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Original Articles

NAD(P)H: Quinone oxidoreductase 1 overexpression in hepatocellular carcinoma potentiates apoptosis evasion through regulating stabilization of X-linked inhibitor of apoptosis protein

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ABSTRACT

NAD(P)H: quinone oxidoreductase 1 (NQO1) is an antioxidant enzyme which is associated with poor prognosis in human breast, colon, lung and liver cancers. However, the molecular mechanisms underlying the pro-tumorigenic function of NQO1 remains unclear. This study investigated the function of NQO1 in the context of hepatocellular carcinoma (HCC) development. We found that NQO1 was frequently up-regulated in human liver cancer, and its high expression level was correlated with the tumor stage and low survival rate of HCC patients. Loss-of-function of NQO1 inhibited growth in HCC cells with increased apoptosis *in vitro*, and suppressed orthotopic tumorigenicity *in vivo*. Mechanistically, high level of NQO1 in HCC cells enhanced protein stability of X-linked inhibitor of apoptosis protein (XIAP) by increasing its phosphorylation at Ser 87. Reintroduction of wile type XIAP and the phospho-mimic mutants $XIAP^{S87D}$ significantly reversed NQO1 knock-down/out induced growth inhibition and apoptosis. In mouse model with orthotopically implanted hepatocarcinoma, NQO1 suppression and NQO1 inhibitor suppressed tumor growth and induced apoptosis. NQO1 plays an important role in sustaining HCC cell proliferation and may thus act as a potential therapeutic target in HCC treatment.

1. Introduction

Being the second-leading cause of cancer death among males in the world, hepatocellular carcinoma (HCC) is one of the most common fatal cancers worldwide [1]. With an estimated number of 782,500 new liver cancer cases and 745,500 deaths occurred worldwide in 2012, China accounted for about 50% of the total number of cases and deaths [1]. Most HCC patients are diagnosed at a late stage when it is no longer amenable to curative surgical resection or liver transplantation due to late symptom presentation and frequent metastasis. Currently, treatment options for inoperable HCC patients are very limited. The multi-kinase inhibitor sorafenib is the only FDA-approved drug available for advanced HCC patients. The survival benefit of sorafenib, however, is unsatisfactory. Evasion of death is a hallmark of cancer cells and is crucial for both carcinogenesis and resistance to anticancer therapies. Defects in apoptotic pathways contribute to tumor development and sustained tumor growth. Importantly, apoptosis is one of the major cancer cell death mechanisms in anti-cancer therapy, therefore, understanding the molecular characteristics and mechanisms in death evasion of cancer cells is required for identifying new therapeutic

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targets for HCC.

NAD(P)H: quinone oxidoreductase 1 (NQO1) is a homodimeric flavoprotein that catalyzes the two-electron reduction of quinones to hydroquinones and is a key component of cellular antioxidant defense system [2]. NQO1 is upregulated in many human cancers, including lung cancer [3], breast cancer [4], uterine cervical cancer [5], cholangiocarcinoma [6], pancreatic cancer [7] and prostate cancer [8]. Upregulation of NQO1 protects cells against various cytotoxic quinones and oxidative stress. NQO1-deficient mice show increased sensitivity to chemical-induced skin cancer [9,10]. Nrf2-induced upregulation of NQO1 in renal epithelial cells protects cells from ischemia-reperfusion injury [11]. NQO1 inhibition alters redox homeostasis in prostate cancer cells which promotes androgen-independent cell survival via opposing effect on NF-κB and p53 function [8]. In addition to its oxidoreductase activity, NQO1 has been shown to stabilize variety of proteins by inhibiting their proteasomal degradation. NQO1 binds to HIF1α and inhibits its polyubiquitination and proteasome-mediated degradation in colorectal cancer and breast cancer cells [12]. While NQO1 inhibits the degradation of tumor suppressor $p33^{ING1b}$ in melanoma cells, ultraviolet irradiation stabilizes $p33^{ING1b}$ by inducing its phosphorylation which enhances the interaction between $p33^{ING1b}$ and NQO1 [13]. However, the molecular pathogenesis of NQO1 in hepatocellular carcinoma have not been explored.

In this study, we reported that NQO1 was frequently upregulated in a subset of HCC tissues and cell lines. Furthermore, we identified the oncogenic function of NQO1 in facilitating both *in vitro* and *in vivo* growth of HCC cells through XIAP–dependent apoptotic pathway. Collectively, this study reveals a novel mechanism that implicates NQO1 as an important player in the development of HCC.

2. Materials and methods

2.1. Cell culture and human samples

HepG2 and PLC/PRF/5 cells were obtained from American Type Culture Collection. Huh-7 cell line was obtained from the Heath Science Research Resource Bank. MIHA cell line was obtained from Professor Ben C.B. Ko (The Hong Kong Polytechnic University). Huh-7 and PLC/PRF/5 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco BRL). HepG2 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum. All cells were cultured in a humidified incubator at 37 °C with 5% CO2. Cells were authenticated by short-tandem repeat (STR) fingerprinting by Beijing Microread Genetics Company Limited recently.

All HCC samples and their adjacent non-tumorous liver tissues were collected from Chinese patients at the Frist Affiliated Hospital of Chongqing Medical University. After surgical resection, all specimens were either snap-frozen immediately in liquid nitrogen and stored at −80 °C or fixed in buffered 10% formalin for paraffin embedding. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of Chongqing Medical University.

2.2. Plasmids and antibodies

NQO1 short hairpin RNA (shNQO1-1 and shNQO1-2), or non-targeting shRNA (shCont) were purchased from Shanghai Genechem Company Limited. Sequences of shNQO1-1 and shNQO1-2 are 5′-CGA GTCTGTTCTGGCTTATAA-3'and 5′-CATGTTATCAAATCTGGGTAT-3'. The pCMV6-NQO1 plasmid (SC119599) was purchased from OriGene Technologies (Rockville, MD). pcDNA3-Xiap-Myc (#11833) was obtained from Addgene (Cambridge, MA). Mouse anti-NQO1 (#3187), rabbit anti-XIAP (#14334), rabbit anti-Cleaved PARP (#5625) were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-GAPDH (sc-365062) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-XIAP (TA325979S) was purchased from OriGene Technologies. Rabbit anti-NQO1 (11451-1-AP) was purchased from Proteintech (Chicago, USA). Dicoumarol (287897) was purchased from MERCK. Cycloheximide (97064–722) was purchased from AMRESCO (WA, USA). DCPIP(33125) were purchased from Sigma. Blasticidin S HCl (R210-10) was purchased obtained from Invitrogen (Carlsbad, CA). NEMI(A600450) was purchased from Sangon Biotech (Shanghai, China).

2.3. Co-immunoprecipitation assay and immunoblotting analysis

For immunoprecipitation, cells were lysed with RIPA lysis buffer containing inhibitor cocktail (Roche Diagnostics). The cell lysates were then precipitated using protein magnetic beads (Millipore) according to the manufacturer's instructions. The bound proteins were resolved by SDS-PAGE and immunoblotted with antibodies as indicated. Signals were detected by ECL western blotting reagents (Millipore). Signal intensity was quantified by ImageJ software (National Institutes of Health).

2.4. RNA extraction and real-time quantitative PCR

Total RNA extraction and cDNA synthesis were carried out as described before (10). Relative quantification of gene expression was carried out by using SYBR green (Roche, Germany) with β-actin mRNA as an internal control. Values represent the means ± the standard deviations (SD) of three independent experiments. The expression values of target genes were calculated using the $2^{-\triangle\triangle Ct}$ method. The primers used were listed in the Supplementary Table S1.

2.5. Cell proliferation assay and cell apoptosis

Cell proliferation in response to NQO1 silencing or overexpressing was determined by trypan blue exclusion assay (Thermo Fisher Scientific, Waltham, MA). DNA synthesis was examined using Click-iT® EdU Imaging Kit (Invitrogen). Cell apoptosis was detected by flow cytometry with propidium iodide (PI)/Annexin V double staining.

2.6. Immunohistochemistry

The paraffin-embedded sections were deparaffinized and rehydrated. Tissue antigens were retrieved with a microwave oven in sodium citrate buffer (10 mmol/L, pH 6.0), followed by quenching of endogenous peroxidase activity by hydrogen peroxide. The sections were incubated with primary antibodies (anti-NQO1, 1:50; anti-XIAP, 1:50; anti-cleaved PARP, 1:100; anti-Ki67, 1:100). DAB staining was used for detecting immunoreactivity (Dako, Carpinteria, CA). Counterstaining was performed using hematoxylin. The scoring of NQO1 in HCC tissues was carried out by two independent pathologists according to the proportion of tumor cells with positive cytoplasmic staining: 0 (< 10%), 1(10–30%), 2(30–50%), 3(> 50%).

2.7. In vivo orthotopic liver injection model

Five or six-week-old male BALB/c nude mice were used for *in vivo* experiments. Briefly, 1×10^6 Huh-7 cells were resuspended in 30 μl DMEM/Matrigel (1:1 mixture). The left lobe of liver was gently exposed and the resuspended cells were injected into lobe after anaesthetization. Immunohistochemistry was performed and integrated optical density (IOD) was measured as reported previously [14].

2.8. Study approval

All animal experiments were performed according to the protocol approved by the Chongqing Medical University Animal Care Committee and in accordance with NIH guidelines.

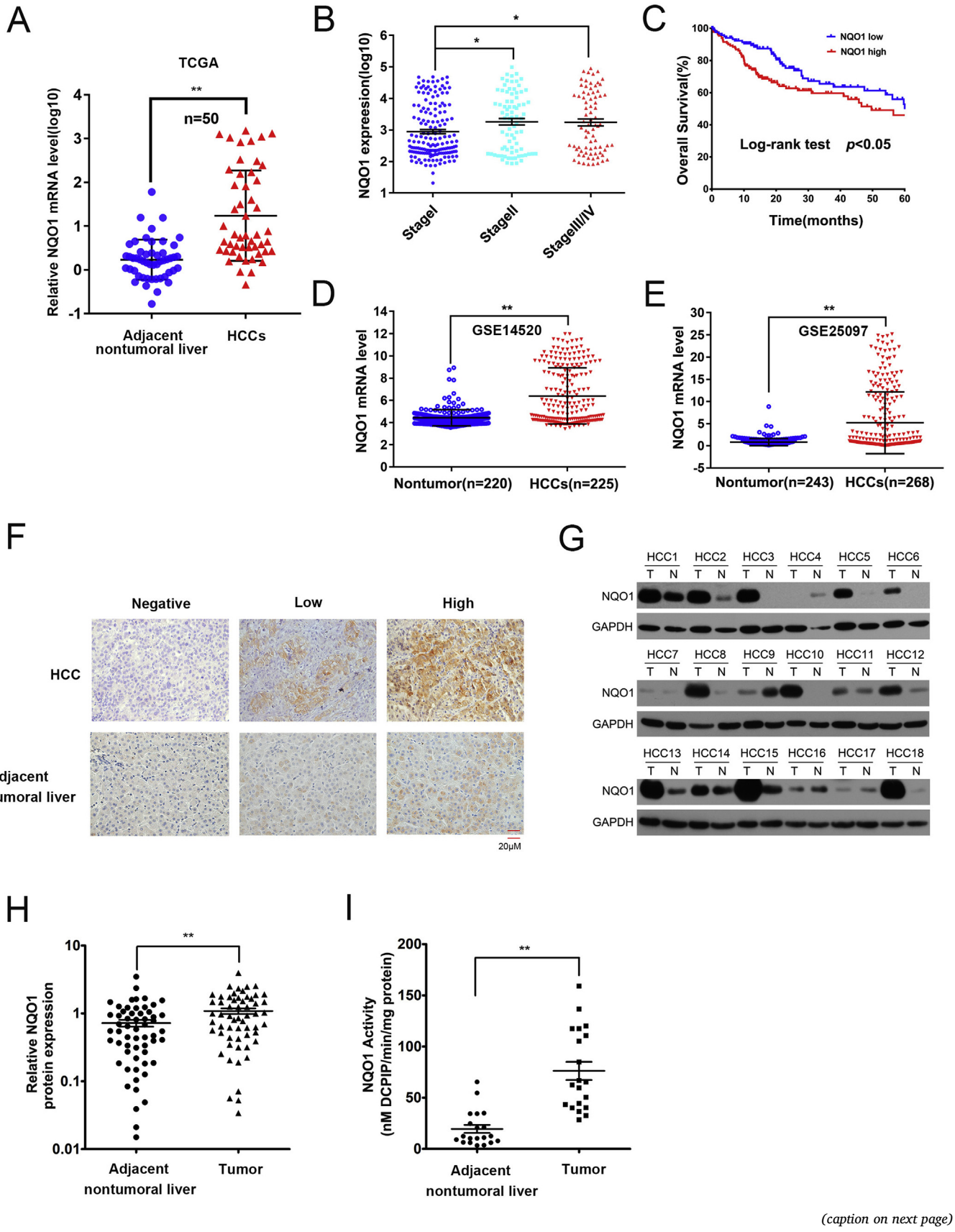

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Fig. 1. NQO1 expression in human HCC. (A) The mRNA level of NQO1 in 50 paired HCC tissues and paired adjacent non-tumoral liver of TCGA database. (B) Relative NQO1 expression in the TCGA cohort with different tumor stages (I or II or III-IV). (C) Kaplan-Meier analysis of the correlation between NQO1 expression and overall survival in the TCGA cohort. Log-rank tests were used to determine statistical significance. (D–E) The NQO1 expression in the GEO cohort. The microarray data was obtained from the NCBI GEO database (accession numbers GSE14520; GSE25097). The relative expression of NQO1 mRNA in noncancerous tissues (Nontumor) and patients with HCC (Tumor) were illustrated by scatterplot. **p < 0.01. (F) Representative image of IHC staining of NQO1 in 60 pairs primary HCC tissues and their adjacent non-tumoral liver tissues. Magnification, × 400. (G) Immunoblotting analysis of NQO1 expression in 18 representative HCC tissues (T) and adjacent non-tumoral tissues (N). GAPDH was used as a loading control. (H) Quantitative analysis of NQO1 protein levels in paired HCC tissues(n = 60). (I) NQO1 activity was measured in paired frozen HCC tissues and adjacent non-tumoral livers as the rate of dicoumarol-inhibitable DCPIP reduction.

2.9. Statistical analysis

The independent *t*-test was used for continuous parametric data, and Mann-Whitney *U* test was used for continuous nonparametric data. Correlations between NQO1 and individual clinicopathologic parameters were evaluated using a nonparametric chi-square test. The Kaplan-Meier method was used to estimate the survival rates for NQO1 expression. Equivalences of the survival curves were tested by log-rank statistics. A value of $p < 0.05$ was considered significant (*$p < 0.05$; **$p < 0.01$). All statistical analyses were performed using SPSS 19.0.

3. Result

3.1. NQO1 is frequently upregulated in human HCCs

From RNA-sequencing data on 50 pairs of primary human HCCs and their adjacent non-tumoral livers of The Cancer Genome Atlas (TCGA), we found that NQO1 was one of the leading genes that was significantly upregulated in HCC tissues (Fig. 1A). By analyzing the correlation between NQO1 expression and clinicopathological features using TCGA HCC data set, we found that NQO1 expression increased with HCC development from stage I to III/IV (Fig. 1B). Importantly, patients with high NQO1 expression levels in tissue had significantly shorter overall survival rates than those with low NQO1 expression ($p < 0.05$, Fig. 1C). In addition, by interrogating two public gene expression data sets from the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) (accession number: GSE14520; GSE25097), high expression of NQO1 was also found in HCC samples compared with adjacent non-tumoral livers tissues (Fig. 1D and E).

Next, we further confirmed the expression of NQO1 in another 60 paired paraffin-embedded HCC tissues and adjacent non-tumoral livers collected in our laboratory by using immunohistochemistry. Immunohistochemical detection showed cytoplasmic staining of NQO1 in HCC tissues (Fig. 1F). NQO1 immunoreactivity was graded as negative (score 0), low (scores 1–2) or high (score 3). Positive NQO1 staining was 29 out of 60 (48.3%) as observed in HCC tissues, whereas the positive staining was 7 out of 60 (11.6%) as found in adjacent non-tumoral livers. NQO1 expression was further analyzed in 60 pairs of frozen HCC and adjacent non-tumoral liver tissues by using immunoblotting analysis. The results showed that NQO1 overexpression was 62% (37 out of 60) as detected in HCCs compared with their adjacent non-tumoral liver tissues (Fig. 1G and Supplementary Fig. S1). The average protein level of NQO1 in HCC group was significantly higher than that in adjacent non-tumoral group (Fig. 1H). Furthermore, the enzymatic activity of NQO1 was examined in 20 paired frozen HCC tissues and adjacent non-tumoral livers. Higher NQO1 activity was observed in tumor tissues compared to non-tumoral liver tissues (Fig. 1I).

3.2. Loss function of NQO1 inhibits proliferation and induces apoptosis in HCC cells

The above data suggested that NQO1 might play an important role in HCC development. Accordingly, we investigated the function of NQO1 in HCC cells using both *in vitro* and *in vivo* assays. To address the function of NQO1 in cell proliferation, we suppressed NQO1 expression via lentivirus-mediated shRNA interference or CRISPR/Cas9-mediated knock-out (Fig. 2A and B and Supplementary Fig. S2). NQO1 knock-down/knock-out significantly decreased cell growth in 3 HCC cell lines (PLC/PRF/5, Huh-7, and HepG2) (Fig. 2A and B). Soft agar assay indicated that NQO1 knock-down/knock-out cells exhibited decreased anchorage-independent growth of HCC cells (Fig. 2C). Flow cytometry was further performed to determine whether NQO1 played a role on the regulation of cell death. Annexin V/PI assay confirmed that NQO1 knock-down or knock-out significantly increased apoptotic rate in PLC/PRF/5 and Huh-7 cells (Fig. 2D).

We further examined whether NQO1 could regulate tumor growth of HCC *in vivo*. NQO1 knock-out Huh-7 cells or control cells were xenografted into nude mice subcutaneously. The volume and weight of tumors derived from NQO1-depleted cells were reduced significantly compared with control cells (Fig. 3A and B). An orthotopic liver implantation experiment was performed to examine the effect of NQO1 knock-out in HCC tumorigenicity. Interestingly, NQO1 knock-out reduced growth of tumors formed in the liver compared to control (Fig. 3C). Immunohistochemistry assay confirmed that tumor formed by NQO1 knock-out cells showed decreased staining of a cell proliferation marker (Ki67), and increased staining of cleaved-PARP (Fig. 3D).

3.3. Ectopic expression of NQO1 promotes cell growth in immortalized liver cell line

We analyzed the cellular gain-of-function phenotype in immortalized liver cell line (MIHA) to address the oncogenic function of NQO1. Overexpression of wild type NQO1 significantly enhanced cell proliferation and colony formation abilities of MIHA cells, however, mutant NQO1 (Y127/129A) with reduced NADH-binding capacity had no effect on cell growth (Fig. 4A and B). Consistently, ectopic expression of NQO1 markedly enhanced DNA synthesis as measured by EdU staining (Fig. 4C). In addition, we examined the potential role of NQO1 in DNA-damaging agent doxorubicin-induced apoptosis. As expected, wild type NQO1 (but not mutant) caused cellular resistance to apoptosis in MIHA cells under doxorubicin treatment (Fig. 4D).

3.4. NQO1 stabilizes XIAP by increasing its phosphorylation

To explore the underlying molecular mechanism of how NQO1 regulates apoptosis, tandem mass tags (TMTs) quantitative mass spectrometry between NQO1 knock-out cells and control parental cells was performed. A total of 89 up-regulated proteins and 121 down-regulated proteins in NQO1 knock-out cells were identified. Gene ontology analysis showed that some proteins were significantly associated with regulation of cell death (Supplementary Fig. S3A). The KEGG pathway analysis further showed that NQO1 knock-out primarily affected the signaling pathways protein sets, such as ECM-receptor interaction, PI3K-Akt signaling pathway and apoptosis (Supplementary Fig. S3B). Among the altered proteins that are involved in regulation of apoptosis, XIAP was the leading gene that was significantly downregulated in NQO1 knock-out cells (Supplementary Fig. S3C). We then performed real-time PCR and western blot analysis to validate the above screening data. NQO1 knock-down/knock-out resulted in markedly decreased protein level of XIAP in HCC cells, whereas no effect was observed on

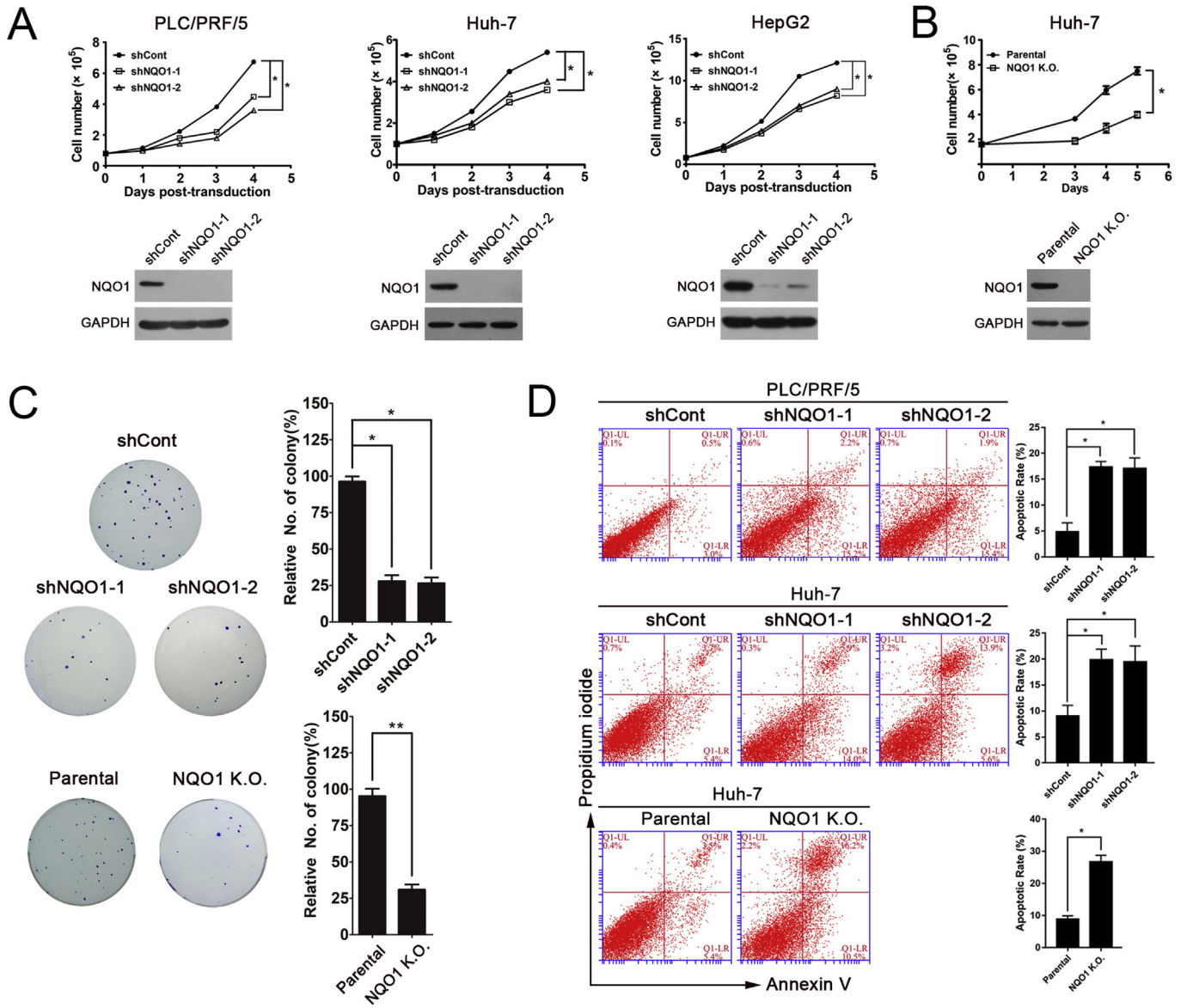

Fig. 2. NQO1 knock-down/knock-out inhibits HCC cell proliferation *in vitro*. (A) Trypan blue exclusion assay for PLC/PRF/5, Huh-7 and HepG2 cells transduced with lentivirus expressing NQO1-targeting shRNA (shNQO1-1 and shNQO1-2) versus control (shCont) shRNA. Cell numbers were determined at the indicated number of days. Plots represented cumulative cell numbers versus days in culture. (B) The trypan blue exclusion assay for NQO1 knock-out Huh-7 cells. (C) Soft agar assay NQO1 knock-down/knock-out cells were grown for 3 weeks under antibiotic selection. Columns showed the mean values of triplicate experiments. (D) NQO1 knock-down/knock-out HCC cells were analyzed by flow cytometry after annexin V/PI staining. Data are mean ± SEM of n = 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

XIAP mRNA level (Fig. 5A and B). In contrast, NQO1 restoration in NQO1 knock-out cells increased XIAP protein level (Fig. 5B). To further validate the effect of NQO1 on XIAP expression, pharmacologic inhibitor dicoumarol that competing with NAD(P)H for binding to NQO1 was used. Consistently, dicoumarol treatment resulted in decreased expression of XIAP in HCC cells (Fig. 5C). These data demonstrated that NQO1 exerted its regulation function on XIAP at post-transcription level. XIAP has been reported to directly inhibit active caspase-7 and caspase-9 [15,16]. Consistently, decreased XIAP expression was accompanied with increased active caspase-7 and caspase-9 in NQO1 knock-down/knock-out cells (Fig. 5D). Next, we sought to determine whether NQO1 affected XIAP protein stability and NQO1 knock-down cells were treated with cycloheximide to block *de novo* protein synthesis. The half-life of XIAP protein was significantly decreased in NQO1 knock-down cells when compared to its control cells (Fig. 5E), indicating the stabilization of XIAP protein by NQO1. The effect of NQO1 depletion on XIAP was blocked in the presence of proteasome inhibitor MG132 (Fig. 5F), suggesting NQO1 stabilized XIAP protein via inhibiting proteasome-mediated degradation.

Phosphorylation of XIAP determines its protein stability and autoubiquitination activity. Thus, we examined whether NQO1 had an effect on XIAP phosphorylation in HCC cells. Depletion or deficiency of NQO1 decreased phospho-XIAP (pSer87) in HCC cells whereas NQO1 overexpression increased XIAP phosphorylation (Fig. 6A). Decreased phospho-XIAP was also observed in dicoumarol-treated cells (Fig. 6B). To further determine whether Ser87 of XIAP was involved in NQO1 signaling, we mutated XIAP Ser87 or Ser430 to a non-phosphorylatable alanine residue. NQO1 overexpression increased wild type XIAP and $XIAP^{S430A}$ protein, whereas it had no effect on $XIAP^{S87A}$, confirming that phosphorylation of XIAP at Ser87 was a physiological target of NQO1 (Fig. 6C). These findings suggest that the anti-apoptotic activity of NQO1 may be associated with the inhibition of XIAP phosphorylation

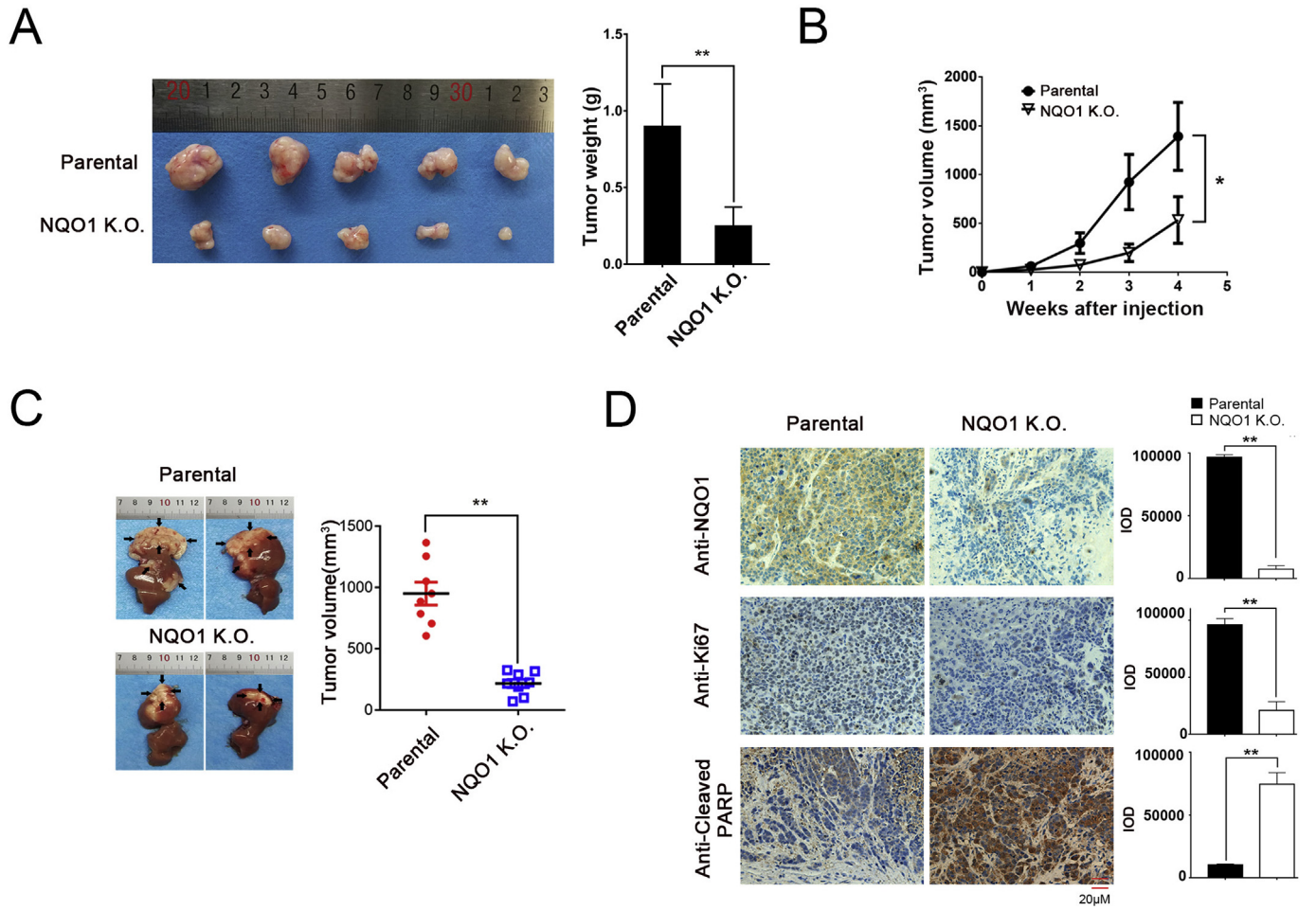

Fig. 3. NQO1 knock-out suppresses orthotopic tumorigenicity *in vivo*. (A–B) The effect of NQO1 knock-out on subcutaneous tumor growth. Huh-7 NQO1 knock-out cells or parental cells were injected subcutaneously into 5-week-old nude mice. At 4 weeks after implantation, the animals were sacrificed, and the tumor masses were excised. Tumor weights(A) and tumor volume (B) in different groups of nude mice over a 4-week time course were analyzed. Data are mean ± SEM. (C) The effect of NQO1 knock-out on orthotopic liver cancer. Tumor volume was measured and analyzed at 35 days after orthotopic injection with indicated Huh-7 cells into the liver of nude mice. (D) Representative images of immunohistochemical staining of NQO1, Ki67 and cleaved PARP in tumor xenografts counterstaining with hematoxylin. Magnification, × 400. Data are mean ± SEM.

and expression. To test this possibility, we overexpressed XIAP expression in PLC/PRF/5 cells that were stably expressing shNQO1. Ectopic expression of wild type XIAP (but not $XIAP^{S87A}$) significantly restored cell proliferation and antagonized apoptosis induced by NQO1 silencing (Fig. 6D). The phospho-mimic mutants $XIAP^{S87D}$ exhibited higher ability to increase cell growth and decrease apoptosis of NQO1-depleted cells (Fig. 6E). These data suggested NQO1 regulates XIAP phosphorylation and protein stability.

3.5. NQO1 deficiency or inhibitor suppressed tumor growth of HCC in vivo

To further verify the oncogenic function of NQO1, NQO1 knock-out cells overexpressing either XIAP or empty vector, or parental cells were orthotopically injected into nude mice. In this model, we proved that knock-out of NQO1 significantly reduced the size of HCC tumors formed in the orthotopic liver microenvironment compared to the parental cells (Fig. 7A). Importantly, reintroduction of XIAP blocked the growth inhibitory effect in tumors formed by NQO1 knock-out cells (Fig. 7A). NQO1 knock-out in tumor tissues was associated with increased level of cleaved PARP and decreased level of the cell proliferation marker ki67, and these effects were partially reversed by XIAP (Fig. 7B).

The anticancer activity of pharmacologic inhibitor dicoumarol was examined *in vivo*. Starting from day 14 post-tumor implantation, dicoumarol was administered i.p. (34 mg/kg) and was supplemented continually in the drinking water (180 mg/L). Compared to the control group, administration of dicoumarol effectively reduced tumor growth (Fig. 7C). Dicoumarol reduced the level of ki67 but increased cleaved-PARP level in the tumors (Fig. 7D).

3.6. The correlation between NQO1 and XIAP expression in clinical tissues

To further investigate the regulation of XIAP by NQO1, we analyzed the mRNA levels of NQO1 and XIAP in HCC samples and compared with adjacent nontumoral livers tissues by interrogating 2 public gene expression data sets from the GEO database. Correlative analysis confirmed no correlations between NQO1 and XIAP (Fig. 8A). The protein levels of NQO1 and XIAP were examined in 24 paired HCC samples and adjacent non-tumoral livers tissues (Fig. 8B and C). Interestingly, correlative analysis indicated that NQO1 protein showed a significant positive correlation with XIAP expression (Fig. 8B and C). These data demonstrated the correlation between NQO1 and XIAP in human HCC tissues.

4. Discussion

NQO1 is a ubiquitously expressed homodimeric flavoprotein that provides variety of cellular functions, such as xenobiotic detoxification,

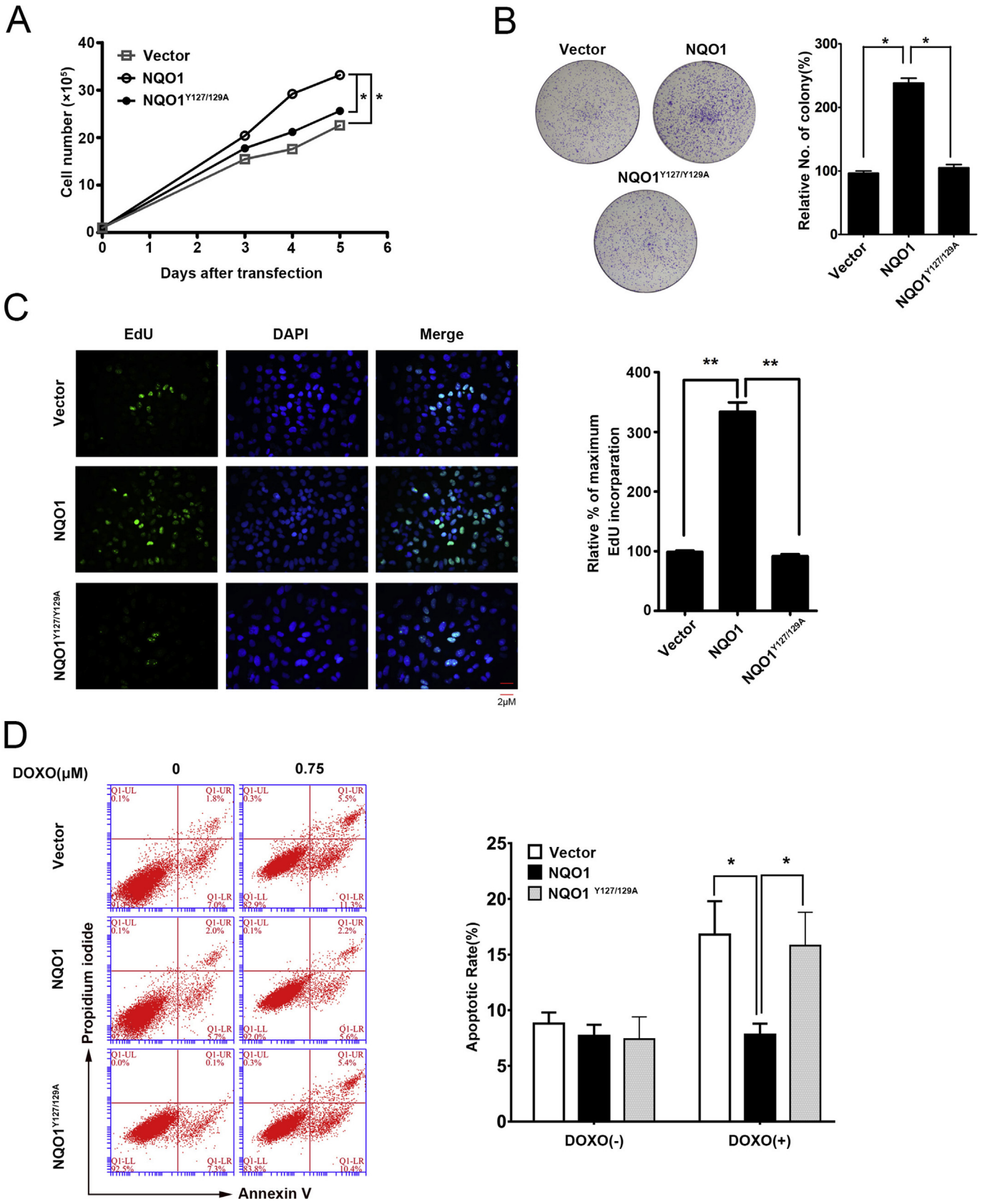

Fig. 4. NQO1 overexpression promotes proliferation of MIHA cell and depends on its binding to NADH. (A) Trypan blue exclusion assay for MIHA cells transfected with construct expressing wild type NQO1 or mutant NQO1(NQO1$^{Y127/129A}$) or empty vector. Cell numbers were counted at the indicated days. (B) Colony formation assay for MIHA cells transfected with the indicated plasmids. Cells were cultured for 2 weeks in the presence of blasticidin (3.5 μg/mL) and stained with crystal violet. Quantification of colonies were obtained from macroscopically visible colonies in each well and expressed as percentage relative to the control group. (C) EdU incorporation assay of MIHA cells overexpressing wild type NQO1 or mutant NQO1 (NQO1$^{Y127/129A}$) or empty vector. (D) MIHA cells overexpressing wild type NQO1 or NQO1$^{Y127/129A}$ or vector under treatment with doxorubicin (0.75 μM) for 48 h, were analyzed by flow cytometry after annexin V/PI staining. Data are mean ± SEM of n = 3 independent experiments.

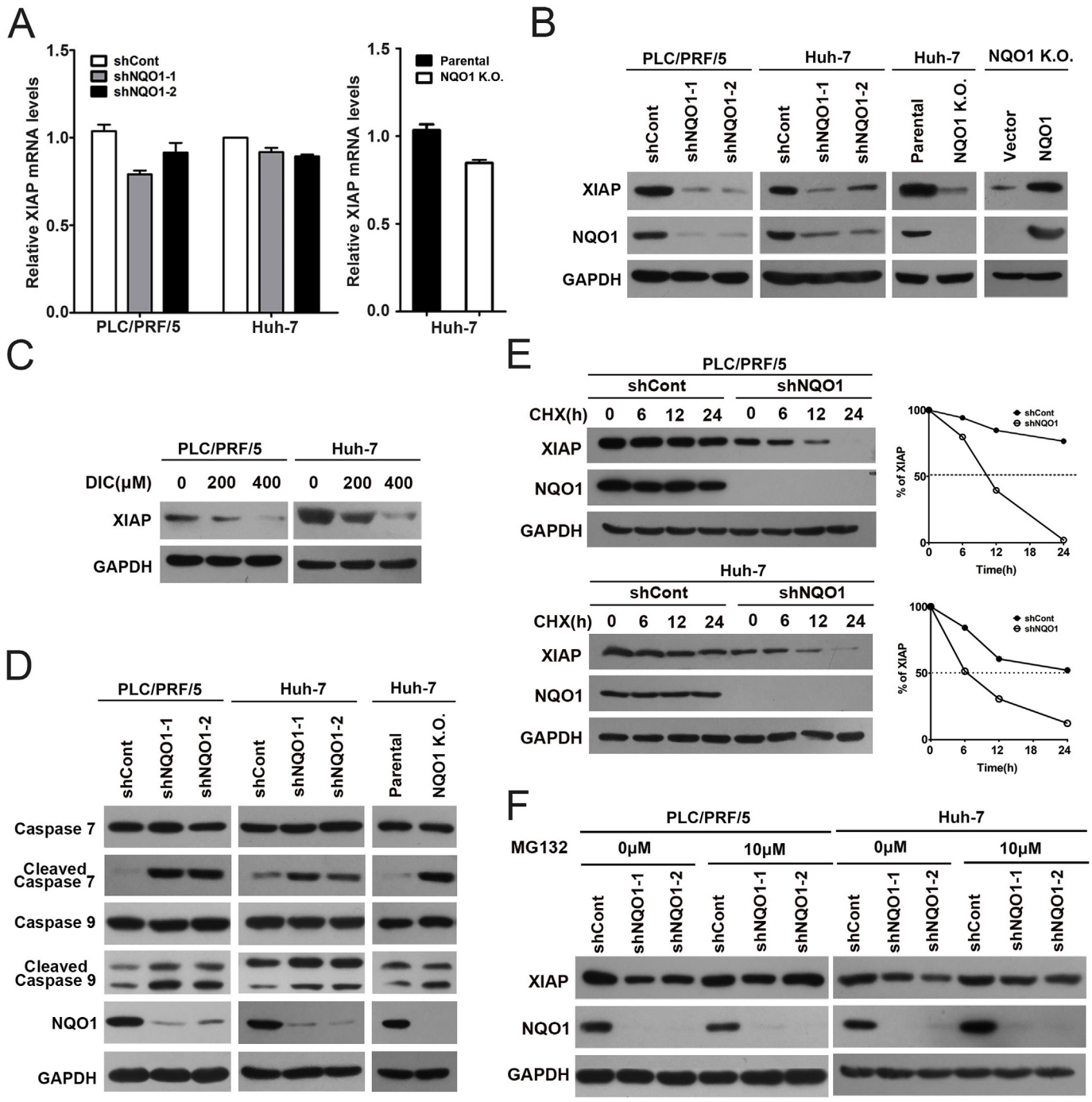

Fig. 5. NQO1 knock-down/out decreased the protein stability of XIAP (A) Real-time PCR for XIAP mRNA level in NQO1 knock-down or out cell. (B) Immunoblotting analysis for XIAP in NQO1 knock-down/knock-out cells or NQO1 knock-out cells transfected with vector expressing NQO1. GAPDH was used as a loading control. (C) Immunoblotting analysis for XIAP in PLC/PRF/5 and Huh-7 cells treated with dicoumarol (200 μM or 400 μM) for 4 h. (D) Immunoblotting analysis for caspase 7, cleaved-caspase 7, caspase 9 and cleaved-caspase 9 in NQO1 knock-down/knock-out cells. (E) The protein level of XIAP was examined by immunoblotting analysis. PLC/PRF/5 and Huh-7 cells were transduced with lentivirus expressing shNQO1 or shCont and incubated with 10 μg/ml cycloheximide, and then harvested at the indicated times. XIAP protein levels was quantified using ImageJ with band intensities normalized to GAPDH (band intensity at t_0 was defined as 100%). (F) Immunoblotting analysis for XIAP in NQO1 knock-down cells treated with proteasome inhibitor MG132 (10 μM) for 8 h.

superoxide scavenging, maintenance of endogenous antioxidants, and proteasomal degradation. Accumulating evidences have shown that NQO1 is expressed at a relatively high level in many solid tumors such as breast cancer [4], lung cancer [3], uterine cervical cancer [5], cholangiocarcinoma [6], pancreatic cancer [7] and prostate cancer [8]. High-level expression of NQO1 protein is significantly correlated with poor differentiation, lymph node metastasis, advanced clinical stage and poor prognosis [3,4]. Regarding its role in HCC, the polymorphism of NQO1 C609T has been implicated in the development of HCC [17]. Lin and colleagues recently have reported that NQO1 is significantly upregulated in HCC where its expression is significantly associated with tumor size, venous infiltration, late tumor stage and survival rate [18]. However, the function of NQO1 in HCC has not been well defined. Consistent with other studies, we found that NQO1 mRNA level was significantly upregulated in majority of HCC tissues compared with adjacent non-tumoral liver tissues by utilizing publicly available data

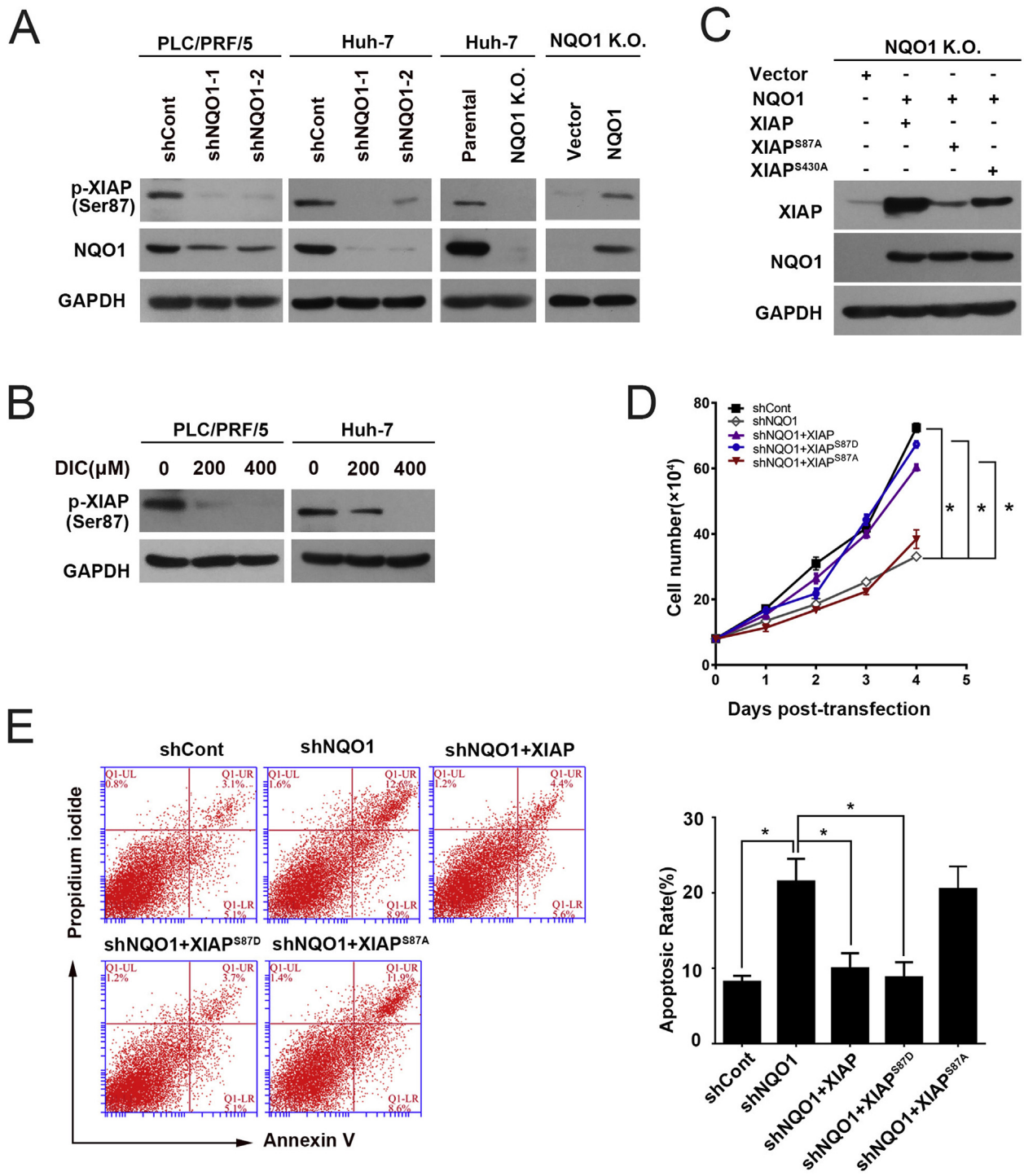

Fig. 6. NQO1 stabilizes XIAP by increasing its phosphorylation. (A) Immunoblotting analysis for phospho-XIAP (pSer87) in NQO1 knock-down/knock-out cells or NQO1 knock-out cells transfected with vector expressing NQO1. GAPDH was used as a loading control. (B) Immunoblotting analysis for phospho-XIAP (pSer87) in PLC/PRF/5 and Huh-7 cells treated with dicoumarol (200 μM or 400 μM) for 4 h. (C) Immunoblotting analysis for XIAP in PLC/PRF/5 cells co-transfected with NQO1 and wild type XIAP or XIAP^{S87A} or XIAP^{S430A}. (D–E) Trypan blue exclusion assay (D) and flow cytometry (E) were performed to analyze the NQO1-depleted PLC/PRF/5 cells transfected with either wild type XIAP, XIAP^{S87A} or XIAP^{S87D}. Data are mean ± SEM of n = 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sets. Increased NQO1 expression was significantly associated with tumor stage and poor overall survival rate. Concordantly, upregulation of NQO1 protein level was further confirmed in 60 paired HCC tissues and adjacent non-tumoral livers, in which NQO1 showed a mainly cytoplasmic staining pattern in HCC tissues. In addition, the enzymatic activity of NQO1 was also found to be markedly increased in HCC tissues compared to adjacent non-tumoral livers in this study. These data indicated that NQO1 might be an independent biomarker for prognostic evaluation of HCC, and it may act as an oncogene in the tumorigenesis and malignant progression of HCC.

The imbalance between proliferation and cell death is a pro-tumorigenic stimulation in human hepatocarcinogenesis [19]. The disruption of balance between death and survival is mainly due to overactivation of anti-apoptotic signals in HCC. At present, the function of

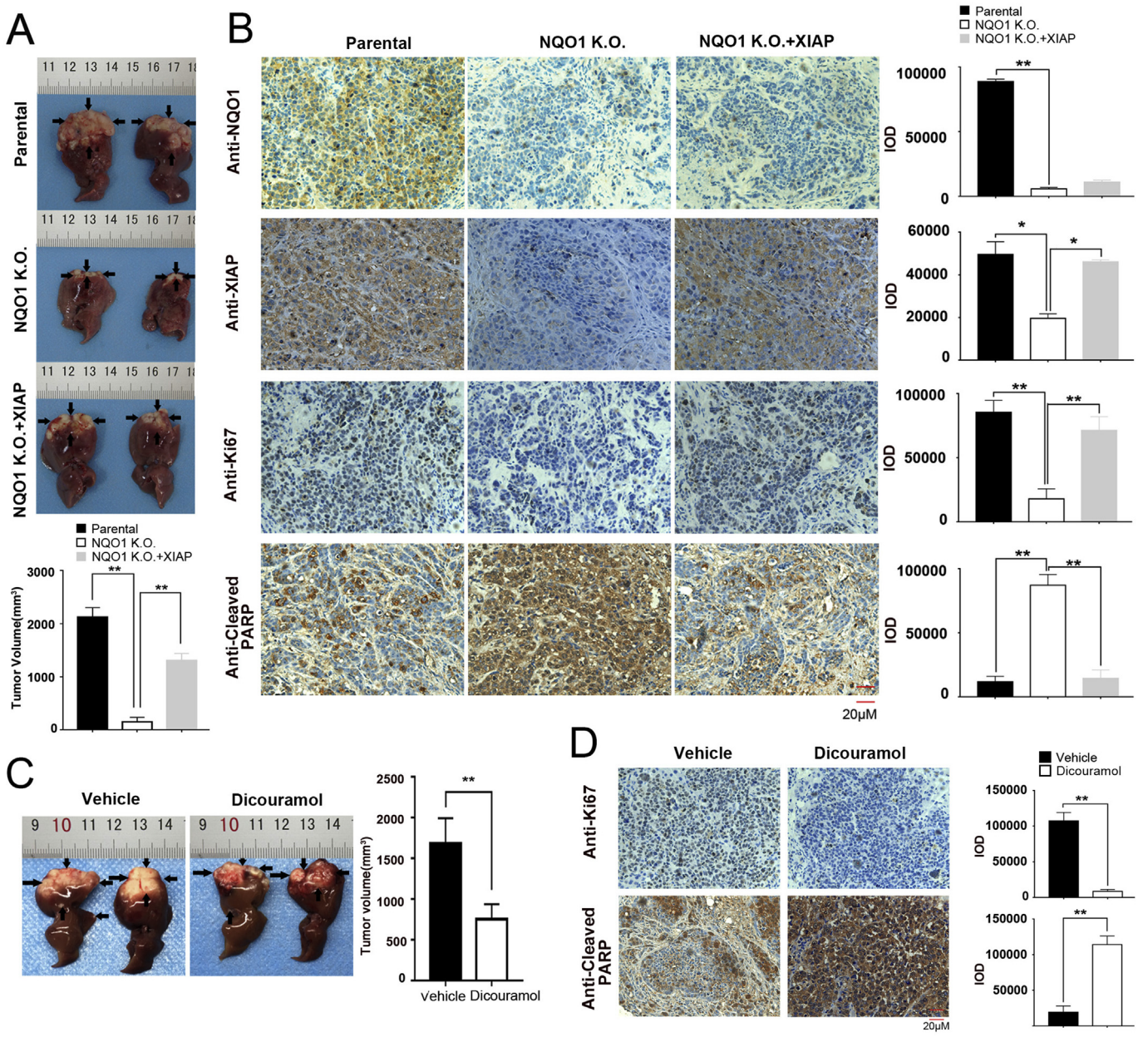

Fig. 7. Anticancer activity of NQO1 deficiency or inhibition in orthotopic tumor. NQO1 knock-out Huh-7 cells, parental cells or NQO1 knock-out cells stably overexpressing XIAP were injected orthotopically into 5-week-old nude mice. At 5 weeks after implantation, the animals were sacrificed, and the tumor masses were excised. (A) Representative images of tumors formed (Left panel) and tumor volume in different groups of nude mice (Right panel). (B) Representative images of immunohistochemical staining of NQO1, XIAP, Ki67 and cleaved PARP in tumor xenografts counterstaining with hematoxylin. Magnification, × 400. (C) Representative images of tumors formed and tumor volume in nude mice after administration of dicoumarol or vehicle. At day 14 post-tumor implantation, dicoumarol was administered i.p. (34 mg/kg) and was supplemented continually in the drinking water (180 mg/L). (D) Representative images of immunohistochemical staining of Ki67 and cleaved PARP in dicoumarol-treated or control tumor xenografts. Data are mean ± SEM.

NQO1 on cell survival and apoptosis remains controversial. Pharmacologic inhibitor of NQO1 protects the neuronal cells from AF64A induced-cell death [20] and blocks p53-mediated apoptosis in γ-irradiated normal thymocytes through inducing p53 degradation [21]. Moreover, NQO1-mediated activation of AMPK pathways contributes to apoptosis of cancer cell under oxygen-glucose deprivation [22]. Paradoxically, upregulation of NQO1 by nuclear factor-erythroid-related factor 2 (Nrf2) is involved in the cellular antioxidant defense system and anti-apoptosis process [23,24]. In this study, we found that NQO1 suppression or deficiency markedly inhibited cell proliferation and induced apoptosis in HCC cells, while NQO1 overexpression in immortalized liver cells caused resistance to chemotherapeutic agents-induced apoptosis. Our data support the oncogenic function of NQO1 as an anti-apoptotic factor in HCC cells, which might also contribute to the resistance of liver cells to therapeutic stress.

We further found that NQO1 suppression induces apoptotic process in HCC cells via promoting proteasome degradation of XIAP. XIAP is well known for its anti-apoptotic function in regulating cell survival, and as a direct and competitive inhibitor of the enzymatic activity of caspases through binding to their catalytically active sites [25]. XIAP contains three BIR domains and a RING motif at its C-terminus. Previous structural and biochemical studies have shown that the linker preceding the BIR2 domain of XIAP directly blocks the active sites of caspase-3 and caspase-7, while the BIR3 domain sterically hinders caspase-9 dimerization and its activation. The RING domain of XIAP may act as an E3 in the ubiquitination pathway to mediate auto-

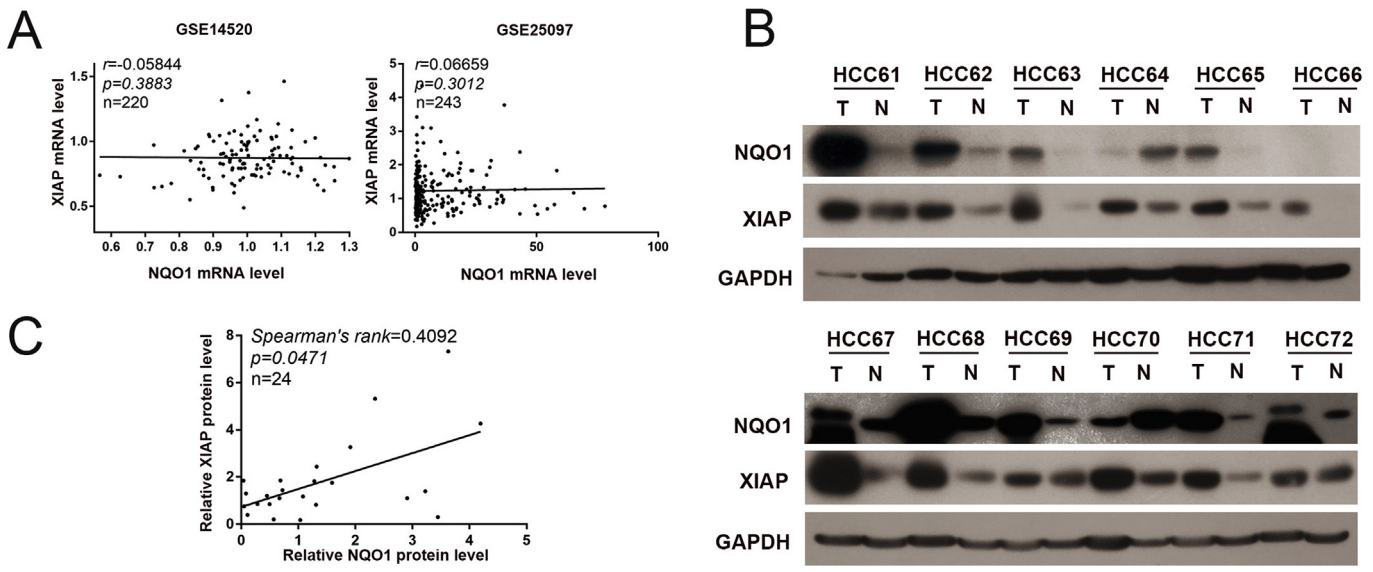

Fig. 8. The correlation between NQO1 and XIAP expression in clinical tissues (A) Correlation analysis for the mRNA levels of NQO1 and XIAP in HCC samples and adjacent non-tumoral livers tissues by interrogating two public gene expression data sets from the GEO database (accession number: GSE14520; GSE25097). (B) Immunoblotting analysis of NQO1 and XIAP expression in 12 representative HCC tissues (T) and adjacent non-tumoral tissues (N). (C) Correlation analysis for the protein levels of NQO1 and XIAP in 24 paired HCC samples and adjacent non-tumoral liver tissues.

ubiquitination and ubiquitination of proteins involved in apoptosis [26,27], such as caspase-3 [28], Smac [29] and apoptosis-inducing factor (AIF) [30]. In this study, we found that NQO1 suppression or NQO1 inhibitor reduced XIAP protein level via proteasome-mediated degradation. Phosphorylation of XIAP determines its protein stability and modulated its autoubiquitination/ubiquitination activity. XIAP has been reported to be phosphorylated at serine 87 by AKT or serine 430 by TANK-binding kinase 1 (TBK1) or IκB kinase IKK [31,32]. Mechanistic study further revealed NQO1 deficiency decreased the level of phosphorylated XIAP at ser87 in the first BIR domain (BIR1). Moreover, decreased XIAP expression was accompanied with increased active caspase-7 and caspase-9 in NQO1 knock-down/knock-out cells. Previous study showed the interaction between the BIR1 domain of XIAP and the N-terminal domain of TAB1, which is an upstream adapter for the activation of the kinase TAK1 [33]. Structure analysis found that BIR1 forms a conserved dimer and this dimer is observed in the crystal structure of the BIR1–TAB1 complex. Disruption of BIR1 dimerization abolishes XIAP-mediated TAK1 activation [34]. Interestingly, AKT phosphorylated XIAP at S87, a residue near the BIR1 dimerization interface and phosphorylation of S87 has been reported to modulate BIR1 dimerization. Therefore, it is possible that XIAP protein stabilization by S87 phosphorylation may be due to inhibition of BIR1 dimerization. On the other hand, BIR2 and BIR3 are critical for the interaction with either caspase- 3 and -7 (BIR2) or caspase-9 (BIR3), respectively. In this study, increased active caspase-7 and caspase-9 were observed in NQO1 knock-down/knock-out cells with decreased XIAP phosphorylation. One possibility is that reduced phosphorylation of XIAP in NQO1 knock-down/knock-out cells resulted in decreased XIAP protein, which consequently led to decreased XIAP binding to active effector caspases and thereby increased apoptosis. However, whether the phosphorylation of XIAP at S87 can affect the ability of XIAP to bind to active effector caspases needs further investigation. Collectively, these data revealed that NQO1 overexpression in HCC cells facilitates the survival of tumor cells via stabilizing XIAP protein to restrict the apoptotic pathway. However, the mechanism by which NQO1 regulated phosphorylation of XIAP needs our further investigation.

In conclusion, we have characterized the oncogenic function of NQO1 in HCC where NQO1 exerts its pro-tumorigenic function through regulating XIAP signaling cascade. Our findings highlight the importance of the NQO1 in HCC carcinogenesis and provide useful information for the development of mechanism-based cancer prevention strategies.

Conflicts of interest

Authors declare that there are no competing interests.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.053.

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