RESEARCH ARTICLE

NQO1 Expression Correlates with Cholangiocarcinoma Prognosis

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Abstract

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

Keywords: NADPH-quinone oxidoreductase 1-drug metabolizing enzyme antioxidant-cytoprotection

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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 (Patel, 2011). The incidence of this cancer is very high in regions of northeast Thailand, Cambodia, and Laos, where the prevalence of liver fluke infection is very high (Sripa and Pairojkul, 2008; Kamsa-ard et al., 2011). *Opisthorchis viverrini* infection is one of the important risk factors of CCA probably through chronic inflammation-induced oxidative stress and nitrosative stress (Coussens and Werb, 2002; Pinlaor et al., 2004), since chronic inflammation predisposes to several types of cancers. Activated inflammatory cells release a variety of inflammatory cytokines such as IL-1β, IFN–γ, TNF-α and inflammatory mediators, including nitric oxide and prostaglandin E2 which act as tumor promoters (Dijkstra et al., 2004; Ohshima et al., 2005). CCA is an aggressive malignancy characterized by the resistance to the current chemotherapy and radiotherapy in vast majority of cases (Patel, 2011). Diagnosis of CCA is very difficult because of the nonspecific clinical manifestations and the lack of appropriate biomarkers (Khan et al., 2005; Patel, 2011). 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 term survival (Khan et al., 2005). 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 (Patel, 2011).

NAD(P)H:quinone oxidoreductase-1 (*NQO1*) is a ubiquitous flavoprotein that functions as an antioxidant enzyme (Siegel et al., 2004). The enzyme catalyzes two electron reduction of quinones to hydroquinones, thus avoids a one electron reduction and associated redox cycling which generates reactive oxygen species (ROS) (Dinkova-Kostova, 2010). Functions of NQO1 include xenobiotic detoxification, superoxide scavenger and the maintenance of endogenous antioxidant vitamins (Siegel et al., 2004). The antioxidant role of NQO1 was suggested by evidences such that the disruption of *NQO1* gene (Radjendirane et al., 1998) or genetic polymorphism increased the risk of chemical-induced toxicity and cancers (Saldivar et al., 2005; Yang et al., 2012). Several

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lines of evidences indicate that cancer chemoprevention afforded by regular intake of dietary phytochemicals involves with induction of the phase II enzymes including NQO1 (Surh et al., 2008). It is conceivable that NQO1 plays an important role in protecting normal cells against oxidative injury and carcinogenesis.

Over-expression of *NQO1* mRNA in tumor tissue compared to the surrounding normal tissue was reported in liver cancer (Cresteil and Jaiswal, 1991), lung cancer (Kolesar et al., 2002), pancreatic cancer (Logsdon et al., 2003), and over-expression of NQO1 detected by immunohistochemistry in breast, ovary, thyroid, adrenal, colorectal and bladder was also observed (Siegel and Ross, 2000). These circumstantial evidences suggest that over-expression of NQO1 may confer cytoprotection for tumor cells against oxidative stress and cause cells resistant to anticancer agents (Danson et al., 2004). The treatment of CCA based on targeting NQO1 has been shown to be a potential strategy to overcome the resistance in CCA (Buranrat et al., 2010). In this study we analyzed *NQO1* expression in CCA tumor and apparently normal adjacent tissues and examined whether *NQO1* expression could be a prognostic marker of CCA in association with the overall survival.

Materials and Methods

Subjects

Subjects were patients admitted at Sringarind Hospital, Faculty of Medicine, Khon Kaen University for the treatment of cholangiocarcinoma. All patients have been diagnosed and confirmed by histopathological examination. Their average age was (mean±SD) 56.1±9.4 years, with 27 males and 16 females. Their survival time ranged from 36 days to 1,120 days, at which time some subjects were still alive. In this study, nine patients (22.5%) received adjuvant chemotherapy after surgery. Tumor tissues were collected from surgical resection of tumor mass for histopathological examination and other tissue samples were kept in liquid nitrogen before uses for extraction of RNA and enzymatic assay.

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 forty three samples, thirty samples were available as the pair of tumorous and non-tumorous tissues. Samples of the same tissue section were used for assays of NQO1 activity and total mRNA. Patients who died within 2 weeks after operation were excluded from this study.

Immunohistochemistry

CCA tissue sections were cut from archival paraffin blocks. Sections were deparaffinized in xylene and rehydrated through descending alcohol series to distilled water. 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. Immunohistochemistry analysis of NQO1 was performed using mouse monoclonal IgG against NQO1 (sc-32793, Santa Cruz, CA, USA) 1:100 dilution for an overnight at 4°C, followed by Immunodetection with Envision system-HRP labeled polymer anti-mouse (Dako) for 1 hr. Sections were counterstained with hematoxylin, and 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 60 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 (Prochaska and Santamaria, 1988) with slight modifications (Prawan et al., 2009). 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 substrate. 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 in 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 second 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 10xPCR 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 as an internal control. The PCR primer sequences were: *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. Assay was performed in triplicate. 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 preset 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

Characteristics of subjects are shown in Table 1. There were 8 long survivors, and 6 out of 8 patients had a papillary type CCA by histopathology. 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 ($p<0.001$) (Figure 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 or *NQO1* mRNA expression. The immunohistochemical staining in tumor

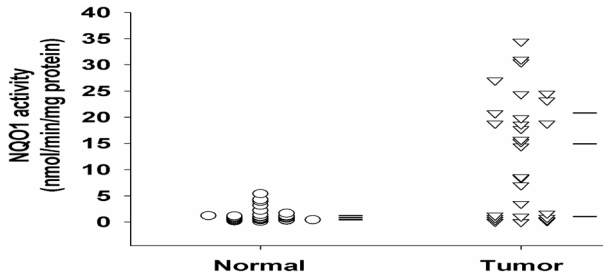

Figure 1. Activity of NQO1 in Tissues from Normal and Tumor Sections of CCA Patients. Surgical liver specimens from CCA patients were sectioned for tumor and adjacent normal tissues. Tissues were homogenized for preparation of cytosolic fraction to determine NQO1 activity by the enzymatic coupling assay.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

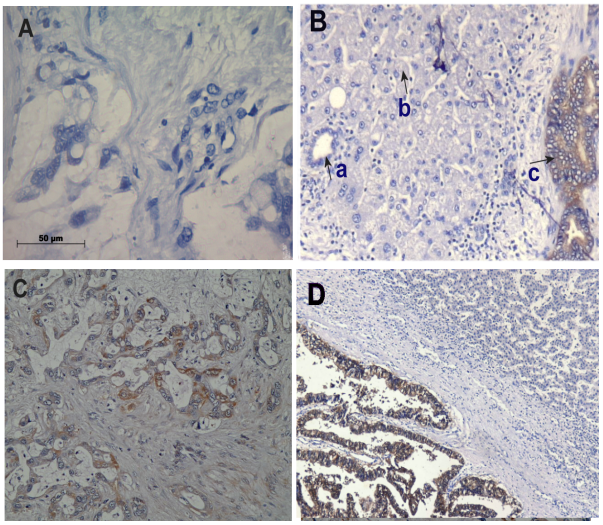

Figure 2. Immunohistochemical Staining of NQO1 in CCA Sections. A) The negative control of NQO staining. B) The non-tumor tissue stained very weakly with NQO1, a: non-tumor bile duct, b: liver parenchyma, and c: bile duct tumor stained strongly with NQO1. C) The well-differentiated adenocarcinoma type.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

Table 1. The Characteristics of CCA Patients in this Study and in Relation to *NQO1* Expression Levels

Characteristics		No.	*NQO1* expression Low	*NQO1* expression High	p-value
Age (year±SD)	56±9	43	57.5±7.0	53.0±13.4	0.15
Gender	Female	16	10	6	0.5
	Male	27	20	7	
Status	Death	35	24	11	0.72
	Alive	8	6	2	
Gross type	Mass forming	24	17	7	0.98
	Periductal infiltrating	17	12	5	
	Intraductal growth	1	0	1	
Histological type	Papillary	22	16	6	0.66
	Non-papillary	21	14	7	
	Well-differentiated	16			
	Moderately-differentiated	3			
	Poorly-differentiated	2			
Metastasis	Present	14	9	5	0.69
	Not establish	27	19	8	
Surgical margin	R0	18	13	5	0.77
	Not R0	25	17	8	

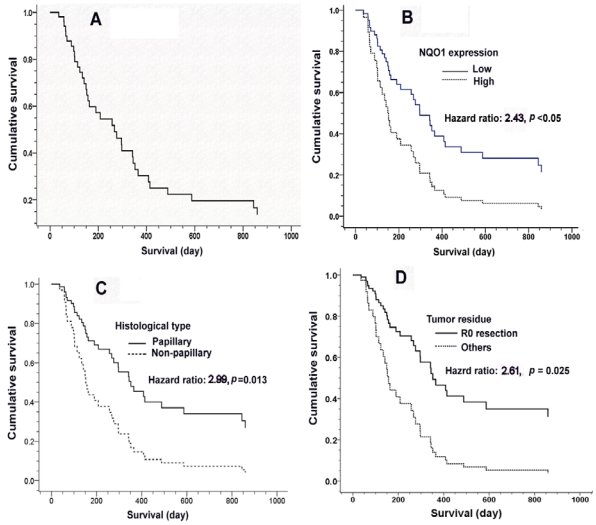

Figure 3. Cumulative Survival Curves for Intrahepatic Cholangiocarcinoma. A) The survival function at mean of covariates. B) Survival function stratified by the levels of *NQO1* mRNA expression. C) Survival function stratified by histological. D) Survival function type stratified by surgical margin. 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. All samples were performed in triplicate assay. 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	2.74	1.23-6.07	0.013
Gender	Female	1		
	Male	1.02	0.49-2.42	0.84
Histological types	Papillary	1		
	Non-papillary	2.99	1.39-6.42	0.005
Metastasis	Presence	1		
	No evidences	1.25	0.56-2.79	0.58
Surgical margin	R0	1		
	Not R0	2.61	1.11-6.14	0.027
NQO1 mRNA expression	Low	1		
	High	2.43	1.12-5.28	0.025

and non-tumor areas are shown in Figure 2. The normal issue showed liver parenchyma and small bile ducts only weakly stained with NQO1 in cytosol (Figure 2B). NQO1 is strongly stained in tumors with tubular type as well as papillary type of adenocarcinoma (Figure 2C and D).

NQO1 expression and association with clinicopathologic variables

Expression levels of *NQO1* mRNA were measured by reverse-transcription real-time PCR. Subjects at the forth quartile of *NQO1* expression were assigned to high expression group (13 subjects) and the rest were low expression group (30 subjects). There was no significant correlation between the level of *NQO1* and the histological type of tumors, and evidence of metastasis (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 days with 95%CI of 131-403 days. Using Kaplan-Meier and log-rank analysis, the median survival time of CCA patients with the high and low expression of *NQO1* were of 149.0 days (95%CI: 102-196 days) and 342.0 days 95%CI: 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 day (95%CI: 98-352 days, respectively. In the univariate analysis, none of variables was 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, histological type, gender and tumor residue at surgical margin were significant predictors of the survival time of CCA patients (Figure 3). CCA patients with low expression of *NQO1* have longer survival than who have high expression, with the hazard ratio of 2.43 ($p<0.05$). The prognosis of patients having papillary adenocarcinoma was better than the patients having other histological types of CCA with the hazard ratio of over 2.99 (Table 2). The patients with residual tumor (surgical margin positive) were at risk with the hazard ratio of 2.61 when compared with complete excision of tumor.

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 (Surh et al., 2008). However, in certain circumstances, NQO1 also functions to protect tumor cells, as it is over-expressed in some cancers (Cresteil and Jaiswal, 1991; Kolesar et al., 2002; Danson et al., 2004). Our results revealed that *NQO1* expression by immunohistochemical staining and NQO1 activity in tumor tissues were significantly higher than the normal adjacent tissues. *NQO1* mRNA expression in tumor tissue 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. 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* and other antioxidant genes where they provide cytoprotection and resistance to stress (Kensler and Wakabayashi, 2010).

In a previous study, Strassburg et al. (2002) reported the expression of NQO1 and NQO2 in bile duct tumor was comparable to that of normal bile duct tissues. In contrast to 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. (2002) observed in only four CCA patients and use of gall bladder as the representative normal biliary tissues. The small sample size with different control specimen might cause the differences in results.

Recently Wakai et al. (2011) reported that the loss of NQO1 expression evaluated by immunohistochemical technique was associated with the poor prognosis of intrahepatic CCA. This report is in contrast with our finding where low *NQO1* expression is associated with longer survival than the high expression. The discrepancy of findings may be due to the different study populations. CCA patients in our report were from the Northeast region of Thailand where liver fluke infection is probably the most important causative agent of CCA (Green et al., 1991; IARC, 1994). On the other hand, Wakai et al. (2011) studied on Japanese patients where opisthorchiasis is not a risk factor. Related to this, Jinawath et al. (2006) reported that liver fluke-associated and non-liver fluke associated intrahepatic CCA showed significantly different gene expression profiles in such that, xenobiotic metabolizing enzyme genes were over-expressed in Thai CCA patients, whereas growth factor signaling genes were over expressed in Japanese patients (Jinawath et al., 2006). A larger population is deemed necessary to clarify an association of *NQO1* and the prognosis of CCA. It also 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 (Subimerb et al., 2010). 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 (Guglielmi et al., 2009). Our study is consistent with the concept of patients without R0 excision are at higher risk than those with 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 provide protection 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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