT1A), a key enzyme controlling FAO. And NQO1 promotes the PAAD progression via CPT1A-mediated FAO. In short, our findings identify CPT1A-depedent FAO as an essential metabolic pathway for NQO1 to promote PAAD process. Targeting NQO1/CPT1A in PAAD to attenuate proliferation and dissemination is a potential approach to promote a better antitumor effect and improve patient outcomes. Key words: NQO1; PAAD; metastasis; fatty acid oxidation; CPT1A
degradation and promoting ATP and NADPH production\textsuperscript{14}. The association between altered lipid metabolism mediated by FAO and tumor progression has been established\textsuperscript{15,16}. It was found that carnitine palmitoyltransferase 1A-mediated (CPT1A, a rate-limiting FAO enzyme) FAO could drives colon cancer peritoneal metastasis by inhibiting anoikis\textsuperscript{17}. Wang \textit{et al.} revealed that the STAT3/CPT1B (a member of the CPT1 family) mediated FAO promotes breast cancer cells stemness and chemoresistance\textsuperscript{18}. It was also showed the mechanism of YY1/PGC-1β regulated medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), the key enzymes of FAO, thereby accelerated hepatocarcinogenesis\textsuperscript{19}. However, the specific regulatory mechanism between NQO1 and FAO in PAAD needs further study.

Immunohistochemistry (IHC) staining results proved and analyzed the clinicopathological significance of NQO1/CPT1A expression in patients with PAAD. At the same time, our team revealed that the high expression of NQO1 can promote cell growth and metastasis. Moreover, the NQO1/CPT1A axis affects the development of PAAD by regulating FAO, which provides potential molecular markers for PAAD treatment in the future.

2 Materials And Methods

2.1 Tissue Samples

According to the Declaration of Helsinki, patients organized for this study provided informed consent, and the patients agreed to use their tumor tissue samples for scientific research. PAAD tissue microarrays were purchased from Shanghai Outdo Biotech Co. Ltd. (Shanghai, China). The classification standard of tumors in this study was to assess tumor differentiation according to the classification standard of the World Health Organization (WHO). This study was reviewed and approved by the Institutional Ethics Committee of Yanbian University School of Medicine.

2.2 Bioinformatics analysis


2.3 Cell culture
BxPC-3 and MIA PaCa-2 cell lines were obtained from the American Type Culture Collection (ATCC) in 2021, which the cells were authenticated by short tandem repeat. The cells were routinely cultured in DMEM Medium (Gibco Laboratories, Grand Island, NY), which were supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100U/mL penicillin and 100µg/mL streptomycin in a 5% CO₂ incubator at 37°C. Before selecting cell lines for experiment, mycoplasma detection has been carried out, and aseptic operation has been strictly observed in the culture process.

### 2.4 Stable cell line generation

The RNAi target sequence and vector of NQO1 gene were designed and constructed by Hesheng Gene Technology Co., LTD (Beijing). The plasmid vector maps of sh-Con, shNQO1, Vector and NQO1 overexpressed were shown in Supplement Fig. 1. Before lentivirus transfection, the adherent cells were seeded into a six-well plate at a density of 6×10⁴. After lentivirus infection, single-cell clonal strains are selected for 14 days under the action of 2µg/mL puromycin, so that a stable expression cell line can be established better. Finally, it was further verified by western blot experiment.

### 2.5 Western Blot

Proteins were extracted from pancreatic cancer cells using RIPA lysis buffer containing phosphatase inhibitors and protease inhibitors, and make a standard curve by BCA method, and determine the protein concentration. Electrophoresis was performed on 8%-10% SDS-PAGE and the gel was transferred to a polyvinylidene fluoride (PVDF) membrane. Then PVDF was sealed in 5% skim milk and incubated with primary antibody in a refrigerator at 4°C overnight. The second antibody was incubated at room temperature for 1-2h. Finally, an enhanced chemiluminescence (ECL) kit was used for exposure the blot.

### 2.6 Reagents

NQO1 (1:1000, mouse anti-NQO1, Abcam), E-cadherin (1:1000, rabbit anti-E-cadherin, Abcam), Vimentin (1:1000, rabbit anti-Vimentin, Abcam), Snail (1:1000, rabbit anti-Snail, Abcam), CPT1A (1:1000, rabbit anti-CPT1A, Abcam), LCAD (1:1000, rabbit anti-LCAD, Proteintech), MCAD (1:1000, rabbit anti-MCAD, Proteintech) and Actin (1:3000, mouse anti-Actin).

### 2.7 Immunohistochemistry (IHC)

The expression of proteins in tissues was detected by immunohistochemistry SP two-step method. The staining steps: 4µm thick serial sections of the tissue, conventional deparaffinization, gradient ethanol hydration, antigen retrieval in hot citrate buffer, dropwise addition of the primary antibody NQO1 (diluted Ratio 1:200), incubate overnight at 4°C. The next day, horseradish peroxidase labeled secondary antibody, DAB developed, counterstained with hematoxylin and sealed with neutral gum. In order to prove the specificity of NQO1 antibody immunohistochemistry, sections with strong NQO1 protein staining were selected and PBS was used as a negative control instead of the primary antibody. Scoring according to the intensity of cell staining: 0 points for no staining, 1 point for light yellow, 2 points for brownish yellow, and 3 points for brown. Scoring based on the percentage of positive cells: 0 to 5% of positive cells are 0
points, 6–25% is 1 point, 26–50% is 2 points, 51–75% is 3 points, and 76–100% is 4 points. Multiply the two score results: 0 is divided into (-), 1 to 4 are divided into (+), 5 to 8 are divided into (++), 9 to 12 divided into (+++); where ≤ 4 is divided into NQO1 low expression, > 4 is divided into NQO1 high expression. In order to make the statistical results more objective, the TissueFAXS Cytometry tissue quantitative technology was adopted, and the TissueFAXS Panoramic tissue cell quantitative analysis system was used for tissue chip analysis and statistics. In this study, the average intensity of DAB in the mask of NQO1 cells was analyzed.

2.8 Immunofluorescence (IF)

Cells were fixed with 4% paraformaldehyde for 15min and rinsed by PBS; permeated with 0.5% Triton X-100 for 30min then block with 5% bovine serum albumin (BSA) for 1h at room temperature. Incubated in a refrigerator at 4°C overnight with NQO1 antibody (1:1000). The next day in sequence: PBS rinse 3 times, 5min each time; immunofluorescence secondary antibody (1:400) incubation for 1h, nuclei were stained with 4', 6-diamidyl- α phenylindole (DAPI) solution and then captured under a fluorescence microscope.

2.9 Colony formation assay

Cells (1000 cells) were seeded in 6-well plates in triplicate and incubated. After 2 weeks, cells were fixed by 4% paraformaldehyde and stained with hematoxylin for 20 min. Then, stained cells were washed with PBS. The positive clones were observed under the microscope, that is, each clone had more than 50 cells, and the number of clones was counted (about 0.3-1.0mm in size).

2.10 MTT assay

PAAD cells were inoculated into 96-well plates at 100µL per well (5×10³ cells). Detected cell absorbance values at 0h, 24h, 48h and 72h respectively. And each concentration had 5 parallel multiple holes. Add 100µL of MTT solution per well to each well, continue to incubate for 4h, discard the supernatant, add DMSO and shake for 10min, measure the absorbance with a full-wavelength microplate reader at 570nm wavelengths, and further calculate according to the formula cell survival rate.

2.11 Wound healing assay

The cells at the logarithmic growth stage with a density of about 80% were inoculated in 6-well plates and starved in serum-free medium. The cells were cut straight with 200µL sterile spear tip, cleaned with PBS for three times, and photographed under fluorescence microscope. After incubation for 24h, photos were taken to record the width of scratch and calculate the healing rate of scratch.

2.12 Transwell assay

5×10⁴ cells were diluted with 100µL FBS-free DMEM medium and placed in transwell upper chamber, and medium containing 20% serum was added to the lower chamber. After 24h culture in the incubator, the chamber was taken out, fixed with methanol, hematoxylin crystallization was used to stain,
decolorization by PBS and sealing the transwell by neutral balsam mounting medium, observation and photo taken under a microscope.

2.13 5-ethynyl-29-deoxyuridine (EdU) assay

Cells were pulsed with 5-ethynyl-2-deoxyuridine (EdU Kit, RiboBio, Guangzhou, China), for 2h before fixation in 4% paraformaldehyde and subsequent EdU detection per the manufacturer’s instructions. In this experiment, 50µM EdU medium was prepared by diluting EdU solution in the ratio of 1000:1.

2.14 FAO and ATP production testing

For FAO testing, kit was purchased from Shanghai Enzyme Linked Biotechnology Co., LTD and is only used for one-step sandwich enzyme-linked immunosorbent assay (ELISA). Samples, standard substances and HRP labeled detection antibodies were added to the coated micropores which were precoated with FAO captured antibodies, and then incubated and washed. After removing the unbound enzyme conjugate, the substrate is added to react with the enzyme conjugate simultaneously to produce color. Measure the absorbance (OD value) at 450nm with a microplate reader and calculate the concentration. ATP kit was purchased from Nanjing Jiancheng Biological Engineering Institute Co., LTD. After cell samples were collected, washed and broken in sequence, ATP production was detected according to the instructions of the kit.

2.14 Immunoprecipitation (Co-IP) and detection of ubiquitination

Pretreatment of protein A/G magnetic beads: Put the magnetic beads on the magnetic frame, add PBS, mix and clean gently, the supernatant was discarded, cleaned three times, sealed with 0.2% BSA, mixed at 4°C and sealed for 1h. Conventional cell lysis, protein extraction, placed on ice standby. After removing PBS, the magnetic beads were mixed with cell lysate and incubated at 4°C for 1h. The supernatant was placed in a new EP tube, and the target antibody and IgG were added correspondingly (as the control group). The supernatant was mixed at 4°C and incubated overnight. The next day, the closed magnetic beads were added into the EP tube and incubated at room temperature for 6h. Discard the supernatant and added 200µL to the EP tube, 4°C gently mixed, 3 times, 5min each time, added appropriate amount of eluent into EP tube, incubating at room temperature for 20min. Added 20µL 3×SDS buffer solution, 95°C metal bath for 5min, samples were obtained for loading, and detected by western blot. For ubiquitination experiment, cells were treated with or without MG132 (10µM) for 6h before harvested in IP lysis buffer, followed by above steps and analysis by Western blot for Ubiquitin (Santa Cruz Biotechnology).

2.15 In vivo models

For the subcutaneous tumor-bearing models, 3x10^6 BxPC-3 cells with differential expression of NQO1 were injected subcutaneously. One week later, used a vernier caliper to monitor the growth of the xenograft. Used calipers to measure the length and width of the tumor to monitor the tumor volume, and used the formula to calculate \( V = 0.5 \times \text{length} \times \text{width}^2 \). For the metastasis model, 1x10^6 BxPC-3 cells were
injected into the vein of nude mice through the tail vein. Further immunohistochemical staining was performed to detect the expression levels of NQO1, CPT1A, Vimentin, E-cadherin and Ki67 in tumor sections. Animal experiments were carried out in accordance with the procedures and procedures of the Animal Ethics Committee of Yanbian University. The mice were sacrificed 6 weeks later, and the lungs were surgically removed and stained by HE.

2.16 Statistical Analysis

All results were analyzed using SPSS 26.0 software and GraphPad Prism 8.0 software for statistical data analysis. Biochemical experiments were performed in triplicate, and at least three independent experiments were evaluated. The average value of independent experiment data ± standard deviation (SD). Comparative analysis of each group used Student t test. A value of \( P < 0.05 \) was considered statistically significant.

3 Results

3.1 NQO1 is upregulated in PAAD and correlates with the poor outcome

To determine the expression of NQO1 in tissues, gene expression databases (GEPIA and TIMER) were applied and revealed that NQO1 was upregulated in the PAAD and other cancers (Fig. 1A). Moreover, GEPIA, ENCORI, UCSC Xena and UALCAn databases utilized to validate the expression of NQO1 in PAAD tissues, which showed that NQO1 expression was higher in PAAD than normal pancreatic tissues. Interestingly, Oncomine database found that compared with pancreatic duct and pancreas, NQO1 expression was elevated in PDAC tissues (Fig. 1B). The correlation between NQO1 expression and overall survival (OS) was explored using Oncolnc, HPA, GEPIA and Kaplan-Meier survival analysis disclosed that PAAD patients with lower NQO1 expression had a longer OS than patients with higher NQO1 expression \( (P < 0.05, \text{Fig. 1C}) \). Similarly, longer RFS was statistically observed in PAAD patients with lower NQO1 expression. Therefore, it is convincing that NQO1 plays a vital role in influencing the survival of PAAD patients.

3.2 NQO1 overexpression is associated with LN metastasis in PAAD patients

In order to further study the role of NQO1 in PAAD, IHC detected the expression level of NQO1 in 81 pairs of PAAD tissues and normal pancreatic tissues. NQO1 protein is negatively expressed in non-tumor pancreatic tissues and positively expressed in PAAD tissues. At the same time, IHC staining results found that NQO1 protein is mainly located in cytoplasm and minorly in nucleus of pancreatic cancer cells. The results were consistent with HPA database (Fig. 2A). Notably, NQO1 cellular masks DAB mean intensity was markedly up-regulated in PDAC tissues compared with adjacent paracous tissue by TissueFAXS (Fig. 2B). Mosaic results showed that NQO1 expression was only correlated with LN metastasis \( (P = \))
0.0149). However, there are no correlations between the expression level of NQO1 and age \((P = 0.1000)\), gender \((P = 0.6739)\), tumor location \((P = 0.2508)\), tumor size \((P = 0.6777)\), histological grade \((P = 0.6772)\), clinical stage \((P = 0.2931)\) (Fig. 2C). Therefore, the above results all indicated that the high expression of NQO1 is closely related to the metastatic ability of PAAD patients.

### 3.3 NQO1 promotes tumorigenicity of tumors **in vivo** and **in vitro**

To further clarify the biological role of NQO1 in PAAD, stable transfected cell lines were established and confirmed by western blot (Fig. 3A). MTT and colony formation experiments revealed that NQO1 knockdown significantly inhibited the proliferation of BxPC-3 and MIA Paca-2 cells, and overexpression of NQO1 promoted the proliferation of cells (Fig. 3B-C). Consistently, EdU assay revealed that NQO1 knockdown markedly inhibited the ability of cell DNA replication, whereas NQO1 overexpression was opposite (Fig. 3D).

Mouse xenograft models were showed that the average tumor volume and weight of mice in the shNQO1 group were significantly reduced, compared with the control group (Fig. 3E-G). IHC staining of Ki67 also found that the proliferation index of mice in the shNQO1 group was significantly lower than the expression level of the control group (Fig. 3H). On the contrary, the overexpression group significantly induced tumorigenicity compared with the vector group. The above results all indicated that NQO1 affects the cell proliferation ability of PAAD.

### 3.4 NQO1 regulates EMT process and affects cell migration and invasion ability

According to Fig. 2, overexpression of NQO1 protein was positively correlated with LN metastasis of PAAD. **In vitro** and **in vivo** tests were used to detect the influence of NQO1 differential expression on the metastasis ability of PAAD. As shown in Fig. 4A-D, wound healing and transwell assays demonstrated that overexpression of NQO1 could promote the migration and invasion of PAAD cells, inversely, the result was opposite after knockdown of NQO1. Western blot detected that in shNQO1 cells, the level of epithelial markers (E-cadherin) was increased, accompanied by a decrease in the levels of mesenchymal markers (Vimentin, Snail), while NQO1 overexpression cells had the opposite result (Fig. 4E). IF staining was consistent with the western blot (Fig. 4F). In addition, IHC staining of subcutaneous tumors found that the expression level of E-cadherin in the knockdown NQO1 group was increased, accompanied by a decrease in the expression level of Vimentin, vice versa (Fig. 4G). All in all, these results demonstrated that NQO1 protein plays an important role in the invasion and metastasis of PAAD.

### 3.5 NQO1 promotes the progression of PAAD through FAO

Function enrichment analysis from public databases proved NQO1 was closely related to regulation of catabolic process and protein catabolic process (Fig. 5A). We analyzed the co-expression pattern of
NQO1 in PAAD through the LinkFinder module of LinkedOmics. As shown in Fig. 5B, all genes significantly related to NQO1 were differentiated by Pearson test. The results showed that the dark red dots gene was positively correlated with NQO1 gene, while the dark green dots gene was negatively correlated with NQO1 gene. Heat maps showed that the top 50 genes were positively and negatively correlated with NQO1 gene respectively. Further analysis of these genes significantly related to NQO1 in biological processes, cellular components and molecular functions revealed that some genes played important roles in FAO (Figure. 5C). Therefore, we explored whether NQO1 could regulate FAO in PAAD. To confirm this hypothesis, we detected the FAO and ATP levels in BxPC-3 and MIA PaCa-2 cells. The results showed that after NQO1 knockdown, the levels of FAO and ATP had a downward trend compared with the control group. When NQO1 was overexpressed, the results were opposite (Fig. 5D-E). Western blot proved that knockdown NQO1, the level of FAO markers (LCAD, MCAD, CPT1A) were decreased, vice versa (Fig. 5F). IHC of subcutaneous tumor tissues of mice showed that the expression levels of related markers (CPT1A, MCAD, LCAD) in shNQO1 group were significantly lower than those in the control group. The overexpression group significantly induced tumorigenicity compared with the vector group. (Supplement Fig. 2). After further adding FAO inhibitor Etomoxir (ETX,15µmol/L), we found that ETX could reduce the abilities of cell proliferation and migration which caused by NQO1 overexpression (Fig. 5G-I). Consistently, western blot revealed that the expression levels of FAO markers, Vimentin and Snail were downregulated and E-cadherin was enhanced after the addition of ETX (Fig. 5J). These results expounded that NQO1 promoted PAAD cell growth, migration, and EMT process in a manner dependent on the FAO pathway.

### 3.6 NQO1/CPT1A axis promotes the development of PAAD.

Relevance analysis found that the NQO1 was positively connected with CPT1A base on ChIPBase, GEPIA and TIMER databases (Fig. 6A). GEPIA database showed that the mRNA expression of CPT1A in tumor tissues was significantly higher than normal tissues, especially in PAAD (Fig. 6B). As show in Fig. 2A, the IHC staining result revealed that CPT1A was negative expression in pancreatic tissues, but positive or strongly positive in PAAD tissues, which was consistent with NQO1 in PAAD (Fig. 6C). Co-localization IF staining showed that NQO1 and CPT1A co-located in the nucleus and cytoplasm (Fig. 6D). At the same time CoIP test in BxPC-3 and MIA PaCa-2 cells showed that NQO1 and CPT1A could both pull down each other (Fig. 6E). These results indicated that NQO1 could interact with CPT1A. Furthermore, ubiquitination experiment showed that CPT1A ubiquitination was significantly increased in sh-NQO1 cells (Fig. 6F), suggested that NQO1 regulates CPT1A degradation through ubiquitin modification. According to above the results, NQO1 might regulate the FAO and development of PAAD via CPT1A, rescue experiments were performed to examine the hypothesis. Colony formation and EdU experiments showed that knockdown of CPT1A partly inhibited the proliferation of PAAD cells which caused by NQO1 overexpression (Fig. 6G-H). Wound healing and transwell experiments were consistent with the EdU results (Fig. 6I-J). Western blot results revealed that while CPT1A knockdown, the expression of E-cadherin was up-regulated, meanwhile, mesenchymal and FAO makers were downregulated (Fig. 6K). These results suggested that NQO1 regulates the malignant progression of PAAD through CPT1A.
4 Discussion

A number of major studies have confirmed that NQO1 regulated cell chromatin binding protein, gene expression and DNA damage\textsuperscript{20}. It promotes p53 accumulation in an MDM2 and ubiquitin independent manner, thereby enhancing the cellular senescence phenotype\textsuperscript{21}. In recent years, it has been reported that NQO1 is closely related to the occurrence and development of cancer. Park \textit{et al.} revealed that the recurrence free survival (RFS) and overall survival (OS) of patients with NQO1 overexpression were shorter than those with NQO1 low expression, and it was interrelated to the poor prognosis of adenocarcinoma\textsuperscript{22}. Moreover, NQO1 could promote the growth and aggressiveness of HCC, and the underlying mechanism involved NQO1-derived amplification of ERK/p38-NRF2 signaling\textsuperscript{23}. Although NQO1 has been studied and identified in humans for many years, but there is still a lack of research report on the functional mechanism of NQO1 in PAAD. In this study, ectopic expression of NQO1 could improve cell growth rate and tumor formation \textit{in vitro} and \textit{in vivo}, it was accordance with the results of immunohistochemistry staining. In other hand, overexpression of NQO1 stimulates the metastatic potential of PAAD cells, and silencing NQO1 has the opposite effects. Overall, our functional experimental analysis shows that NQO1 promotes the occurrence and development of PAAD, and that NQO1 is closely related to poor prognosis in patients with PAAD, which may be a potential prognostic marker of PAAD.

Cell function experiment and public databases confirmed that FAO as an essential metabolic pathway for NQO1 to promote PAAD process. Catabolism of FAO may promote occurrence and development of tumors, but the mechanism research of FAO and NQO1 in PAAD was rarely reported. Initial study hinted that FAO as one of the ways of cell energy supply, the change of FAO will also affect the autophagy clearance of macrophages. Inhibition of FAO could promote the fusion of autophagosomes and lysosomes, increase the number of autophagosomes in cells, and significantly down regulate the retention of tumor cells. Moreover, FAO plays an important role in carcinogenic signaling pathway, lipid homeostasis and tumor microenvironment remodeling. Cheng \textit{et al.} reported FAO inhibitor Etomoxir induced bladder cancer developed with alterations in fatty acid metabolism associated gene expression\textsuperscript{24}. In the present study, we showed that the level of FAO and ATP were up/down-regulated by NQO1 expression, and the protein expressions of FAO markers (CPT1A, LCAD and MCAD) were also consistent. Moreover, rescue experiments showed that FAO inhibitor ETX partially inhibited the PAAD oncogenic effects of NQO1 induced of cells. Most importantly, the key enzymes of FAO might be the downstream targets of NQO1.

Studies have reported that the key enzymes of FAO are closely related to the occurrence and development of multiple tumors, especially pancreatic cancer\textsuperscript{25}. Yu \textit{et al.} found that LCAD catalyzes a key step in mitochondrial FAO, and plays an important tumor-promoter in esophageal squamous carcinoma\textsuperscript{26}. The existing literature pointed that MCAD could promote the EMT process of breast cancer cells and improved the migration and invasion ability\textsuperscript{27}. In our study, the results of correlation analysis Co-IP and protein ubiquitination experiment showed that NQO1 has a stronger correlation with CPT1A, and interacts with CPT1A. CPT1A is an important rate limiting enzyme for FAO, which localizes to the outer mitochondrial
membrane, and it is responsible for fatty acid transport into mitochondria for further oxidation by converting acyl-CoAs into acylcarnitines. Lin et al. iRGD-modified exosomes effectively deliver CPT1A siRNA to colon cancer cells, reversing oxaliplatin resistance by regulating fatty acid oxidation\textsuperscript{28}. Further study revealed that depletion of CPT1A could consequently inhibit FAO pathway and decrease NQO1-induced cell growth and motility capability in PAAD cells. Therefore, we illuminated the molecular mechanism of NQO1 in promoting PAAD progress by up-regulating CPT1A-mediated FAO.

Collectively, we reported that NQO1/CPT1A was frequently overexpressed in PAAD, and played a crucial instigator in PAAD cell proliferation and metastasis by activating FAO pathway. Importantly, administration of shNQO1/sh-CPT1A and FAO inhibitor ETX could suppress the oncogenicity of PAAD, which may lead to develop a novel therapeutic approach in PAAD treatment (Fig. 7).

**Declarations**

**Ethics approval and consent to participate**

According to the Declaration of Helsinki, patients organized for this study provided informed consent, and the patients agreed to use their tumor tissue samples for scientific research. This study was reviewed and approved by the Institutional Ethics Committee of Yanbian University School of Medicine.

**Consent for publication**

Applicable.

**Competing interests**

No potential conflicts of interest were disclosed.

**Funding**

This research was supported by the National Natural Science Foundation of China (No. 82160552), the National Natural Science Foundation of Jilin Province (No.210101207), Horizontal Science and Technology Projects (natural science) of Yanbian University and Siping Central People's Hospital.

**Authors’ contributions**

RX conducted all analysed and interpreted data, conducted literature search, wrote the manuscript; YL conducted validation and critically reviewed the manuscript; LM, YS, H.L and D.Y contributed to supervised the research project, critically reviewed the manuscript; YY and T.J contributed to the study design, supervised writing of the manuscript, critically reviewed the paper, supervised the research project. All authors read and approved the final manuscript.

**Acknowledgements**
Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**References**


Figures
Figure 1

NQO1 is upregulation in PAAD and correlates with a poor outcome. (A) The expression of NQO1 in PAAD and other tumors was analysed by GEPIA and TIMER databases. (B) NQO1 mRNA expression levels of PAAD and normal pancreas in GEPIA, ENCOR1, UCSC Xena, TCGA and Oncomine (Pancreatic data were derived from Grutzmann Pancrease Statistics, and Pancreatic data from Badea Pancreas Statistics.)
Reporter of both statistics is 215019_s_at) databases. (C) Correlation between NQO1 expression and survival rate in PAAD patients assessed by Oncomine, HPA database, GEPIA and KM-plotter.

Figure 2

NQO1 is closely related to the malignant progression of PAAD. (A) NQO1 expression in PAAD and normal pancreatic tissues was detected by IHC. (B) NQO1 cellular masks DAB mean intensity in PDAC tissues.
Figure 3

**NQO1 enhances tumorigenesis *in vitro* and *in vivo*.** (A) Western blot was performed to test the stable establishment effect of NQO1 knockdown and overexpress in PAAD cell lines. (B-D) Cell proliferation ability was tested by MTT (B), colony formation (C) and EdU assays (D). (E-G) The nude mice xenograft
model evaluated the effect of NQO1 differential expression on tumor volume and weight. (H) IHC staining showed NQO1 and Ki67 expression in tumor specimens from xenografts.

Figure 4

**NQO1 induces the migration, invasion and promotes EMT process.** (A-B) Wound healing demonstrated NQO1 affects lateral migration of PAAD cells, cells were cultured under medium with (A) or without (B)
serum. **(C-D)** Transwell assay showed NQO1 affects longitudinal migration ability of PAAD cells. **(E)** Western blot assay was used to detect the effects of NQO1 differential expression on the protein expression level of EMT markers. **(F-G)** IHC and IF staining were used to observe protein expression and localization of EMT markers in PAAD cells after NQO1 knockdown and overexpression.
NQO1 promotes the progression of PAAD through FAO. (A) GO enrichment analysis showed that NQO1 was involved in a variety of biological functions. Wherein, the size of the circle represents the number of differentially expressed genes involved in the enrichment pathway, while the corresponding p-value is represented by the color of the circle, and the darker the color, the more significant the difference. (B-C) LinkedOmics database enrichment analysis of NQO1 related (D-E) FAO and ATP levels of PAAD cells were detected by kits. (F) FAO markers (LCAD, MCAD and CPT1A) expression level was analyzed by western blot. (G-I) MTT, colony formation and wound healing were used to test the proliferation and migration abilities of NQO1 overexpression cell after adding FAO inhibitor ETX. (J) The expression levels of FAO and EMT markers were measured by western blot. *p < 0.05 compared with vector group and #p< 0.05 compared with NQO1 group.
Figure 6

**NQO1/CPT1A axis promotes the development of PAAD.** (A) ChIPBase, GEPIA and TIMER databases were used to analyze the relation between NQO1 and CPT1A. (B) GEPIA database showed the expression level of CPT1A in tumor tissues and normal tissues. (C) IHC staining of CPT1A in PAAD and paired normal pancreatic tissues. (D) IF showed the co-localization of NQO1 and CPT1A in the nucleus and cytoplasm. (E) Co-immunoprecipitation detected the interaction between NQO1 and CPT1A. (F) The ubiquitination of
CPT1A was examined by ubiquitin experiments. (G-H) The proliferation ability of NQO1 overexpression cells after knockdown CPT1A was detected by colony formation and EdU assays. (I) Lateral migration ability of cells was detected by wound healing assay. (J) Transwell experiment showed longitudinal migration ability of cells. (K) Western blot demonstrated the changes of EMT and FAO markers expression after CPT1A knockdown. *p < 0.05 compared with vector group and #p< 0.05 compared with NQO1 group.

Figure 7

Schematic model showed that CPT1A-depedent FAO as an essential metabolic pathway for NQO1 to promote PAAD progression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUPPLEMENT.docx