Histol Histopathol (2009) 24: 1223-1232
DOI: 10.14670/HH-24.1223

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Analysis of NQO1 polymorphisms and p53 protein expression in patients with hepatocellular carcinoma

Mei-Miao Chiu[1]*, Ying-Ju Ko[1]*, Ann-Ping Tsou[2], Gar-Yang Chau[3] and Yat-Pang Chau[1]

[1]Institute of Anatomy and Cell Biology, School of Medicine, National Yang-Ming University, [2]Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University and Department of Surgery and [3]Taipei-Veterans General Hospital, Taipei, Taiwan

*Both authors contribute equally

Summary. NAD(P)H: quinone oxidoreductase 1 (NQO1), a cytosolic enzyme which catalyzes the two-electron reduction of quinone compounds, has been suggested to prevent the generation of semiquinone free radicals and reactive oxygen species, thus protecting cells from oxidative damage. However, the enzymatic activity of NQO1 strongly depends on the individual genetic polymorphism of the NQO1 gene. A common NQO1 polymorphism is a C to T transition at position 609, which results in an inactive enzyme. Recent studies showed that NQO1 is an important enzyme for stabilizing p53 protein, which is involved in anti-tumorigenesis. Thus, the lack of enzymatic activity in the homozygous C609T NQO1 polymorphism may play a pivotal role in tumor development.

This study aimed to investigate the relationship between C609T NQO1 polymorphism and p53 expression in human hepatocellular carcinoma (HCC). Genotyping of NQO1 was performed on 100 HCC specimens by PCR-RFLP analysis. In addition, NQO1 and p53 protein expression in HCC samples at different TNM stages was determined by immunohistochemistry. Our data showed that (1) the frequency of C609T NQO1 was significantly increased in TNM stage III HCC patients; (2) no significant association was found between p53 expression and C609T polymorphism of NQO1 gene; and (3) a tumor/non-tumor (T/N) ratio > 1.27 of NQO1 expression revealed by real-time qPCR analyses was positively correlated with poorer survival in patients with tumors >5 cm, suggesting that an increase of NQO1 expression may be an indicator of advanced tumor progression. This study provides important information about NQO1 genotypes and its expression to HCC tumor development and progression.

Key words: Hepatocellular carcinoma, NAD(P)H, Quinone oxidoreductase 1 (NQO1), P53 gene

Offprint requests to: Yat-Pang, Chau., Ph.D., Institute of Anatomy and Cell Biology, School of Medicine, National Yang-Ming University, 155, 2nd Sec., Li-Nung Street, Shih-Pai, Taipei, Taiwan 112, Republic of China. e-mail: leonchau@ym.edu.tw

Gar-Yang, Chau, Division of General Surgery, Department of Surgery, Taipei Veterans General Hospital-Taipei, 201 Sec. 2, Shih-Pai Road, Taipei, Taiwan 217, Republic of China. e-mail: gychau@vghtpe.gov.tw

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and its incidence continues to increase. Important risk factors for HCC include chronic infection with hepatitis B or hepatitis C virus and chemical carcinogens, including aflatoxin contamination of food. Recent studies have demonstrated an association between genetic background and environmental factors in the development of HCC within ethnic human populations (Pang et al., 2003; Herath et al., 2006; Teufel et al., 2007), but the molecular mechanisms that lead to the development and progression of HCC remain unclear (Miyasaka et al., 2001) .

The cytosolic enzyme NAD(P)H: quinone oxidoreductase 1 (NQO1), an antioxidative enzyme, is important in the detoxification of environmental carcinogens (Joseph et al., 2000; Iskander and Jaiswl 2005). It catalyzes the two-electron reduction of quinone compounds and prevents the production of semiquinone free radicals and reactive oxygen species, thus protecting cells from oxidative damage. However, the activity of the NQO1 enzyme strongly depends on polymorphisms at the NQO1 locus (Rauth et al., 1997). The major polymorphism involves a single C to T substitution at nucleotide 609 of exon 6, which causes a Pro187Ser amino acid change, resulting in three phenotypes, the wild type phenotype with complete enzyme activity, the

heterozygous phenotype with an approximately 3-fold lower activity, and the homozygous mutant (null) phenotype with no enzyme activity. The lack of NQO1 enzymatic activity due to the homozygous mutant (null) phenotype is associated with an increased risk of various cancers, including renal, urothelial, and cutaneous basal cell carcinoma (Sunga et al., 2002; Zhang et al., 2003). Furthermore, NQO1-knock-out mice have been shown to be more susceptible to chemical-induced skin cancer (Long et al., 2000, 2001).

The *p53* gene is one of the major tumor suppressor genes in humans, and mutations in this gene are one of the most common genetic events that occur in human cancers (Hupp 1999; Malanga and Althaus, 2005). When normal cells are subjected to stress signals, such as DNA damage or oxidative stress, the p53 gene is activated, resulting in transcription of downstream genes that coordinate either growth arrest of the cell or apoptosis, thus preventing the proliferation and clonal expansion of damaged cells. Mutation or deletion of p53 and consequent loss of p53-dependent function leads to increased susceptibility to neoplasia (Stewart and Pietenpol, 2001; Giono and Manfredi, 2006). The study of small molecules and proteins that increase p53 stability and, as a result, protect cells against cancer progression is a currently active area of research.

Recently, it has been proposed that *NQO1* is activated by many of the same stresses that activate the *p53* gene, and that NQO1 protein stabilizes wild type p53 protein (Asher et al., 2002a). These authors examined p53 stability and function in cells transfected with human NQO1 and showed that transfected wild type NQO1 protein, but not C609 T mutant NQO1 protein, stabilizes wild type p53 in HCT-116 cells. Accordingly, cotransfection of p53 null HCT-116 cells with wild-type p53 and wild-type NQO1, but not mutant NQO1, can stabilize endogenous as well as transfected wild-type p53 protein (Tsvetkov et al., 2005). A redox mechanism was proposed for p53 stabilization which relies upon NQO1-dependent NAD(P)H oxidation (Asher et al., 2000b). Moreover, high levels of NQO1 gene expression have been observed in tumors of the liver, lung, colon, and breast compared to normal tissues of the same origin (Joseph et al., 1994; Strassburg et al., 2002; Begleiter et al., 2003). NQO1 gene expression is increased not only in established tumors, but also in developing tumors (Aleksunes et al., 2006; Lyn-Cook et al., 2006; Liu et al., 2007), indicating a role in cellular defense during tumorigenesis.

It has been proposed that low molecular weight substances can diffuse from tumor cells into the surrounding normal cells and increase *NQO1* expression (Begleiter et al., 2003). NQO1 expression in, and the association of the C609T polymorphism with HCC have not been investigated. The purpose of this study was to examine the expression of the C609T *NQO1* polymorphism and p53 protein in HCC tumor specimens and adjacent liver tissue and the association between the NQO1 C609T genotype and HCC. C609T NQO1 genotypes were determined by PCR-RFLP analysis in patients with HCC, and NQO1 and p53 protein expression in different TNM stages in HCC patients was determined by immunohistochemistry. This study provides important information about the role of NQO1 gene expression and C609T polymorphism in HCC development and progression.

Materials and methods

Tissue preparation

In this study, paired samples of HCC and nontumorous liver tissues were obtained from patients who underwent curative hepatic resection for HCC at the Department of Surgery, Taipei Veterans General Hospital (Taipei, Taiwan). This study was approved by the Committee for the Conduct of Human Research at the Taipei Veterans General Hospital. The diagnosis of HCC was confirmed by histological examination of surgically resected specimens. Tumor and paired nontumor liver tissues were obtained immediately after surgical resection and the nontumor liver tissues were taken more than 1 cm away from the HCC, to ensure that tumor or non-tumor samples were not mixed. The resected tumor tissues were stored at -80°C in our tissue bank until used. One hundred patients were selected from the patients who underwent hepatic resection between Jan 1992 and Dec 1998. Patients who received any preoperative treatment, such as chemotherapy, ethanol injection, or transarterial chemoembolization, were excluded. The pathologist confirmed the diagnosis of HCC by histological examination of surgically resected specimens and that the peri-tumoral tissues were free of malignant cells. From these patients, we selected 30 from each TNM stage (stage I, II, and III). Before and after operation, the patients' detailed clinicopathological features, including hepatitis markers, serum, α-fetoprotein levels, tumor size, tumor number, vascular invasion, tumor differentiation, capsular infiltration, and postoperative survival data, were prospectively collected in a computer-based database. The degree of differentiation of the tumor was determined according to the Edmondson-Steiner classification. The patients were classified according to the new AJCC/UICC TNM staging system.

Reverse transcription-polymerase chain (RT-PCR) reaction and Real-time quantitative PCR methods

For RT-PCR analysis of liver tissue, total RNA was extracted with Super RNApure^{TM} Kit (Genesis Biotech Inc., Taipei, Taiwan). First-strand cDNA was synthesized from total RNA with Superscript™ II Reverse Transcriptase (Invitrogen Corporation., Carlsbad, CA) using oligo(dT) as primers. Semi-quantitative evaluation of *NQO1* mRNA transcription by RT-PCR was performed using the following primers: NQO1 gene (sense: 5'-TCCTCAGAGTGGCATTC

TGC-3', antisense: 5'-TCTCCTCATCCTGTACCTCT-3'). Glyeraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense: 5'-CAACTACATGGTTTA CATGTTC-3', antisense: 5'- GCCAGTGGACTCCA CGAC-3) was used as the internal control. The PCR amplification was done by preincubation for 4 min at 94°C, denaturation at 95°C for 60s, annealing at 60°C for 55s and extension at 72°C for 60s for 35 cycles, with a final extension of 5 min at 72°C. Real-time quantitative PCR analysis was performed using the fluorescent dye SYBR Green methodology with the DyNAmo™ Flash SYBR Green qPCR kit (Finnzymes, Inc., Massachusetts, USA) and DNA Enghine Peltier Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). Quantitative evaluation of NQO1 mRNA transcripts were performed using the following primers: NQO1 gene (sense: 5'-CTGGTTTGAGCGAGTGTT CA-3' and antisense: 5'-CGGAAGGGTCCTTTGTC ATA-3'). RNA samples were also amplified using 18S gene primers (sense: 5'- TTTCGCTCTGGTCCGTCT TG-3' and antisense: 5'- TTCGGAACTGAGGCCAT GAT-3') as control. The thermal cycling conditions for real-time PCR were 95°C for 10 minutes, and 40 cycles of melting (95°C, 15s) and annealing/extension (60°C, 60s). Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA tested with an active reference, 18S.

NQO1 activity assay

For determination of NQO1 activity, samples were homogenized in buffer containing Tris (25 mM) and sucrose (500 mM), pH 7.4 on ice and then centrifuged (14,000xg, 4°C) for 20 min. Supernatants from all samples were transferred to clean tubes and frozen (-80°C) until the time of assay. Samples were thawed on ice and protein concentration was determined using a BCA protein assay with bovine serum albumin as the reference standard. NQO1 activity was calculated by measuring the colorimetric oxidation of NADH to NAD+ using DCPIP as a substrate. The reaction mixture in the 1 ml reaction contained 25 mM Tris (pH 7.4), 200 μM NADH, 0.07% bovine serum albumin (w/v), 40 μM DCPIP, and 20 μg of sample. Enzyme activity was determined by monitoring the enzyme-dependent decrease in absorbance at 600 nm for 30s at 27°C in the presence or absence of 20 μM/L dicumarol. The results were expressed as nanomole of DCPIP reduced per minute per milligram of protein. Each sample was performed in triplicate.

Immunohistochemistry

Five micron sections were used for immunohistochemical staining to examine the expression of NQO1 and p53. The tissue sections were first treated with 3% hydrogen peroxide for 10 min, then, after washing, were subjected to antigen retrieval by boiling for 3x15 min in 0.06 M citric acid buffer (pH 6.0) in a 500 W microwave oven. They were then incubated for 10 min at room temperature with a blocker of non-specific binding (Zymed Lab, CA), then incubated for 16 h at 4°C with mouse antibodies against human NQO1 (Ab2346, Abcam, Cambridge, England) or human wild-type p53 protein (Ab-5, Calbiochem, CA) or human mutant-type p53 protein (Do-7, NeoMarker, CA), all at a 1:50 dilution. The sections were then incubated sequentially at room temperature with the non-specific binding blocker for 10 min, biotinylated universal IgG for 10 min, streptavidin peroxidase conjugate for 10 min, and AEC substrate chromogen for 5 min, then counterstained with hematoxylin for 5 min. A carcinoma sample with confirmed NQO1 protein expression was used as the positive control in every immunohistochemical experiment. Cell culture supernatant from a nonspecific IgG1 secreting hybridoma was used instead of primary antibody in the negative controls. The percentage of chromogen-labeled tumor cells were scored independently by two pathologists as 0 (0-4% positive tumor cells), I (5-25%), II (26-50%), III (51-75%), or IV (76-100%). The intensity of NQO1 immunoreactivity in tumor and nontumor cells in specimens was also scored independently by a senior pathologist as 0, I, II, III or IV.

PCR-RFLP method

PCR-RFLP was used to detect the C609T NQO1 polymorphism in the tumor and liver tissue. In brief, DNA isolated from frozen tissue using a DNA extraction kit (Promega) was subjected to two rounds of PCR amplification using a nested primer strategy. In the first round of PCR, a 334 bp product was amplified using 35 cycles of 94°C for 1 min, 60°C for 30s, and 72°C for 45s using the forward primer GAGACGCTAGCTCTGAAC TGAT and the reverse primer CTGCCTGGAAGTTTG GTCA. In the second round of PCR using the forward primer ATTTGAATTCGGGCGTCTGCTG and the reverse primer TCTAGTGTGCCTGAGGCCTCC and 30 cycles of PCR (94°C for 1 min, 63°C for 30s, and 72°C for 30s), a clean product of 217 bp was obtained from all specimens tested. Digestion of this PCR product with Hinf1 generated fragments of 217 bp (wild type); 217, 161, and 56 bp (heterozygote); or 161 and 56 bp (homozygous mutant), which were separated on a 3% agarose gel.

Statistical analysis

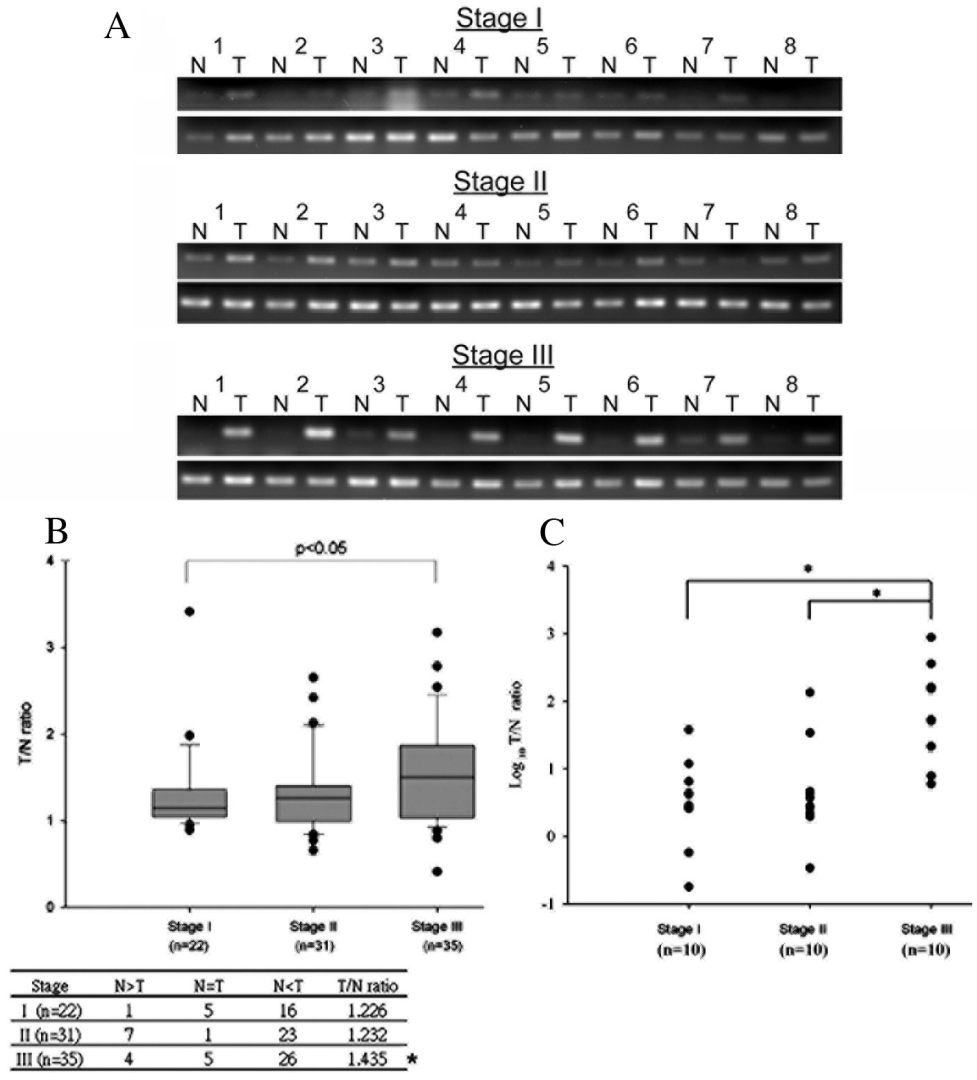
Comparison of the NQO1 genotype distribution in the study groups was performed by means of two-sided contingency tables using the Chi-squared test. Survival rates were calculated using the Kaplan-Meier method, and survival curves compared between different NQO1 genotype groups using the log-rank test. A probability level of 5% was taken as statistically significant. Statistical analysis was performed using the SPSS

software package (10.0 version).

Results

RT-PCR, real time-qualitative PCR and immunohistochemical analyses of NQO1 expression in HCC and paired normal tissues

To assess NQO1 gene expression in the tumor and paired nontumor parts of HCC tissues, RT-PCR was performed using NQO1-specific primers to detect NQO1 transcription in 100 HCC samples. Analysis of the PCR products demonstrated increased NQO1 transcript levels in most tumors compared to the paired nontumor part of the samples (Fig. 1A). Laser densitometry quantification and calculation of the band intensity relative to that of the GADPH band showed significantly greater NQO1 expression in the tumor tissue than the nontumor liver tissue. The ratio of the intensity of the NQO1 PCR product/GADPH PCR product in HCC (T) to that of the NQO1 PCR product/GADPH PCR product in paired normal liver tissue (N) (T/N ratio) was 1.226 in stage I, 1.232 in stage II, and 1.435 in stage III (Fig. 1B). To further confirm the increase of NQO1 expression in advanced stages of HCC, we included the Real-time qPCR analyses in this study (Fig. 1C). Statistical analysis showed a significant difference of NQO1 expression in HCCs of stage I and III as well as stage II and III, suggesting that upregulation of NQO1 transcription may be involved in the progression of HCC (Fig. 1B,C). Immunohistochemical results for the NQO1 protein levels showed that lack of NQO1 immunreactivity in normal or nontumorous liver tissues. Of the 69 HCC under investigation, 54 tumors (79%) were negative for NQO1 expression and only 15 tumors (21%) were distinctly positive for NQO1 protein immunostaining (Table 1A). The percentage of NQO1-positive cell number in HCC samples showed that 56



Stage	N>T	N=T	N<T	T/N ratio
I (n=22)	1	5	16	1.226
II (n=31)	7	1	23	1.232
III (n=35)	4	5	26	1.435 *

Fig. 1. RT-PCR analysis of NQO1 expression in HCC patients. **A.** NQO1 mRNA levels in nontumor liver tissue and paired tumor HCC tissue in TNM stage I, II, and III patients. **B.** Semi-quantitative analysis of NQO1 mRNA levels in nontumor liver tissue and paired tumor HCC tissue in HCC patients. Upper panel: The boxes represent the median values and the 25th and 75th percentiles for the T/N ratio, and the bars indicate the 90th and 10th percentiles. Lower panel: The T/N ratio is significantly increased ($p<0.05$) in stage III HCC patients compared to stage 1. **C.** Real-time quantitative PCR analysis of NQO1 mRNA levels in different stages of HCC patients (n=30). Each point in the graph represents the ratio of NQO1 mRNA expression in the tumor to that in corresponding nontumor liver tissue. Note that the T/N ratio of NQO1 mRNA expression is significantly increased ($p<0.05$) in stage III HCC patients compared to stage 1 and stage 2.

tumors were negative or less than 5% positive tumor cells (category 0), four (5.8%) were in category I (5-25% positive tumor cells), one (1.5%) was in category II (26-50% positive tumor cells), three (4.35%) were in category III (51-75% positive tumor cells) and five (7.25%) were in category 1 (76-100% positive tumor cells) (Fig. 2; Table 1B). Results from RT-qPCR and immunohistochemistry suggested that NQO1 in HCC tissues compared to the adjacent nontumor tissues was dramatically upregulated at the transcription levels but not increased at the translation levels.

Relationship between the NQO1 activity and NQO1 polymorphisms

Since HepG2 cells are wild-type and Mahlava and Huh-7 cells are heterozygous for polymorphism in NQO1, these cells were used to test the NQO1 enzymatic activity. Immunoblot analysis demonstrated the expression of NQO1 protein in these cell lines. The enzyme activity assay significantly showed that NQO1 activity in Mahlava and Huh-7 cells with heterozygous genotype is 13-15 fold less than that of the cells (HepG2) with wild type NQO1 (Fig. 3A).

NQO1 genotyping analyses in HCC tissues

Genotyping of NQO1 polymorphism was performed in 100 HCC and paired nontumor liver tissues. The frequencies and distributions of the NQO1 genotypes were 80% (20/25) wild type (C/C), 12% (3/25) heterozygous (C/T), and 8% (2/25) homozygous mutant

Table 1. Intensity of NQO1 immunoreactivity **(A)** and percentage of NQO1-positive cell number **(B)** in tumor tissues of hepatocellar carcinoma from different TNM stages.

(A)

Tumor tissues		NQO1 intensity				
Stage	No. of cases	0	1	2	3	4
I	23	18 (78.26%)	3 (13.04%)	2 (8.7%)	0 (0%)	0 (0%)
II	23	17 (73.91%)	5 (21.74)	1 (4.35%)	0 (0%)	0 (0%)
III	23	19 (82.6%)	2 (8.7%)	2 (8.7%)	0 (0%)	0 (0%)
Total	69	54 (78.26%)	10 (14.5%)	5 (7.25%)	0 (0%)	0 (0%)

(B)

Tumor tissues		Percentage of NQO1-positive cell number				
Stage	No. of cases	0	1	2	3	4
I	23	18 (78.26%)	1 (4.35%)	1 (4.35%)	1 (4.35%)	2 (8.7%)
II	23	19 (82.6%)	1 (4.35%)	0 (0%)	2 (8.7%)	1 (4.35%)
III	23	19 (82.6%)	2 (8.7%)	0 (0%)	0 (0%)	2 (8.7%)
Total	69	56 (81.16%)	4 (5.8%)	1 (1.5%)	3 (4.35%)	5 (7.25%)

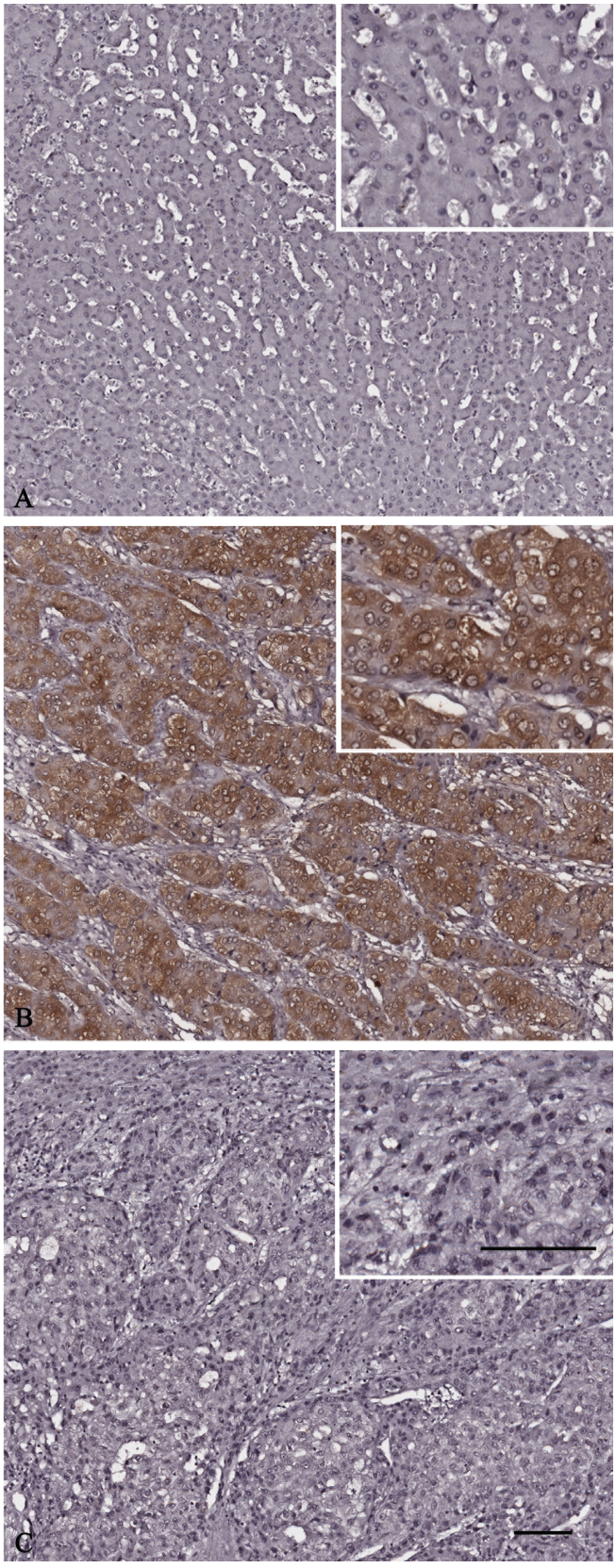


Fig. 2. Immunohistochemical staining for NQO1 in **(A)** nontumor liver tissue and **(B)** the tumor HCC tissues in patient with wild type (C/C) and **(C)** the tumor HCC tissues in patient with mutant type (T/T). Inset: higher magnification of the section. Bar: 100 μm.

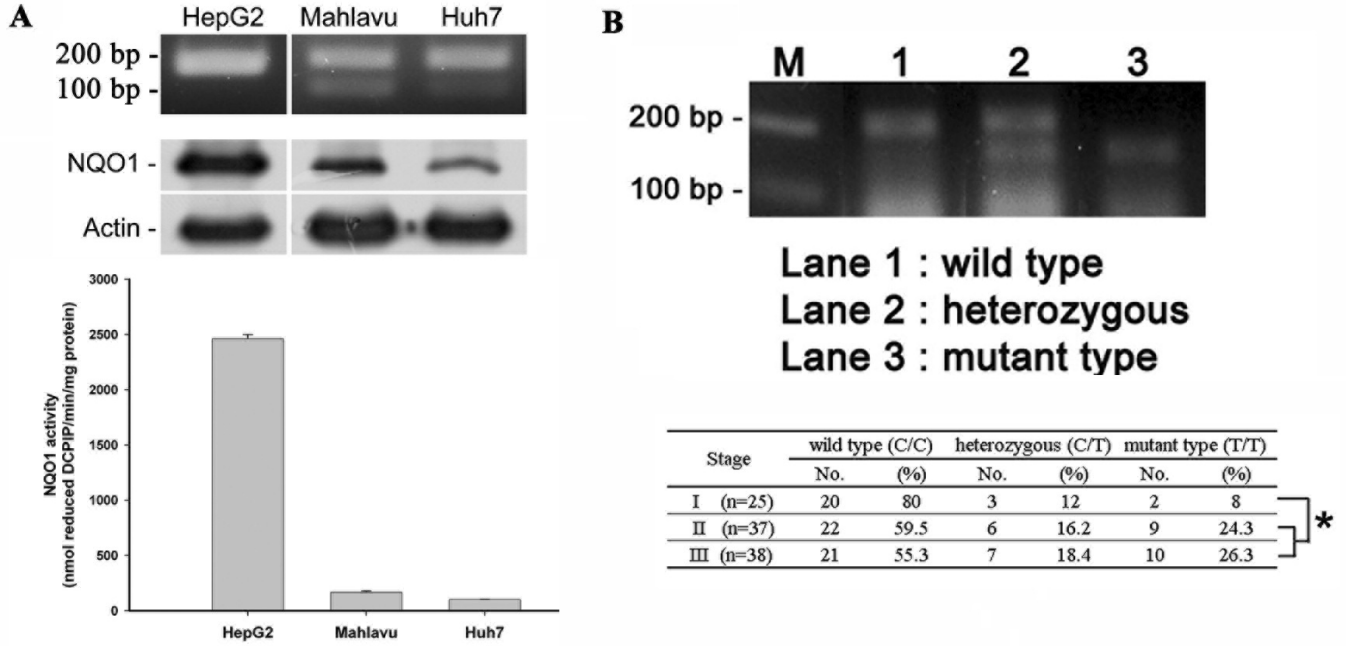
(T/T) in stage I; 59.5% (22/37) wild type, 16.2% (6/37) heterozygous, and 24.3% (9/37) homozygous mutant in stage II; and 55.3% (21/38) wild type, 18.4% (7/38) heterozygous, and 26.3% (10/38) homozygous mutant in stage III. As shown in Fig. 3, individuals carrying the homozygous mutant generally had an advanced stage of HCC.

Immunostaining analyses of p53 expression in HCC patients

To examine p53 status in HCC patients, two monoclonal mouse antibodies against human p53 protein were used, one against the normal form of p53 protein (Ab-5) and the other against the mutant form (DO-7). The binding of these two antibodies is shown in Fig. 4. Immunostaining with antibody Ab-5 was only detected in HCC tissues expressing the wild-type p53 genotype (Fig. 4C,D), while DO-7 antibody immunoreactivity was detected in HCC tissues with the mutant p53 genotype (Fig. 4E,F). Staining with both antibodies was seen in HCC tissues expressing the heterozygote p53 genotype (Fig. 4G,H). No p53 protein immunoreactivity was detected in human normal liver tissue in negative controls (Fig. 4A,B). The examination of p53 status by immunohistochemistry in 98 HCC cases showed 44% (43/98) were wild-type, 26.5% (26/98) mutant-type, and 29.5% (29/98) had both types of p53, showing that more than 56% of HCC cases carried the p53 mutation (Fig. 4I). However, there was no significant association between NQO1 and p53 genotypes in HCC using the chi-squared test.

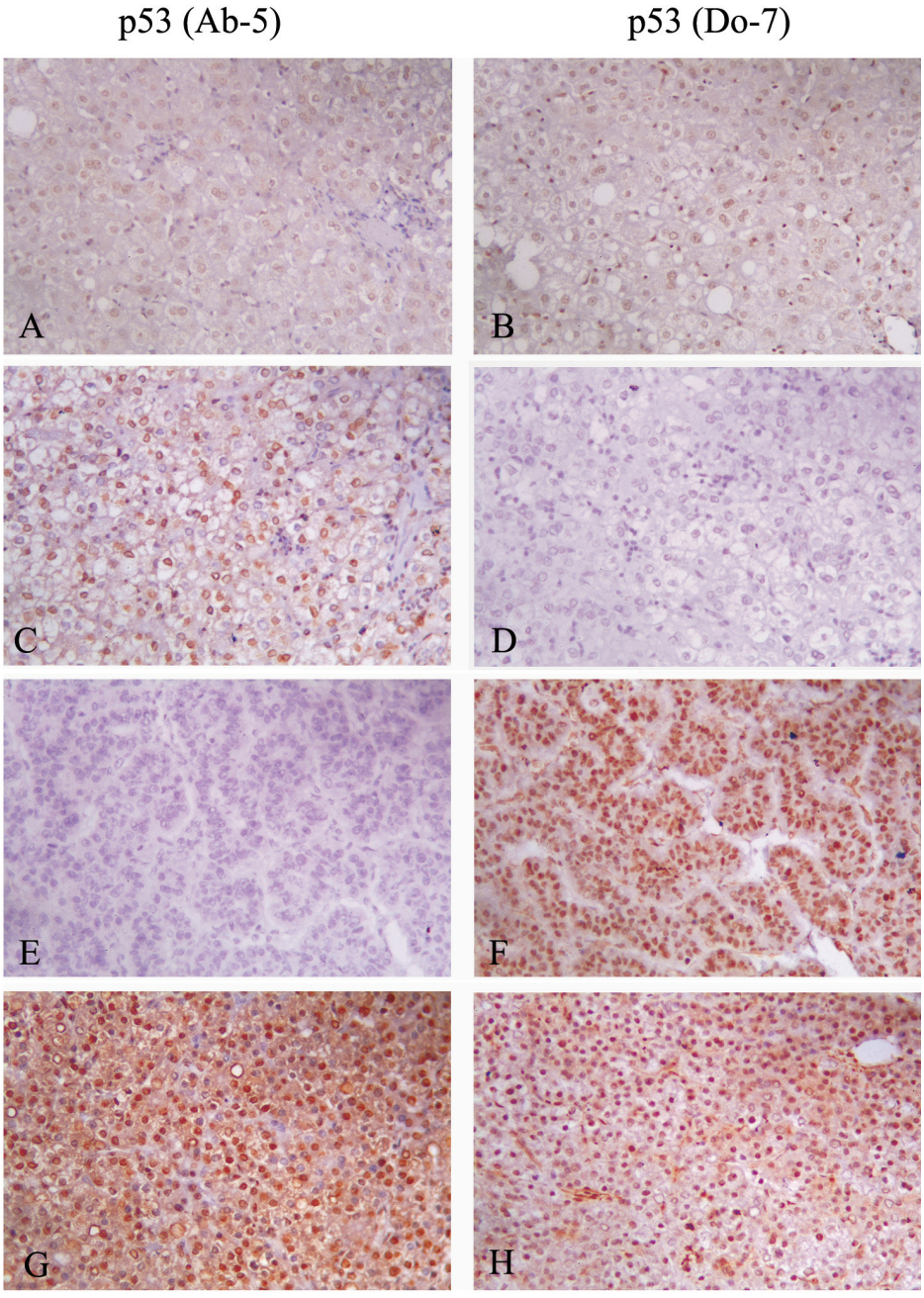
Correlation of the NQO1 T/N ratio with clinico-pathological factors and prognosis

Eighty-three HCC patients were categorized into two groups on the basis of their tumor NQO1 gene expression. Group 1 had an NQO1 T/N ratio <1.27 (n=41) and group 2 an NQO1 T/N >1.27 (n=42). We analyzed the association between the T/N ratio and clinicopathological parameters, including sex, hepatitis B surface antigen status, anti-HCV antibodies, serum AFP levels, ICG-15 value, serum albumin levels, serum total bilirubin levels, serum alanine aminotransferase levels, tumor size, tumor number, liver cirrhosis, and tumor p53 expression. The results are summarized in Table 2. Significant associations were found between the T/N ratio and patient age and tumor size. Patients with a T/N ratio <1.27 were significantly older and had smaller tumors than those with a T/N ratio >1.27. By the time of this study, the median follow-up was 48 months (range 1 to 110 months) after surgery. Patients with wild type p53 as shown by immunohistochemistry had a significantly better postresectional prognosis than those with the heterozygous/mutant type (p=0.012) (Fig. 5). In patients with a tumor size >5 cm, those with an NQO1 T/N ratio <1.27 had a significantly better prognosis than those



Stage	wild type (C/C)		heterozygous (C/T)		mutant type (T/T)		
	No.	(%)	No.	(%)	No.	(%)	
I (n=25)	20	80	3	12	2	8	
II (n=37)	22	59.5	6	16.2	9	24.3	*
III (n=38)	21	55.3	7	18.4	10	26.3	*

Fig. 3. Relationship of polymorphism and the enzyme activity of NQO1. **A.** NQO1 genotyping by PCR-RFLF analysis showed that the HepG2 cells are wild type (C/C), whereas Mahlava and Huh7 cells are heterozygous type (C/T). Immunoblot analysis demonstrated the expression of NQO1 protein in all cell lines. Note that the NQO1 enzyme activity of Mahlava and Huh-7 cells is less 13-15 fold than that of HepG2 cells by NQO1 enzyme activity assay. **B.** NQO1 genotyping by PCR-RFLF and the distribution of NQO1 polymorphisms in different TNM stages of HCC patients. *: p<0.05 between the indicated stages.



I

NQO1 \ p53	wild	heterozygous	mutant
Wild (n=67)	31 (46.27%)	16 (23.88%)	20 (29.85%)
Heterozygous (n=14)	5 (35.71%)	5 (35.71%)	4 (28.58%)
Mutant (n=17)	7 (41.18%)	8 (47.06%)	2 (11.76%)

Fig. 4. Immunohistochemical staining with anti-p53 antibodies (Ab-5, wild-type; and DO-7, mutant-type) in tumor liver tissues (**A-H**). **A and B.** Immunostaining of HCC tissue without anti-p53 antibody (Ab-5 or Do-7) served as negative control. **C and D.** Homozygous wild-type p53 HCC cells expressing wild-type p53 protein were immunostained by Ab-5 antibody, but not by DO-7 antibody. **E and F.** Homozygous mutant p53 HCC cells expressing mutant-type p53 protein were immunostained by DO-7 antibody, but not by Ab-5 antibody. **G and H.** Heterzygous p53 HCC cells expressing both wild-type and mutant p53 proteins were immunostained by Ab-5 and DO-7 antibodies. **I.** Analysis of the relationship between the NQO1 polymorphism and p53 status in different TNM stages of HCC patients.

Table 2. Comparison of clinical characteristics of HCC patients according to NQO1 T/N ratio.

	NQO1 T/N ratio <1.27 (n=41)	NQO1 T/N ratio ≥ 1.27 (n=42)	P
Age	65.7±11.2	59.3±11.9	0.014*
Male	37 (90.2%)	38 (90.5%)	0.971
HBsAg (+)	26 (63.4%)	28 (66.7%)	0.756
Anti-HCV (+)	12 (29.3%)	8 (19.0%)	0.276
α-fetoprotein, mg/dL	1336±3867	10045±53113	0.298
ICG-15, %	12.2±10.6	11.4±6.6	0.702
Albumin, g/dL	4.0±0.4	4.0±0.4	0.563
Total bilirubin, mg/dL	1.0±0.5	0.9±0.3	0.191
ALT, U/dL	55±51	58±47	0.770
Tumor size, cm	5.3±3.4	7.5±5.0	0.022*
Single tumor	28 (68.3%)	20 (47.6%)	0.057
Liver cirrhosis	22 (53.7%)	14 (33.3%)	0.062
Tumor P53 expression			0.277
Wild type	11/20 (55.0%)	17/24 (70.8%)	
Heterozygous/Mutant	9/20 (45.0%)	7/24 (29.2%)	

*: statistically significant.

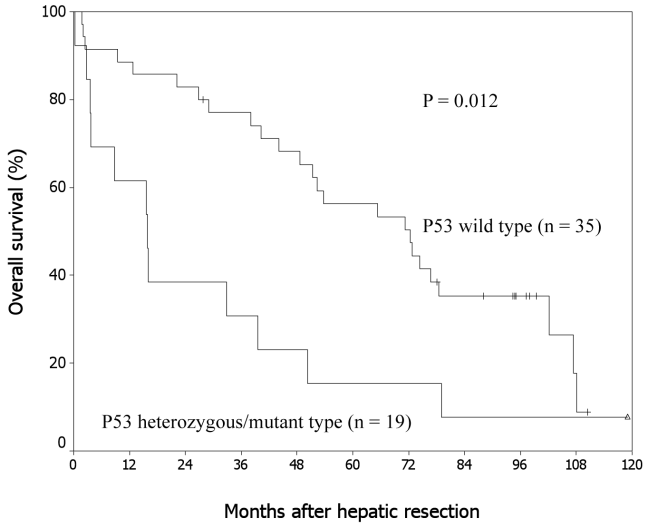


Fig. 5. Comparison of the overall survival rate of HCC patients with wild-type p53 or heterozygous/mutant type p53. Patients showing wild type p53 immunohistochemical staining (upper line) have a significantly better postresectional prognosis than patients with heterozygous/mutant type expression (lower line) (p=0.012).

with a T/N ratio >1.27 (p=0.036) (Fig. 6).

Discussion

NQO1 has been suggested to prevent the generation of free radicals, thus protecting cells against oxidative stress, but this is controversial. NQO1 overexpression in tumors has been demonstrated, especially in lung (Kiyohara et al., 2005), pancreatic (Lyn-Cook et al., 2006), and breast cancers (Siegel and Ross, 2000), and is indicated as a risk factor in these cancers. However, de

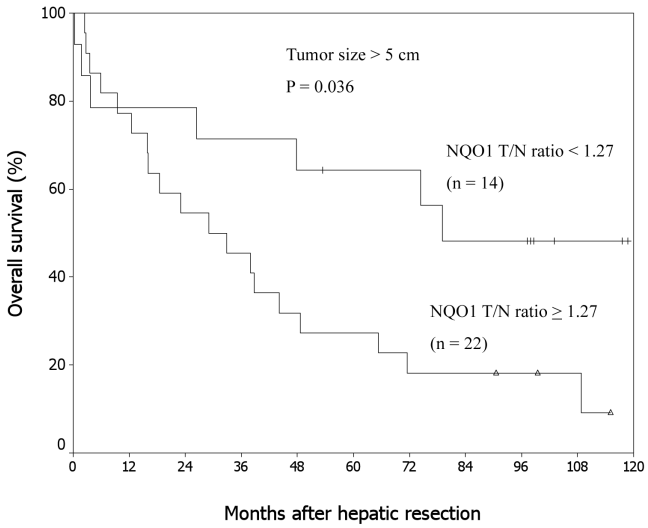


Fig. 6. Kaplan-Meier analysis of survival in HCC patients with a tumor >5 cm and an NQO1 mRNA T/N ratio of <1.27 or ≥ 1.27. In patients with a tumor size >5 cm, those with an NQO1 mRNA T/N ratio <1.27 (upper line) have a significantly better prognosis than those with a T/N ratio >1.27 (lower line) (p=0.036).

Hann et al. (2006) examined the relationship between NQO1 activity and protection against the cytotoxic effects of menadione and found that baseline NQO1 levels in normal liver, kidney, small intestine, colon, and lung are generally below the 'lower protection threshold', and that to achieve NQO1 levels above this threshold would require a 5- to 20-fold increase. Under normal physiological conditions, NQO1 is only weakly expressed in the liver (Siegel and Ross, 2000). Although NQO1 expression can be markedly upregulated after injury caused by alcohol (Schlager and Powis, 1990), tert-butylhydroquinone (Tanaka et al., 2007), or acetaminophen (Aleksunes et al., 2006), NQO1 activity in tumor tissue is only increased 3.8- to 6 fold compared to normal tissue (de Haan et al., 2006). Another problem is that over 20% of Asians, but only 4.4% of Caucasians, have the homologous C609T NQO1 polymorphism (Danson et al., 2004). C609T NQO1 results in a lack of enzymatic activity, so it would be expected that individuals with the homologous C609T NQO1 would have a higher susceptibility to developing cancer. Recent studies have shown that deletion of the NQO1 gene in mice increases sensitivity to menadione-induced cytotoxicity (Radjendirane et al., 1998) or benzene-induced toxicity (Bauer et al., 2003) and causes myelogenous hyperplasia (Long et al., 2002). Furthermore, a correlation between NQO1 polymorphism and cancer incidence has been found in patients with lung cancer (Sungata et al., 2002), upper gastrointestinal cancer (Sarbia et al., 2003), or colorectal cancer (Harth et al., 2000). However, whether NQO1 overexpression is a risk factor in human HCC is not clear.

Our present RT-PCR and real-time qPCR studies demonstrated an overexpression of NQO1 transcript levels and genotype analysis, showing an elevated level of *NQO1* C609T polymorphism in HCC tumor tissues (Fig. 1), respectively. These results are consistent with the findings in lung (Kiyohara et al., 2005), pancreatic (Lyn-Cook et al., 2006), and breast cancers (Siegel and Ross, 2000). Moreover, the high level of this C609T polymorphism correlated strongly with a more advanced TNM stage of HCC (Fig. 3), indicating that the overexpression of C609T NQO1 mRNA may be a prognostic factor of advanced HCC. Immunohistochemical investigations showed that the significant increase of NQO1 protein was only found in the tumor specimens with wild genotype (C/C), but weak or no NQO1 protein immunoreactivity was detected in non-neoplastic liver tissue, as well as in HCC tumor tissue in individuals carrying the heterozygous (C/T) or homozygous (T/T) null genotypes (Fig. 2). Additionally, only 21.7% of examined samples (15/69 specimens) had distinctly higher expression of NQO1 protein at different TMN stages (Table 1). Since the protein turnover studies showing the mutant-type NQO1 protein compared with that of the wild-type protein exhibited a dramatically decreased stability (half-life time 1.5 h vs 18 h) because of ubiquitination and proteosomal degradation, the loss of NQO1 immunoreactivity in HCC tissues of patients with C/T and T/T genotypes may be due to the instability and rapid degradation of mutant NQO1 protein (Siegel et al., 2001; Zhang et al., 2003).

Recent findings have shown that NQO1 can interact with p53 and protect it against 20S proteasomal degradation, leading to stabilization and activation of p53 in tumor cells (Saldivar et al., 2005; Gong et al., 2007). Inhibition of NQO1 activity by dicoumarol or curcumin induces p53 degradation and inhibits p53-dependent apoptosis (Asher et al., 2002b). NQO1 knockdown by specific small-interfering RNA reduces basal levels of p53 protein (Asher et al., 2002a), and NQO1-deficient (null) mice exhibit reduced p53 protein levels and decreased apoptosis in the bone marrow (Long et al., 2002). Since approximately 80% of patients with HCC have a p53 mutation (Sheen et al., 2003), detection of p53 mutant protein expression is commonly used as a marker for predicting the prognosis of HCC patients (Qin et al., 2002). In this study, we first investigated the association between p53 status and NQO1 polymorphism. Although our results showed upregulation of p53 transcripts and expression of mutant p53 in 56% of HCC patients (61/108), suggesting an important role of the p53 gene in HCC development, the association between the NQO1 polymorphism and p53 status did not reach statistical significance. Similar results have been reported in patients with bladder carcinoma (Martone et al., 2000) or Leber's hereditary optic neuropathy (Ishikawa et al., 2005).

In our study, the NQO1 T/N expression ratio was associated with tumor size and postresectional prognosis (Fig. 6). A T/N ratio >1.27 was seen in patients with a larger tumor and was related to a poorer survival in patients with a tumor >5 cm, suggesting that overexpression of *NQO1* transcription might be associated with the clinicopathological malignant characteristics of tumors. These findings indicated that NQO1 polymorphism correlated with more aggressive tumor behavior with a more advanced TNM stage of HCC. High levels of NQO1 expression might function as a prognostic index and could be associated with tumor progression in human HCC.

In summary, our study shows that (1) NQO1 expression in HCC tissues is higher than in paired nontumor liver tissues; (2) Since the NQO1 C609T polymorphism is lack of insufficient enzyme activity against carcinogenicity or mutagenicity, the overexpression of C609T NQO1 is suggested to be a prognostic factor of HCC; and (3) there is no association between the NQO1 polymorphism and p53 status in patients with HCC.

Acknowledgements. We thank Drs. An-Hang, Yang, Shyh-Haw Tsai and Annie Fan-Yau Lee (Department of Pathology, Taipei Veterans General Hospital) for their support and helpful discussion. This work is supported by grants NSC 93-2314-B075-093 and NSC 96-2752-B010-002-PAE from the National Science Council, Taiwan.

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Accepted April 8, 2009