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NQO1*2 [NAD(P)H: quinone oxidoreductase 1] polymorphism and its influence on acute leukemia risk

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Abstract
NAD (P) H: quinone oxidoreductase 1 (NQO1) is an enzyme that protects cells against mutagenicity from free radicals and toxic oxygen metabolites. The gene coding for NQO1 has polymorphism at nucleotide position 609(C-T) of the human cDNA. Heterozygous individuals (C/T) have intermediate activity and homozygotes for the variant allele (T/T) are deficient in NQO1 activity. In previous studies, genotypes conferring lower NQO1 activity have been associated with an increased risk of acute leukemia. The present study includes 297 acute leukemia cases comprising of 151 acute lymphocytic leukemia (ALL), 146 acute myeloid leukemia (AML) and 220 control samples for analysis of NQO1*2 polymorphism using PCR-RFLP method. The NQO1*2 polymorphism was significantly associated with acute leukemia development (χ2- 31.614; df-2, p - < 0.000) with respect to clinical variables. Mean WBC, Blast %, LDH levels were increased in both ALL and AML cases with TT genotype. 50% of AML cases failed to achieve complete remission towards therapy. There was significant reduction in mean DFS (Disease Free Survival) in both ALL and AML cases with TT genotype (21.18m, 8.31m). Our results suggest that TT genotype might be considered as a risk genotype for development of acute leukemia and is associated with poor prognostic markers.

Keywords: NQO1*2; AML; ALL; polymorphism; DFS.

Introduction

NQO1 is a flavo protein that catalyzes the two-electron reduction of quinone compounds and prevents the generation of semiquinone free radicals and reactive oxygen species, thus protecting cells from oxidative damage (Winski *et al*., 2002). On the other hand, NQO1 also catalyzes the reductive activation of quinoid chemotherapeutic agents and of environmental carcinogens such as nitrosamines, heterocyclic amines and cigarette smoke condensate (Larson *et al*., 1999). The gene, located at 16q22.1, is composed of 6 exons and 5 introns spanning 17,881 base pairs. It is induced by synthetic antioxidants and cruciferous vegetables and offers protection against oxidative stress (Benson *et al*., 1980).

Two polymorphisms described in the *NQO1* gene are C609T *(NQO1*2*) and C465T (*NQO1*3*) substitutions which lead to P187S and R139W amino acid replacements respectively. C465T causes reduction in enzyme activity, whereas the C609T results in complete loss of enzymatic activity due to protein instability (Krajinovic, 2005). People who were homozygous for the variant allele completely lack *NQO1* activity, and heterozygotes had low to intermediate activity compared with people with the wild type (Siegel *et al*., 1999). The incidence of this polymorphism varies widely by race (Kelsey *et al.*, 1997) and associations were observed between the presence of variant alleles in lung and urological cancers (Rosvold *et al.*, 1995).

NQO1 was constitutively expressed in most tissues, including the bone marrow, where the expression was thought to be highly inducible by xenobiotics with quinine moieties and was up regulated during the times of oxidative or electrophilic stress (Ross *et al.*, 2004). *NQO1* expression was also up regulated in tumor tissues as the result of hypoxia (Waleh *et al.*, 1998). The variant *NQO1* C609T polymorphism conferred an increased risk to develop leukemia, supporting the role for *NQO1* substrates (benzene and related compounds) and oxidative stress as determinants of ALL. The presence of the *NQO1*2* (Rothman *et al.*, 1997) was reported to confer an increase in the risk of both AML and ALL. Hence, in the present study, an attempt was made to evaluate the role of NQO1*2 (C609T) Polymorphism in determining the risk for developing acute leukemia as well as its progression.

Materials and Methods

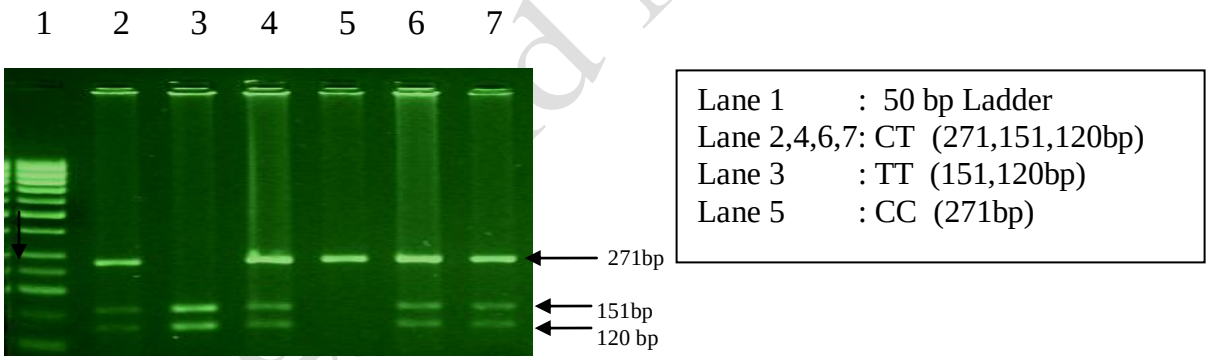
297 acute leukemia cases comprising of 151 acute lymphocytic leukemia (ALL), 146 acute

myeloid leukemia (AML), were recruited from NIMS (Nizam's Institute of Medical Sciences), Hyderabad. 5ml of blood sample was collected into EDTA vacutainers from these cases and also from 220 age and sex matched healthy controls. Patient's clinical data like WBC count, Blast%, Platelet count, Haemoglobin %, LDH levels, Complete Remission (CR) response to therapy and Disease Free Survival (DFS) was noted from the tumor registry files with the help of medical oncologist. Genomic DNA was isolated by using salting-out method (Nuremberg and Lahari, 1991) and used for genotyping of NQO1*2 polymorphism through PCR-RFLP analysis.

*Genotyping of NQO1*2 polymorphism*

Genomic DNA was amplified using gene specific primer sequences**:** Forward: 5'- AAG CCC AGA CCA ACT TCT -3', Reverse: 5'- TCT CCT CAT CCT GTA CCT CT -3'. The 25µl PCR reaction mixture consisted of approximately 100-150ng of genomic DNA, 15 pmol/l of each primer, 200µmol/l of dNTPs, 20 mmol/l of Tris HCl, 50 mM of KCl, 2.5 mmol/l of $MgCl_2$, 5% dimethyl sulfoxide (DMSO), 0.5 U of Taq DNA polymerase and deionized water (varied). The PCR Cycling conditions included initial denaturation at 94^0 C for 2 minutes followed by 40 cycles of denaturation at 94^0C for 50 seconds, annealing at 52^0C for 50 seconds, extension at 72^0C for 30 seconds and final extension at 72^0C for 10 minutes. After amplification, a 271 bp PCR product was subjected to restriction digestion using of Hinf1 enzyme (New England Biolabs, USA). The samples were genotyped on 3% agarose gel. The C609T transition creates Hinf1 restriction site so that 271 bp PCR product give rise to two fragments of sizes 151 and 120 bp.

Gel photograph of NQO1*2 polymorphism



Statistical analysis

All the required statistical analysis were performed using SPSS 15.0 version (Statistical Package for the Social Science). Chi square test was calculated to test the significance of genotype association with the occurrence of acute leukemia and its prognosis. All the p values were two sided and the level of significance was taken as $p < 0.05$.

Results

In the present study, the frequency of TT homozygous genotype was significantly elevated in both ALL, AML as compared to control group **(χ2- 7.778; df- 2, p = 0.020*; χ2- 31.614; df-2, p < 0.000) (Table 1)** which might indicate the functional significance of the polymorphism in leukemiogenesis. The inability of TT genotype to detoxify the xenobiotics with quinine moieties which were up regulated during the time of oxidative stress might lead to DNA damage and leukemiogenesis. The genotypic distribution of *NQO1*2* showed significant deviation from HWE in both disease and control group **(χ2-29.05) (Table 1)** which might be due to presence of selective forces or method of sampling.

Table 1: Genotype distribution of NQO1*2 Polymorphism in acute leukemia and controls.

NQO1	Genotype Frequency			Total	Allele Frequency	
	CC	CT	TT		C	T
	n %	n %	n %			
ALL	57 (37.7)	60 (39.7)	34 (22.5)*	151	0.58	0.42
AML	44 (30.1)	42 (28.8)	60 (41.1)**	146	0.45	0.55
Total	101(34.0)	102 (34.3)	94 (31.6)	297	0.51	0.49
Controls	115 (52.3)	70 (31.8	35 (15.9)	220	0.68	0.32

* ALL vs Controls $\chi 2$- 7.778; df- 2, p =0.020*
**AML vs Controls $\chi 2$- 31.614; df-2, p=0.000*
Cases vs Controls $\chi 2$ - 22.885; df-2, p=0.000*

Hardy Weinberg Equilibrium $\chi 2$ for i) ALL Cases $\chi 2$- 5.25
ii) AML Case $\chi 2$-25.47*
iii) Total cases $\chi 2$-29.05*
iV) Controls $\chi 2$-15.64*

Odds ratios (OR)

ALL: Disease versus Controls

CC vs. CT (OR) 0.578* 95% CI (0.363to 0.92) p < 0.05
CT vs.TT (OR) 0.882 95% CI (0.492 to 1.58)
CC vs. TT (OR) 0.504* 95% CI (0.284 to 0.896) p < 0.05

AML: Disease versus Controls

CC vs. CT (OR) 0.636 95% CI (0.378 to 1.06)
CT vs.TT (OR) 0.36* 95% CI (0.208 to 0.621) p < 0.05
CC vs. TT (OR) 0.231* 95% CI (0.135 to 0.388) p < 0.05

Table 2a: NQO1*2 Polymorphism and sex of the proband in ALL group.

ALL	Genotype Frequency						Total	Allele Frequency	
	CC		CT		TT				
	n	%	n	%	n	%		C	T
Males	44	42.7	38	36.9	21	20.4	103	0.612	0.388
Females	13	27.1	22	45.8	13	27.1	48	0.5	0.5
$\chi 2$-3.43; df-2, (p-0.18)									
OR (CI 95%) : CC Vs CT : 1.9595 (from 0.8704 to 4.4114) *OR (CI 95%) : CT Vs TT : 1.0693 (from 0.4487 to 2.5483)* *OR (CI 95%) : CC Vs TT : 2.0952 (from 0.8283 to 5.2999)*									

Table 2b: NQO1*2 Polymorphism and sex of the proband in AML group.

AML	Genotype Frequency						Total	Allele Frequency	
	CC		CT		TT				
	n	%	n	%	n	%		C	T
Males	29	32.2	25	27.8	36	40	90	0.461	0.539
Females	15	27.8	16	29.6	23	42.6	54	0.426	0.574
χ2-0.31; df-2 , (p-0.85)									
OR (CI 95%) : CC Vs CT : 1.2373 (from 0.5109 to 2.9965) *OR (CI 95%) : CT Vs TT : 0.9983 (from 0.441 to 2.2599)* *OR (CI 95%) : CC Vs TT : 1.2352 (from 0.5474 to 2.7873)*									

Table 3a: NQO1*2 Polymorphism and age at onset in ALL group.

ALL	Genotype Frequency						Total	Allele Frequency	
	CC		CT		TT				
	n	%	n	%	n	%		C	T
<10 yrs	15	34.1	18	40.9	11	25.0	44	0.545	0.455
10-20 yrs	27	40.3	22	32.8	18	26.9	67	0.567	0.433
>20 yrs	15	37.5	20	50.0	5	12.5	40	0.625	0.375
χ2-4.61; df-4, (p-0.32)									

Table 3b: NQO1*2 Polymorphism and age at onset in AML group.

AML	Genotype Frequency						Total	Allele Frequency	
	CC		CT		TT				
	n	%	n	%	n	%		C	T
<20 yrs	6	23.1	10	38.5	10	38.5	26	0.423	0.577
20-30 yrs	21	38.2	13	23.6	21	38.2	55	0.5	0.5
>30 yrs	17	27.0	18	28.6	28	44.4	63	0.413	0.587
χ2-3.47; df-4, (p-0.48)									

Table 4a: Mean values of clinical variables with respect to NQO1*2 Polymorphism in ALL group.

Clinical variables	CC		CT		TT		Total
	Mean ±SE	n	Mean ±SE	n	Mean ±SE	n	
Mean Age	15.32±1.08	57	16.75±1.57	60	13.85±1.27	34	151
Mean WBC (Thousand)	46.15±6.19	57	52.40±9.80	60	67.97±16.63*	34	151
Mean Blast %	49.95±4.18	57	46.80±4.49	60	55.00±5.60*	34	151
Mean Platelet count(lakhs)	0.90±0.10	57	0.90±0.09	60	0.72±0.10	34	151
Mean HB	8.53±0.30	57	9.02±0.34	60	9.31±0.51	34	151
Mean LDH	753.11±84.80	57	683.08±57.99	60	1034.0±169.9*	34	151
Mean DFS	28.73±2.54	52	29.79±2.83	53	21.18±2.67	33	138

***** $p < 0.05$ as compared to CC genotype

Table 4b: Mean values of clinical variables with respect to NQO1*2 Polymorphism in AML group.

Clinical variables	CC		CT		TT		Total
	Mean ±SE	n	Mean ±SE	n	Mean ±SE	n	
Mean Age	33.82±2.34	44	29.15±2.27	41	31.98±1.96	59	144
Mean WBC (Thousand)	48.87±11.56	44	38.72±9.16	41	60.49±10.97*	59	144
Mean Blast %	59.89±4.80	44	60.05±3.78	41	62.32±3.21	59	144
Mean Platelet Count (Lakhs)	0.78±0.12	44	0.82±0.17	41	1.33±0.21	59	144
Mean HB	8.46±0.30	44	8.23±0.47	41	8.21±0.31	59	144
Mean LDH	394.70±44.08	44	510.93±65.55	41	517.31±42.64*	59	144
Mean DFS	12.33±1.75	24	15.23±2.09	26	8.31±1.11	36	86

* $p < 0.05$ significant as compared to CC genotype

Table 5: NQO1*2 Polymorphism and complete remission rates in ALL and AML groups.

NQO1							
ALL	**CC**		**CT**		**TT**		**Total**
	n	**%**	**N**	**%**	**n**	**%**	
CR +VE	52	38.0	53	38.7	32	23.4	137
CR -VE	3	60	1	20	1	20	5
χ2- 1.072; df-2, (p-0.585)							
AML							
CR +VE	19	28.8	23	34.8	24	36.4	66
CR -VE	12	30.0	8	20.0	20	50.0	40
χ2- 3.006; df-2, (p-0.222)							

The heterozygous CT genotype frequency was elevated in female ALL patients (45%) compared to male patients (36.9%) **(Table 2a).** The risk genotype (TT) frequency did not show association with sex in ALL and AML. Although significant association of male sex with ALL and AML was observed in the present data, the lack of association of sex with TT genotype indicated that the influence of TT genotype in conferring risk to acute leukemia might be independent of sex. There was no significant association of age at onset with *NQO1*2* polymorphism in ALL and AML **(Table 3a, 3b).** The elevated frequency of TT genotype among early onset ALL (26.9%) and CT with late age onset ALL (12.5%) was noted **(Table 3a, 3b).** Specific trends in age at onset distribution with respect to *NQO1*2* polymorphism was not observed in AML.

Clinical variables such as Mean WBC, Blast %, LDH levels was increased in ALL and only Mean WBC and LDH levels were elevated in AML cases with TT genotype. 50% of AML patients with TT genotype failed to achieve complete remission. There was significant reduction in mean DFS in both ALL and AML cases with TT genotype. Earlier studies by Krajinovic *et al.* (2002) had reported that the *NQO1*2* polymorphism reduces survival

probability in children with AML on the basis of drug associated toxicity.

Discussion

A case control study carried out in benzene exposed workers in China showed an increased risk of hematotoxicity and leukemia in individuals harboring *NQO1*2* allele (Rothman *et al*., 1997). Several studies carried out in adults from various ethnic groups revealed an association of the *NQO1*2* polymorphism with t-AML/t-MDS, patients with t-AML/t-MDS homozygosity of *NQO1*2* conferred a 2.6 fold increase in the risk of developing t-AML compared with those carrying the wild type allele (Naoe *et al*., 2000). Additionally this polymorphism was strongly associated with t-AML carrying abnormalities of chromosome 5 and 7 (Larson *et al*., 1999). Further, the patients with defective *NQO1* treated with standard dose chemotherapy were most at risk of telomere attrition and a possible switch to clonal haematopoiesis. This might in turn predispose such patients to the development of t-AML/MDS.

The low *NQO1* activity confers a significantly increased risk of contracting AML with inv(16) [inversion 16] (Smith *et al.,* 2001). The obvious explanation for the strong association between AML case with inv(16) (Smith *et al.,* 2001) and lower *NQO1* activity is that certain substances that are normally detoxified by NQO1 are highly effective at causing inv(16) (Smith *et al.,* 2001) However, it is of interest that the *NQO1* gene is located on chromosome 16q22.1, one of the break points for the inv(16) rearrangement(Smith *et al.,* 2001). It is possible that one copy of the *NQO1* gene is disrupted by the rearrangement, with the result that heterozygotes would have null *NQO1* activity in leukemic cells with the inv(16) (Smith *et al.,* 2001).

This loss of activity could be strongly associated with the production of secondary genetic changes caused by exposure to *NQO1* substrates after an inv(16) (Smith *et al.,* 2001) has arisen leading to a leukemic clone. The association between null or low *NQO1* activity and adult leukemia is that *NQO1* protein expressed in peripheral blood cells and bone marrow progenitors is normally very low, but is highly inducible.

Apart from its inducibility, the presence of *NQO1* in other cells such as the bone marrow, stroma, hepatocytes, where it is highly expressed might be important in protecting against leukemiogenesis. Low *NQO1* activity caused by the inheritance of one or more mutant C609T allele was associated with increased risk of *de novo* acute leukemia in adults (Smith *et al.,* 2002).

Previous studies had also reported the *NQO1* C609T polymorphism was associated with risk of childhood and adult ALL, as well as *de novo* and therapy associated AML (Rothman *et al*., 1997; Larson *et al*., 1999; Wiemels *et al.,* 1999; Naoe *et al*., 2000; Krajinovic *et al*., 2002). *NQO1*2* (T) allele was more prevalent in children with ALL carrying mixed lineage leukemia (MLL) gene translocations as compared to controls (Smith *et al.,* 2002). Moreover, the presence of at least one *NQO1*2* (T) allele conferred 2.7 fold increase in the risk of ALL / MLL positive cases. Contrary to earlier reports, two European studies reported lack of association between the *NQO1* variant and the incidence of pediatric ALL, or AML. However, the modulating role of NQO1 in the pathogenesis of pediatric sporadic Burkitts lymphoma was suggested (Sirma *et al*., 2004; Kracht *et al*., 2004).

Conclusion

In conclusion, our results suggest that the TT genotype might be considered as a risk genotype for development of acute leukemia and is associated with poor prognostic markers.

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