

Carcinogen Switching NQO1 Gene Polymorphism Study to Find Out the Risk Population in Vindhyan Region



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Abstract

The NQO1 is a gene catalyses an enzyme capable of maintaining quinones in their reduced form, therefore detoxify them. DT-diphorase (NQO1) converts toxic benzoquinone into hydroquinone in an incumbent two electron reduction. Only two polymorphic variants of NQO1 have been identified, a C-to-T change at nucleotide 609 yields a serine substitution and a T -to-C change at nucleotide 464 results in tryptophan replacement of Arginine. Some synthetic antioxidants such as Butyrate Hydroxyanisole and extracts of cruciferous vegetables, including broccoli have been emerges to be a potent inhancers of NQO1. This inhancibility has led to the suggestion that NQO1 can play an possible role in cancer chemo-prevention and therapy.

Keywords: Carcinogen switching, NQO1, Polymorphism, PCR, RFLP, Genotyping

Abbreviations: PAH: Polycyclic Aromatic Hydrocarbon; NQO1: DT-diphorase; HNSCC: Head and Neck Squamous Cell Carcinoma; DNA: Deoxyribonucleic Acid; PCR: Polymerase Chain Reaction; MW: Molecular Weight

Introduction

The NQO1 is an important carcinogen metabolizing gene, as responsible for the metabolism of highly ubiquitous environmental carcinogen like Polycyclic Aromatic Hydrocarbon (PAH) which is also the most lethal constituent of cigarette smoke. The genotype and allelic frequencies of three Polycyclic aromatic Hydrocarbon (PAH) metabolizing genes were determined in a sample of typical population, which were stratified into three groups (Hindu, Muslim and Tribal) based on their ethnicity. DT-diphorase is an enzyme which has known because of its ability to detoxify a number of natural and Synthetic Compounds and also to activate certain anti- cancer factors [1]. Among Northern Europeans and Caucasian Americans, the gene frequency is 0.79 for the wild-type allele and 0.21 for the mutated allele [2,3]. DT-diphorase (NQO1) converts toxic benzoquinone into hydroquinone in an obligate two electron reduction [4].

This reaction is in competition with one-electron reduction reactions by Cytochrome P-450, producing the semiquinones, which generate free radicals and reactive oxygen species via redox cycling. The NQO1 is capable of maintaining these quinones in their reduced form, therefore detoxify them [5,6]. Only two polymorphic variants of NQO1 have been identified,

a C-to-T change at nucleotide 609 yields a serine substitution and a T -to-C change at nucleotide 464 results in tryptophan replacement of Arginine. The C 609 T effectively inactivates the enzyme due to decreased catalytic activity and stability of NQO1 protein [7,8].

The frequency of the mutated allele is known to be slightly higher among African Americans and considerably higher among Hispanics and Asians [9]. NQO1 enzyme activity is found to be normal in individuals with wild-type alleles. It is variably reduced in individuals who are hetero-zygotes for the polymorphism [10]. The NQO1 protein and activity are absent in those who are homozygous for the point mutation [11]. It is reported that inhibition of NAD (P) H: quinone oxidoreductase 1 (NQO1) activity by dicoumarol induces degradation of p53, indicating that NQO1 plays a role in p53 stabilization, which has a crucial role in cell cycle regulation [12]. In various studies NQO1 null alleles has shown to be associated with benzene related leukemia, cancer of lung, and colon. A study suggests that NQO1 can modulate the susceptibility for breast cancer [13-16]. Chang Gun Cho and co - workers concluded that NQO1 139Arg allele were associated with tobacco dose-dependent increase

in risk of head and neck squamous cell carcinoma (HNSCC) and NQO1 genotype polymorphisms may play an important role in the development of smoking related HNSCC [17]. However, Hongwei Chen and coworkers have found no relation between NQO1 null allele and lung cancer [18]. But this not renders the significance of NQO1. It is also a highly inducible enzyme, synthetic antioxidants such as Butyrate Hydroxyanisole and extracts of cruciferous vegetables, including broccoli have been shown to be potent inducers of NQO1. This inducibility has led to the suggestion that NQO1 can play an important role in cancer chemo-prevention and therapy.

Material and Methods

Study population

In our study we have selected the vindhyan region, the heart land of India. This is constructed by four district of Madhya Pradesh and is the oldest habitat of man kind. The Kol, Gond, Bhil and Baiga are the native tribe of this region. Modern population is dominated by Hindu and Mohammedan religions. We have selected the 409 normal healthy individuals who were occupationally (or non-occupationally PAHs (Polycyclic Aromatic Hydrocarbons) exposed. All the subjects were the regular resident of the Vindhyan region (Table 1).

Table 1: Clinical feature of study population.

Clinical feature	Hindu	Muslim	Tribal
Total no.	311	25	73
Sex ratio (Male:Female)	136:175	9:16	34:39
Age	33.43±13.96	35.24 ±15.68	35.52±15.30
Mean±SD	32	32	30
Median	Nov-73	16-73	16-74
Age range			
Smoker (No.)	39	8	16

Blood collection and plasma/serum separation

Approx. 5ml. of Venous blood samples of each individual were collected in storage vials containing EDTA as anticoagulant (50µl. EDTA for 1ml. blood). Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min at 4 °C.

Deoxyribonucleic acid (DNA) isolation

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller [7].

Detection of NQO 1 (Hinf I) polymorphism via PCR-RFLP

A C-to-T change at nucleotide 609 in NQO 1 genes yields a serine substitution. Which create a Hinf I cleavage site. Primer was designed to amplify 172 bp sequence to study this polymorphism.

Genotyping

Genotyping was carried out by polymerase chain reaction (PCR) and restriction enzyme digestion. The NQO1

polymorphism was detected using the following primers: NQO Hinf I F- 5'AAG CCC AGA CCA ACT TCT3' and NQO Hinf I R- 5'ATT TGA ATT CGG GCG TCT GCT G3'. The oligonucleotides sequences (primers) used were those described by Jianhui Zhang et al (171): The PCR was carried out in a final volume of 25 µl, containing 100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X Taq polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concent ration 1X; Genetix Biotech Asia Pvt. Ltd., India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of sificic forward and reverse primers and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

Thermal profile used for the amplification of desired segment of gene was as follows: Initial denaturation at 95 °C for 5 min and 30 cycles of denaturation at 94 °C for 45 Sec, annealing at 62.9 °C for 45 sec and extension at 72 °C for 1 min, followed by final extension at 72 °C for 07 min. PCR products were separated on 2% agarose gel (2%w/v, Sigma) using a 100 bp molecular weight (MW) marker to confirm the PCR product size of 172 bp. For restriction digestion of the C -to-T substitution at Nucleotide 609 non-coding region, which creates an Hinf I restriction enzyme cleavage site.

The reaction mix included 0.2 of 10,000U/ml HinfI restriction enzyme (final concentration 2.5 U), 2.5 of 10X GENAI Buffer c (final concentration 1X; 50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9(10.0 of PCR product and 10 of sterile water. Reaction was incubated for 24 hrs at 37 °C for complete digestion. 10 of digested PCR product was loaded on 2% Agarose gel. Electrophoresis was done at 80 V in 1 X Tris-borate EDTA buffer (89mM Tris pH 7.6, 89 mM Boric acid, 2 mM EDTA pH 8.0) buffer.) A 100 bp gene DNA ladder (genetix india) was run concurrently as molecular weight marker. The gel was than stained with ethidium bromide (10mg/ml). The products were visualized using an ultraviolet trans illuminator. The gel picture was captured using a digital camera and gel documentation software (Vilber Lourmate, Cedex I , France).

Statistical analysis

Statistical analysis was done by comparing the distribution of genotype frequencies and allele frequencies for all polymorphisms in all groups of study subjects. The proportions of different genotypes for a gene in a population are known as genotype frequencies. The proportion of a genotype in a sample will be the ratio of the number of individuals having that genotype of the total number of individuals in the sample. The proportions of different alleles for a gene present in a population are known as allele frequencies. The proportion of an allele in a sample will be the ratio of number of occurrences of the investigated allele in the population to the total number of alleles. Data was analyzed using Microsoft Excel 2002, Microsoft Corporation and graphPad Instat program (GraphPad Software, San Diego California USA, Copyright 1992-1998 GraphPad Software Inc. (www.graphpad.com).

Results

Hardy - Weinberg equilibrium

The genotype frequencies of each study group were tested to be in accordance with Hardy - Weinberg equilibrium using chi square test for independence. All the calculated values were compared with tabulated values and found that all the frequencies were in Hardy- Weinberg equilibrium (Table 2).

Table 2: Values of Chi square test for Hardy-Weinberg equilibrium for NQO1 genes in study population.

Gene polymorphism	Study Groups (χ^2 Values)		
	Hindu	Muslims	Tribal's
NQO1 Hinf I	0.000255	0.11347	0.151241

Determination of NQO1 Hinf I polymorphism, genotypes / Alleles frequencies and their association between study groups

In our investigation the polymorphism of NQO1 genes

studied by PCR - RFLP. The C to T substitution at nucleotide 609 creates the restriction site for Hinf I. The PCR products of Wild type allele (CC) were 172 bp long and were un-digestable by Hinf I at standard condition. Heterozygous condition (CT) was appeared as the production of 172 bp, 131bp and 41bp long DNA fragment after the digestion with Hinf I. The Null alleles (TT) were detected as the 131bp and 41bp long fragments after digestion with Hinf I at standard condition. The genotype frequency (in %) of CC, CT and TT Genotypes scored 69%, 24% and 07% respectively in hindues. In Muslims it is recorded CC = 68%, CT= 20% and TT= 12%. In Tribal population genotype frequencies were found CC = 70%, CT = 23% and TT = 07% (Table 3) (Figures 1 & 2). The association of genotype frequency distribution in all three groups (Hindu, Muslim and Tribals) were studied and as recorded, there is no statically significant differences exist as the χ^2 and P values were $\chi^2 = 0.7994$ (P = 0.6705) between Hindu - Muslim, $\chi^2 = 0.7042$ (P = 0.7032) between Muslim - Tribal and $\chi^2 = 0.05735$ (P = 0.9717) between Hindu - Tribals (Table 3).

Table 3: Frequency distribution and association of Genotype and alleles of NQO1 genes in study groups.

NQO1 genes	Study Groups					
	Hindu		Muslim		Tribal	
Genotypes	No.	%	No.	%	No.	%
CC	213	69	17	68	51	70
CT	75	24	5	20	17	23
TT	23	7	3	12	5	7
χ^2 Values			P- Values			
Hindu- Muslim	0.7994				0.6705 (NS)	
Muslim- Tribal	0.7042				0.7032(NS)	
Hindu-Tribal	0.05735				0.9717(NS)	
Allels	Hindu Frequency %		Muslim Frequency %		Tribal Frequency %	
C	81		78		82	
T	19		22		18	
P- Values (Fisher's exact test)						
Hindu - Muslim	0.7264 (NS)					
Muslim - Tribal	0.5963 (NS)					
Hindu - Tribal	1.0000 (NS)					

NS = Non Significant, P = Probability.

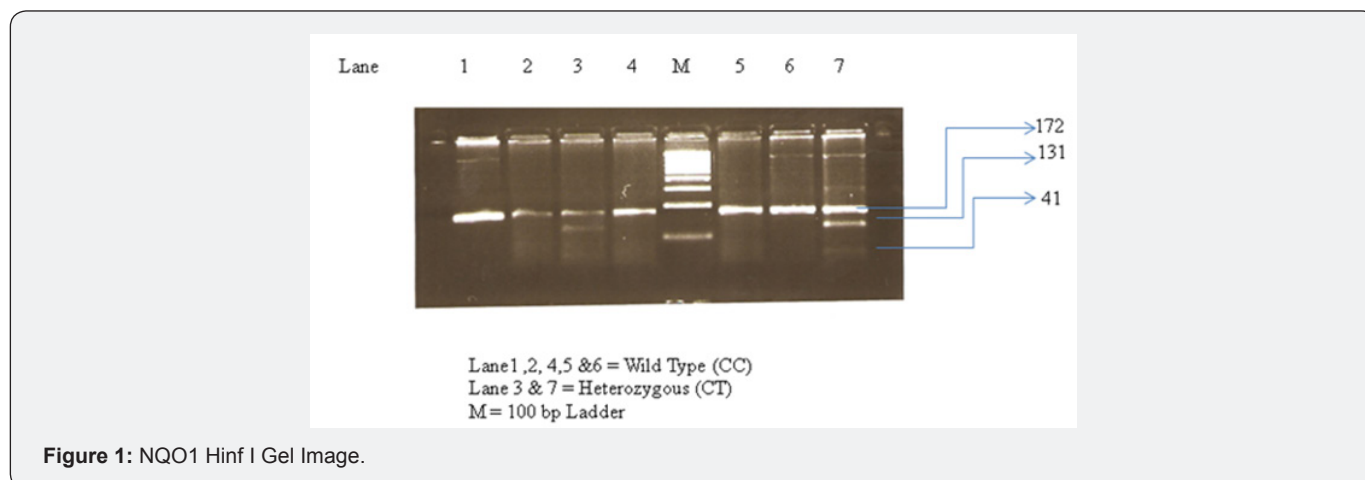


Figure 1: NQO1 Hinf I Gel Image.

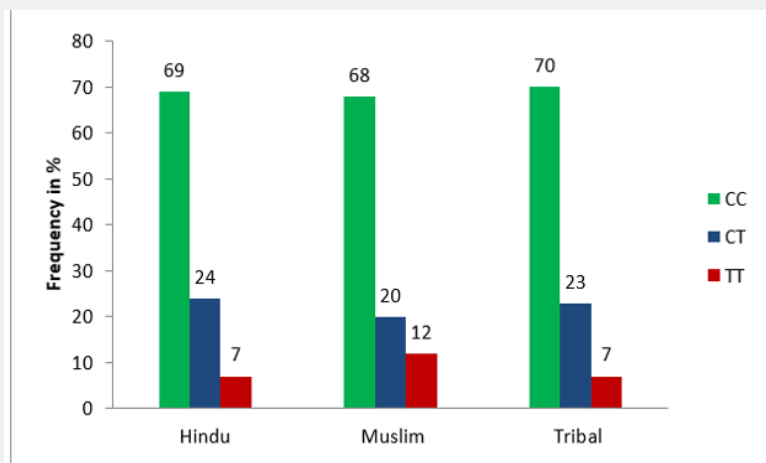


Figure 2: NQO1 Genotype frequency in (in %) study groups (Hindu, Muslim & Tribal).

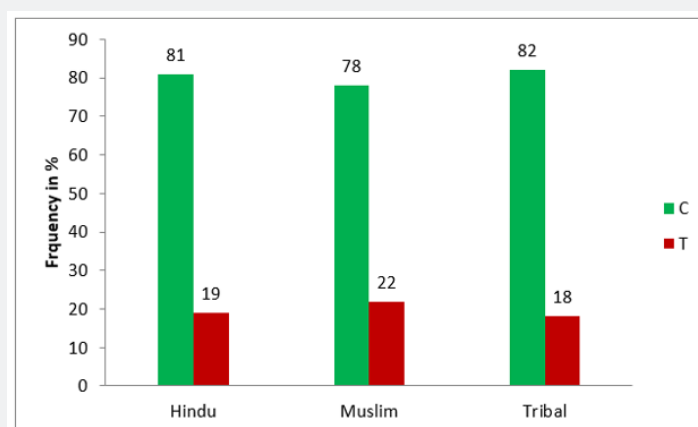


Figure 3 : NQO1 Allelic frequency in (in %) study groups (Hindu, Muslim & Tribal).

The distribution of C and T alleles in various groups were scored C = 81% and T = 19% in Hindus, C = 78% and T=22% in Muslims and C = 82%, T = 18% in Tribal population (Table 3) (Figure 3). The association between Allelic distribution in all three groups were studied by Fisher’s exact test and values obtained were, P = 0.7264 between Hindu – Muslim, P = 0.5963 between Muslim – Tribal and P = 1.0000 between Hindu – Tribal. All the values were non significant which showed that the frequency distribution of C and T allele in all three groups were statically not different (Table 3).

Discussion

The population of India is always being the part of research due to its large population, unique culture and diversity. Indian population can be distinguished as the castes, tribes and religious communities. Tribes represent approx. 8.2% of the total population of India. There are currently about 530 tribal groups in India. Madhya Pradesh (MP) is the second largest Indian state by area, is located in the central part and is homeland of several caste and tribal groups. In past three decades the information about carcinogen metabolism has been improved. Due to this information the interest has grown about study of polymorphism among carcinogen activating and de-activating

genes. In this respect we have conducted a broad polymorphism screening study in population of Vindhyan Region.

The total 409 individuals were screened for the NQO1 Hinf I polymorphism, in which 311 individuals were Hindu, 25 individuals were Muslim and 73 individuals were Tribal. The NQO1 Hinf I genotype distribution pattern varies by ethnicity in world human population. In our study population (Hindu, Muslim and Tribal) it was observed as CC = 69%, CT = 24% and TT = 07% in Hindu, which was found corresponding to Tribal population where CC = 70%, CT = 23% and was TT = 07% observed. There was a slight difference scored between these two groups and Muslim as genotype frequencies observed were CC = 68%, CT = 20% and TT = 12% in Muslim, which showed the slight increase in homozygosity and higher frequency of TT (Mutant) genotype than Hindu and Tribal. The NQO1 Hinf I genotype frequency of our population did not statically different with reported in Maharastrian Indians where CC = 52.3%, CT = 42.48 and TT = 5.18% scored [19]. But an increase in Heterozygosity observed in Maharastrian Indian than our population. It could be due to random mixing of various ethnic groups in Maharastrian population in modern age. But increase in heterozygosity surprised us because the marriage between the close relatives in Maharastrian is common.

The frequencies observed in our population did not match with the Caucasian, Chinese and Koreans where frequencies reported were CC = 79%, CT = 16% and TT = 05% in Caucasian [20], CC = 34%, CT = 49.7% and TT = 16.3% in Chinese and CC = 94.5%, CT = 5.2% and TT = 0.3% in Koreans [21,22]. This showed that these populations are distinct from our study population. The Swedish population showed a slight difference with our population where the CC = 69.4, CT = 28.9% and TT = 1.7% scored [23]. But the distribution of TT (Mutant) allele was less in Swedish than our population. The genotype frequencies of our population also match with the population of different Iranian ethnic groups [24], when compared, where the CC = 59.5%, CT = 31% and TT = 9.5% in Fars, CC = 64%, CT = 24% and TT = 12% in Mazzandarani and CC = 69%, CT = 28.6%, TT = 2.4% in Turk recorded. But an Increase in TT (Mutant) genotype was scored in our population and in Two Iranian ethnic groups (Fars and Mazzandarani) than Turk and other world population. The results were in agreement with the phenomena of common ancestry of Indian and Iranian population.

The allelic distributions were found in accordance with others finding in Caucasian population (C = 81.1% and T = 18.9%) [25], as there were no significant differences were calculated, where in our population it was recorded as C = 81% and T = 19% in Hindu, C = 78% and T = 22% in Muslim and in Tribal it was C = 82% and T = 18%.

Conclusion

The significant differences were seen when results from our finding were compared with others finding in various Asian population, where the frequency of C allele were 57% and T allele were 43% calculated in previous finding by Kiyohara et al. [14]. This difference were also evident when our results of our population compared with individual finding of Choi et al. [25] in Korean and Fowke et al. [26] in Chinese population, where the frequencies were recorded as C = 58.5% and T = 41.5% in Korean and frequency of C allele were scored 59.3% and T allele were scored 40.7% in Chinese. These results were not surprised us because in calculation of NQO1 Hinf I allele the Kiyohara et al. [14] did not included the frequencies of Indian population and his finding were solely based on the results from Korean, Chinese and Japanese population, which are totally distinct from our population and belongs to different race. This finding suggests that the Indian population can not co-related with whole Asian population.

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