

Differential Gene Expression of *NAD(P)H*:Quinone Oxidoreductase and *NRH*:Quinone Oxidoreductase in Human Hepatocellular and Biliary Tissue

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ABSTRACT

NAD(P)H:quinone oxidoreductase (NQO1) and dihydronicotinamide riboside:quinone oxidoreductases (NQO2) are cytosolic flavoproteins that catalyze the two-electron reduction of quinones and quinoid compounds to hydroquinones, thereby promoting detoxification and preventing the formation of highly reactive oxygen species, which lead to DNA and cell damage. Two NQO isoforms, designated NQO1 and NQO2, have been cloned and sequenced. To elucidate their role in carcinogenesis, the gene expression of human NQO1 and NQO2 in paired normal and tumor tissue samples was examined. Quantitative triplex reverse transcriptase polymerase chain reaction was employed to analyze NQO1 and NQO2 mRNA expression in normal hepatic and biliary tissue as well as in cholangiocellular carcinomas (CCC), hepatocellular carcinomas (HCC), and focal nodular hyperplasias (FNH). Coexpression of β -actin RNA was used as an internal reference standard and linear ranges of

transcript amplification were established for each sample. In normal hepatocellular tissue, the two NQO isoforms were differentially regulated, with a higher expression of NQO2 than NQO1. Malignant hepatocellular tissue (HCC), however, displayed up-regulation of NQO1 and down-regulation of NQO2. Regulation of either transcript was not seen in benign hepatocellular tumor tissue (FNH), which indicates a reciprocal control of NQO genes in hepatocarcinogenesis. Normal biliary tissue expressed a significantly higher level of NQO1 transcripts compared with normal liver, whereas biliary NQO2 levels were significantly lower than in hepatocellular tissue. Comparing the levels of expression in normal and malignant biliary tissue (CCC), no significant differences were noted between the expression levels of either transcript. Thus, this study provides evidence for differential hepatic and biliary regulation of both NQO1 and NQO2.

Quinones are a class of compounds ubiquitous in nature; they are natural byproducts of plants and vegetables. Humans are exposed to these agents either through dietary intake of naturally occurring quinones or through inhalation of airborne environmental contaminants generated through various combustion processes, such as automobile exhaust and cigarette smoke. Quinones undergo metabolism by either a one- or two-electron reduction. One-electron reduction is carried out by the cellular reductases, such as xanthine oxidoreductase, ubiquinone oxidoreductase, cytochrome P450 reductase, and cytochrome b5 reductase. This process generates semiquinone radicals, which can undergo redox cycling in the presence of molecular oxygen leading to the formation of reactive oxygen species (e.g., superoxide anion, perhydroxyl radical, hydrogen peroxide, hydroxyl radical). Reactive oxygen species promote oxidative damage. In contrast,

two-electron reduction is catalyzed by the *NAD(P)H*/dihydronicotinamide riboside (NRH):quinone oxidoreductases (NQOs), producing hydroquinones. These metabolites are more stable and can also be targeted for additional metabolism through conjugation with glutathione or glucuronic acid. In promoting obligatory two-electron reduction, quinone oxidoreductases prevent the formation of reactive semiquinone intermediates. Thus, they play an important role in cellular detoxification (Lind et al., 1982; Thor et al., 1982; Joseph and Jaiswal, 1994) and, by preventing the generation of reactive oxygen species, are considered part of the human antioxidant defense system.

Quinone oxidoreductases were originally felt to use NADH and NADPH as electron donors (Ernster and Navazio, 1958) in the reduction of quinones to hydroquinones (Iyanagi and Yamazaki, 1970). It is now known that *NAD(P)H*:quinone oxidoreductase 1 (NQO1) uses *NAD(P)H*, whereas *NRH*:quinone oxidoreductase 2 (NQO2) uses the nonbiogenic cosub-

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ABBREVIATIONS: NRH, dihydronicotinamide riboside; NQO1, *NAD(P)H*:quinone oxidoreductase 1; NQO2, dihydronicotinamide riboside:quinone oxidoreductase 2; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma; FNH, focal nodular hyperplasia; CCC, cholangiocellular carcinoma; RT, reverse transcriptase; bp, base pair(s); kb, kilobases.

strate NRH as the electron donor (Wu et al., 1997). Recombinant studies have concluded that human NQO1 and NQO2 possess approximately 49% amino acid sequence identity. Although the genes for *NQO1* and *NQO2* are on different chromosomes, there are regions of considerable nucleotide conservation, indicating that the two genes are evolutionarily conserved. However, because there is considerable amino acid divergence between the proteins and the requirements for electron donation are served by different cosubstrates, expression experiments have demonstrated that NQO1 and NQO2 exhibit remarkable differences in substrate specificity. Although it is believed that the formation of hydroquinones leads to detoxification, two-electron reduction catalyzed by both NQO1 and NQO2 can also activate certain substrates, such as quinone-containing antitumor drugs. The end result of such metabolism is the formation of highly reactive alkylating species, which readily associate with cellular macromolecules to initiate a cytotoxic reaction.

Because quinone oxidoreductases have been speculated to serve a role in genoprotection, studies in both humans and rodents have been undertaken to examine the relationship between these proteins and the incidence of certain diseases. In one study, NQO1 activity was estimated to be absent in 4% of the population (Edwards et al., 1980). This could be attributed to a C-to-T missense mutation at codon 187 (Pro to Ser), where the allele carrying the P187S change has been found to be inactive in cells that are homozygous (T/T) for the mutation (Traver et al., 1992, 1997). Linkage studies examining the P187S null allele in humans with cancers of the lung, kidney, prostate and gastrointestinal tract have been investigated and the results linking this mutation to disease have been inconclusive. Although several studies show no correlation, others have indicated that the P187S allele may be positively linked (Chen et al., 1999; Larson et al., 1999; Lin et al., 1999). Interestingly, the P187S null allele (T/T) has been associated with benzene toxicity (Rothman et al., 1997) in humans (Moran et al., 1999), which is characterized as an elevated risk of developing benzene-induced leukemia. In addition, the metabolites of benzene that accumulate in the bone marrow, including the hydroquinone and benzoquinone, are also positive regulators of NQO1, leading to transcriptional activation of the gene. It has also been shown that T/T genotype individuals are resistant to induction and thus express little NQO1 RNA, indicating that a regulatory polymorphism may also be involved in eliciting individuals susceptible to benzene toxicity (Pink et al., 2000). Additional evidence that NQO1 participates in detoxifying quinones comes from recent efforts to generate *NQO1*-null mice. Mice lacking NQO1 (*NQO1*^{-/-}) exhibit an increased sensitivity to the toxic effects of menadione, indicating that NQO1 supports a protective role against oxidative stress. Although the *NQO2* gene does show a restriction fragment length polymorphism (Jaiswal et al., 1990; Long and Jaiswal, 2000), no studies have been conducted linking this genotype to cancer or toxicity in humans.

Natural cellular defenses against environmental insults can be attributed to the recruitment of appropriate detoxification systems within the cell as well as an understanding of the tissue-specific distribution of these enzymes. NQO1 has been shown by direct immunohistochemistry to be expressed in many normal human tissues as well as in solid tumors from thyroid, adrenal, breast, ovarian, colon, cornea, and

non-small-cell lung cancers (Siegel et al., 1998; Siegel and Ross, 2000). Comparing normal and malignant tissue for quinone reductase activities, NQO was reported to be up-regulated in malignant tissue of the colon, breast, lung and liver and down-regulated in solid tumors of the stomach and kidney (McGinty et al., 1973; Koudstaal et al., 1975; Schor and Cornelisse, 1983; Schlager and Powis, 1990). Elevation of NQO1 RNA in solid tumors has also been demonstrated (Cresteil and Jaiswal, 1991; Belinsky and Jaiswal, 1993), but regulation in tumors is not a universal phenomenon. Immunohistochemical analysis did not detect NQO1 protein in small-cell lung cancer or carcinoid lung tumors (Siegel and Ross, 2000), and no differences were observed in DT-diaphorase (NQO1) activity in colon between normal and tumor tissue (De Waziers et al., 1991). Several studies indicate that NQO1 levels in the gastrointestinal tract are highest in stomach and ileum, whereas levels are low in colon and liver tissue (Schlager and Powis, 1990; Siegel and Ross, 2000).

Limited studies have been undertaken to examine NQO2 expression in human tissues. Using Northern blot analysis, NQO2 was found to be expressed heart, brain, lung, liver, kidney, and skeletal muscle, but no RNA was detected in placenta (Jaiswal, 1994). No previous study has investigated NQO2 enzyme activity or mRNA expression in malignant human tissues.

A comparative analysis of NQO1 and NQO2 would be of particular interest in metabolically active tissue. Liver tissue is differentiated into two metabolically active cell types involved in detoxification and excretion: hepatocytes and cholangiocytes. Different malignant tumors develop from these cell types, offering the unique opportunity to examine the regulation of NQO1 and NQO2 in normal and malignant tissues of related function and origin. In this study, we developed a triplex reverse transcriptase PCR method and employed Southern and Northern blot analyses to investigate the expression of both NQO1 and NQO2 mRNA in normal and malignant hepatocellular and cholangiocellular tissue as well as in benign hepatocellular tumors.

Materials and Methods

Tissue Procurement. Paired tissue samples of normal and tumor tissue were obtained from 19 patients undergoing hemihepatectomy or liver transplantation (University of Hannover Medical Center in Hannover, Germany) for HCC ($n = 12$ patients, age 58 ± 10.4 years (mean \pm S.D.), 11 men, 1 woman), FNH ($n = 3$ patients, mean age 37.3 ± 14.5 years, all women), and CCC ($n = 4$ patients, mean age 56.8 ± 18.5 years, three men, one woman). Only tissue without any visible sign of necrosis was collected. Diagnosis was established through histological examination. In every case, the sample pair of normal and tumor tissue was taken from the same resection specimen, immediately snap-frozen in liquid nitrogen, and continuously stored at -80°C until analysis.

Isolation of RNA. The isolation of RNA was based on the protocol of Chomczynski and Sacchi (1987) and has been described in detail previously (Strassburg et al., 1997b). Approximately 200 mg of frozen tissue sample were pulverized in liquid nitrogen in a mortar. The frozen tissue powder was immediately lysed in 1 ml of acidic phenol/guanidine thiocyanate solution (TriPure; Roche Molecular Biochemicals, Mannheim, Germany). Samples were not allowed to thaw at any point of the procedure. RNA was extracted by addition of chloroform, resuspended in 50 μl of diethylpyrocarbonate-treated water and frozen at -80°C until analysis. Quantity and purity of the RNA were determined by spectrophotometry at $A_{260\text{ nm}}$ and $A_{280\text{ nm}}$. Pos-

sible DNA contamination was monitored by employing PCR-primer pairs for amplification of β -actin, NQO1 and NQO2 cDNA that span several exon/intron junctions leading to amplification products of different sizes if generated from genomic or cDNA templates.

Reverse Transcription of RNA. Three micrograms (in 5 μ l of water treated with diethyl pyrocarbonate) of RNA were denatured at 70°C for 10 min in the presence of 1 μ l of oligo(dT)12–18 (0.5 μ g/ μ l) followed by chilling on ice for 2 min. In a volume of 19 μ l containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM MgCl₂, 10 mM dithiothreitol, and 0.5 mM each dNTP, the RNA was incubated at 42°C for 5 min before the addition of 1 μ l of reverse transcriptase (200 units/ μ l SuperScript II RT; Invitrogen, Carlsbad, CA). The final volume of 20 μ l was incubated at 42°C for 50 min followed by 70°C for 15 min. The incubations were then chilled on ice before use in PCR.

Triplex RT-PCR Amplification of NQO1, NQO2 and β -Actin Transcripts. Six primers were generated for the amplification of human NQO1, NQO2 and β -actin by automated phosphoramidite chemistry at the UCSD Cancer Center Molecular Biology Core facility. PCGene and GenBank Blastn software were employed to exclude cross-reactivity and self-complementarity of primers. Primer melting temperatures ranged from 54–63°C. All of the following accession and base-pair numbers refer to sequences deposited in GenBank: β -actin sense primer (accession number M10278, bases 942–962), 5'-ggcggccaccacatgtacctt-3'; β -actin antisense primer (accession number M10278, bases 1123–1143), 5'-aggggcccggactctgctact-3'; NQO1 sense primer (accession number J03934, bases 92–113), 5'-gaggactcttcaactatgcc-3'; NQO1 antisense primer (accession number J03934, bases 437–457), 5'-cctttgtcatatgagcagc-3'; NQO2 sense primer (accession number J02888, bases 214–234), 5'-ggaacccaagtcttcaacgg-3'; NQO2 antisense primer (accession number J02888, bases 816–836), 5'-tgggtcttctctcagatgg-3'. Primers for β -actin have been published previously (Strassburg et al., 1997b).

NQO1, NQO2, and β -actin were coamplified in a volume of 96 μ l containing 1.5 mM MgCl₂, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.0% Triton X-100, 0.2 mM concentration of each dNTP, 1.0 μ M concentration of each primer, 4 units of VENT DNA polymerase (New England Biolabs, Beverly, MA) and 0.625, 1.25, 2.5, or 5 μ l of cDNA solution. The mixture was incubated at 94°C for 3 min followed by 30 cycles of 94°C (30 s), 57°C (30 s), and 72°C (30 s) and a final 7-min incubation at 72°C. β -Actin primers were added to a concentration of 0.2 μ M each after the first six cycles for a final reaction volume of 100 μ l. Triplex RT-PCR reactions for each sample were performed in duplicate in a PerkinElmer GeneAmp PCR System 2400. PCR products were stored at –20°C. Linear kinetics for all three coamplified products were documented by amplification using different dilutions of cDNA template in all studied samples and by terminating PCR amplification reactions after 26, 28, 30, 32, and 34 cycles. Independence of triplex coamplification product generation was documented by comparison with single or duplex PCR amplification of the same amplicons.

Quantitation of PCR Products. Fifteen microliters of each PCR reaction was resolved in a 2% agarose gel containing 1 μ g/ml ethidium bromide. Gels were photographed using Polaroid (Cambridge, MA) type 665 positive/negative film and negatives were used to quantify bands by laser densitometry (LKB 2222–020 UltoScan XL densitometer; LKB, Bromma, Sweden). The results for human β -actin were used as internal standard for each sample. Arbitrary units were calculated relative to β -actin.

Cloning and Analysis of the 366-bp NQO1 Transcript and the 623-bp NQO2 Transcript. After synthesis of cDNA from total RNA, the 366- and 623-bp transcripts were PCR-amplified separately. The NQO1 sense primer (5'-gaggactcttcaactatgcc-3') and the NQO2 sense primer (5'-ggaacccaagtcttcaacgg-3') were designed to incorporate a *Hind*III restriction endonuclease cleavage site 5' to the respective 366- or 623-bp transcript. The NQO2 antisense primer (5'-tgggtcttctctcagatgg-3') contained an *Xba*I site 3' to the NQO2 transcript. The NQO1 antisense primer (5'-cctttgtcatatgagcagc-3') was not altered. The amplified NQO1 and NQO2 PCR products were

column-purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The NQO1 PCR product was digested with *Hind*III and cloned into the *Hind*III/*Sma*I sites of the pBluescript KS⁺ vector (Stratagene, La Jolla, CA), whereas the NQO2 PCR product was digested with *Hind*III/*Xba*I and cloned into the *Hind*III/*Xba*I sites of the pBluescript KS⁺ vector. Ligation products were transformed into *Escherichia coli* MN522 cells, positive clones selected and sequences were determined by dideoxy sequence analysis (Sanger et al., 1977).

Southern Blot Analysis. Triplex RT-PCR products were separated in a 1% agarose gel. The DNA was transferred to a nitrocellulose membrane (Immobilon-NC; Millipore, Bedford, MA) after incubating the gel for 20 min in a buffer containing 1.5 M NaCl and 0.5 N NaOH followed by neutralization for 45 min in a buffer containing 1.5 M NaCl and 1 M Tris-HCl, pH 7.4. Hybridization was performed with the nick-translated ³²P-labeled (Amersham Biosciences, Piscataway, NJ) NQO1 transcript. Approximately 10⁸ cpm/ml was used in hybridization. Membranes were washed with 2 \times standard saline citrate/0.1% SDS at room temperature and 0.1 \times standard saline citrate/0.1% SDS at 42°C (1 \times standard saline citrate = 15 mM sodium citrate, pH 7.0, containing 150 mM NaCl). Dried membranes were exposed to X-ray film then stripped by two washes with boiling 0.1% SDS solution of 15 min each, rehybridized with the ³²P-labeled NQO2 transcript, and again exposed to X-ray film. After stripping for a second time, rehybridization with a ³²P-labeled full-length human β -actin probe was followed by a third exposure to X-ray film.

Northern Blot Analysis. Twenty micrograms of total RNA were separated in a 1% denaturing agarose gel containing 7% formaldehyde and transferred to a nitrocellulose membrane. Hybridization was performed with the nick-translated, ³²P-labeled NQO1 transcript overnight. Dried membranes were exposed to X-ray film at –80°C. For rehybridization, the membranes were then stripped by washing with boiling 0.1% SDS solution, rehybridized with the ³²P-labeled NQO2 transcript, and exposed to X-ray film. Once again, stripping was carried out, followed by rehybridization with a ³²P-labeled full-length human β -actin probe.

Results

Expression of the NQO1 and NQO2 Genes in Normal and Tumorigenic Tissue. To examine the expression of NQO1, NQO2, and β -actin genes in human tumor and normal tissue, a triplex RT-PCR amplification protocol was developed so that levels of gene expression were quantitated during the linear amplification phase of each transcript. PCR primers were selected yielding amplification products of the following sizes: 202 bp (β -actin), 366 bp (NQO1), and 623 bp (NQO2). Each primer pair was designed to anneal specifically to different exons.

NQO1, NQO2, and β -actin transcripts were analyzed in 12 HCC, 3 FNH, and 4 CCC tissue samples and paired with expression patterns from corresponding normal tissue that was taken adjacent to each tumor. Analysis of PCR products from normal liver and biliary tissue (Fig. 1) demonstrated NQO2 to be expressed in both, with the relative abundance of NQO2 being slightly lower in biliary epithelium ($p < 0.001$). However, laser densitometry quantification and calculation of band intensities relative to β -actin documented significantly greater expression of NQO1 ($p = 0.007$) in normal biliary tissue than in hepatocellular tissue. This is somewhat in contrast to analysis of bile duct epithelium by immunohistochemistry, where only trace amounts of NQO1 were detected (Siegel and Ross, 2000).

Comparison of malignant HCC with surrounding normal hepatocellular tissue demonstrated a dramatic increase in

NQO1 transcript levels in HCC, whereas the NQO2 transcript levels were mostly unchanged or slightly decreased (Fig. 2). These results were confirmed by Southern blot hybridization of the PCR products (Fig. 2, right) and by Northern blot analysis (Fig. 3). Two RNA signals of 1.2 and 2.7 kb were detected by Northern blot analysis with the NQO1 probe as described previously (Jaiswal et al., 1988), a result that corresponds to the use of multiple polyadenylation signals (Jaiswal, 1991), whereas a single 1.2-kb band was identified for NQO2 (Jaiswal, 1994).

In contrast to their regulation in HCC, triplex RT-PCR amplification of NQO1, NQO2 and β -actin from samples of the benign liver tumor FNH did not demonstrate appreciable regulation of NQO transcripts (Fig. 4, right). Furthermore, differential regulation of the NQO1 and NQO2 RNA transcripts was not found between normal biliary tissue and the malignant CCC (Fig. 4, left).

Summation of the results quantified by laser densitometry and analyzed for statistical significance by *t* test is shown in Fig. 5. In a comparison between normal hepatocellular tissue and HCC, there was a statistically significant up-regulation of NQO1 ($p < 0.001$) and down-regulation of NQO2 ($p = 0.01$). Regulation of either transcript between normal liver tissue and FNH (NQO1, $p = 0.26$; NQO2, $p = 0.49$) as well as between normal biliary tissue and CCC (NQO1, $p = 0.97$; NQO2, $p = 0.58$) was not statistically significant (Fig. 5, FNH and CCC, respectively).

Discussion

Metabolism by quinone oxidoreductases is considered to bypass the generation of reactive oxygen species from quinone derivatives through a unique two-electron reduction that facilitates the detoxification and elimination of xenobiotic and carcinogenic compounds (Lind et al., 1982; Thor et al., 1982; Prestera et al., 1993; Joseph and Jaiswal, 1994).

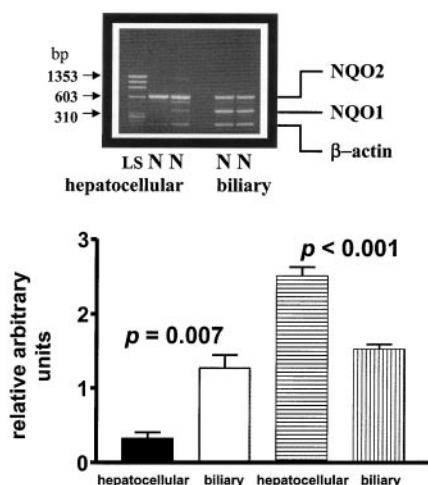


Fig. 1. Triplex RT-PCR amplification comparing NQO1 and NQO2 regulation in normal hepatocellular and normal biliary tissue. Each PCR reaction was carried out with 2.5 μ l of cDNA solution for 30 cycles. Top, PCR amplification results from two normal hepatocellular and two normal biliary tissue samples. Bottom, statistical analysis of triplex RT-PCR amplification results from all normal hepatocellular ($n = 16$) and normal biliary ($n = 4$) tissue samples. A statistically significant up-regulation of NQO1 ($p = 0.007$) and down-regulation of NQO2 ($p < 0.001$) between normal hepatocellular and biliary tissue was documented. ■, NQO1-hepatocellular; ■, NQO1-biliary; ■, NQO2-hepatocellular; ■, NQO2-biliary. LS, length standard (Φ X174RF DNA/*Hae*III fragments).

This process therefore plays an important role among the cellular metabolic defense mechanisms. On the other hand, NQOs have been implicated in the metabolic activation of chemotherapeutic agents (Riley and Workman, 1992). To initiate experiments studying their role in cancer development, data on specific NQO tissue expression was examined.

For further definition of the potential role that NQOs assume in the metabolism of xenobiotic compounds, expression studies in tissue types distinguished by a high degree of metabolic activity are of particular interest. Liver tissue exhibits this property; furthermore, it is differentiated into two major specialized cell types: hepatocytes and cholangiocytes.

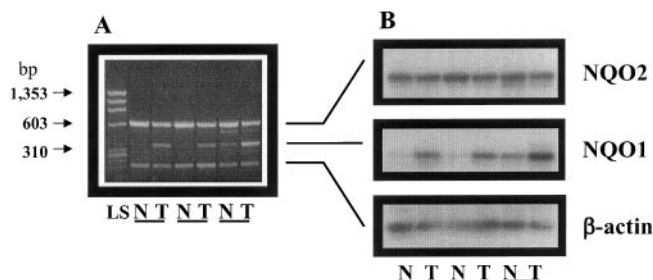


Fig. 2. Triplex RT-PCR (A) and Southern blot analysis (B) comparing NQO1- and NQO2-expression in paired normal (N) and malignant (T) liver tissue samples (HCC) from three patients (pairs underlined). In every PCR reaction, 2.5 μ l of cDNA was used as template and amplification was performed for 30 cycles. PCR products were transferred from an agarose gel to a nitrocellulose filter. NQO1 (366 bp) and NQO2 (623 bp) cDNA fragments were cloned, sequenced, and consecutively used as 32 P-labeled probes for Southern blot hybridization followed by hybridization with a full-length human β -actin probe. LS, length standard (Φ X174RF DNA/*Hae*III digest).

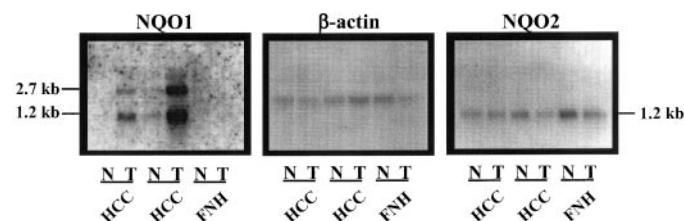


Fig. 3. Differential expression of the NQO1 and NQO2 genes as detected by Northern blot analysis. Total RNA (20 μ g) from paired tumor (T) and the corresponding normal (N) hepatocellular tissue of two patients with HCC and one patient with FNH were electrophoresed and the RNA transferred to nitrocellulose paper. Left, NQO1. Two RNA signals of 1.2 and 2.7 kb are detected. Center, β -actin. Right, NQO2.

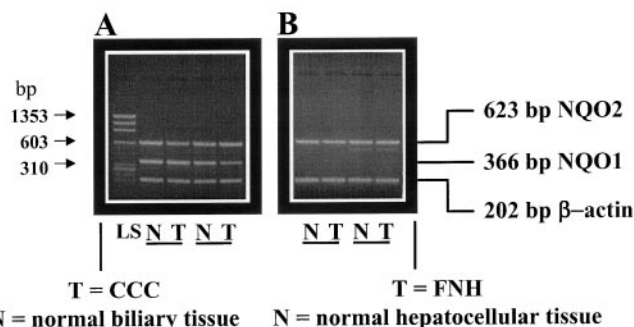


Fig. 4. Triplex RT-PCR amplification of NQO1, NQO2, and β -actin from paired normal and tumor tissue samples. Each reaction contained 2.5 μ l of cDNA solution as template and was carried out for 30 cycles. A, PCR results of two sample pairs comparing amplification from normal gallbladder tissue (N) and CCC (T). B, PCR results of two sample pairs comparing amplification from normal hepatocellular tissue (N) and FNH (T). LS, length standard (Φ X174RF DNA/*Hae*III digest).

These characteristics provide the means for a tissue-specific and, with both benign and malignant tumors arising from the same cellular origin, tumor-specific analysis of NQO1 and NQO2 expression in metabolically active tissue. We demonstrate the parallel investigation of *NQO1* and *NQO2* gene expression in hepatocellular and cholangiocellular tissue by means of a quantitative one-step triplex RT-PCR method that permits the specific identification and amplification of the two NQO transcripts.

Analysis of NQO1 expression in liver tissue demonstrated low NQO1 transcript levels in normal hepatocellular tissue and a statistically significant up-regulation in malignant

HCC, confirming previous observations on the expression of NQO1 in liver tumors (Cresteil and Jaiswal, 1991; Belinsky and Jaiswal, 1993). In comparison, the predominant quinone oxidoreductase in liver is NQO2. Expression of NQO2 was observed to be slightly higher in normal hepatocellular tissue compared with malignant hepatocellular tumor tissue (Figs. 2, 3, and 5). While NQO1 is dramatically regulated in hepatocellular carcinoma, no statistically significant regulation of either transcript was observed between normal hepatocellular tissue and FNH, a benign hepatocellular tumor (Figs. 4 and 5). It remains unclear whether the regulatory changes in HCC are early events in carcinogenesis or consequences of cancer development, but these results demonstrate that the process of carcinogenesis affects primarily NQO1.

Compared with the regulation observed for NQO1 in HCC and not in FNH, the *UDP-glucuronosyltransferase 1A* (*UGT1A*) locus has previously been demonstrated to be down-regulated in HCC in contrast to no regulation in FNH tumor tissue (Strassburg et al., 1997a). In addition, regulation in premalignant liver adenoma tissue was observed, suggesting that *UGT1A* regulation is an early event in hepatocarcinogenesis. Interestingly, *UGT1A* down-regulation was not only seen in malignant liver tumors of hepatocellular origin but also in those of cholangiocellular origin. Quinone oxidoreductases, however, display a regulatory difference between hepatocellular and cholangiocellular tissue. Whereas NQO1 and NQO2 expression was demonstrated to be regulated in HCC, a more individual regulatory pattern was observed in CCC leading to no statistically significant regulatory results (Fig. 5). This regulatory difference between liver and biliary tissue was even more striking when normal tissue samples were compared. The NQO1 transcript was distinctly more abundant in cholangiocellular than in hepatocellular tissue (Fig. 1), whereas expression of the NQO2 transcript is abundant in both hepatocellular and cholangiocellular tissue. These data correlate with a report by Martin et al. who observed a higher tissue activity of DT-diaphorase (NQO1) in a gallbladder tissue sample than in normal liver tissue.

Because of elevations in NQO1 activity in solid tumors relative to surrounding normal tissue, this activity has been exploited for catalyzing the two electron reduction of antitumor agents with the hopes of being effective cytotoxic agents (Ross et al., 2000). Indeed, experiments using cell lines that overexpress NQO1 have demonstrated a linkage to the cytotoxic actions of NQO1 antitumor substrates (Kelland et al., 1999; Pink et al., 2000; Okamura et al., 2000), although certain cells with elevated levels of NQO1 seem to be resistant to NQO1-mediated cytotoxicity (Brunton et al., 1998). It is also important to note that although *NQO1* gene expression may be elevated in liver tumors, other factors, such as the inheritance of the NQO1 P187S (T/T) allele, render the protein inactive (Traver et al., 1997; Siegel et al., 1999), thus making it unlikely that the antitumor compounds will be effectively metabolized to cytotoxic agents. Our studies indicate that not all malignant tumors, such as CCC, would be an appropriate target for NQO1-mediated bioactivation of antitumor agents, because there is little difference in NQO1 expression between normal and malignant tissue. In addition, identifying novel cosubstrates for the activation of potential antitumor substrates by NQO2 may also be a chal-

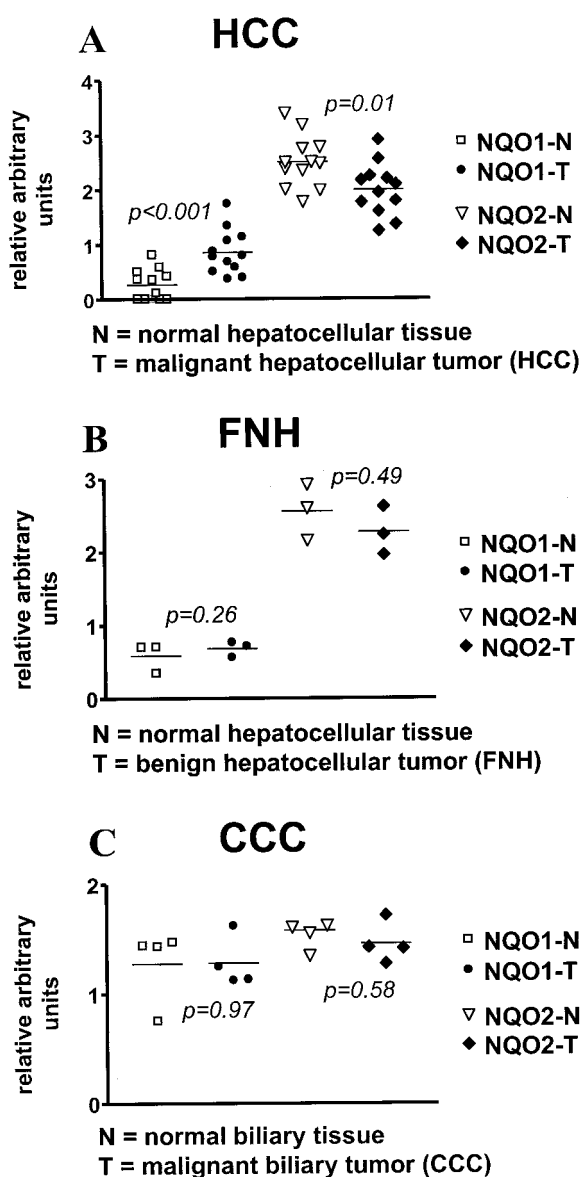


Fig. 5. Statistical analysis of triplex RT-PCR results. NQO1, NQO2, and β -actin transcripts were coamplified from total RNA of 12 HCC samples (A), 4 CCC samples (B), 3 FNH samples (C), and the corresponding normal tissue samples. PCR products were quantitated by laser densitometry, arbitrary units relative to β -actin levels were calculated and data pairs (N/T) were analyzed by *t* test. Each data point represents the mean of two amplifications from the same RNA. Lines represent the mean of each data set. The differential up-regulation of NQO1 and down-regulation of NQO2 in HCC was statistically significant (NQO1, $p < 0.001$; NQO2, $p = 0.01$).

lenge because NQO2 does not seem to be significantly regulated between normal and malignant tissues.

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