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Original Article

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

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Abstract

NQO1, a cytosolic enzyme, is closely related to the progression of cancers and poor outcome of cancer patients. However, the molecular biological mechanism of NQO1 tumorigenicity in pancreatic adenocarcinoma (PAAD) has not been clearly understood. In this study, we demonstrate the molecular mechanism of NQO1 in PAAD proliferation, metastasis and fatty acid oxidation (FAO). Multiple databases and western blot analysis show that NQO1 is overexpressed in PAAD and associated with lymph node metastasis and shorter survival. Furthermore, *in vitro* and *in vivo* experiments reveal that 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. Therefore, Co-IP and a series of rescue experiments demonstrate that NQO1 promotes PAAD progression via CPT1A-mediated FAO. Our findings identify CPT1A-dependent FAO as an essential metabolic pathway for NQO1 to promote the PAAD process. Targeting the NQO1/CPT1A/FAO axis in PAAD to attenuate proliferation and dissemination is a potential approach to promote a better antitumour effect and improve patient outcomes.

Key words NQO1, PAAD, metastasis, fatty acid oxidation, CPT1A

Introduction

Pancreatic adenocarcinoma (PAAD) is the third leading cause of cancer-related death, with a five-year survival rate of approximately 11% [1]. 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 [2,3]. This may be partly due to the complexity and heterogeneity of PAAD. Therefore, the exploration of a promising biomarker that regulates the biological dynamics and behavior of PAAD will contribute remarkably to optimizing treatment.

The gene of NAD(P)H:quinone oxidoreductase-1 (NQO1), also known as DT diaphorase, is located on chromosome 16q22 and consists of six exons and five introns [4,5]. 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 [6]. In addition, more reports have found that NQO1 is closely related to clinical significance and molecular mechanisms in different malignant tumors [7]. Jiang *et al.* [8] found that the expression of NQO1 protein in gastric cancer tissue is higher than that in normal gastric tissue and is closely related to the patients' tumor stage and age. Hirose *et al.* [9] found that the positive expression of NQO1 in colorectal and liver metastatic tumor cells may be an important factor for poor prognosis after hepatectomy. Yang *et al.* [10] 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 [11]. Previous studies have revealed that the high expression of NQO1 is positively correlated with glucose metabolism and tumor progression. However, the relationship between NQO1 and the metabolic mechanism of PAAD progression has not yet been reported.

Dysregulated metabolism, such as aerobic glycolysis and fatty acid metabolism, is recognized as the hallmark of cancer development [12,13]. Fatty acid oxidation (FAO) is a major pathway regulating fatty acid degradation and promoting ATP and NADPH production [14]. The association between altered lipid metabolism

mediated by FAO and tumor progression has been established [15,16]. It was found that carnitine palmitoyltransferase 1A-mediated (CPT1A, a rate-limiting FAO enzyme) FAO could drive colon cancer peritoneal metastasis by inhibiting anoikis [17]. Wang *et al*. [18] revealed that STAT3/CPT1B (a member of the CPT1 family)-mediated FAO promotes breast cancer cell stemness and chemoresistance. The mechanism by which YY1/PGC-1β regulates medium-chain acyl-CoA dehydrogenase (MCAD) and long-chain acyl-CoA dehydrogenase (LCAD), the key enzymes of FAO, thereby accelerating hepatocarcinogenesis was also shown [19]. However, the specific regulatory mechanism between NQO1 and FAO in PAAD needs further study.

Herein, we analysed the clinicopathological significance of NQO1/CPT1A expression in patients with PAAD. At the same time, we revealed that 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.

Materials and Methods

Tissue samples

All patients agreed to use their tumor tissue samples for scientific research and signed the consent forms to participate in this study. PAAD tissue microarrays were purchased from Shanghai Outdo Biotech (Shanghai, China). The classification standard of tumors in this study was used 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 and was conducted in compliance with the tenets of the Declaration of Helsinki. All animal procedures were performed under the ethical guidelines of the Laboratory Animal Center of Yanbian University.

Bioinformatics analysis

The Oncomine (https://www.oncomine.org/resource/login.html), ENCORI (https://starbase.sysu.edu.cn/), TIMER (http://timer.cistrome.org/), UCSC Xena (http://xena.ucsc.edu/), UALCAN (http://uaLcan.path.uab.edu) and GEPIA websites (gepia.cancer-pku.cn) were used to explore NQO1 mRNA expression in PAAD tissues and adjacent normal tissues. The relationship between NQO1 expression and patient survival was searched in Kaplan-Meier Plotter (http://kmpLot.com/anaLysis/index.php?p=service), UALCAN, OncoLnc (http://www.oncolnc.org/), GEPIA and The Human Protein Atlas (HPA, https://www.proteinatlas.org/). The STRING (https://string-db.org/cgi/input.pl) and LinkedOmics (http://www.linkedomics.org/login.php) databases were used to analyse the involvement of NQO1 in the biological process of PAAD. The GEPIA, TIMER, cBioPortal (http://www.cbioportal.org), and CHIPBase (https://rna.sysu.edu.cn/chipbase/index.php) databases were used to explore genetic correlation analysis.

Cell culture

BxPC-3 and MIA PaCa-2 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA), and the cells were authenticated by short tandem repeats. The cells were routinely cultured in DMEM (Gibco, Grand Island, USA), which was supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Before selecting cell lines for the experiment, mycoplasma detection was carried out, and strict aseptic operation was performed in the culture process.

Stable cell line generation

The RNAi target sequence and vector of the NQO1 gene were designed and constructed by Hesheng Gene Technology (Beijing, China). The plasmid vector maps and gene sequences of sh-Con, shNQO1, Vector, and NQO1 lentiviruses are shown in Supplementary Figure S1 and Supplementary Table S2. Before lentivirus transfection, the adherent cells were seeded into a six-well plate at a density of 6×10^4 cells/well. After lentivirus infection, single-cell clonal strains were selected for 14 days under the action of 2 μg/mL puromycin so that a stable expression cell line could be established. Finally, the transfection results were further verified by western blot analysis.

Western blot analysis

Proteins were extracted from pancreatic adenocarcinoma cells using RIPA lysis buffer (CWBIO, Beijing, China) containing phosphatase inhibitors and protease inhibitors. The protein concentration was determined using BCA reagent (CWBIO). Protein samples were separated by 8%–10% SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, USA). Then, PVDF membrane was blocked in 5% skim milk and incubated with mouse anti-NQO1 (1:1000; Abcam, Cambridge, USA), rabbit anti-E-cadherin (1:1000; Abcam), rabbit anti-Vimentin (1:1000; Abcam), rabbit anti-Snail (1:1000; Abcam), rabbit anti-CPT1A (1:1000; Abcam), rabbit anti-LCAD (1:1000; Proteintech, Chicago, USA), rabbit anti-MCAD (1:1000; Proteintech), or mouse anti-actin (1:3000; Santa Cruz Biotech, Santa Cruz, USA) primary antibody in a refrigerator at 4°C overnight, followed by incubation with corresponding HRP-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (Beyotime, Shanghai, China) at room temperature for 1-2 h. Finally, an enhanced chemiluminescence (ECL) kit (CWBIO) was used for the visualization of the blots.

Immunohistochemistry (IHC) analysis

The expressions of proteins in tissues were detected by immunohistochemistry staining [20]. The staining steps were as follows: 4-μm-thick serial sections of the tissue were conventionally deparaffinized, followed by gradient ethanol hydration and antigen retrieval in hot citrate buffer. Sections were incubated with mouse anti-NQO1 antibody (1:200; Abcam) overnight at 4°C, and then incubated with HRP-labelled secondary antibody (ZSGB-BIO, Beijing, China). DAB was used for colour development. Sections were counterstained with hematoxylin (BaSO, Zhuhai, China) and sealed with neutral gum. 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.

NQO1 staining of tissue sections of PAAD patients was judged by two methods, including the panoramic microscope of the TissueFAXS Plus scoring system (TissueGnostics, Vienna, Austria) and pathologists scoring. The TissueFAXS Plus scoring system was performed according to the mean intensity of the NQO1 cellular mask DAB. NQO1 staining of tissue sections of PAAD patients or xenograft tumors was judged by pathologists. Two pathologists scored all the specimens in a blinded manner using a coupled scoring system combining staining intensity and area extent. The

scoring method followed a previously described standard [21]. The scores of staining intensity were set based on the following criteria: 0, no staining; 1, light yellow; 2, brownish yellow; and 3, brown. The scores of positive cell percentage were defined according to the following criteria: 0, 0 to 5% positive cells; 1, 6% to 25% positive cells; 2, 26% to 50% positive cells; 3, 51% to 75% positive cells; and 4, 76% to 100% positive cells. The two score results were multiplied to obtain a final score: 0 (–); 1 to 4 (+); 5 to 8 (++); and 9 to 12 (+++), where a final score ≤4 was defined as NQO1 low expression and a final score > 4 was defined as NQO1 high expression.

Immunofluorescence (IF) microscopy

Cells were fixed with 4% paraformaldehyde for 15 min, rinsed with PBS, permeated with 0.5% Triton X-100 (CWBIO) for 30 min and then blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature. The cells were incubated in a refrigerator at 4°C overnight with anti-NQO1 antibody (1:1000; Abcam). The next day, the sections were rinsed with PBS 3 times for 5 min each time and incubated with Alexa Fluor 488-labelled and 568-labelled goat anti-rabbit or mouse IgG secondary antibodies (1:400; Invitrogen, Carlsbad, USA) for 1 h. The nuclei were stained with 4′,6-diamidyl-α phenylindole (DAPI) solution and then images were captured under an SP5II fluorescence microscope (Leica, Wetzlar, Germany).

Colony formation assay

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

MTT assay

PAAD cells were inoculated into 96-well plates at 100 μL per well (5×10^3 cells). MTT solution (100 μL, 5mg/mL; Solarbio, Beijing, China) was added at 0 h, 24 h, 48 h and 72 h and incubated for another 4 h. Then the supernatant was discarded and 100 μL DMSO was added to each well to dissolve the formazan crystal. After 10 min, the absorbance of each well was measured at 570 nm with a full-wavelength microplate reader (Tecan, Männedorf, Switzerland), and the cell growth curve was plotted with time as the abscissa and absorbance as the ordinate.

Wound healing assay

Cells at the logarithmic growth stage with a density of approximately 80% were inoculated in 6-well plates. When cells were grown to confluency in a monolayer, a straight scratch line was created with a 200-μL sterile tip, cell debris were washed away with PBS, and immediately photographed under an IX73 microscope (Olympus, Tokyo, Japan) to record the initial width of the scratch wound. After incubation for 24 h, photos were taken again to record the width of the scratch wound. The healing rate of the scratch was calculated using the following formula: Wound healing (%) = $(\text{Width}_{0\ \text{h}} - \text{Width}_{24\ \text{h}} / \text{Width}_{0\ \text{h}}) \times 100\%$.

Transwell migration and invasion assay

In the migration assay, a total of 5×10^4 cells were suspended in 100 μL FBS-free DMEM and placed in Millicell culture inserts (EMD Millipore, Billerica, USA), and medium containing 20% serum was added to the lower chamber. After 24 h of culture in the incubator, the chamber was removed and the migrated cells on the transwell were fixed with methanol and stained with Haematoxylin. After wash with PBS, the transwell was sealed with neutral balsam mounting medium. Then cells were observed and photographed, and cell number was counted under a BX53 microscope (Olympus).

The invasion assay was performed inserting 100 μL Matrigel (1 mg/mL; Corning, Corning, USA) diluted by serum-free medium precooled at 4°C was uniformly coated with Millicell culture inserts, then incubated at 37°C for 4–5 h and dried into a gel. The following steps were the same as the migrationassay.

EdU assay

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

Measurement of FAO and ATP production

To detect FAO, an FAO ELISA kit (Shanghai Enzyme Linked Biotechnology, Shanghai, China) was used for one-step sandwich ELISA. Briefly, samples, standard substances and HRP-labelled 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. The absorbance (OD value) at 450 nm was measured with the microplate reader, and the concentration was calculated.

After cell samples were collected, washed and broken in sequence, ATP production was detected using an ATP kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Co-immunoprecipitation (Co-IP) and detection of ubiquitination

Co-IP was performed with anti-NQO1 (1:1000, Santa Cruz, Heidelberg, Germany) and anti-CPT1A (1:1,000; Abcam, Cambridge, UK) antibodies. Cells were lysed with IP lysis buffer (Beyotime, China). The supernatant, which was collected from centrifugation, was incubated with the anti-NQO1 and anti-CPT1A antibody respectively, overnight at 4°C followed by protein A/G agarose beads (Santa Cruz Biotechnology) for 6 h. Then washed beads with the cold lysis buffer, mixed with 2× SDS buffer (Santa Cruz Biotechnology), and heated denaturation, followed by western blotting analysis.

For the ubiquitination experiment, cells were treated with or without MG132 (10 μM; ApexBio, Houston, USA) for 6 h before being harvested for immunoprecipitation and western blot analysis using anti-Ubiquitin antibody (Santa Cruz Biotechnology).

In vivo models

Twenty-four 4- to 5-week-old male BALB/c nude mice (Vital River Laboratory Animal Technology, Beijing, China) were randomly assigned into four groups ($n = 6$) and placed in specific pathogen-free conditions. For the subcutaneous tumor-bearing models, 3×10^6 BxPC-3 cells with differential expression of NQO1 were

injected subcutaneously. One week later, a Vernier calliper was used to monitor the growth of the xenograft. Callipers were used to measure the length and width of the tumor to monitor the tumor volume, and the formula: $V = 0.5 \times \text{length} \times \text{width}^2$ was used for calculation. At the end of experiment, tumor tissues were collected for subsequent immunohistochemical staining to detect the expression levels of NQO1, CPT1A, Vimentin, E-cadherin and Ki67 in tumor sections.

For the metastasis model, 1×10^6 BxPC-3 cells were injected into the tail vein of nude mice. Six weeks later, mice were sacrificed and the lungs were surgically removed and stained with HE. Animal experiments were carried out in accordance with the procedures of the Animal Ethics Committee of Yanbian University.

Statistical analysis

All results were analysed using SPSS 26.0 software (SPSS, Chicago, USA), GraphPad Prism 8.0 software (GraphPad software Inc, San Diego, USA), and ImageJ software (NIH, Bethesda, USA). The χ^2 test or Fisher's exact test was used to analyse the correlation of clinicopathological parameters. T tests for independent means were used for group comparisons. Group comparisons for continuous data were performed by the Mann-Whitney U test or one-way ANOVA. All experiments were performed in triplicate. $P < 0.05$ was considered statistically significant.

Results

NQO1 is upregulated in PAAD and correlates with poor outcome

To determine the expression of NQO1 in tissues, gene expression databases (GEPIA and TIMER) were applied, which revealed that NQO1 was upregulated in PAAD and other cancers (Figure 1A). Moreover, the GEPIA, ENCORI, UCSC Xena and UALCAN databases were utilized to validate the expression of NQO1 in PAAD tissues, which showed that NQO1 expression was higher in PAAD than in normal pancreatic tissues. Interestingly, the Oncomine database showed that NQO1 expression was elevated in PDAC tissues compared with that in pancreatic duct and pancreas tissues (Figure 1B). The correlation between NQO1 expression and overall survival (OS) was explored using Oncolnc, HPA, GEPIA and Kaplan-Meier survival analyses, which revealed that PAAD patients with lower NQO1 expression had a longer OS than patients with higher NQO1 expression ($P < 0.05$, Figure 1C). Similarly, longer RFS was 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.

NQO1 overexpression is associated with LN metastasis in PAAD patients

To further explore the role of NQO1 in PAAD, IHC was used to detect the expression level of NQO1 in 81 pairs of PAAD tissues and normal pancreatic tissues. NQO1 protein is negatively expressed in nontumor pancreatic tissues and positively expressed in PAAD tissues. At the same time, IHC staining results showed that the NQO1 protein is mainly located in the cytoplasm and minorly in the nucleus of pancreatic cancer cells (Figure 2A). The results were consistent with the HPA database (Figure 2A). Notably, NQO1 cellular masks DAB-mean intensity was markedly upregulated in PAAD tissues compared with that in adjacent paracous tissues as revealed by TissueFAXS (Figure 2A,B). Mosaic results showed that NQO1 expression was only correlated with LN metastasis ($P = 0.0149$). However, there were 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 ($P = 0.6772$), or clinical stage ($P = 0.2931$) (Figure 2C and Supplementary Table S1). Therefore, the above results indicated that the high expression of NQO1 is closely related to the metastatic ability of PAAD patients.

NQO1 promotes tumorigenicity *in vivo* and *in vitro*

To further clarify the biological functions of NQO1 in PAAD, we screened the protein expression of NQO1 in PAAD cell lines (PATU-8988, MIA PaCa-2, Panc-1, BxPC-3) and found that it was the lowest in the MIA PaCa-2 cell line and the highest in the BxPC-3 cell line (Supplementary Figure S2). Subsequently, stably transfected cell lines were established and confirmed by western blot analysis (Figure 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 (Figure 3B,C). Consistently, the EdU assay revealed that *NQO1* knockdown markedly inhibited cell DNA replication, whereas NQO1 overexpression had the opposite effect (Figure 3D).

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

NQO1 regulates the EMT process and affects cell migration and invasion ability

Subsequently, we focused on the functions of NQO1 in the metastasis of PAAD cells. As expected, knockdown of *NQO1* significantly inhibited the lateral and longitudinal migration capacities of PAAD cells compared with control groups, as demonstrated in the wound healing, migration, and invasion transwell assays, but had opposite results in NQO1-overexpressed cells (Figure 4A–D). Interestingly, western blot analysis showed that the epithelial marker E-cadherin was upregulated in the shNQO1 group compared with that in the control group. Conversely, mesenchymal markers (Vimentin, Snail) were downregulated in parallel with NQO1 expression and vice versa (Figure 4E). Immunofluorescence (Figure 4F) and IHC staining of xenografted tumor sections (Figure 4G) further verified our findings that E-cadherin was enhanced in cells or tissues with NQO1 depletion but weakened in those with NQO1 overexpression. Overall, these results demonstrated that the NQO1 protein plays an important role in the EMT progression and metastasis of PAAD.

NQO1 promotes the progression of PAAD through FAO

It is well established that alteration of FAO affects tumor development [22]. Functional enrichment analysis from public databases proved that NQO1 was closely related to the regulation of catabolic processes and protein catabolic processes (Figure 5A). We analysed the coexpression pattern of NQO1 in PAAD through the

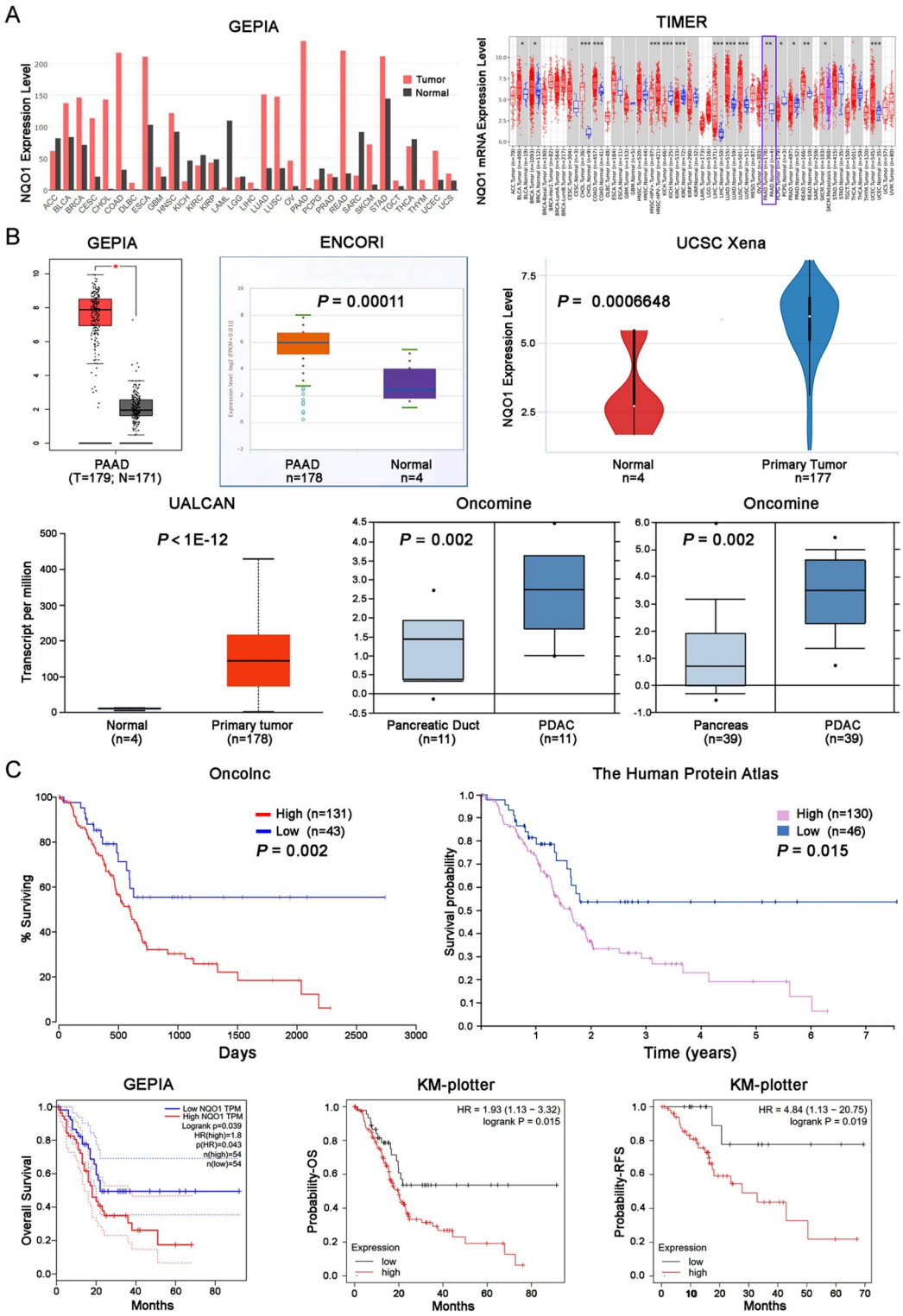

Figure 1. NQO1 is upregulated in PAAD and correlates with a poor outcome (A) The expression of NQO1 in PAAD and other tumors was analysed by the GEPIA and TIMER databases. (B) NQO1 mRNA expression levels of PAAD and normal pancreas in GEPIA, ENCOR1, UCSC Xena, TCGA and Oncomine (Pancreatic data were 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 OncoInc, HPA database, GEPIA and KM-plotter.

LinkFinder module of LinkedOmics. As shown in Figure 5B, all genes significantly related to NQO1 were differentiated by Pearson's test. The results showed that the dark red dot gene was positively correlated with the *NQO1* gene (Supplement Table S2), while the

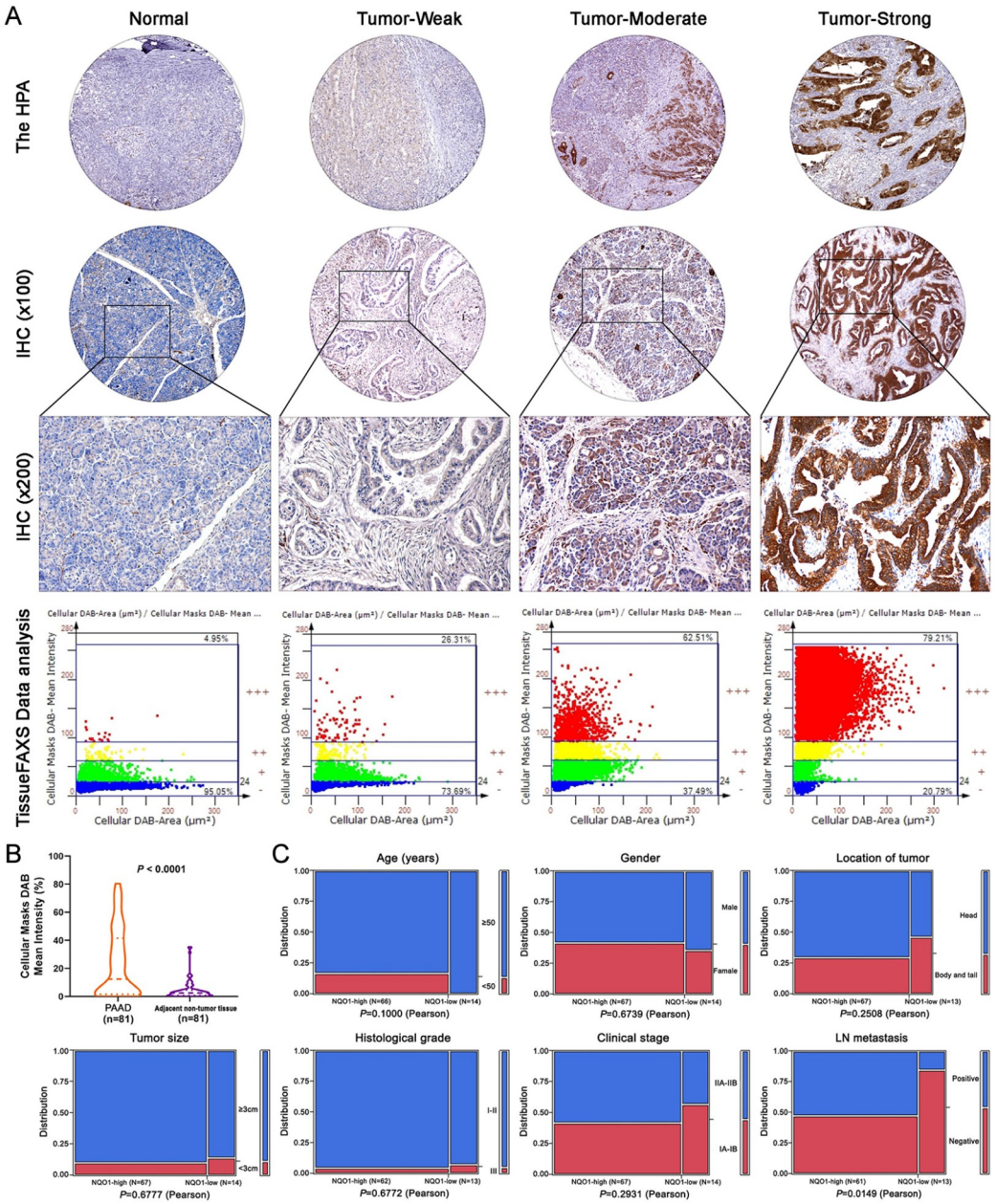

Figure 2. NQO1 is closely related to the malignant progression of PAAD (A) NQO1 expression in PAAD and normal pancreatic tissues was detected by IHC. (B) NQO1 cellular masks DAB mean intensity in PAAD tissues. (C) Mosaic map showing NQO1 and clinicopathological analysis of PAAD patients.

dark green dot gene was negatively correlated with the *NQO1* gene (Supplement Table S3). Heatmaps showed that the top 50 genes were positively and negatively correlated with the NQO1 gene. Further analysis of these genes significantly related to NQO1 in biological processes, cellular components and molecular functions revealed that some genes play important roles in FAO (Figure 5C). Therefore, we explored whether NQO1 could regulate FAO in PAAD. To confirm this hypothesis, we detected 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 showed a downwards trend compared with those in the control group. When NQO1 was overexpressed, the results were the opposite (Figure 5D,E). Western blot analysis proved that *NQO1* knockdown decreased the levels of FAO markers (LCAD, MCAD, CPT1A) and vice versa (Figure 5F). IHC of subcutaneous tumor tissues of mice showed that the expression levels of related markers (CPT1A, MCAD, LCAD) in the shNQO1 group were significantly lower than those in the control group. The overexpression group showed significantly increased tumorigenicity compared with the vector group (Supplement Figure S3). After treatment with the FAO inhibitor etomoxir (ETX, 15 μM), we found that ETX could reduce the abilities of cell proliferation and migration caused by NQO1 overexpression (Figure 5G–I). Consistently, western blot analysis revealed that the expression levels of the FAO markers Vimentin and Snail were downregulated and that

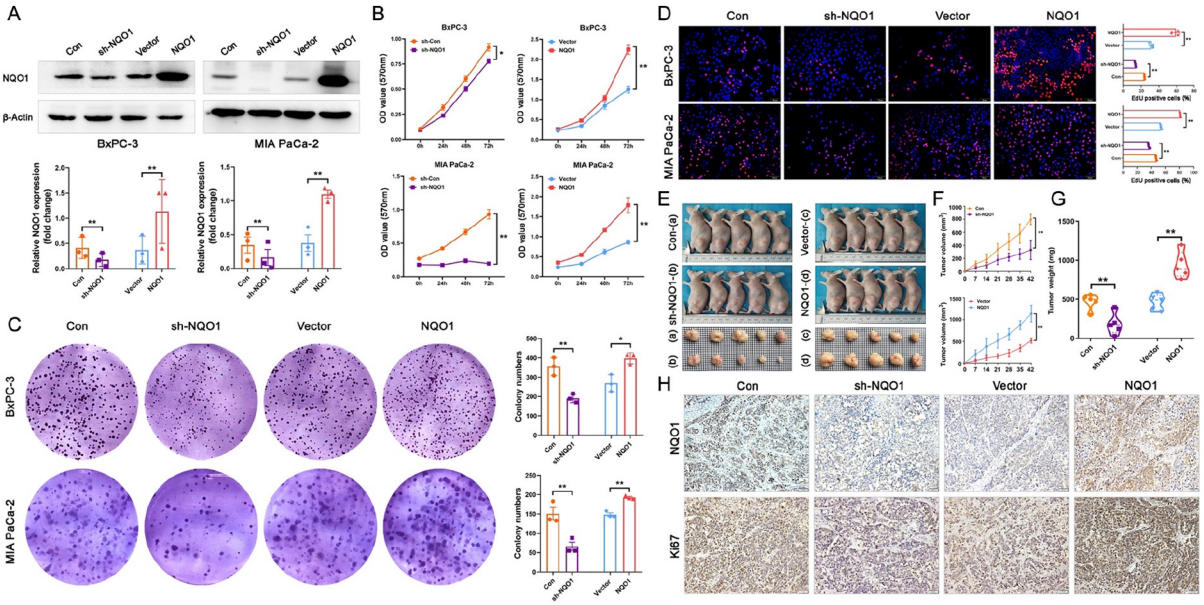

Figure 3. NQO1 enhances tumorigenesis *in vitro* and *in vivo* (A) Western blot analysis was performed to verify the stable establishment of NQO1 knockdown and overexpression in PAAD cell lines. (B–D) Cell proliferation ability was measured by MTT (B), colony formation (C) and EdU assays (D). (E–G) The nude mouse xenograft model was used to evaluate the effect of NQO1 differential expression on tumor volume and weight. (H) IHC staining showed NQO1 and Ki67 expressions in tumor specimens from xenografts.

of E-cadherin was enhanced after the treatment with ETX (Figure 5J). These results revealed that NQO1 promotes PAAD cell growth, migration, and EMT in a manner dependent on the FAO pathway.

NQO1/CPT1A axis promotes the development of PAAD

Wang *et al.* [23] reported that CPT1A-mediated FAO activation is an essential metabolic pathway for treating metastatic CRC. Therefore, we inferred that NQO1/FAO might also accelerate EMT through CPT1A in PAAD cells. To assess this hypothesis, the correlation between NQO1 and CPT1 family members was predicted using the GEPIA website. The results showed that NQO1 was only positively related to CPT1A in PAAD tissues (Supplementary Figure S4), indicating that NQO1 might regulate CPT1A expression in PAAD cells. Relevance analysis further verified the interaction between NQO1 and CPT1A (Figure 6A). The GEPIA database showed that the mRNA expression of CPT1A in tumor tissues was significantly higher than that in normal tissues, especially in PAAD (Figure 6B). As shown in Figure 2A, the IHC staining results revealed that CPT1A was negatively expressed in pancreatic tissues but positively or strongly expressed in PAAD tissues, which was consistent with NQO1 in PAAD (Figure 6C). Colocalization IF staining showed that NQO1 and CPT1A colocalized in the nucleus and cytoplasm (Figure 6D). At the same time, a co-IP assay in BxPC-3 and MIA PaCa-2 cells showed that NOQ1 and CPT1A could both pull down each other (Figure 6E). These results indicated that NQO1 could interact with CPT1A. Furthermore, ubiquitination experiments showed that CPT1A ubiquitination was significantly increased in sh-NQO1 cells (Figure 6F), suggesting that CPT1A is a potential effector of the NQO1/FAO axis in the regulation of progression and metastasis in PAAD.

Given that NQO1 could regulate FAO in a CPT1A-dependent manner, rescue experiments were performed to examine the effect of *CPT1A* silencing in NQO1-overexpressing cells (Figure 6F). Colony formation and EdU experiments showed that knockdown of *CPT1A* partly inhibited the proliferation of PAAD cells caused by NQO1 overexpression (Figure 6G,H). Similarly, wound healing and transwell assays revealed that CPT1A depletion decreased the migration capability of PAAD cells compared with NQO1-overexpressing cells (Figure 6I,J). In addition, the related markers of FAO and EMT were significantly changed in shCPT1A cells (Figure 6K). Accordingly, NQO1 activates FAO by interacting with CPT1A, and NQO1/CPT1A/FAO participates in the progression and metastasis of PAAD.

Discussion

Studies have confirmed that NQO1 regulates cell chromatin binding protein, gene expression and DNA damage [24]. It promotes p53 accumulation in an MDM2- and ubiquitin-independent manner, thereby enhancing the cellular senescence phenotype [25]. In recent years, it has been reported that NQO1 is closely related to the occurrence and development of cancer. Park *et al.* [26] revealed that the recurrence-free survival (RFS) and overall survival (OS) of patients with NQO1 overexpression were shorter than those with low NQO1 expression, which was interrelated with the poor prognosis of adenocarcinoma. Moreover, NQO1 could promote the growth and aggressiveness of HCC, and the underlying mechanism involved NQO1-derived amplification of ERK/p38-NRF2 signaling [27]. Although NQO1 has been studied and identified in humans for many years, there is still a lack of research reports on the functional mechanism of NQO1 in PAAD. Here, we revealed that high expression of NQO1 could predict the poor prognosis of patients with PAAD and was related to LN metastasis and shorter survival of patients. In addition, we focused on the regulatory mechanism of NQO1 on EMT, metastasis, and FAO in depth in PAAD.

Cancer cells require FAO for ATP, energy, membrane synthesis, and lipid signals to achieve rapid proliferation, which is essential for

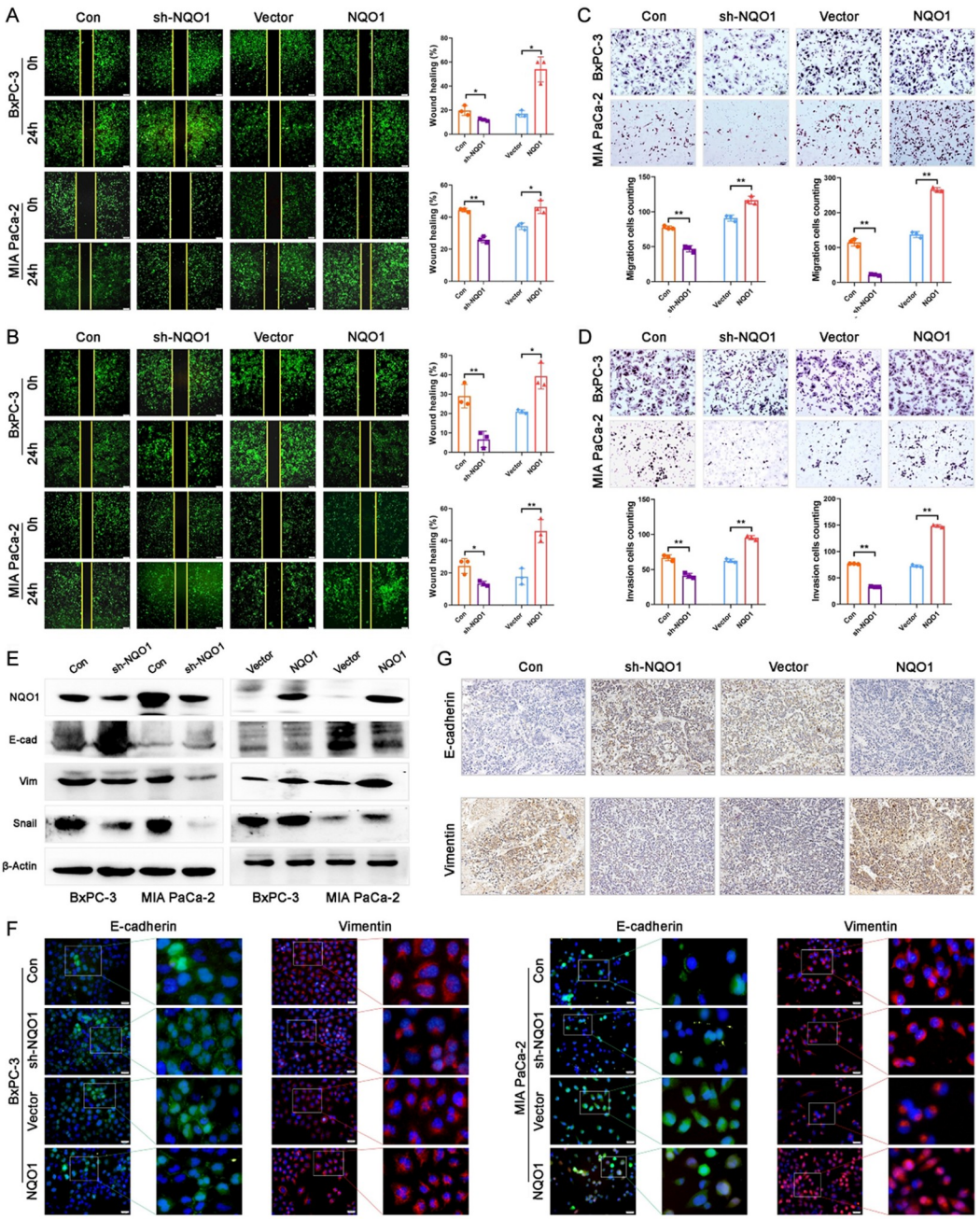

Figure 4. NQO1 induces migration and invasion and promotes the EMT process (A,B) Wound healing assays demonstrated that NQO1 affects the lateral migration of PAAD cells. Cells were cultured in medium with (A) or without (B) serum. (C,D) Transwell assays showed that NQO1 affects the longitudinal migration ability of PAAD cells. (E) Western blot analysis was used to detect the effects of NQO1 differential expression on the protein expression levels of EMT markers. (F,G) IHC and IF staining were used to observe the protein expression and localization of EMT markers in PAAD cells after NQO1 knockdown and overexpression.

metastasis and colonization of distant organs [17], especially in PAAD cells [28]. Our experiments also confirmed that FAO is an essential metabolic pathway by which NQO1 promotes the PAAD process, and the levels of FAO and ATP were up/downregulated by NQO1 expression. The protein expression levels of FAO markers (CPT1A, LCAD and MCAD) were also consistent. Rescue experiments showed that the FAO inhibitor ETX partially inhibited the PAAD oncogenic effects of NQO1 in cells. Moreover, FAO plays an important role in the carcinogenic signaling pathway, lipid homeostasis and tumor microenvironment remodelling. As one of the mechanisms of cell energy supply, the change in 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 downregulate the retention of tumor cells. Cheng *et al.* [29] reported that the FAO inhibitor etomoxir induced bladder cancer development with alterations in fatty acid metabolism-associated gene expression. Meanwhile, our previous study demonstrated that as a metabolic

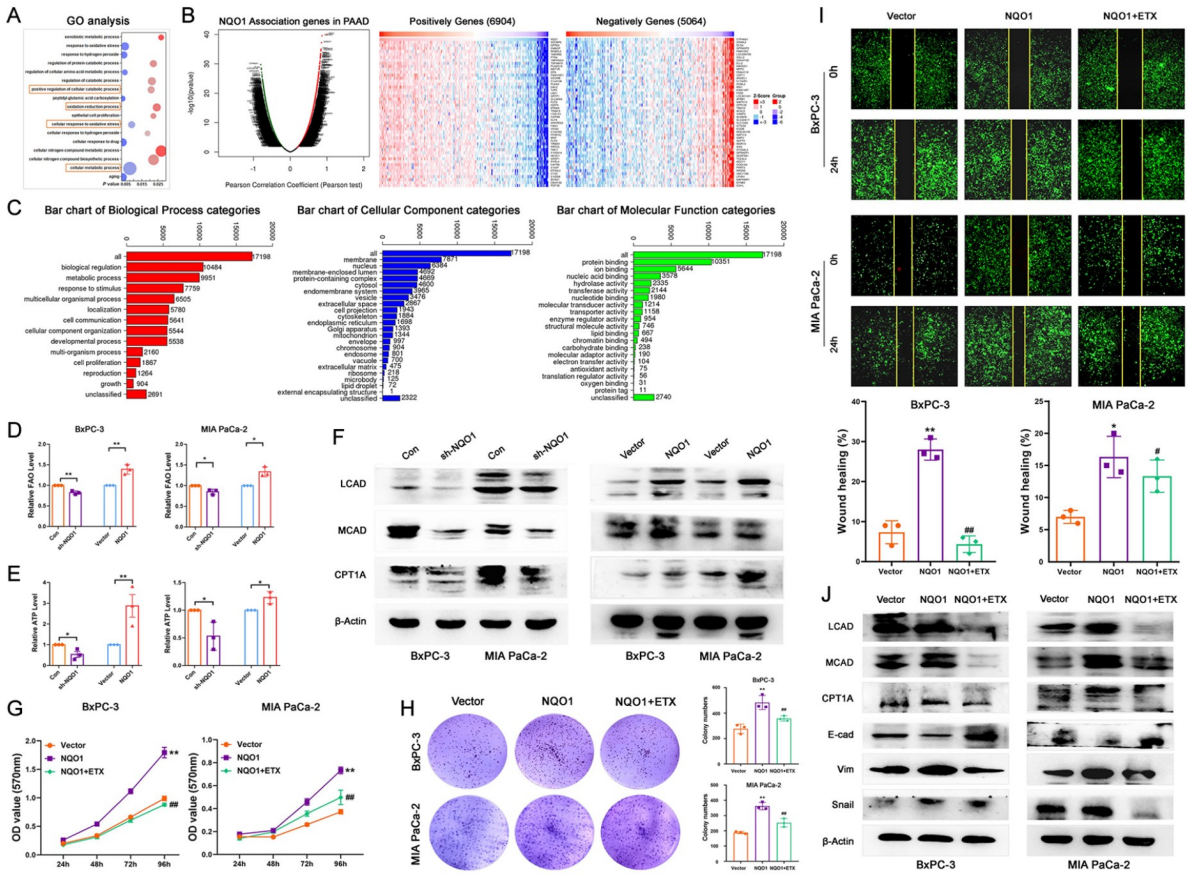

Figure 5. NQO1 promotes the progression of PAAD through FAO (A) GO enrichment analysis showed that NQO1 was involved in a variety of biological functions. The size of the circle represents the number of differentially expressed genes involved in the enrichment pathway, while the corresponding P value 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. (D,E) FAO and ATP levels in PAAD cells were detected by using their corresponding kits. (F) FAO marker (LCAD, MCAD and CPT1A) expression levels were analysed by western blot analysis. (G–I) MTT, colony formation and wound healing assays were used to test the proliferation and migration abilities of NQO1-overexpressing cells after treatment with the FAO inhibitor ETX. (J) The expression levels of FAO and EMT markers were measured by western blot analysis. $^{*}P<0.05$ compared with the vector group and $^{\#}P<0.05$ compared with the NQO1 group.

activator, NQO1 could activate lipid metabolism reprogramming and promote tumor progression in HCC [20], and similar results were reported in breast cancer by Yang *et al.* [10]. However, the exact mechanism of NQO1 in the transition from glycolysis to lipolysis in tumors needs further in-depth study.

It is widely known that the key enzymes of FAO are closely related to the occurrence and development of multiple tumors, especially pancreatic cancer [28]. Zhao *et al.* [30] found that LCAD catalyzes a key step in mitochondrial FAO and plays an important tumor promoter role in esophageal squamous carcinoma. The literature indicates that MCAD can promote the EMT process of breast cancer cells and improve their migration and invasion abilities [31]. CPT1A is the first 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 [32]. Several studies have exploited that CPT1A facilitates FAO, contributing to ATP and NADPH production [33, 34]. Here, the results of correlation analysis Co-IP and protein ubiquitination experiments showed that NQO1 has a stronger correlation with CPT1A and interacts with CPT1A. Further study revealed that depletion of CPT1A could consequently inhibit the FAO pathway and decrease NQO1-induced cell growth and motility in PAAD cells. One hypothesis is consistent with our data (Figure 6), and iRGD-modified exosomes effectively deliver CPT1A siRNA to colon cancer cells, reversing oxaliplatin resistance by regulating FAO [35]. Huang *et al.* [36] demonstrated that a lack of CPT1A could result in reduced levels of FAO and ATP, thereby exacerbating the oxidative stress induced by platinum. Moreover, our results extend these findings by demonstrating that CPT1A could reverse the tumor promotion of NQO1 and can also be a cell autonomous trait associated with tumor cell regulation of rate-limiting enzymes involved in FAO (e.g., MCAD and LCAD). In terms of mechanism, this study revealed that NQO1/FAO could influence CPT1A stability by ubiquitylation, and the NQO1/CPT1A/FAO axis could regulate the growth, EMT, and a certain degree of lipid catabolism of PAAD cells.

Collectively, we demonstrated that NQO1/CPT1A is frequently overexpressed in PAAD tissues and that their overexpression is related to the poor prognosis of patients with PAAD. Furthermore, we provided a conceptual framework in which NQO1 overexpression promotes FAO in a CPT1A-dependent manner. NQO1/CPT1A/FAO accelerates proliferation and EMT by enhancing lipid catabolism in PAAD. Importantly, these findings indicate that the NQO1/CPT1A/FAO axis is closely related to the progression of

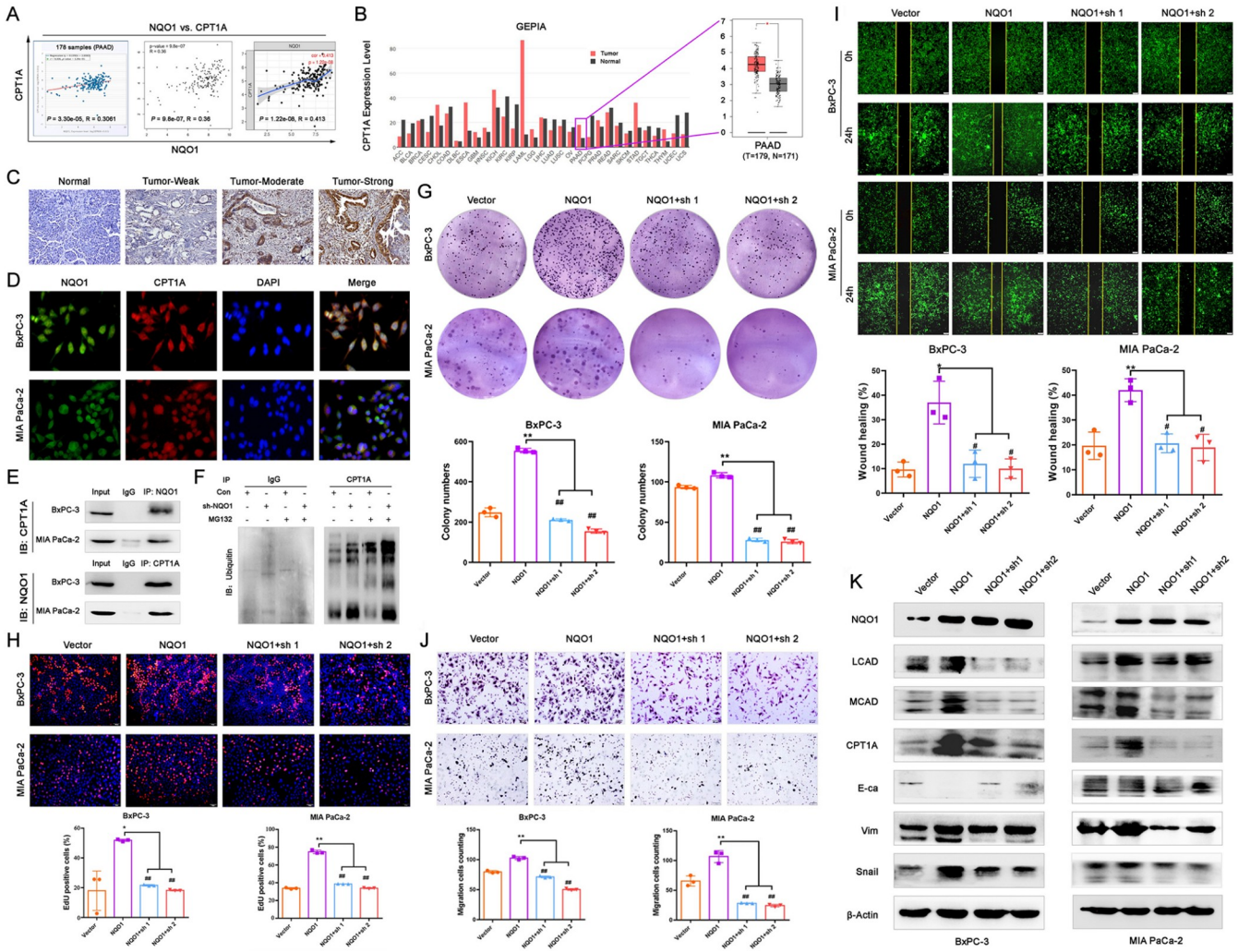

Figure 6. The NQO1/CPT1A axis promotes the development of PAAD (A) ChIPBase, GEPIA and TIMER databases were used to analyse the relationship between NQO1 and CPT1A. (B) The GEPIA database showed the expression level of CPT1A in tumor tissues and normal tissues. (C) IHC staining of CPT1A in PAAD and paired normal pancreatic tissues. (D) IF showed the colocalization of NQO1 and CPT1A in the nucleus and cytoplasm. (E) Coimmunoprecipitation detected the interaction between NQO1 and CPT1A. (F) The ubiquitination of CPT1A was examined by ubiquitin experiments. (G,H) The proliferation ability of NQO1-overexpressing cells after CPT1A knockdown was detected by colony formation and EdU assays. (I) The lateral migration ability of cells was detected by wound healing assay. (J) Transwell experiments showed the longitudinal migration ability of cells. (K) Western blot analysis demonstrated the changes in EMT and FAO marker expression after CPT1A knockdown. $^{*}P<0.05$ compared with the vector group and $^{\#}P<0.05$ compared with the NQO1 group.

PAAD and might be a potential biomarker for prognostic evaluation and targeted therapy of patients with PAAD.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biphysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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