No Association of NAD(P)H: quinone oxidoreductase 1 (NQO1) C609T Polymorphism and Risk of Hepatocellular Carcinoma Development in Turkish Subjects

Hikmet Akkız1, Süleyman Bayram1,2*, Aynur Bekar1, Ersin Akgöllü1, Yakup Ülger1, Berrin Yalınbaş Kaya1, Macit Sandıkçı1, Burhan Özdi1

Abstract

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In this study, we aim to evaluate the association of NQO1 C609T with the risk of hepatocellular carcinoma (HCC) development among Turkish population. NQO1 C609T polymorphism was investigated in 167 confirmed subjects with HCC and 167 cancer-free control subjects matched on age, gender, smoking and alcohol consumption by using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. There is no association between the allele or genotype of NQO1 C609T polymorphism and HCC development risk in the Turkish subjects examined (p>0.05). Our result demonstrate for the first time that the NQO1 C609T polymorphism is not a genetic susceptibility factor for HCC in the Turkish population. Independent studies are need to validate our findings in a larger series, as well as in patients of different ethnic origins.

Keywords: Hepatocellular carcinoma - NQO1 C609T polymorphism - susceptibility - case-control study

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the worldwide and the third leading cause of cancer death. Because of its high fatality rates, the incidence and mortality rates are approximately equal (Parkin et al., 2005). It is now well established that multiple risk factors contribute to hepatocarcinogenesis, including chronic HBV or HCV infection, cirrhosis, carcinogen exposure (such as aflatoxin B1), excessive alcohol drinking, and a number of genetic and epigenetic alterations (Farazi and DePinho, 2006; El-Serag and Rudoph, 2007). However, HBV and HCV infections are the major cause of HCC, only a fraction of infected patients develop HCC during their lifetime; therefore the identification of other risk characteristics to stratify those individuals into high-risk populations is needed. As in many cancers, genetic polymorphisms of the genes involved in multistage of hepatocarcinogenesis may determine individual’s susceptibility to the development of HCC (Akkız et al., 2009; 2010). The identification of single nucleotide polymorphisms that affect gene function or expression and contribute to HCC susceptibility is important as it may help to predict individual and population risk and clarify pathophysiologic mechanisms relevant to HCC.

The human NAD(P)H:quinone oxidoreductase 1 (NQO1, Enzyme Commission (EC) number 1.6.99.2) formerly referred to as diphtheria toxin diaphorase (DT-diaphorase), is an important homodimeric flavin adenine dinucleotide (FAD)-containing enzyme (Lind et al., 1990). Several lines of evidence have indicated that NQO1 enzyme may have a protective effect against carcinogenicity, mutagenicity and cytotoxicity caused by quinones and their metabolic precursors (Lind et al., 1982; Thor et al., 1982; Morrison et al., 1984). This cytosolic flavoenzyme is ubiquitously expressed in most tissues that catalyzes the two-electron reduction of endogenous and exogenous quinoid compounds into their reduced form, such as hydroquinones (Ross et al., 1993).
Thus, NQO1 protects cells from oxidative damage to DNA and other cellular macromolecular constituents by preventing generation of reactive oxygen species (ROS) through the reduction of highly toxic quinoid compounds (Nebert et al., 2002). However, the activity of the NQO1 enzyme strongly depends on polymorphisms at the NQO1 locus. There have been more than 93 single nucleotide polymorphisms (SNPs) identified in NQO1 gene. The most widely studied SNP of NQO1 is a cytosine (C) to thymine (T) transition at nucleotide position 609 exon 6 of NQO1 cDNA that encodes for a proline (P) to serine (S) substitution at position 187 in amino acid sequence of the protein (Traver et al., 1997). The reference number of this SNP in the database of the National Center for Biotechnology Information (NCBI) is rs1800566. It has been shown that the lack of protein as a result of the NQO1 TT genotype appears to be due to accelerated degradation T allele of NQO1 protein mediated by the polyubiquitination and proteasomal system (Siegel et al., 2001). Compared with wild type genotype (CC) the enzyme activity of homozygous variant genotype (TT) has only 2% to 4% of the quinone reductase activity, whereas the heterozygote variant has a 3-fold decrease in enzyme activity level (Siegel et al., 1999), so it would be expected that individuals with the homologous NQO1 C609T polymorphism would have a susceptibility developing cancer. A wide variation of the of allelic frequency of NQO1 609T allele has been observed across ethnic groups ranging from 0.22 to 0.45 (Nebert et al., 2002).

The homozygous TT genotype is as rare as 2-5% in Caucasians and Blacks but as frequent as 20% in Asians (Nebert et al., 2002).

NQO1 C609T polymorphism has been associated with an increased risk of various cancers including, bladder (Park et al. 2003), gastric (Sarbia et al., 2003; Zhang et al., 2003), cervical (Niwa et al., 2005), renal (Schulz et al., 1997), urothelial (Schulz et al., 1997; Wang et al., 2008), colorectal (van der Logt et al., 2006; Lafuente et al., 2000; Begleiter et al., 2006), breast (Menzel et al., 1999; Soucek et al., 2004), esophageal (Sarbia et al., 2003; Zhang et al., 2003) and both pediatric (Wiemels et al., 1999; Krajnovic et al., 2002) and adult leukemias (Naoe et al., 2000; Smith et al., 2001). In contrast, no association was found in renal (Longuemaux et al., 1999), esophageal (Hamajima et al., 2001), breast cancer (Hamajima et al., 2002; Siegelmann et al., 2002), prostate (Steiner et al., 1999; Hamajima et al., 2002), gastric (Hamajima et al., 2002), head and neck cancers (Begleiter et al., 2005) adult gliomas (Peters et al., 2001), lymphomas (Hamajima et al., 2002; Soucek et al., 2002) and conflicting results have been published for lung cancer (Kiyohara et al., 2005) and esophageal cancer (Hamajima et al., 2002; Sarbia et al., 2003; Zhang et al., 2003; di Martino et al., 2007).

NQO1 enzyme is implicated in stabilization of the p53 tumor suppressor and protect it against 20s proteasomal degradetion, resulting in increased apoptosis in carcinogen-initiated colonic epithelial cells, which prevents these cells from progressing to a neoplastic state (Asher et al., 2001; Gong et al., 2007; Long 2nd et al., 2002). Thus, reduced NQO1 activity due to NQO1 C609T polymorphism may decrease the level of p53, reduce apoptosis and increase genomic instability in hepatocyte increasing risk of susceptibility HCC. Recent findings have shown that NQO1 expression is upregulated statistically significant in HCC when compared with normal hepatocellular tissue, suggesting that NQO1 plays an important role in cellular defense during hepatocellular carcinogenesis (Strassburg et al., 2002; Chiu et al., 2009). Chiu et al., (2009), reported that high levels of NQO1 might function as a prognostic factor and could be associated with tumor progression in human HCC. Moreover, the findings of that study indicated that T variant allele of NQO1 C609T polymorphism correlated with more aggressive tumor behavior with a more advanced Tumor-Node-Metastasis (TNM) stage of HCC (Chiu et al., 2009). Hence, NQO1 C609T polymorphism may be a relevant candidate SNP for HCC susceptibility. According to our recent knowledge, no research has been done to evaluate NQO1 C609T polymorphism and risk of HCC development. To test the hypothesis that the polymorphism of NQO1 C609T is associated with risk of developing HCC, we performed genotyping analysis using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay in a hospital-based case-control study of 167 confirmed HCC patients and 167 age, gender, smoking and alcohol consumption matched cancer-free controls in Turkish population.

Materials and Methods

Study population

The study was approved by the Committee for Ethics of Medical Experiment on Human Subjects, Faculty of Medicine, Çukurova University, Adana. Submission of the individuals to the study was conditioned by an obtained written informed consent form regarding the use of their blood samples for research studies. The study proceeded in agreement with the Helsinki declaration approved on the World Medical Association meeting in Edinburgh. Blood samples were collected from 167 consecutive patients with HCC seen in the department of gastroenterology and general surgery between September 2005 and April 2010. During the same time, 167 unrelated community residents with no evidence of hepatocellular or other cancer who entered the hospital for health check-ups were enrolled as the control group. The 167 cancer-free control subjects did not have a history of liver disease and had no serological evidence of hepatitis B or C virus infection. Each control was pair-matched by sex, age (±3 years), smoking and alcohol consumption to a patient with HCC. These characteristics allowed us the choice of a control population without any possible risk bias for HCC. The HCC diagnostic criteria was based on the guideline proposed by European Association for the Study of the Liver (EASL) (Bruix et al., 2001). We gave a diagnosis of HCC when a patient had one or more risk factors (i.e., HBV or HCV infection, or cirrhosis) and one of the following: >400 ng/mL α-fetoprotein (AFP) and at least one positive finding following examination using spiral computed tomography (CT), contrast-enhanced dynamic MRI, or hepatic angiography; or <400 ng/mL α-fetoprotein and at least two findings following CT, MR,
or hepatic angiography. A positive HCC finding using dynamic CT or MRI is indicative of arterial enhancement followed by venous washout in the delayed portal/venous phase. In addition; we performed histopathological diagnosis for cases that did not fulfill all of the clinical noninvasive diagnostic criteria of HCC. Cirrhosis was diagnosed with liver biopsy, abdominal sonography, and biochemical evidence of parenchymal damage plus endoscopic esophageal or gastric varices (Tsai et al., 1994). Patients with cirrhosis were classified into the three Child-Pugh grades based on their clinical status (Pugh et al., 1975). Serum HBsAg and Anti-HCV were assessed using an immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Serum AFP concentration was measured by microparticle enzyme immunoassay (Abbott Laboratories, AXSYM, USA). Heavy alcohol intake was defined as a daily minimum consumption of 160 g alcohol for at least eight years.

Technicians who performed the blood tests were blinded to the identity and disease status of participants. Peripheral blood samples were taken from patients and controls, and blood specimens, including white blood cells and serum, were frozen at -20°C until analysis.

**DNA extraction**
A 5 mL sample of venous blood was collected from each subject into a test tube containing EDTA as anticoagulant. Genomic DNA was extracted from peripheral whole blood using High Pure PCR Template Preparation Kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer’s protocol.

**Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis**

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis with an internal check on digestion was performed to determine the genotype of the polymorphism of NQO1 gene, as described previously (Tijhuis et al., 2008). The 415 bp fragment encompassing the C to T polymorphic site in the NQO1 gene was amplified using specific primers 5'-TGA GAAGCC CAG ACC AACT TT-3' and 5'-GAA GGA AAT CCA GGC TAA GGA-3'. Amplification was performed in GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Singapore) with 100 ng of genomic DNA, 25 pmol of each primer, 200 µM total dNTP, 1.5 mM MgCl2, 1X PCR buffer and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: an initial melting step of 5 min at 95°C; 35 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C; a final elongation step of 7 min at 72°C. After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 10 units HinfI (from A Escherichia coli strain that carries the HinfI gene from Haemophilus influenzae Rf (ATCC 49824)) enzyme at 37°C (New England BioLabs, Beverly, MA) and electrophoresed on 3% agarose gel containing 0.5µg/ml ethidium bromide and visualized under UV illumination. HinfI recognizes 5' - GANTC -3' and so the transition from wild type -GAA CCT- to -GAA TCT- in the variant creates new HinfI restriction site. The

**Statistical analysis**

The sample size was calculated using the QUANTO 1.1 program (hydra.usc.edu/gxe). The desired power of our study was set at 80%. Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 10.0). Differences in the distributions of demographic characteristics between the cases and controls were evaluated using the Student’s t-test (for continuous variables) and χ2 test (for categorical variables). The observed genotype frequencies were compared with expected values calculated from Hardy–Weinberg equilibrium theory (p² + 2pq + q² = 1; where p is the frequency of the wild-type allele and q is the frequency of the variant allele) by using a χ²-test with degree of freedom equal to 1 among cases and controls, respectively. Pearson’s χ² test was used to determine whether there was any significant difference in allele and genotype frequencies between patients and controls. The associations between NQO1 C609T genotypes and the risk of HCC were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from conditional logistic regression analyses. Statistical modeling was performed on the relative risk of the TT genotype or the TC genotype against the CC genotype independently. Furthermore, to estimate the recessive or dominant effect of NQO1 genotype on risk, statistical modeling was performed on the relative risk of the CC genotype against the CT+TT genotype or the CC+CT genotype against the TT genotype. Probability levels less than 0.05 were used as a criterion of significance.

**Results**

**Characteristic of study population**
Table 1 shows selected characteristic of cases and controls. There were a total of 167 cases and 167 controls, all of whom were Turkish. The cases and controls were matched well on age and gender. The cases and controls were also matched on smoking status and alchol consumption by study design and there were similar percentages of smokers and alcohol drinkers in cases and the controls.

**Genotype frequency distribution**

The frequency distributions of the different genotype and allele for NQO1 C609T polymorphism are shown in Table 2. Genotyping results showed that the allelic frequencies of case subjects (C609, 0.73; T609, 0.27) were not different from those of the control subjects (C609, 0.76; T609, 0.24) p=0.33. The genotypic frequencies of the control (n=167, χ²=0.008, df =1, p=0.93) were
Association between NQO1 C609T Genotype and HCC Risk

To determine if there was a statistically significant increase risk of HCC development according to the NQO1 genotypes, we conducted logistic regression analysis (Table 2). Logistic regression analysis showed that individuals with one or two copies of T variant allele exhibited with 1.28 (95% CI = 0.82-2.00, p=0.28) and 1.24-fold (95% CI = 0.48-3.20, p=0.66) increased HCC risk, respectively, although neither association reached statistical significance. Study subjects combined with the NQO1 CT and TT genotypes had a non-significant association with HCC. The NQO1 CT and TT genotypes had a non-significant association with the OR of the TT genotype was 1.12 (95% CI = 0.44-2.83, p=0.81).

The NQO1 C609T genotypes distributions in HCC cases and controls stratified by gender, HBV and HCV infection status. Subgroup analyses revealed that the effect of gender and viral infection status were not significantly different among NQO1 C609T genotypes (data not given). In addition to this, we compared the age at diagnosis for HCC patients who had different genotypes of the arithmetic mean ages and SD for CC, CT and TT were 59.02±12.39, 60.79±10.44 and 62.00±11.43 years old, respectively. There was no significant difference between them by t-test (p=0.54).

Discussion

ROS can induce genetic mutations as well as chromosomal alterations and thus contribute cancer development in multistep carcinogenesis (Wiseman et al., 1996). Most of HCC cases arise in the setting of chronic hepatitis virus infection and ROS generated during persistent inflammation induces continuous cell death and regeneration. This leads to genetic damage and may result in the initiation of HCC (Farazi and DePinho, 2006; El-Serag and Rudolph, 2007). A major mechanism regulating neoplasia is the balance between phase I xenobiotic metabolic enzymes and phase II xenobiotic metabolic enzymes such as NQO1 (Lind et al., 1982; Ross et al., 1993). Thus, disruption of the detoxification enzyme by NQO1 C609T polymorphism may cause excessive ROS and result in the initiation of HCC. Indeed, Iizuka et al., (2002), reported that blockade of the detoxification system was a a common pathway during carcinogenesis and/or progression of hepatitis B-related and hepatitis C-related HCCs.

This molecular epidemiological study investigated whether the functional NQO1 C609T polymorphism could have an impact on susceptibility to HCC. NQO1 was selected as candidate gene because high levels of NQO1 gene expression and correlation of NQO1 C609T polymorphism with aggressive tumor behavior and advanced TNM stage have been observed in HCC (Strassburg et al., 2002; Chui et al., 2009), suggesting that the lack of NQO1 may play a role in the pathology of this disease. Thus, the presence of an NQO1 inactivating C609T polymorphism may represent a genetic factor that predisposes individuals to HCC. The C609T base pair alteration leading to loss enzymatic activity of NQO1 was first discovered within a colorectal cancer cell line and later identified as a common polymorphism (Traver et al., 1997), to the best of our knowledge, the possible role of NQO1 C609T as a risk factor for hepatocellular carcinogenesis has not been previously studied and our results suggest that NQO1 C609T allele and genotype variations do not influence susceptibility to this malignancy despite NQO1 expression is upregulated statistically significant in HCC. In addition to this, our findings agree with some studies that observed no associations of the NQO1 C609T polymorphism with renal, esophageal, breast, prostate, gastric, head and neck cancers, adult gliomas and lymphomas (Longuemaux et al., 1999; Steiner et al., 1999; Peters et al., 2001; Hamajima et al., 2002; Siegelmann et al., 2002; Soucek et al., 2002; Begleiter et al., 2005). Nonetheless, it should be noted that some studies, including bladder, gastric, cervical, renal, urothelial, colorectal, breast, esophageal cancers and both pediatric and adult leukaemias have found association with NQO1 C609T polymorphism (Schulz et al., 1997; Wiemels et al., 1999; Naoe et al., 2000; Smith et al., 2001; Krajinovic et al., 2002; Park et al., 2003; Sarbia et al., 2003; Zhang et al., 2003; Menzel et al., 2004; Niwa et al., 2005; Begleiter et al., 2006; van der Logt et al., 2006; Wang et al., 2008). In fact, lack of association found may suggest that the NQO1 C609T polymorphism selected for this study do not exert any particular biological effect in HCC.
pathogenesis within the Turkish population. Because, the contribution of genetic polymorphisms to the risk for cancer may be dependent on the studied population and distinct carcinogenesis process of different types of cancer, as well as on several environmental and other factors that influence the population. Geographic or ethnic differences have been reported regarding the genotype frequency of several polymorphisms (Nebert et al., 2002).

To date, the role of NQO1 C609T polymorphism has been examined in numerous case-control cancer studies. Nevertheless, results have often been conflicting, and some report strong-to-moderate associations, while others show no elevation of risk (Schulz et al., 1997; Longuemaux et al., 1999; Steiner et al., 1999; Wiemels et al., 1999; Naoe et al., 2000; Peters et al., 2001; Smith et al., 2001; Hamajima et al., 2002; Krajnovic et al., 2002; Park et al., 2003; Siegelmann et al., 2002; Soucek et al., 2002; Sarbia et al., 2003; Zhang et al., 2003; Nebert et al., 2004; Begleiter et al., 2005; Niwa et al., 2005; Begleiter et al., 2006; van der Logt et al., 2006; Wang et al., 2008). These conflicting results may be due to relatively small samples size, or the lack of proper controls for other environmental exposures that are also risk factors for cancer. In addition that, these contradictory results may be explained by the fact that NQO1 may play a protective role when acting on certain substrates but may contribute to carcinogenesis when acting on other substrates (Hajos et al., 1991; Ross et al., 2000; Nebert et al., 2002).

Limitation of the present study is that it was hospital-based case-control study, and patients were selected at a single institution (Çukurova University, Balcali Hospital) and thus may have been unrepresentative of hepatocellular

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (%)</th>
<th>Controls (%)</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) mean (±SD) (range)</td>
<td>59.95±11.51</td>
<td>59.11±11.31</td>
<td>NS</td>
</tr>
<tr>
<td>Male sex</td>
<td>132 (79.0%)</td>
<td>132 (79.0%)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Ever</td>
<td>81 (48.5%)</td>
<td>81 (48.5%)</td>
<td></td>
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<tr>
<td>Never</td>
<td>86 (51.5%)</td>
<td>86 (51.5%)</td>
<td></td>
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<tr>
<td>Alcohol status</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Drinker</td>
<td>50 (29.9%)</td>
<td>50 (29.9%)</td>
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<tr>
<td>Non-drinker</td>
<td>117 (70.1%)</td>
<td>117 (70.1%)</td>
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<tr>
<td>Viral Infection</td>
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<td></td>
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<tr>
<td>HBsAg positive</td>
<td>97 (58.1%)</td>
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<tr>
<td>Anti-HCVAb positive</td>
<td>42 (25.1%)</td>
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<td></td>
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<tr>
<td>Both positive</td>
<td>2 (1.2%)</td>
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<tr>
<td>Both negative</td>
<td>26 (15.6%)</td>
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<tr>
<td>Liver cirrhosis</td>
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<tr>
<td>Present</td>
<td>138 (82.6%)</td>
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<tr>
<td>Absent</td>
<td>29 (17.4%)</td>
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<tr>
<td>Child-Pugh classification</td>
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<tr>
<td>A</td>
<td>25 (18.1%)</td>
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<tr>
<td>B</td>
<td>49 (35.5%)</td>
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<tr>
<td>C</td>
<td>64 (46.4%)</td>
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<tr>
<td>α-Fetoprotein (ng/mL)</td>
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<tr>
<td>&lt;100</td>
<td>74 (44.3%)</td>
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<tr>
<td>100-400</td>
<td>25 (15.0%)</td>
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<tr>
<td>&gt;400</td>
<td>68 (40.7%)</td>
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<tr>
<th>Table 2. Association Between C609T Polymorphism of NQO1 Genotype and Hepatocellular Cancer Risk</th>
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<tbody>
<tr>
<td>Cases (%), n = 167</td>
</tr>
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<td>---------------------</td>
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<tr>
<td>Allele frequency</td>
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<tr>
<td>General genotype</td>
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<td>Dominant genotype</td>
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<td>Recessive genotype</td>
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*Data were calculated by logistic regression analysis; Abbreviations: C=allel of NQO1 with proline at codon 187; T=allel of NQO1 with serine at codon 187; OR=odds ratio; CI=confidence interval
carcinoma patients in the general population. In addition it should be noted that the control subjects were recruited at the same hospital. However, in the control group, the agreement between the observed distribution of NQO1 C609T genotype frequencies with the expected according to the Hardy-Weinberg equilibrium model suggested no selection bias. This study is limited by the relatively small number of cases and controls. Therefore further studies with a larger number of subjects are needed to clarify this issue. We also limited our study to Turkish population due to previous reports of significant differences in the prevalence of the homozygous NQO1 T609 allele with approximately 2-5% found in Caucasian compared to 20% found in Asian (Nebert et al., 2002). Variation in allele frequency between different ethnic groups has been observed for NQO1 C609T polymorphism (Neber et al., 2002). Although the origin and consequence of the ethnic differences are not clear, it may explain the inconsistent findings between different studies.

In conclusion; our results demonstrate for the first time that the NQO1 C609T polymorphism have not been any major role in genetic susceptibility to hepatocellular carcinogenesis within the studied population. Because this is the first report concerning the NQO1 C609T polymorphism and the risk of HCC in the literature, further independent studies are required to validate our findings in a larger series, as well as in patients of different ethnic origins and to better understand NQO1 C609T polymorphism and susceptibility HCC.

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References


**NQO1 C609T Polymorphism and HCC in Turkish Subjects**


