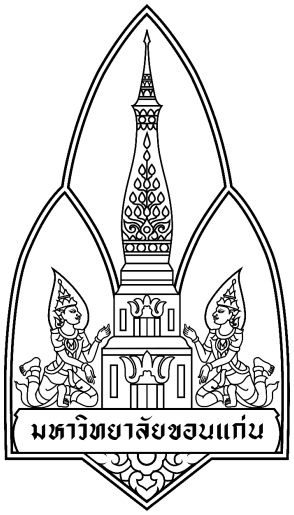

รายงานการวิจัย

บทบาทของเอนไซม์ NQO1 ต่อความไวต่อมะเร็ง พยากรณ์โรค และเป้าหมายของยารักษามะเร็ง 2

Role of NQO1 in cancer susceptibility, prognosis and as a target for chemotherapy II

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สารบัญ

โครงการวิจัย บทบาทของเอนไซม์ NQO1 ต่อความไวต่อมะเร็ง พยากรณ์โรค และเป้าหมายของยารักษามะเร็ง 2

(Role of NQO1 in cancer susceptibility, prognosis and as a target for chemotherapy II)

NQO1 expression is correlated with prognosis in cholangiocarcinoma

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บทคัดย่อ

มะเร็งท่อน้ำดีเป็นมะเร็งตับที่มีอุบัติการณ์ต่ำมากและมีพยากรณ์โรคที่เลวมาก ระบาดวิทยาของมะเร็งนี้มีความปรปรวนสูง อุบัติการณ์ของโรคพบสูงสุดที่ภาคตะวันออกเฉียงเหนือของประเทศไทยบางพื้นที่ในกลุ่มประเทศอาเซียตะวันออกเฉียงใต้และจีน ปัจจุบันยังไม่มีตัวบ่งชี้ทางชีวภาพเพื่อการวินิจฉัยและพยากรณ์โรคที่เชื่อถือได้ NADPH-quinone oxidoreductase 1 (NQO1) เป็นเอนไซม์ที่เปลี่ยนแปลงสารภายนอกร่างกายที่มีบทบาทในการเปลี่ยนแปลงสารเคมีก่อความเครียด และต้านออกซิเดชัน เอนไซม์ออกฤทธิ์ปกป้องเนื้อเยื่อปกติ แต่เพราะ NQO1 มีการแสดงออกสูงในมะเร็งหลายชนิด บ่งถึงบทบาทเพื่อการดำรงอยู่ของเซลล์มะเร็ง ตัวอย่างชิ้นเนื้อมะเร็งจากผู้ป่วยได้รับการวิเคราะห์พบว่าการทำงานของเอนไซม์ NQO 1 ในเนื้อเยื่อมะเร็งมีค่าสูงกว่าเนื้อเยื่อปกติรอบๆมาก การย้อมสีของเนื้อเยื่อมะเร็งโดยเทคนิค immunohistochemistry พบว่าเนื้อเยื่อมะเร็งมีการติดสีที่ชัดเจนมาก ในขณะที่เนื้อเยื่อปกติรวมถึงท่อน้ำดีและเนื้อเยื่อตับติดสีน้อยมาก การแสดงออกของ NQO1 mRNA ในเนื้อเยื่อมะเร็งจากคนไข้ 43 คนพบว่า ผู้ที่มีการแสดงออกของ NQO1 สูง จะมีพยากรณ์ของการมีชีวิตอยู่รอดต่ำ โดยมีค่า Cox proportional hazard ratio 2.40, $p<0.05$ นอกจากนี้จุลกายวิภาคของเนื้อเยื่อชนิดไม่ใช่ papillary adenocarcinoma เป็นตัวบ่งชี้อิสระถึงความพยากรณ์โรคที่เลว โดยมี hazard ratio 2.79, $p<0.05$ การแสดงออกของ NQO1 อาจสามารถใช้เป็นตัวบ่งชี้ทางชีวภาพของพยากรณ์โรคมะเร็งท่อน้ำดี

Abstract

Cholangiocarcinoma (CCA) is a rare type of liver cancers with very poor prognosis. The prevalence of CCA is markedly variable with the highest incidence in northeast Thailand followed by other parts of Southeast Asia and China. Currently there is still no reliable biomarkers for the diagnosis or treatment. NADPH-quinone oxidoreductase 1 (NQO1) is a xenobiotic metabolizing enzyme played roles in detoxifying chemical stressors and antioxidants thereby providing cytoprotection in normal tissues. However, NQO1 is over-expressed in some cancers, suggesting its roles in carcinogenesis and/or cancer development. In this study, we examined NQO1 activity in the surgical specimens from CCA patients. The NQO1 activity in tumor area was much higher than that in the adjacent normal tissues. The immunohistochemistry revealed the strong staining in tumor area, whereas the non-tumor bile ducts and liver parenchyma were weakly stained. The NQO1 mRNA expression levels in tumor tissues were widely variable among 43 patients, but the significant association was observed in between the high level of NQO1 expression and the shorter overall survival times with the Cox proportional hazard ratio of 2.40, $p<0.05$. By histological typing, non-papillary adenocarcinoma was an independent predictor for poor prognosis with the hazard ratio of 2.79, $p<0.05$. The NQO1 expression level may be served as a prognostic biomarker of CCA

Introduction

Cholangiocarcinoma (CCA) is a malignant neoplasm originating from the bile duct epithelium. Although CCA is a rare cancer worldwide, the incidence and mortality rates have grown up in the US, United Kingdom, Japan and Australia (1). The incidence of this cancer is very high in regions of northeastern Thailand, Cambodia, and Laos, where the prevalence of liver fluke infection is very high (2-3), Opisthorchis viverrini (OV) infection is one of the important risk factor of CCA probably through chronic inflammation induced oxidative stress and nitrosative stress (4-5), since chronic inflammation predisposes to several types of cancers Activated s inflammatory cells release a variety of inflammatory cytokine such as IL-1β, IFN–γ, TNF-α and several inflammatory mediators, such as nitric oxide (NO) and prostaglandin E2 (6-7). CCA is an aggressive malignancy characterized by the resistance to the current chemotherapy and radiotherapy in vast majority of cases (1). Diagnosis of CCA is very difficult because of the nonspecific clinical manifestations and the lack of appropriate biomarkers (1, 8). Complete surgical excision with negative tumor margin, solitary lesion, absence of lymph node involvement, and lack of vascular invasion are the best predictors for long tern survival (8). However, current predictors of pathological and operative staging strategies do not accurately predict long-term prognosis of CCA patients. The other markers for diagnosis or treatment such as serum markers of CA 19-9 and CEA are of very limited value (1).

NAD(P)H:quinone oxidoreductase-1 (NQO1) is a ubiquitous flavoprotein that functions as an antioxidant enzyme (9). The enzyme catalyzes two electron reduction of quinines to hydroquinones, thus avoids a one electron reduction and associated redox cycling

which generates reactive oxygen species (ROS) (10-11). Functions of NQO1 include xenobiotic detoxification, superoxide scavenger and the maintenance of endogenous antioxidant vitamins (9). The antioxidant role of NQO1 was suggested by the evidences such that the disruption of NQO1 gene (12) or genetic polymorphism increased the risk of chemical-induced toxicity and cancers (13-14). Several lines of evidences indicate that cancer chemoprevention afforded by regular intake of dietary phytochemicals involves with induction of the phase II enzymes including NQO1 (15). It is conceivable that NQO1 plays an important role in protecting normal cells against oxidative injury and carcinogenesis.

Over-expression of NQO1 in tumor tissue compared to the surrounding normal tissue was reported in liver cancer (16), lung cancer (17), pancreatic cancer (18), breast, ovary, thyroid, adrenal, colorectal and bladder (19). These circumstancial evidences suggest that over-expression of NQO1 may confer cytoprotection for tumor cells against oxidative stress and cause cells resistant to anticancer agents (20). The treatment of CCA based on targeting NQO1 has been shown to suggested as a potential strategy to overcome the resistance in CCA (21). In addition, NQO1 may be a prognostic marker of CCA because its expression in the CCA tissues is associated with the disease progression.

Materials and Methods

Tissue samples

Forty-three tissue sections from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University. The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research. Written informed consent was obtained from all patients. Among 43 samples, 30 samples were available as the pair of tumorous and non-tumorous tissues. Patients who died within 2 weeks after operation were excluded in this study.

Immunohistochemistry

CCA tissue sections were cut from archival paraffm blocks. Sections were deparaffinized in xylene and rehydrated through descending alcohol series to distilled water. Sections were then placed in a 10 mM citric acid solution (pH 6.0) and microwaved for 10 min. Sections were cooled to room temperature and then wash with PBS for 3 times. Endogenous peroxidase activity was eliminated by placing sections in 3% hydrogen peroxide for 30 min. Sections were rinsed in PBS for 10 min and then blocked in 5% milk in PBST for 20 min. Immunodetection of NQO1 was performed using mouse monoclonal IgG against NQO1 (sc-32793) purchased from Santa Cruz Biotechnilogy, Inc. (California, USA) and then diluted further with an equal part of PBS. Serial sections of each tissue sample were incubated with anti-NQO1 antibodies. Sections were incubated with 200 µL of 1:100 diluted anti-NQO1 antibody for overnight at 4°C. Sections were washed in PBST for three 5-rain cycles. Immunodetection was performed using an envision system-HRP labeled polymer anti-mouse (Dako) for 1 hr. Peroxidase substrate kit (DAB Staining Kit; Vector Laboratories, Inc. Burlingame, CA) were used. DAB/hydrogen peroxide solution was prepared according to the manufacturer, and 350 µL of solution was added to each section for 5-7 min. Sections were then rinsed in distilled water, counterstained with hematoxylin, dehydrated, and mounted. Sections were photographed.

NQO1 activity in tissues.

Tissues was thawed, cut into small pieces in ice-cold 1.15% (w/v) KCl in 0.1 M phosphate buffer pH 7.4 and homogenized with Utra-Turrax homogenizer. Homogenates were centrifuged at 100,000 g at 4°C for 6 min. Finally, supernatant was collected for the cytosol fraction and the microsomal pellet was resuspended by homogenizing the pellet in the same buffer. Cytosol and microsomal fractions were preserved with 20% (v/v) glycerol. Aliquots of samples were stored at -70°C until use.

NQO1 activity was measured according to the previously described method (22) with slight modifications (23). Cytosol samples were assayed by coupling reaction using menadione and MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) as the substrates. Tissue cytosol was added to reaction mixture containing with Tris 25 mM, pH 7.4, bovine serum albumin 67 mg/mL, 0.015% tween-20, 50 µM FAD, 1 mM glucose-6-phosphate (G6P), 30 µM NADP, 2 unit/mL G6P-dehydrogenase, 0.03 mg/mL MTT and 0.24 µM menadione. The assay was performed under the presence and absence of 50 µM dicoumarol. The enzyme activity was measured as a rate-kinetics at a wavelength of 620 nm, and the readings were made at 30 sec intervals for 5 min. The initial velocity of dicoumarol-suppressible kinetics was calculated as the NQO1 activity using the extinction coefficient of formazan of MTT of 11,300 M^{-1} cm^{-1}.

RNA preparation and reverse transcription-polymerase chain reaction

Total RNA was extracted from tumor tissues using Trizol reagent following the manufacturer's instruction. Total RNA (1 µg) was reverse-transcribed in 20 µL containing 0.5 µg of oligo$(dT)_{15}$ primer, 20 U of RNasin® ribonuclease inhibitor and 200 U of ImProm-II ™ reverse trancriptase in 10 x PCR buffer, 3 mmol/L $MgCl_2$, and 1 mmol/L dNTPs. The first-strand cDNA was synthesized at condition of 42°C for 60 min. The reverse transcription products were served as a template for real-time PCR. PCR amplification was performed using specific primers for the NQO1 using beta-actin the internal control. The PCR primer sequences were as follows: NQO1; forward primer: 5' GGC AGA AGA GCA CTG ATC GTA 3', NQO1; reverse primer: 5' TGA TGG GAT TGA AGT TCA TGG C 3', GenBank accession number BC007659.2, with the expected amplicon size of 159 bp, beta-actin; forward primer: 5' TGC CAT CCT AAA AGC CAC 3', Beta-actin; reverse primer: 5' TCA ACT GGT CTC AAG TCA GTG 3', GenBank accession number NM_001101.3 with amplicon size of 290 bp. The real-time PCR, based on SYBR Green, was carried out in a final

volume of 20 μL containing 1x SYBR Green master mix, 0.5 μmol/L of each NQO1 or beta-actin primers. Thermal cycling was performed for each gene in duplicate on cDNA samples in 96-wells reaction plate using the ABI 7500 Sequence Detection system (Applied Biosystems). The negative control, set up by substituting the template with deionized H_2O and that routinely had a high Ct value which represented the lower detection limit, was included in the experimental runs. Real-time PCR was conducted with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 45 s. To verify the purity of the products, a melting curve analysis was performed after each run. Upon completion of 40 PCR amplification cycles, there was a dissociation step of ramping temperature from 60 to 95°C steadily for 20 min, while fluorescence signal was continually monitored for melting curve analysis. The relative expression ratio (R) of the target genes was calculated based on the efficiency (E) and CT deviation and expressed as the ratio to the reference gene. The corresponding real time PCR efficiencies were calculated according to the equation $E = 10^{[-1/slope]}$. All data were analyzed using Sequence Detector Software Version 1.4 (Applied Biosystems).

Statistical analysis

Data are expressed as mean ±SD. Student's *t*-test was used to determine significant differences between tumor and normal tissues. The level of significance was preseted at $P<0.05$. Cross tabulations were analyzed with the chi-square test for association of NQO1 mRNA expression and the pathological features of patients. The Kaplan-Meier survival curves were generated for patients who having high and low expression of NQO1 mRNA, histological types of papillary type and other. Hazard ratios and p-values for comparisons of patients having high and low NQO1 expression were calculated based on multivariate Cox

proportional hazards model, adjusting for their age, sex, histological types, tumor residue and metastasis. The analyses were conducted by using Stata software version 7.0

Results

Characteristics of patients

CCA tissues used in this study were from patients with an average age of (mean ± SD) 56.1 ± 9.4 years. They were 27 males and 16 females (Table 1). Their survival time ranged from 36 days to 1,120 days, at which time points the subjects were still alive. There were 8 long survivors, and 6 out of 8 patients had a papillary type CCA by histopathology. In this study, nine patients (22.5%) received adjuvant chemotherapy after surgery. Patients in this series were initially evaluated as the candidates for complete excision. However, only 18 (42%) patients were proven to have been achieved complete resection with negative margin (R0) by histopathology, while all the others have extensive invasion and tumor residue at the surgical margin.

NQO1 activity and immunohistochemical localization in CCA tissue

NQO1 activity in tumor tissues was much higher than that in non-tumor adjacent tissues ($p < 0.001$) (Fig. 1). The median and 25% and 75% percentiles of NQO1 activities were 14.9, 1.0 and 20.8 nmol/min/mg protein for tumor tissues and 0.81, 0.44 and 1.21 nmol/min/mg protein for normal tissues. There was no association between NQO1 activity and survival time nor NQO1 mRNA expression. The immunohistochemical staining in tumor and non-tumor areas are shown in Fig.2. The normal issue showed liver parenchyma and small bile ducts only weakly stained with NQO1 in cytosol (Fig.2B). NQO1 is strongly stained in tumors with tubular type as well as papillary type of adenocarcinoma (Fig 2 C and D).

NQO1 expression and association with clinicopathologic variables

Expression levels of NQO1 mRNA were measured by reverse-transcription real-time PCR. There were no significant correlations between the high level of NQO1 and the histological types of tumors, evidences of metastasis, and gender (Table 1). The age of patients between NQO1 high and low groups was also of no difference.

Survival time analysis

The median survival time of the patients in this series was 267: (95% CI) 131 - 403 days. Using Kaplan-Meier and log-rank analysis, the median survival time of CCA patients with the high and low expression levels of NQO1were of 149.0: (95% CI) 102 - 196 days and 342.0: 228 - 456 days, respectively. The median survival time of the patients having papillary and non-papillary types by histology were 297: (95% CI) 219 -375 days and 157: 1147 -200 days, respectively. In the univariate analysis none of variables were a significant predictor for survival.

The multivariate Cox proportional hazard analysis was performed in patients to explore the impact of NQO1 expression after adjusting for age, gender, histological type, metastasis and presence of tumor residue. The levels of NQO1 expression, the histological type, genderand tumor residue at surgical margin were significant predictors of the long-term survival of CCA patients (Fig. 3). CCA patients with low expression of NQO1 have longer survival than who have high expression, with the hazard ratio about 2.23 ($p< 0.05$) (Table 2). The prognosis of patients having papillary adenocarcinoma was better than the patients having other histological types of CCA with the the hazard ratio of over 2.4 (Table 2). The patients with residual tumor (surgical margin positive or metastasis) were at risk with the

hazard ratio of about 1.9 when compared with complete excision of tumor, although this did not reach a statistical significance.

Discussion

NQO1 functions as xenobiotic metabolizing and antioxidant in normal cells. NQO1 gene is one of the down-stream genes regulated by Nrf2 - antioxidant-response-element (Nrf2-ARE) signaling pathway, which is an important target for cancer chemoprevention of various phytochemicals (15). However, in certain circumstances, NQO1 also functions to protect tumor cells, as it is over-expressed in some cancers (16-17, 20). Our results revealed that, both by semi-quantitative measurement of the mRNA expression level and by immunohistochemical staining, CCA tissues express high NQO1 activity compared with adjacent normal tissues and NQO1 mRNA expression level can be an independent predictor of long-term survival.

In this study, NQO1 activity was very high in CCA tumor tissues compared to the normal liver tissue. There was a wide variation in the activity of tumors, whereas there was much less in normal tissues. The survival analysis suggested patients with high NQO1 expression have poor prognosis. However, there was lack of correlation between the activity and mRNA expression of NQO1. The high expression might not directly represent the activity observed. Alternatively, NQO1 expression may represent the activity of Nrf2-ARE signaling system, where the pathway could regulate a number of downstream genes including NQO1 where they provide cytoprotection and resistance to stress (24).

In contrast to our results, Strassburg et al (25) reported the expression of NQO1 and NQO2 in bile duct tumor was comparable to that of normal bile duct tissues. In our study, high NQO1 immunohistochemical staining was observed in CCA tissues, whereas surrounding tissues including normal bile ducts and liver parenchyma were very weakly

stained. Since Strassburg et al. (25) observed only 4 CCA patients using gall bladder as the representative normal biliary tissues, such small number of observation with different control specimen might cause the different results.

Recently Wakai et al. (26) reported that the loss of NQO1 expression evaluated by immunohistochemical technique was associated with the poor prognosis of intrahepatic CCA. This report is incompatible with our finding where low NQO1 expression is associated with longer survival than the high expression. It should be noted that populations in the studies were from different parts of the world where the risk factors and etiology of the disease are considered to be different. CCA patients in our report were from the Northeast region of Thailand where liver fluke infection is probably the most important causative agent for CCA (27-28). On the other hand, Wakai et al. studied on Japanese patients where opisthorchiasis is not a risk factor. Related to this, Jinawath et al (29) reported that liver fluke-associated and non-liver fluke associated intrahepatic CCA showed significantly different gene expression profiles. For example, xenobiotic metabolizing enzyme genes were over-expressed in Thai CCA patients, whereas growth factor signaling genes were over expressed in Japanese patients (29). Moreover both studies have been done using rather small size of sample populations, a larger population are deemed necessary to clarify an association of NQO1 and the prognosis of CCA and highlights the need to evaluate the biomarkers under relevant circumstances.

Apart from NQO1, the present results revealed that histological type of CCA tissue could be a significant and independent predictor associated with prognosis of the patients; i.e. the patients having papillary type have better prognosis than those having non-papillary type. Our result is in agreement with the previous report in CCA patients of Thailand (30). Histological type and NQO1 expression are independent parameters of patients'survival, as the histological type is not correlated with NQO1 expression level (Table 1). Residual tumor

after surgical operation is usually regarded as the predictor of poor survival after surgery. The complete excision of tumor with surgical margin negative (R0) is associated with long-term survival (31). Our study is consistent in that patients who could not achieve R0 excision are at higher risk than ones could achieve R0 operation.

In summary, NQO1 acting as xenobiotic metabolizing and antioxidant enzyme was over-expressed in the majority of CCA. Since NQO1 is over-expressed in some other tumors, this enzyme may be protective to cancer cells. The high expression of NQO1 was associated with poor prognosis. Histology of tumors of papillary type and complete surgical removal of tumor mass were significantly associated with good prognosis. Further study with larger population is necessary to clarify the inconsistency among the reports.

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Disclosure of Potential Conflicts of Interest: Authors declare no potential conflicts of interest.

Table 1 The characteristics of CCA patients in this study

			NQO1 expression		*p*-value
Characteristics		No.	Low	High	
Age (year±SD)	56 ± 9	43	57.5±7.0	53.0±13.4	0.15
Gender					
	Female	16	10	6	0.50
	Male	27	20	7	
Status					
	Death	35	24	11	0.72
	Alive	8	6	2	
Gross type	Mass forming	24	17	7	
	Periductal infiltrating	17	12	5	
	Intraductal growth	1	0	1	
Histological type					
	Papillary	22	17	6	0.74
	Non-papillary	21	13	7	
	Well-differentiated (16)				
	Moderately-differentiated (3)				
	Poorly-differentiated (2)				
Metastasis					
	Present	14	9	5	0.48
	Not establish	28	20	8	
Surgical margin					
	R0	18	13	5	0.77
	Not R0	25	17	8	

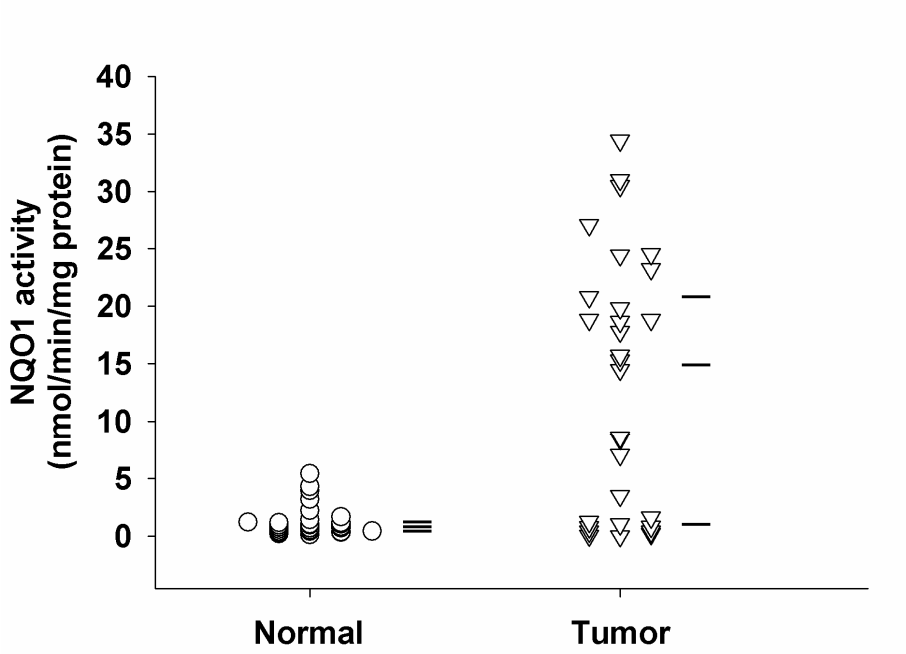

Figure 1. Activity of NQO1 in tissues from normal and tumor sections of CCA patients. Surgical liver specimens from CCA patients were sectioned for tumor and adjacent normal tissues. Tissues were homogenized for preparation of cytosolic fraction to determine NQO1 activity by the enzymatic coupling assay. Small horizontal bars on the right side of the plots represent the percentiles of 25^{th}, 50^{th} and 75^{th}.

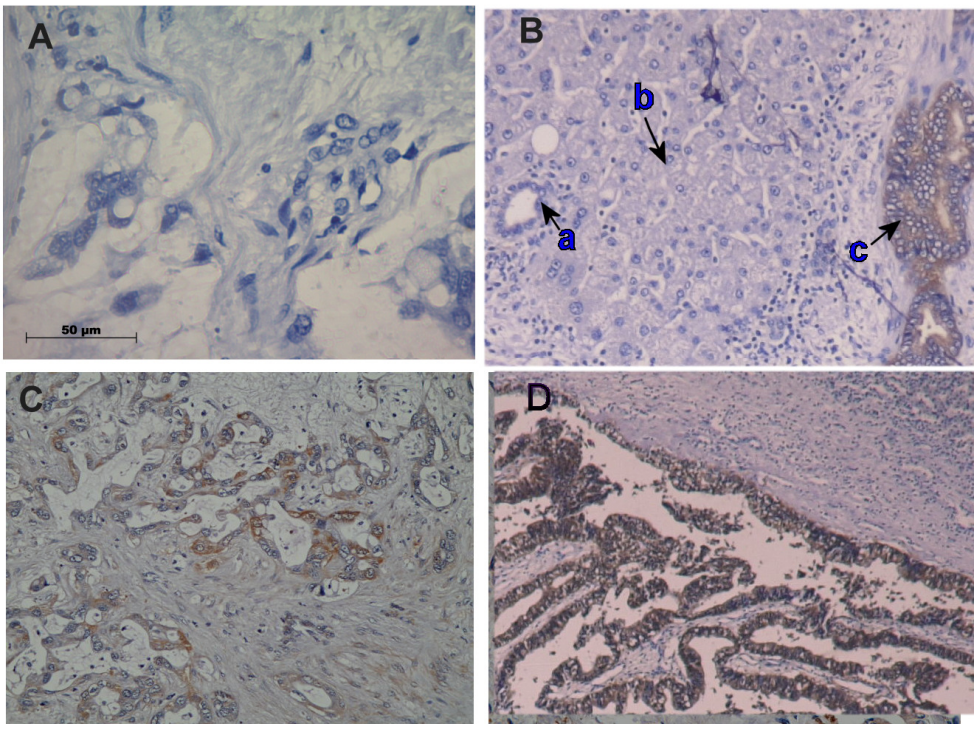

Figure 2 Immunohistochemical staining of NQO1 in CCA sections. A. The negative control of NQO staining. B. The non-tumor tissue stained very weakly with NQO1, a: non-tumor bile duct, b: liver parenchyma, and c: bile duct tumor stained strongly with NQO1. C. The well-differentiated adenocarcinoma type and D. The papillary type. (Original magnification of x40 for A & C, and 10X for B & D)

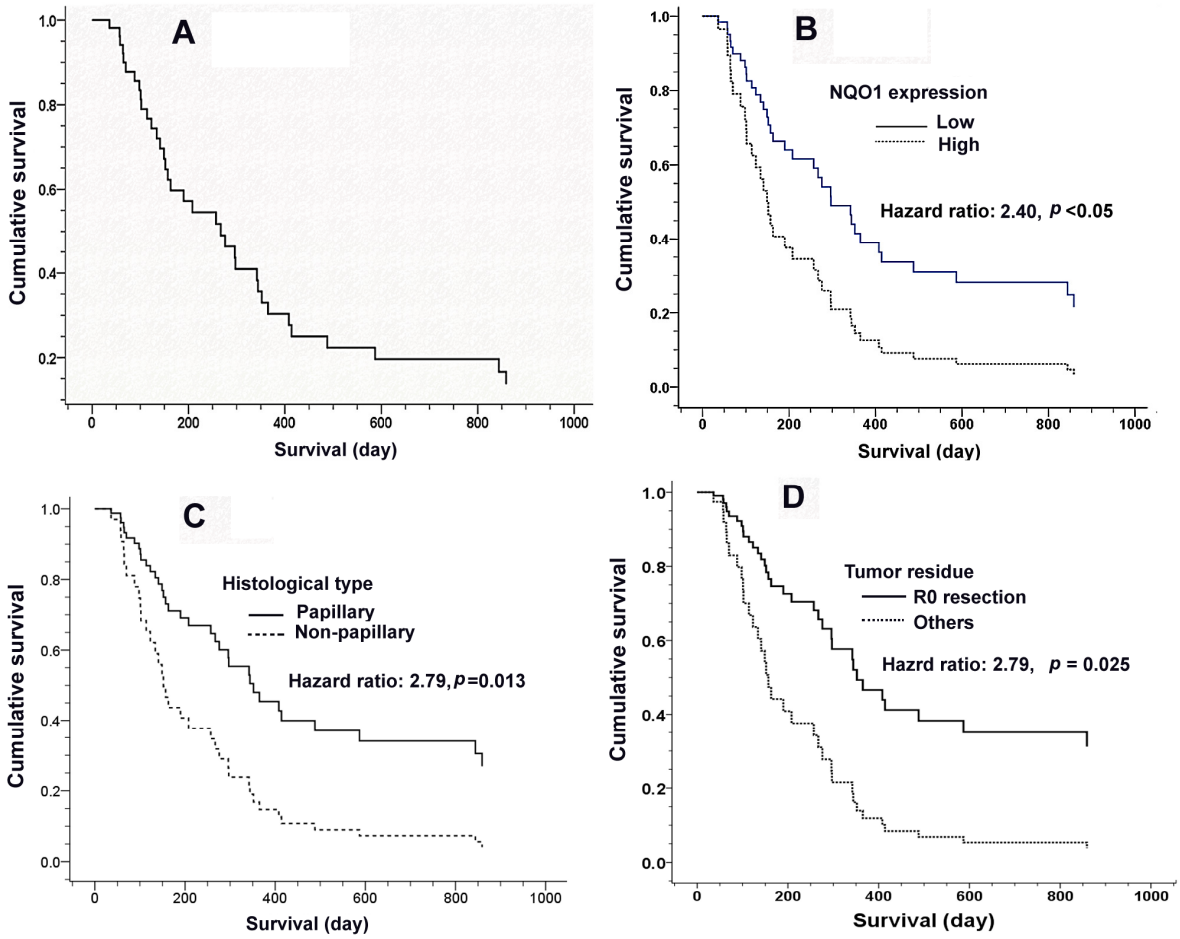

Figure 3 Cumulative survival curves for intrahepatic cholangiocarcinoma. A. The survival function at mean of covariates. B. Survival function stratified by the levels of NQO1 mRNA expression. C. Survival function stratified by histological, and D. Survival function type stratified by surgical margin. Tumor tissues from surgical sections of cholangiocarcinoma patients were prepared for total RNA and analyzed for NQO1 mRNA by reverse-transcription and real-time PCR. Patients with high NQO1 mRNA expression group or histology of non-papillary type or surgical margin showing tumor residue had worse overall survival.

Table 2 Multivariate analysis by COX proportional hazards

Variable	Hazard ratio	95% CI	p-value
Age			
<57	1		
>=57	3.20	1.34 – 7.66	0.009
Gender			
Female	1		
Male	1.12	0.51 – 2.49	0.009
Histological types			
Papillary	1		
Non-papillary	2.79	1.24 – 6.28	0.013
Metastasis			
Presence	1		
No evidences	1.24	0.54 – 2.83	0.604
Surgical margin			
R0	1		
Not R0	2.79	1.38 – 6.85	0
NQO1 mRNA expression			
Low	1		
High	2.40	1.08 – 5.33	0.030

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