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The Predisposing Role of NAD (P) H:Quinine Oxidoreductase Gene Polymorphisms in the Development of Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Objectives: NAD (P) H:Quinone Oxidoreductase (NQO1) protects cells against oxidative stress and toxic quinines which protects cells against mutagenicity of free radicals and toxic oxygen metabolites. In fact, low level of NQO1 activity is often associated with increased risk of developing different types of tumors and with toxic effects linked to environmental quinines. Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. C to T base substitution at nucleotides 609 and 465 of NQO1 cDNA, results in loss of enzyme activity. Low NQO1 activity may play a role in etiology of ALL. In the present study, we investigated the association between the NQO1 polymorphisms and increased risk of ALL in children.

Methods: C609T and C465T polymorphisms of NQO1 were explored using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) assay in 100 pediatric ALL patients and 135 healthy controls.

Results: Although C609T polymorphism is very common among the general population, we found no association between this variant and increased risk for pediatric ALL [odds ratio (OR) = 0.95; 95% confidence interval (95% CI) = 0.55–1.64]. Interestingly the other polymorphic allele of NQO1 (C465T) was strongly associated with pediatric ALL (OR = 7.83; 95% CI = 3.27-18.75).

Conclusion: These findings do not support the predisposing role of NQO1 C609T polymorphism for pediatric ALL. However, The C465T polymorphism was associated with increased risk of pediatric ALL. Further studies with larger sample including evaluating multiple gene-gene interactions seem necessary to validate the exact role of these mutations.

Key Words: *Acute lymphoblastic leukemia – polymorphism – NQO1 – RFLP.*

INTRODUCTION

As a heterogeneous disease that disrupts normal hematopoiesis in acute or chronic form, leukemia accounts for one-third of all cancer cases among patients under the age of 15 years and constitutes the most common type of pediatric cancer **[1]**. Among patients younger than 15 years of age with childhood leukemia, acute lymphoblastic leukemia (ALL) comprises 80% of cases; acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) account for 18% and 2% of cases, respectively. Chronic lymphocytic leukemia (CLL) rarely occurs in children **[2]**. Various studies **[3-5]** have demonstrated polymorphisms that contribute to detoxification of carcinogens are related to the development of various types of cancer. NAD (P) H: Quinone Oxidoreductase 1 (NQO1), (OMIM: 125860), protects cells against oxidative stress and toxic quinines **[6]**. Moreover, it has been shown **[7,8]** that this protein interacts with and stabilizes the tumor suppressor protein p53. Asher et al., **[8]** suggested that exposure to carcinogenic substrates of NQO1 could lead to increased genotoxic damage at lower p53 levels in individuals with lower NQO1 activity (compared with individuals with normal NQO1 activity). NQO1 is expressed in most tissues, including bone marrow, in which expression is thought to be highly inducible and up-regulated during the oxidative process **[9]**. The NQO1 can contribute to the formation of reactive oxidation species via oxidative cycling; therefore, it can act as a pro-oxidant **[10]**. A number of single nucleotide polymorphisms (SNPs) have been identified in NQO1 but only 2 of them, namely, NQO1*2 and NQO1*3, have been implicated as signifying risk of a variety of cancers **[11]**. The NQO1*2 polymorphism (C609T) causes a change in the amino-acid sequence (Pro187Ser)

and a low activity variant enzyme **[12]**. The prevalence of the NQO1 C609T polymorphism is 4.4% in non-Hispanic whites, 5.2% in African Americans, 12.2% in ethnic Japanese, 15.5% in ethnic Mexican-Hispanics, 17.9% in Native Americans, 18.8% in ethnic Koreans, and 22.4% in ethnic Chinese **[9,13,14]**. Another polymorphic variant of NQO1, namely, NQO1*3 (ie, C465T that results in Arg139Trp), causes alternative messenger RNA splice sites that can lead to deletion of exon 4 and the creation of a protein that lacks the quinone binding site **[15,16]**. Heterozygotes for the variant alleles of NQO1 (ie, the C/T genotypes of NQO1 C609T and C465T) display intermediate enzymatic activity, whereas homozygotic alleles (ie, T/T genotypes) display essentially no NQO1 activity **[17]**. The frequency of the NQO1 C465T polymorphism is generally low and ranges from 0% to 5% among different ethnic populations **[18]**. Several reports **[19-21]** about the role of NQO1 in childhood ALL were summarized in a recent meta-analysis **[22]**. Despite the results of these studies, it has been shown **[23,24]** that NQO1 C609T is associated with an elevated risk of nonhematologic malignancies such as urologic and basal cell carcinomas. Association of the NQO1 C465T polymorphism, the other variant form of NQO1, with ALL has been reported in 2 studies **[20,25]**. Herein, we assessed common polymorphisms of NQO1 (C609T and C465T) in relation to pediatric ALL in a case control study. The basic questions in our study were whether these polymorphisms play an important role in susceptibility to ALL and if these SNPs in our population have a high degree of heterozygosity.

MATERIAL AND METHODS

Patient and Control Samples We performed a case-control study with 100 patients with ALL [male/female: 0.72, mean age 6.5 (±5.0) years] and 135 healthy age- and sex-matched individuals without leukemia as the control group [male/female: 0.84, mean age 6.0 (±6.0) years]. Patient samples were diagnosed as ALL through morphological and immunophenotypic assessments and were randomly collected at the time of diagnosis from October, 2010 through November, 2013, at Dammam University of Medical Sciences in Saudi Arabia. Immunophenotypic subtypes of patients with ALL according to French-American-British (FAB) classification were as follows: 41 patients with pre-B ALL; 37 patients with early pre-B ALL; 15 patients with T ALL, and 7 patients with pro-B ALL. The Medical Ethics Committee of the Dammam University of Medical Sciences (DUMS) approved the study; written informed consent was obtained from all patients and healthy control individuals who participated in this study.

Genotyping analysis:

Blood and bone marrow samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA); subsequently, mononuclear cells were purified by Ficoll-Hypaque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) centrifugation, then their DNA was extracted through the standard method **[26]**. Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as described by Eguchi-Ishimae et al., **[25]** Twenty nmol of each primer for NQO1-C609T, forward primer: 5'-CCTCTC TGTGCTTTCTGTATCC-3' and reverse primer: 5'-GATGGACTTGCCCAAGTGATG-3'; for NQO1-C465T, forward primer: 5'-CTAGCTTT ACTCGGACCCACTC-3' and reverse primer: 5'-GCAACAAGAGGGAAGCTCCATC-3' were mixed with 60ng of DNA, 50mM KCl, 10mM tris (hydroxymethyl) aminomethane-hydrogen chloride (Tris-HCl) (pH, 8.3), 2.5 pmol of each deoxynucleoside triphosphate (dNTP), and 1.25 units of Taq polymerase (GeNet Bio, Nonsan, South Korea) in a total volume of 25μL. These samples were subjected to PCR using a TC-512 Techne Thermal Cycler (Bibby Scientific Limited, Staffordshire, England) with initial denaturation at 95ºC for 5 minutes, followed by 35 cycles (94ºC for 1 minute, 60ºC for 45 seconds, and 72ºC for 1 minute), and finally, an extension phase at 72°C for 10 minutes. In the next step, digestion of the PCR products for the NQO1 C609T polymorphism using HinfI (Thermo Fisher Scientific Inc, Waltham, MA) produced 2 bands for homozygous wildtypes (CC; 85 and 214 bp), 4 bands for heterozygotes (CT; 63, 85, 151, and 214 bp), and 3 bands for homozygous mutants (TT; 63, 85, and 151 bp). The PCR products were also digested by HpaII (Thermo Fisher Scientific Inc) to be assessed for NQO1 C465T polymorphism, which generated 2 bands for homozygous wild types (CC; 111and 353 bp), 3 bands for heterozygotes (CT; 111, 353, and 464 bp), and 1 band for homozygous mutants (TT; 464 bp).

Statistical analysis:

Statistical analysis for a different genotype distribution in case individuals versus controls was performed via the C2-test. The same analysis was also used to calculate the significance of differences in allele frequencies between the control and patient groups. Odds ratios (ORs) with 95% confidence intervals (CIs) were estimated by logistic regression. All statistical analyses were performed with SPSS software, version 16.0 (SPSS, Inc, Chicago, IL).

Table (1): Distribution of the NQO1 C609T & NQO1C 465T Genotypes Among ALL Patients and Controls

	CI (95%)	OR	Controls	Patients	Variants
NQO1 C609T	–	1.00*	89 (65.92%)	67 (67.0%)	CC
	(0.56-1.8)	1.01	38 (28.14%)	29 (29.0%)	CT
	(0.19-2.29)	0.66	8 (5.92 %)	4 (4.0%)	TT
	(0.55-1.64)	0.95	46 (34.07%)	33 (33.0%)	CT/TT
Allele-wise comparison (%)	–	1.00	108 (80.00%)	81 (81.0%)	C
	(0.48-1.82)	0.93	27 (20.00%)	19 (19.0%)	T
NQO1 C465T	–	1.00*	128 (94.82%)	70 (70.0%)	CC
	(3.06-23.09)	8.41	5 (3.70%)	23 (23.0%)	CT
	(1.29-31.64)	6.40	2 (1.48%)	7 (7.0%)	TT
	(3.27-18.75)	7.83	7 (5.18%)	30 (30.0%)	CT/TT
Allele-wise comparison (%)	–	1.00	130 (96.30%)	81 (81.0%)	C
	(2.19-16.97)	6.09	5 (3.70%)	19 (19.0%)	T

OR: Odds ratio. CC: Wild type. CI : Confidence interval. CT: Heterozygous. TT: Homozygous mutant.

Table (2): Combined Effects of NQO1 C609T and NQO1 C465T Genotypes on ALL Risk.

CI (95%)	OR	Controls	Patients	Combination
–	1.00*	86 (63.70%)	46 (46.0%)	CC609/ CC465
3.51-44.16	12.46	3 (2.22%)	20 (20.0%)	CC609/ CT465
0.33-42.34	3.73	1 (0.74%)	2 (2.0%)	CC609/ TT465
0.58-2.14	1.12	35 (25.92%)	21 (21.0%)	CT609/ CC465
1.52-36.68	7.47	2 (1.5%)	8 (8.0%)	CT609/ CT465
–	0.00	1 (0.74%)	0 (0.0%)	CT609/ TT465
0.12-3.21	0.62	6 (4.44%)	2 (2.0%)	TT609/ CC465
0.11-30.58	1.87	1 (0.74%)	1 (1.0%)	TT609/ CT465
–	0.00	0 (0.0%)	0 (0.0%)	TT609/ TT465

CI (95%) = Confidence interval. OR = Odds ratio.

RESULTS

All 235 DNA samples (from patients and controls) were successfully genotyped using the PCR-RFLP technique. NQO1 variants genotyping among 135 healthy controls indicated mutant allele frequencies of 20.0% and 3.7% for NQO1 C609T and C465T, respectively, which are comparable with the percentage of NQO1 variants in the control group of a previous study by some of us27 about NQO1 polymorphisms in adult AML patients (21.25% and 2.5% for C609T and C465T variants, respectively). The association between NQO1 variants and ALL risk were assessed by logistic regression. The NQO1 C609T genotypes for the 135 controls were distributed as follows: 65.92% wild type (CC), 28.14% heterozygous (CT) and 5.92% homozygous mutants (TT; the frequencies for CC, CT, and TT genotypes among the 100 patients with ALL were 67.0%, 29.0% and 4.0%, respectively. Regarding NQO1 C465T, genotype distribution among 135 controls was as follows: 94.8% wild type (CC), 3.7% heterozygous (CT), and 1.5% homozygous mutants

(TT), whereas CC, CT, and TT genotype frequencies in the patient group were 70.0%, 23.0% and 7.0%, respectively. The mutant allele frequency between patients was 19.0% for NQO1 C609T and C465T (Table 1).

In addition to evaluation of independent associations between NQO1 C609T and C465T polymorphisms and ALL, we assessed the joint effects of these 2 polymorphisms. Table (2) shows that CC609/CT465 and CT609/CT465 genotypes have significant correlation with pediatric ALL.

DISCUSSION

NQO1 may play a crucial role in protecting cells against cancer. For instance, it seems to not only stabilize the p53 protein 28 but also to contribute to anticancer signaling pathways that are activated by tumor necrosis factor and other inflammatory stimuli **[29]**. In this study, the distribution of alleles and genotype frequencies of NQO1 variants were compared with ALL and controls to find a possible association between these variants and elevated risk of developing ALL. We found no statistically significant association between the NQO1 C609T polymorphism and risk of childhood ALL (CT/TT versus CC; OR, 0.95; 95% CI, 0.55-1.64), whereas the mutant genotypes of NQO1 C465T showed a significant association with risk of ALL (CT/TT versus CC; 7.83; 3.27-18.75).

Chi-square analysis also showed a significant difference in the allele frequencies between the control and patient groups for the C465T polymorphism only (f2 =14.65, df = 1, p<0.001 for the C465T variant; f2 = 0.036, df = 1, p=0.85 for the C609T variant).

Regarding NQO1 C609T, our results contrast with those of previous studies [**20,30,31]** in other populations. Our results indicate that the NQO1 C609T polymorphism is associated with the elevated risk of childhood ALL.

Recently, a family-based study32 suggested that the NQO1 C609T variant was associated with the risk of developing childhood ALL; another study **[33]** performed in 2004 in Turkey did not support the role of the NQO1 C609T polymorphism in the increased risk of pediatric acute leukemia.

In Brazilian children, the NQO1 and myeloperoxidase (MPO) polymorphisms were shown to have a protective function against leukemogenesis **[34]**.

A French-Canadian study **[35]** showed that children carrying at least 1 mutant allele of the NQO1 C609T polymorphism had an increased risk of developing ALL, whereas individuals with wild-type homozygotes seem to be protected against ALL.

Lack of agreement between these studies might be due to differences in the duration of the exposure to the NQO1 substrates and small sample sizes, as well as the demographic stratification that exists in these kinds of studies.

In a HUGE net literature review and meta-analysis **[36]**, it was shown that the NQO1 C609T variant appeared to have no strong association with childhood ALL or AML but may be associated with mixed lineage leukemia–positive childhood leukemia.

In our study, it is noteworthy that the NQO1 C609T variant did not show any effect on ALL in univariant analysis; however, in multivariant analysis, the heterozygous genotype (CT) of NQO1 C609T in combination with the heterozygous genotype (CT) of NQO1 C465T showed an increased risk for ALL (OR, 7.47; 95% CI, 1.52-36.68), in which the main effect might have been created by the CT465 allele. Also, the CC609/CT465 combined genotype was significantly associated with risk of ALL; again, this supports the results of our univariant analysis.

The effect of these polymorphisms in NQO1 may be modified by polymorphisms in other carcinogen metabolizing genes, such as glutathione S-transferase (GST), cytochrome P450 2E1 (CYP2E1), and MPO. Therefore, it is important to study the effects of gene-gene and gene-environment interactions in the development of childhood ALL. In a study in Japan,13 variant alleles of NQO1 C465T also showed a striking positive association with infant ALL, especially in individuals with the chromosomal translocation of t(4;11)(q21;q23).

Other studies support the concept that the etiology of ALL in children is related to genetic variability at more than one gene locus and may

be related to the equilibrium between the metabolic activation and detoxification processes **[37]**. Therefore, it is crucial to study other polymorphisms that could possibly affect the susceptibility to ALL, such as MPO and CYP1E2.

Nevertheless, to yield more findings about the role of NQO1 polymorphisms in the etiology of childhood leukemia, further research, particularly using a larger sample size and sound design, seems to be necessary.

In summary, our study suggests that the mutant allele of the NQO1 C609T polymorphism is not associated with increased risk of pediatric ALL, whereas the NQO1 C465T variant showed a significant association with increased risk of ALL in children. Previous findings **[36]** have suggested that the etiology of ALL cannot be explained by polymorphism at a single locus, perhaps because of complexity in the metabolism of diverse xenobiotic compounds. Therefore, multiple gene-gene interactions should be investigated to enable prediction of the risk of ALL.

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