

RESEARCH ARTICLE

The C609T (Pro187Ser) Null Polymorphism of the NQO1 Gene Contributes Significantly to Breast Cancer Susceptibility in North Indian Populations: a Case Control Study

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Abstract

Background: Worldwide, breast cancer is the most common cancer among women and is a leading cause of cancer death. In the present study, we investigated the NQO1 C609T genotypic and allelic distribution in north Indian breast cancer patients. **Materials and Methods:** The genotypic distribution of the NQO1 C609T polymorphism was assessed in 100 invasive ductal carcinoma (IDC) breast cancer patients and 100 healthy controls using allele specific PCR (AS-PCR). **Results:** A lower frequency of the CC genotype was found in breast cancer patients (24%) than in the controls. On the other hand, TT genotype frequency was also found to be higher in female healthy controls (32%) than the female breast cancer patients (20%). The frequencies of all three genotypes CC, CT, TT in patients were 24%, 56% and 20% and in healthy controls 50%, 22% and 32% respectively. We did not find any significant correlation between the NQO1 C609T polymorphism and age group, grading, menopausal status and distant metastasis. A less significant association was found between the NQO1 C609T polymorphism and the stage of breast cancer ($X^2=5.931, P=0.05$). **Conclusions:** The present study shows a strong association between NQO1 C609T polymorphism with the breast cancer risk in the north Indian breast cancer patients so that possible use as a risk factor should be further expel

Keywords: Breast cancer - NAD(P)H:quinoneoxidoreductase 1 polymorphism - invasive ductal carcinoma - India

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Introduction

Breast cancer is the leading cause of cancer related female deaths all over the world accounting for >1.6% of deaths and case fatality rates are highest in low-resource countries. In India, Breast cancer is the most frequently occurring cancers in females in comparison with other cancers. In India the average age of the high risk group for breast cancer is 43-46 years unlike in the west where women aged 53-57 years are more prone to breast cancer.

NADPH: Quinone oxidoreductase 1(NQO1) gene, originally called DT-diaphorase is located on the 16q22.1. NQO1 gene codes a 274 amino acids (30868 Da) containing cytosolic NQO1 enzyme which catalyzes the two-electron reduction of quinone compounds and prevents the generation of semi-quinone free radicals and reactive oxygen species, thus protecting cells from oxidative damage. Additionally, NQO1 catalyzes the reductive activation of quinoid chemotherapeutic agents

and of environmental carcinogens such as nitrosamines, heterocyclic amines and cigarette smoke condensate (Rauth et al., 1997). There is evidence that oxidative stress, resulting from either an excess of reactive oxygen species (ROS) or a deficiency in antioxidant capabilities, may play a role in the etiology of breast cancer (Nowell et al., 2004, Ambrosone et al., 2000).

NQO1 was found to play an important role in apoptosis and oxidative stress, and the effects of C609T polymorphism were thoroughly investigated in several types of cancers (Lewis et al., 2001, Menzel et al., 2004, Hamajima et al., 2002, Niwa et al., 2005, di Martino et al., 2007). NAD(P)H:quinone oxidoreductase 1 (NQO1) may be particularly relevant for breast carcinogenesis because of its role in reduction of endogenous catechol estrogens generated in the metabolism of estrogen. By catalyzing the obligatory two-electron reduction of catechol estrogens and other quinones, the reactive semiquinone intermediate that drives the Fenton reaction is bypassed, and superoxide

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mediated release of iron from ferritin stores is prevented (Wyllie et al., 1997). The gene coding for NQO1 is subject to polymorphism (C609T) with a C/T base change at position 609 of the human NQO1 cDNA, leading to a change in the amino acid sequence of the protein (P187S).

The C609T polymorphism (rs1800566) in NQO1 (Traver et al., 1997) leads to a proline to serine substitution in the NQO1 protein that results in loss of virtually all enzyme activity due to rapid degradation of the variant enzyme (Ross et al., 1996, Siegel et al., 1999). This major polymorphism involves a single C to T substitution at nucleotide 609 of exon 6 of the NQO1 cDNA that causes a Pro187Ser amino acid change thereby leading to three phenotypes, i.e. wild-type phenotype with complete enzyme activity, heterozygous phenotype with ~3-fold decreased activity and homozygous mutant (null) phenotype with a complete lack of enzyme activity (Kuehl, B.L. et al 1995, Moran, J.L. et al 1999). Decreased NQO1 enzyme activity is caused by enhanced polyubiquitination and proteasomal degradation of the mutant protein (Siegel, D. et al. 2001). In the current study, we investigated the NQO1 C609T genotypic and allelic distribution and its role in breast cancer patients from north Indian population.

Materials and Methods

During 2010 to 2012, from 100 female healthy controls and 100 female patients of confirmed Invasive Ductal Carcinoma (IDC) breast cancer in Department of Surgery, Maulana Azad Medical College, New Delhi assessed on the basis of clinic-pathological findings, a 5 ml sample of peripheral blood was collected from each patient and healthy control. Patients with any previous history of cancer were excluded from this study. This study was approved by the Institutional Ethical Committee of Maulana Azad Medical College, New Delhi. The clinical characteristics of the patients and healthy controls are given in Table 1.

Whole genomic DNA extraction was done from whole blood by DNA extraction kit (Geneaid, India) according to manufacturer's protocol. NQO1 C609T polymorphism was evaluated by the AS-PCR using previously described primers (Hamajima N, 2002). The total volume of the PCR reaction was 25 μ l and the amplification was done using the conditions; 100ng genomic DNA, 0.25 μ l of 25 pmol/ μ l each primers, 2.5 μ l 10 mM dNTPs, 1.5 μ l of 20 mM MgCl₂, 0.3 μ l of 5 U/ μ l Taq polymerase with 2.5 μ l of 10X Taq Buffer (Fermantas). The PCR program for NQO1 C609T polymorphism was initial denaturation at 95 oc for 10 minutes followed by 40 cycles 95 $^{\circ}$ c for 45 sec, 55 oc for 45 sec followed by the final extension at 72 oc for 10 minutes. Agarose gel electrophoresis was performed on the 2% agarose gel for analysis of amplicon product.

Statistical analysis

Statistical analysis was done using SPSS16 and MedCalc software. The estimated risk for both alleles was analyzed using Odd ratios. Standard chi-square test was used to calculate the p-value and the p-value less than 0.05 was taken as significant.

Results

Patients' clinicopathological characteristics are shown in table 1. Of 100 consecutive breast cancer patients blood samples collected 49(49%) patients were below or equal to 45 years age and 51(51%) were above 45. Besides 44(44%) patients were premenopausal status and 56(56%) patients were postmenopausal. Of breast cancer cases 44(44%) were in early (I &II) stages and 56(56%) samples were in advanced stages (III & IV). Histological grading of the patients' tumor showed that 17(17%), 52(52%) and 31 (31%) were in grade I, II and III respectively. Lymph node status of patients showed that 59(59%) were lymph node positive and 7(7%) patients had distant metastasis of total samples. Of the total samples 54(54%) samples were Her2/neu positive.

The genotypic distribution of NQO1 C609T polymorphism in breast cancer patients with respect to female healthy controls showed a significant correlation ($X^2=26.6$, df =2, $p<0.0001$) (Table 2). A lower frequency

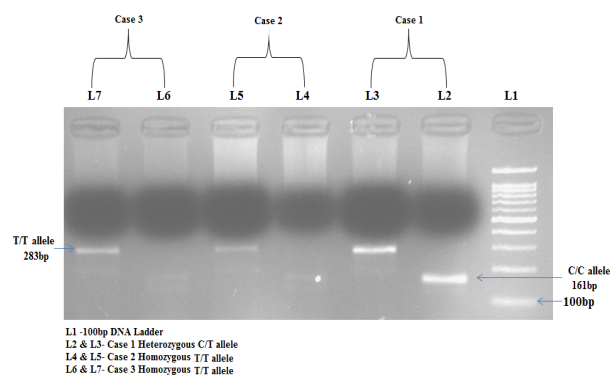


Figure 1. Agarose Gel Electrophoresis of AS-PCR of NQO1 C609T Alleles

Table 1. Clinico-pathological Characteristics of Breast Cancer Patients

Parameters	Patients (%)	Healthy Controls (%)
Patients	100(100)	100(100)
Age Group		
Age >45	49(49)	
Age <45	51(51)	
Menopause		
Pre	44(44)	
Post	56(56)	
Stage		
Early(I & II)	44(44)	
Advanced (III & IV)	56(56)	
Grading		
I	17(17)	
II	52(52)	
III	31(31)	
Lymph Nodes		
Positive	59(59)	
Negative	41(41)	
Her2/Neu		
Positive	54(54)	
Negative	46(46)	
Distant Metastasis		
Positive	7(7)	
Negative	93(93)	

Table 2. NQO1 (C609T) Polymorphism with Different Clinical Features of Breast Cancer Patients

Parameters	Total Samples	Cc	Tc	Tt	X ²	P-Val
Patients	100	24 (24%)	56 (56%)	20 (20%)	26.6	<0.0001
Controls	100	50(50%)	22(22%)	32 (32%)		
Age Group						
Below Or = 45	49	12(24.5%)	26(53.1%)	11(22.4%)	0.44	0.80(Ns)
Above 45	51	12(23.5%)	30(58.9%)	9(17.6%)		
Menopause						
Pre	44	12(27.3%)	23(52.3%)	9(20.4%)	0.55	0.75 (Ns)
Post	56	12(21.5%)	33(58.9%)	11(19.6%)		
Stage						
Early(I & Ii)	44	15(34.1%)	19(43.2%)	10(22.7%)	5.931	0.05
Advanced (Iii & Iv)	56	9(16.1%)	37(66.1%)	10(17.8%)		
Grading						
I	17	4(23.5%)	5(29.4%)	8(47.1%)	Ns	
Ii	52	14(26.9%)	28(53.9%)	10(19.2%)		
Iii	31	6(19.6%)	23(74.2%)	2(6.5%)		
Lymph Nodes						
Positive	59	7(11.9%)	42(71.2%)	10(16.9%)	15.42	0.0004
Negative	41	17(41.5%)	14(34.1%)	10(24.4%)		
Her2/Neu Status						
Positive	54	9(16.6%)	29(54.8%)	16(29.6%)	8.18	0.01
Negative	46	15(32.6%)	27(58.7%)	4(8.7%)		
Distant Metastasis						
Positive	7	0(0%)	6(85.7%)	1(14.3%)		0.23 (Ns)
Negative	93	24(0%)	50(0%)	19(0%)		

Table 3. Comparison of Tumor Grading with Different Genotypes of NQO1 C609T Polymorphism in Breast Cancer Patients

Grade vs Grade	TOTAL PATIENTS	Grading Comparison			P-value
		CC	CT	TT	
I vsII	17	4(23.5%)	5(29.4%)	8(47.1%)	0.09 (NS)
	52	14(26.9%)	28(53.9%)	10(19.2%)	
I vs III	17	4(23.5%)	5(29.4%)	8(47.1%)	0.001
	31	6(19.6%)	23(74.2%)	2(6.5%)	
II vs III	52	14(26.9%)	28(53.9%)	10(19.2%)	0.13(NS)
	31	6(19.6%)	23(74.2%)	2(6.5%)	

of CC genotype was found in breast cancer Patients (24%) than the female healthy controls (50%). On the other hand, TT genotype frequency was also observed to be lower in female breast cancer patients (20%) than the female healthy controls (32%). Frequencies of all three genotypes CC, CT, TT in patients are 24%, 56% and 20% and in healthy controls were 50%, 22% and 32% respectively. We were not able find any significant association between the NQO1 C609T polymorphism and age, histological grading, menopausal status and presence/absence of distant metastasis.

A low significant association was found between the NQO1 C609T polymorphism with the early/advanced stage of cancer ($X^2 = 5.931$, $p=0.05$) (Table 2). Early stage group included Ductal Carcinoma in Situ (DICS) patients in stage I and II of breast cancer. On the other hand, advanced stage group included breast cancer patients in stage III and IV.

During the assessment of correlation between NQO1 C609T Polymorphism with lymph node status of breast cancer patients we observed a highly significant correlation ($X^2=15.42$, $P=0.0004$) (Table 2).

We have found a significant association between NQO1 C609T polymorphism and HER2/neu positive status of breast cancer patients ($X^2=8.18$, $P=0.01$) (Table: 2). During the investigation of association of NQO1 C609T polymorphism with histological tumor grading of breast cancer patients, we observed a significant result during comparison of tumor grade I vs III ($p=0.001$). However, we were not able to find any significant association between tumor grades I vs II ($p=0.09$) or grade II vs III ($p=0.13$) (Table 3).

In the investigation of NQO1 C609T polymorphism with breast cancer risk we have found a highly significant association. We observed significant results in analysis of both allelic as well as genotypic distribution (Table 4). During the comparison of CC homozygous genotype with the TT homozygous genotype we were not able to find any significant results for OR 1.30(0.73-1.91) RR 1.18 (0.73-1.91) ($p=0.48$). In comparison of CC homozygous genotype with the CT heterozygous genotype we found a highly significant association with the OR 5.30 (2.65-10.6) RR 2.21 (1.54-3.56) ($P\leq 0.0001$). During the allelic comparison, the C allele was compared with the T allele

Table 4. Risk of Breast Cancer Associated with the NQO1 C609T Polymorphism

	Breast cancer patients (N=100)		Healthy controls (N=100)		OR (95% CI)	Risk Ratio(RR)	P-Val
	No.	%	No.	%			
	Pro/Pro (CC)	24	-24%	50			
Pro/Ser (CT)	56	-56%	22	-22%	5.30(2.65-10.6)	2.21(1.54-3.56)	<0.0001
Ser/Ser (TT)	20	-20%	32	-32%	1.30 (0.62-2.73)	1.18(0.73-1.91)	0.48 (NS)
Pro/Ser + Ser/Ser (CC + TT)	76		54		2.93(1.61-5.33)	1.80(1.25-2.58)	0.0004
Pro	104		122		1(ref.)	1(ref.)	
Ser	96		86		1.30 (0.88-1.93)	1.14 (0.94-1.39)	0.17 (NS)

Table 5. Allelic Frequencies of NQO1 C609T Polymorphism in Breast Cancer Patients

	Total Samples	Cc	Allelic Frequencies		C Allele Frequency	T Allele Frequency
			Tc	Tt		
Patients	100	24 (24%)	56 (56%)	20 (20%)	0.52	0.48
Controls	100	50(50%)	22(22%)	32 (32%)	0.59	0.41
X ² = 26.6	P-Val ≤0.0001	Df = 2				

and we observed an odd ratio (OR) 1.30 (0.88-1.93) and risk ratio (RR) 1.14 (0.94-1.39) (P=0.17).

Discussion

NAD(P)H: quinone oxidoreductase (NQO1) is an enzyme that is involved in metabolising numerous endogenous quinones. Exchange of Proline at position 187 by Serine leads to a non-functional enzyme (Nebert DW et al. 2002). Individuals homozygous for the 187serine allele have a high risk for aplastic anaemia and leukaemia. Present study showed a strongly positive association between NQO1 C609T polymorphism with breast cancer risk in north Indian breast cancer patients. We have shown a significant association between NQO1 C609T polymorphism and different patients characteristics (stage, lymph node status, Her2/neu status). Another study published by V Singh et al (2011) on north Indian breast cancer patients concluded that NQO1 C609T polymorphism does not play a significant role in breast cancer susceptibility in north Indian women. Fagerholm et al (2008) suggested that NQO1 was a strong prognostic and predictive factor in breast cancer patients receiving anthracyclines, and they also proposed that NQO1 influences the outcome of epirubicin treatment through at least three mechanisms: the TP53 and TNF-NF- κ B pathways and direct detoxification of reactive oxygen species. H-J Menzel et al (2004) studied in breast cancer patients from two different populations and found that the 187S allele was found significantly more frequently in breast cancer patients than in controls. However our results suggest that the heterozygous genotype has a higher risk of breast cancer than homozygous 187S genotype. Our results also suggest that NQO1 C609T polymorphism may be significantly associated with the advancement of breast cancer stage. J Sarmanova et al (2004) has suggested an influence of genetic polymorphisms of xenobiotic-metabolizing enzymes, particularly NQO1, on the susceptibility to breast cancer, possibly by change of the ratio of activation/ detoxification of procarcinogens or

by linkage to another cancer-causative gene(s). Fowke et al (2004) suggested that the use of oral contraceptives was associated with a lower breast cancer risk among women with the T allele of NQO1.

The C609T NQO1 polymorphism is one of very few known and common single nucleotide polymorphisms that completely eliminate enzymatic activity, and its biological impact in vivo is undoubted. The NQO1 enzyme has been increasingly attracting attention in cancer and more roles unrelated to its classical metabolic functions are being constantly discovered. The various functions NQO1 performs are - NQO1 enzyme has the classical role of catalysing the obligatory two electron reduction of a broad range of exogenous and endogenous quinones to their respective hydroquinones, preventing the one electron reduction of these compounds into semiquinones and a variety of reactive oxygen species. NQO1 was found to have an additional antioxidant effect by directly and independently scavenging superoxides, a function that is yet to be established. NQO1 enzyme was recently found to act as a stabilizer for several tumor suppressor proteins (p53, p73, and p33), and this stabilization was shown to be especially prominent under conditions of oxidative stress. As the C609T polymorphism principally affects the susceptibility of the enzyme to degradation in vivo, it affects all aspects of NQO1 function indistinguishably. The largest role of NQO1 in cancer susceptibility is attributable to the interaction of the enzyme with environmental exposure. The high minor allelic frequency of the C609T polymorphism of this 'environmental' enzyme in the Asian population (43%) compared with the Caucasian population (20%) suggests that the environmental exposure is widely different among the two populations. The variation in allele frequency of this polymorphism therefore highlights major environmental differences among the two populations and may in turn partly explain why a significant association was found in Caucasians but not in Asians. Another possible explanation is the widely different genetic structure between the two populations, as the overall effects of NQO1 C609T polymorphism

on cancer susceptibility might be overshadowed or compensated by variants in other metabolic genes. In conclusion, present study demonstrates that the NQO1 C609T polymorphism may increase the risk of breast cancer in north Indian population.

In conclusion, The present study suggests the NQO1 C609T polymorphism as an important genetic factor in the overall risk for developing breast cancer, especially in Indian populations and predicts that oxidative stress-modifying enzymes would be a promising area of further investigation for prognostic markers of breast cancer. However, more association studies are needed to support this finding in various ethnic groups.

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