RESEARCH ARTICLE

Clinical Significance of the NQO1 C609T Polymorphism in Non Small Cell Lung Adenocarcinoma Patients

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Abstract

Background: NAD(P)H:quinone oxidoreductase 1 (NQO1) is part of the antioxidant defence system involved in detoxification. This study aimed to analyze the influence of NQO1 (C609T) genetic polymorphism in non small cell lung cancer (NSCLC)as a putative risk factor. Materials and Methods: Present study included 100 cases of NSCLC (adenocarcinoma) patients and 100 age and sex matched healthy controls. NQO1 (C609T) genotyping was performed by allele specific PCR for assessment of putative associations with clinical outcome and genotypes of. The association of the polymorphism with the survival of NSCLC patients' was analyzed by Kaplan–Meier method. Results: In Indian NSCLC (adenocarcinoma) patients increased risk of developing NSCLC was found to be associated with NQO1 609TT genotype [OR 3.68(0.90-14.98), RR 2.04(0.78-5.31)] for CT [OR 2.91(1.58-5.34), RR 1.74(1.23-2.44) p= 0.0005 for CT], for CT+TT [OR 3.26(1.82-5.82), RR 1.87(1.34-2.61) p<0.0001 for CT+TT]. A significant difference (p=0.0009) was observed in genotype distribution among cases and healthy controls. Patients with CT+TT genotype exhibited a significant poor overall survival compared with patients displaying homozygous CC genotype (p=0.03) and when survival independently compared with CC, TT and CT genotype was also found to be significantly associated (p=0.02). Overall median survival times were CT 6.0 months, TT 8.2 months, and CT + TT (6.4 months)]. Conclusions: The present study revealed that NQO1 CT, TT and CT+TT genotypes may be associated with clinical outcome and risk of developing NSCLC in the Indian population.

Keywords: NSCLC patients - adenocarcioma - NQO1 gene (C609T) polymorphism - AS-PCR.

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Introduction

NAD (P) H: quinone oxidoreductase 1 (NQO1) is involved in antioxidant defence system by detoxification of mutagenic agents and carcinogenic quinones by two electron reduction to hydroquinones (Chen et al., 1999; Hou et al., 2005). NQO1 protects cells from quinones damage by reducing mutagenic and carcinogenic agents and competing for them with p450 reductase to prevent highly reactive semiquinone production (Sarmanova et al., 2004). NQO1 protects cells from oxidative stress by maintaining antioxidant forms of ubiquinone and vitamin E and this enzymatic activity could be enough to protect against carcinogenesis (Chen et al., 1999; Hou et al., 2005). NQO1 gene is mapped on chromosomal 16 location q22.1 and comprised of 6 exons and 5 introns (Guha et al., 2008). NQO1 is a flavoprotein which functions as a homodimer. The physiological dimer has one catalytic site per monomer. Each monomer consists of 273 amino acids. NQO1 is expressed in human epithelial and endothelial tissues and at high levels in many human solid tumors. NQO1 is a cytosolic enzyme, localized in smaller amounts to mitochondria, endoplasmic reticulum and nucleus (Ross et al., 2004; Chao, 2006). C to T Polymorphism at position 609 of exon 6 of the NQO1 that encodes for a proline to serine substitution at position 187 in the amino acid sequence of the protein. Proline to Serine amino acid substitution leads to an extremely unstable NQO1 protein which is rapidly ubiquitinated and degraded by the proteasome (Siegel et al., 2001). Some studies have found that the C609T SNP is associated with an increased risk for several malignancies, eg, colorectal cancer, breast cancer, lung cancer, and bladder cancer (Chao, 2006; Chen, 2012). NAD(P)H:quinone oxidoreductase 1 (NQO1), also known as diphtheria toxin diaphorase (DT-diaphorase), is a cytoplasmic 2-electron reductase, belonging to the NAD(P)H dehydrogenase (quinone) family. NQO1 plays an important role in the aromatic amine metabolism pathway (Menashe et al., 2012). The CC genotype of the NQO1 C609T polymorphism is associated with the risk

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of lung cancer, and that the TT genotype increases the risk of smoking for cancers of the esophagus and lung (Hamajima et al., 2002).

Thus present study aimed to conduct a case-control study to evaluate the relevance of the NQO1 (C609T) polymorphism on the risk and susceptibility of NSCLC in Indian patients.

Materials and Methods

Cases and controls

Present study included 100 newly diagnosed NSCLC (Adenocarcinoma) patients and 100 gender and age (±5 years) matched healthy controls. 3 ml Peripheral blood sample collected from each subjects included in the study. This study was approved by the Institutional Ethics Committee of MAMC, New Delhi and written informed consent was obtained from all subjects. Patient follow-up was obtained through the hospital records and follow-up done from May 2013 to February 2015.

DNA extraction and genotype

Genomic DNA extraction was done by phenol chloroform method from blood samples collected in EDTA vials from cases as well as controls. The C609T polymorphism was performed by allele specific PCR method with NQO1 C609 Forward 5' TAT CAG AGT GTC TTA CTG AGA 3', NQO1C609 Reverse 5' AAT GCT ATA TGT CAG TTG AGG 3'and NQO1T609 Forward 5' GTG GCT TCC AAG TCT TAG AAT3', NQO1T609 Reverse 5' TTT CTA GCT TTG ATC TGG TTG3' (Haruya et al., 2003). PCR was performed in 25 µl reaction volume containing 3 µl of 100 ng template DNA, 0.25 µl, 25 pmol each primer 2.5 µl, 10 mM dNTPs, 1.5 µl of 20mM MgCl2, 0.3 µl of 5 U/ µl Taq polymerase with 2.5 µl of 10X Taq Buffer (Fermantas) and 14.7 µl of nuclease-free ddH^2O. The PCR was performed with initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 95oC for 40 seconds, annealing at 55oC for 40 seconds, extension at 72°C for 40 seconds and the final extension was at 72°C for 10 minutes. PCR products were separated by electrophoresis on a 2 % agarose gel containing ethidium bromide and the amplified sequences 161base pairs for NQO1 609C and 283base pairs for NQO1 609T (Figure 1).

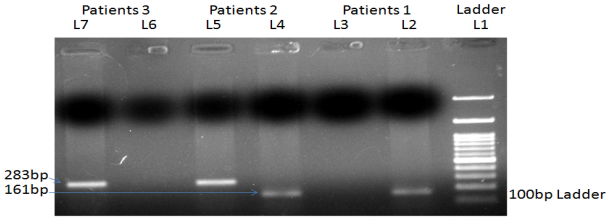

Figure 1. PCR Amplification of Normal and Mutant Alleles of NQO1 (C609T) Polymorphism; by Allele Specific PCR Method. L1:100bp ladder. Patient1; L2 – L3: Normal allele (C) amplified: Patients 1 positive for Homozygous C allele. Patient2; L4 – L5: Both normal (C) and mutant allele (T) amplified: Patients 2 positive for Heterozygous C and T allele. Patient3; L6 – L7: Mutant allele (T) amplified: Patients3 Positive for Homozygous T allele

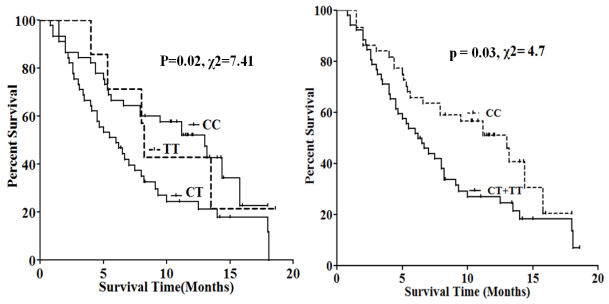

Figure 2. Kaplan–Meier Survival Curves of NSCLC Patients with Respect to NQO1 (C609T) Polymorphism

Statistical analysis

Genotype frequencies between the cases and controls were evaluated using the Chi square test, Hardy-Weinberg equilibrium test used to check the allele frequency and values below 5 were analyzed by Fisher exact test. The associations between NQO1T genotypes and risk of NSCL cancer (Adenocarcinoma) were estimated by computing the odds ratios (ORs), risk ratios (RRs) and risk differences (RDs) with 95 % confidence intervals (CIs). Kaplan-Meier

Table 1. Distribution of selected characteristics among NSCLC patients and healthy controls

Variables	NSCLC patients (%)	Healthy controls (%)
Total no.	100	100
Gender		
Males	71	71
Females	29	29
Age at diagnosis (Years)		
< 55	56	56
> 55	44	44
Mean + SD age (years)	54.37+10.77 (range 32-75years)	54.25+10.82 (range 30-70 years)
Smoking status		
Non smoker	55	55
Smoker smokers	45	45
Current smokers	24	24
Ex- smokers	21	21
Smoking type		
Cig	18	18
Bidi	16	16
Hukka	11	11
Smoking level (pack year)		
Mild (< 10)	23	23
Moderate (< 40)	18	18
Heavy (> 40)	4	4
TNM Stage		
Early (I&II)	30	
Advanced (III&IV)	70	
Distant Metastases		
Positive	44	
Negative	56	
Histopathological Grade		
Grade 1	24	
Grade 2	41	
Grade 3	35	
Pleural effusion		
Yes	15	
No	85	

methods were used to evaluate the relationship between NQO1 genotype and overall survival of NSCLC patients. All statistical analyses were performed using Graph Pad Prism 6.0 or SPSS 16.0.

Results

Study population:

All demographic features of the subjects are depicted (Table 1). In brief, total of 100 Non-small cell lung adenocarcinoma patients and same number of healthy control were analyzed.

Both cases and controls include 71 males and 29 females of age <55 group (56%) and >55group (44%) with mean ±SD in cases of 54.37+10.77 (range 32-75years) and controls of 54.25+10.82 (range 30-70 years). 44% patients were in stage IV,15%, 15% and 26% patients in stage I, II and III respectively while 44% patients had distant metastases. Patients with different pathological grade, grade 1 (well differentiated) includes 24%, grade

Table 2. Genotype frequencies of NQO1 (C609T) among NSCLC Cases and Controls

Variables	CC	CT	TT	p value	C allele frequency	T allele frequency
Patients(n=100)	45(45%)	48(48%)	7(7%)	0.0009	0.69	0.31
Controls(n=100)	71(71%)	26(26%)	3(3%)		0.84	0.16

Table 3. NQO1 Genotype Frequencies in Cases & Controls and Associations with NSCLC Risk

NQO1 (C609T) Genotype	Control (n=100)	Cases (n=100)	OR*(95% CI)	RR**	P value
CC	71	45	Ref (1)	1	
TT	3	7	3.68(0.90-14.98)	2.04(0.78-5.31)	0.09
CT	26	48	2.91(1.58-5.34)	1.74(1.23-2.44)	0.0005
CT+TT	29	60	3.26(1.82-5.82)	1.87(1.34-2.61)	< 0.0001

Table 4. Association and Stratification Analysis of NQO1 (C609T) Polymorphism and NSCLC

Variables	Total	CC Genotype n (%)	CT Genotype n (%)	TT Genotype n (%)	C allele frequency	T allele frequency
Gender						
Male	71	34(47.9%)	33(46.5%)	4(5.6%)	0.5	0.5
Female	29	11(37.9%)	15(51.7%)	3(10.4%)	0.18	0.82
Age (in years)						
< 55	56	27(48.22%)	26(46.43%)	3(5.35%)	0.4	0.6
> 55	44	18(40.90%)	22(50%)	4(9.1%)	0.29	0.71
Smoking status						
Nonsmokers	55	29(52.7%)	21(38.1%)	5(9.2%)	0.39	0.61
Smokers	45	16(35.5%)	27(60%)	2(4.5%)	0.29	0.71
Current Smokers	24	10(41.6%)	13(54.1%)	1(4.2%)	0.17	0.83
Ex-smokers	21	6(28.5%)	14(66.6%)	1(4.83%)	0.13	0.87
Smoking type						
Cigarette	18	7(38.8%)	9(50%)	2(11.2%)	0.64	0.36
Bidi	16	4(25%)	12(75%)	0(%)	0.63	0.38
Hukka	11	5(45.5%)	6(54.5%)	0(%)	0.73	0.27
Smoking level (pack year)						
Mild(<10)	23	10(43.5%)	13(56.5%)	0(%)	0.72	0.28
Moderate(<40)	18	3(16.6%)	13(72.2%)	2(11.2%)	0.53	0.47
Heavy(> 40)	4	3(75%)	1(25%)	0(%)	0.58	0.42
TNM Stage						
Early (I&II)	30	19(63.3%)	10(33.4%)	1(3.3%)	0.8	0.2
Advanced (III&IV)	70	26(37.1%)	38(54.3%)	6(8.6%)	0.64	0.36
Distant Metastases						
Positive	44	17(38.6%)	23(52.3%)	4(9.1%)	0.65	0.35
Negative	56	28(50%)	25(44.6%)	3(5.4%)	0.72	0.28
Histopathological Grade						
Grade I	24	5(20.8%)	17(70.8%)	2(8.4%)	0.6	0.4
Grade II	41	22(53.6%)	16(39.1%)	3(7.3%)	0.73	0.27
Grade III	35	18(51.4%)	15(42.8%)	2(5.8%)	0.73	0.27
Pleural effusion						
No	85	41(48.2%)	38(44.7%)	6(7.1%)	0.71	0.29
Yes	15	4(26.7%)	10(66.7%)	1(6.6%)	0.6	0.4

Table 5. Association between the NQO1 genotype and Clinico-pathologic Characteristics in Cases

Variables	Group I	Group II	OR	RR
Gender	Male	Female		
CC	34	11	Ref (1)	
CT	33	15	1.4(0.56-3.5)	1.09(0.85-1.41)
TT	4	3	2.31(0.44-12)	1.32(0.68-2.56)
Age (in years)	< 55	> 55		
CC	27	18	Ref (1)	
CT	26	22	1.26(0.55-2.89)	1.1(0.77-1.57)
TT	3	4	2(0.39-10.02)	1.4(0.57-3.4)
Smoking status	Non-smokers	Smokers		
CC	29	16	Ref (1)	
CT	21	27	2.33(1-5.37)	1.47(0.99-2.17)
TT	5	2	0.72(0.12-4.17)	0.9(0.53-1.51)
Smoking status	Current Smokers	Ex-smokers		
CC	10	6	Ref (1)	
CT	13	14	1.79(0.50-6.34)	1.29(0.75-2.23)
TT	1	1	1.66(0.08-31.89)	1.25(0.29-5.26)
Smoking type	Cigarette	Bidi + Hukka		
CC	7	9	Ref (1)	
CT	9	18	1.55(0.43-5.54)	1.31(0.60-2.83)
TT	2	0	0.15(0.00-3.81)	0.43(0.25-0.76)
Smoking level (pack year)	Mild(<10)	Moderate(<40) + Heavy(> 40)		
CC	10	6	Ref (1)	
CT	13	14	1.79(0.50-6.34)	1.29(0.75-2.23)
TT	0	2	8.07(0.33-196.3)	-
TNM Stage	Early (I&II)	Advanced (III&IV)		
CC	19	26	Ref (1)	
CT	10	38	2.77(1.11-6.92)	2.02(1.05-3.87)
TT	1	6	4.38(0.48-39.52)	2.95(0.46-18.74)
Distant Metastases	Positive	Negative		
CC	17	28	Ref (1)	
CT	23	25	0.65(0.28-1.5)	0.78(0.48-1.27)
TT	4	3	0.45(0.09-2.28)	0.66(0.31-1.39)
Pleural effusion	Yes	No		
CC	4	41	Ref (1)	
CT	10	38	0.37(0.10-1.28)	0.42(0.14-1.26)
TT	1	6	0.58(0.05-6.15)	0.62(0.08-4.79)

2 (moderately differentiated) includes 41% and grade 3 (Poorly differentiated) includes 35% cases. We included smoker 45% as well as non smoker 55% with different smoking type as cigarette, bidi, and hukka, 18% cases smoked cigarette, 16% cases smoked bidi and 11% cases smoked hukka.

Case-control genotype distribution

This study observed that high percentage of CT (48%) and TT (7%) genotype was found in patients compared to controls CT (26%) and TT (3%) genotype while lower CC (45%) genotype in patients compared to control CC (71%) genotype. The genotype and allele distributions of NQO1 (C609T) in cases and controls are summarized in Table 2 and 4. We observed a statistically significant difference in the frequencies of NQO1 CC, CT and TT genotypes among patients and healthy controls (p=0.0009). The frequency of C allele (fC) was found to be lower among NSCLC patients (0.69) whereas, the higher frequency of C allele (fC) was observed among healthy controls (0.84). However frequency of T allele (fT) was found to be higher among NSCLC patients (0.31) whereas, the lower frequency of T allele (fT) was observed among healthy controls (0.16).

NQO1 (C609T) polymorphism and NSCLC risk

Odds ratio and risk ratio with 95 % confidence intervals was calculated for each group to estimate the degree of association between the NQO1 (C609T) genotype and risk of NSCLC in Indian patients depicted in Table 3 and 5. Compared to the CC genotype, the OR 2.91(1.58-5.34) and 3.68(0.90-14.98), RR 1.74(1.23-2.44) and 2.04(0.78-5.31) for the heterozygous CT and homozygous TT genotypes were estimated, suggesting a possible dominant effect of this polymorphism on NSCLC risk.

NQO1 (C609T) genotypes and survival analysis: Survival analysis was based on the population of 100 NSCLC (Adenocarcinoma) patients. Patients with CC genotype showed 13 months of overall median survival time, Patients with TT genotype showed 8.2 months of overall median survival time while patients with CT genotype showed 6 months of median survival time. When survival calculated together CT + TT genotype also showed 6.35 months of median survival time in comparison of CC genotype and found to be significantly associated(p=0.03). Patients with heterozygosity (CT) exhibited a significant poor overall survival and found to be significantly associated (p=0.02) (Figure 2a, b).

Discussion

NAD (P)H: oxidoreductase (NQO1) is also an important enzyme in xenobiotic metabolism that catalyzes the two electron reduction of carcinogenic quinoid compound to their reduced form such as quinones (Nebert et al., 2002). The activity of NQO1 enzyme depends on polymorphisms at the NQO1 locus. The major polymorphism is C to T substitution at nucleotide 609 of exon 6 resulting in the Proline to Serine amino acid substitution Pro187Ser has previously been reported to reduce quinone reductase activity in vitro (Siegel et al., 2001). The present case-control study of 100 subjects explored the association of NQO1 (C609T) functional polymorphism with risk and prognosis of NSCLC in Indian population. Our results suggest that homozygous NQO1 609TT genotype as the risk of developing NSCLC with approximately more than 3.6-fold increase while heterozygous NQO1 609CT genotype also showed approximately more than 2.9-fold increase than homozygous NQO1 609CC genotype, and were an independent prognostic marker of unfavourable clinical outcome of NSCLC patients. Patients with homozygous TT and heterozygous CT genotype had a higher risk for death than homozygous CC genotype. Patients with heterozygous genotype had at intermediate risk it was observed that 2.04 and 1.74 fold higher risk was observed with TT and CT genotype respectively [table 3]. It was also observed that high T (0.31) allele frequency in case compared to control (0.16).To the best of our knowledge, this is the first study report of genetic association of NQO1 (C609T) polymorphism with the NSCLC risk and prognosis, confirming the possible role of NQO1 gene in the pathogenesis of NSCLC malignancy. NQO1 has been involved to stabilize the p53 tumour suppressor protein by inhibiting its degradation through a direct protein-protein interaction (Asher et al., 2001; Anwar et al., 2003). NQO1 C to T substitution results in poor enzymatic activity and no detectable protein as shown in individuals homozygous for the T allele (Traver et al., 1997). NQO1 variant T allele has been suggested to be a risk factor for lung cancer in Caucasians population (Rosvold et al., 1995; Lewis et al., 2001) and Xu et al. found that both the C/T and T/T genotype produced a higher risk of lung cancer compared with the wild-type genotype (C/C) (Xu LL et al., 2001). NQO1 C to T (C609T) polymorphism results in Proline to Serine amino acid substitution at position 187 (P187S). This substitution results to an extremely unstable NQO1 protein which is rapidly ubiquitinated and degraded by the proteasome (Siegel et al., 2001). It has been found C609T polymorphism is associated with an increased risk for several malignancies, eg, colorectal cancer, breast cancer, lung cancer, and bladder cancer (Chen et al., 1999; Chao et al., 2006; Yuan et al., 2011) while Hamajima et al also suggested that the CC genotype of the NQO1 C609T polymorphism is associated with increased risk of lung cancer, and the TT genotype increases the risk of lung cancers and esophagus cancer (Hamajima et al., 2002). Among patients who developed tumours, induced by chemical carcinogens such as urothelial cancer , renal cancer (Schulz et al., 1997) and leukemias (Wiemels et al., 1999; Larson et al., 1999) the NQO1 609T allele has been found more frequently than among controls. According to Ren et al. (2006). NQO1 C609T in gastric cancer patients is higher than the controls. Individuals with NQO1 (T/C) and NQO1 (T/T) genotypes have higher onset risk with odds ratios of 2.08 and 3.81 respectively (Ren et al., 2006). Su et al. (2012) found that the frequency of NQO1 (T/T) was increased in colon cancer patients with the increased malignance, and the patients with NQO1 (T/T) had higher onset risk to develop colon cancer in well-differentiated, moderately-differentiated and poorly-differentiated colon cancer (Su et al., 2012). According to Kolesar JM NQO1 *2/*2 is associated with poor survival in non-small cell lung cancer patients (Kolesar et al., 2011). Mei-Miao Chiu et al. (2009) also found that Immuno - histochemical examination showed that the significant increase of NQO1 protein was only found in the tumour specimens with wild type CC genotype but weak or no NQO1 protein immunoreactivity was detected in nonneoplastic liver tissue, as well as in HCC tumor tissue in individuals carrying the heterozygous CT genotype (Mei-Miao Chiu et al., 2009).

Conclusion: Present study conclude that NQO1 (C609T) polymorphism contributes to risk of developing NSCLC and heterozygous CT and TT genotype may be associated with higher risk for unfavorable clinical outcome of NSCLC patients. In addition, NQO1 CT and TT genotype also associated with poor survival of Non small cell lung adenocarcinoma patients. Due to the small sample size in the present study, our findings need to be validated by further independent and prospective studies on a larger population.

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