

Association between mitochondrial DNA 4,977 bp deletion and NAD(P)H:quinone oxidoreductase 1 C609T polymorphism in human breast tissues

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Abstract. The NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme is implicated in protection against oxidative stress and carcinogenesis. NQO1 C609T genetic polymorphism was reported to be associated with an increased risk for cancers, including breast cancer. However, there is still lack of evidence whether higher oxidative stress occurs in breast tissues of patients with NQO1 609 C/T and/or T/T genotypes. Mitochondrial DNA (mtDNA) 4,977-bp deletion, the most common mutation of mtDNA, was frequently detected in post-mitotic tissues of aged subjects and associated with oxidative damage. In this study, we detected the mtDNA 4,977-bp deletion in 60 breast cancers and corresponding non-cancerous breast tissues. The incidence of the common 4,977-bp deletion in non-cancerous breast tissues (48.3%) was higher than that in breast cancer (5.0%). Moreover, 63.4% of the breast cancer patients with NQO1 C/T or T/T genotypes had the deletion in their non-cancerous breast tissues. The mtDNA deletion was more frequently detected in breast tissue of NQO1 C/T carriers (65.2%) and T/T carriers (61.1%) as compared with the NQO1 C/C

carriers (15.8%, $P=0.003$). Similar results were observed in the <50 years old (y/o) group ($P=0.06$) and the ≥ 50 y/o group ($P=0.005$). Our findings suggest that mtDNA 4,977-bp deletion associated with NQO1 deficiency is involved in carcinogenesis and progression of breast cancer.

Introduction

NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, EC 1.6.99.2) is a cytosolic flavoenzyme that catalyzes two-electron reduction of a broad range of substrates including quinones, quinone-imines and nitro-compounds (1). The enzyme is generally considered as a detoxification enzyme because of its ability to reduce reactive quinone substrates to less reactive and less toxic hydroquinone derivatives. The two-electron reduction of quinones bypasses semiquinone production and thus prevents the generation of reactive oxygen species (ROS) (2). In addition, NQO1 activity was shown to maintain the reduced and active states of endogenous quinines, such as α -tocopherol quinines and coenzyme Q derivatives, thereby promoting their antioxidant function in membrane (3). Thus, NQO1 appears to protect against oxidative stress and carcinogenesis.

The NQO1 C609T genetic polymorphism has been found to exchange the Proline (P) at position 187 by Serine (S) and results in reduced levels of the NQO1 P187S protein by rapid turnover via the ubiquitin proteasomal pathway (2,4). Cells with the homozygous NQO1 609 T/T genotype have no measurable NQO1 enzyme activity. Several studies suggest an increased risk for cancers in the null genotype carriers, including breast cancers (5,6). Recently, the NQO1 genotype was suggested as a prognostic and predictive marker for breast cancer (7). However, it is still unclear whether higher oxidative stress occurs in breast tissues of patients with the NQO1 609 C/T or T/T genotypes.

Human mitochondrial DNA (mtDNA) is a 16,569-bp circular double-stranded DNA molecule, which contains genes coding for 13 polypeptides involved in respiration and oxidative phosphorylation, 2 rRNAs and a set of 22 tRNAs that are essential for protein synthesis in mitochondria (8).

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Abbreviations: mtDNA, mitochondrial DNA; NQO1, NAD(P)H:quinone oxidoreductase 1; n.p., nucleotide position; ROS, reactive oxygen species; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism

Key words: NAD(P)H:quinone oxidoreductase 1, oxidative stress, mitochondrial DNA deletion, breast cancer

The mitochondrial genome is particularly susceptible to oxidative damage and mutation by ROS and mutagens because of the high levels of ROS and free radical generation and inefficient DNA repair system in the organelle (9-11).

MtDNA 4,977-bp deletion is one of the most frequently observed mtDNA mutations and has a 13 bp direct repeat flanking the 5'- and 3'-end breakpoints at nucleotide position (np) 8470/8482 and np 13447/13459, respectively (12). It has been well established that mtDNA with the 4,977-bp deletion is accumulated with age in post-mitotic tissues of the human (13) and correlates with the oxidative modification (8-OHdG) content of mtDNA (14). In addition, it is increased by environmental insults, such as UV irradiation (15-17), cigarette smoking (18,19) and betel quid chewing (20). Moreover, the mtDNA 4,977-bp deletion is also frequently detected in various types of cancer tissues (20-24). Due to its good correlation with oxidative damage levels in somatic tissues, we hypothesized that the mtDNA deletion would be more frequently detected in breast tissues of the patients with NQO1 609 C/T or T/T genotype than those with C/C genotype.

In this study, we examined the occurrence of the mtDNA 4,977-bp deletion in 60 breast cancers and corresponding non-cancerous breast tissues. The relationship between the mtDNA deletion and the NQO1 genetic polymorphism was analyzed.

Materials and methods

Collection of human breast tissues and DNA extraction. Sixty breast cancers and the adjacent non-cancer breast tissues were obtained from patients with their consent at Taipei Veterans General Hospital. In the 60 breast cancer patients, 32 cases were <50 years old (the early age group) and 28 patients were ≥50 years old (the older age group). The mtDNA alterations in the same set of samples have been reported previously (23). All the tissues were kept in liquid nitrogen immediately after surgical resection according to a protocol approved by the medical Ethics Committee for conducting human research at the hospital. Total DNA of the tissues was extracted by the QIAamp DNA mini kit (Qiagen) according to the instructions of the manufacturer. The final DNA pellet was dissolved in doubly distilled water and stored at -30°C until use.

Analysis of NQO1 C609T polymorphism. The NQO1 C609T genetic polymorphism was analyzed according to the published polymerase chain reaction (PCR)-based restriction fragment length polymorphism (PCR-RFLP) method (25). The primers for the PCR reaction were NQO1 F: 5'-AGT GGC ATT CTG CAT TTC TGT G-3' and NQO1 R: 5'-GAT GGA CTT GCC CAA GTG ATG-3'. PCR was performed in an ABI GeneAmp PCR system 9700 DNA thermal cycler. The reactions were carried out for 35 cycles in a 50 µl reaction mixture containing 200 ng DNA, 200 µM of each dNTP, 40 pmol of each primer, 1.0 U of Taq DNA polymerase and 1X reaction buffer. The PCR cycles consisted of 15-sec denaturation at 94°C, 15-sec annealing at 58°C and 40-sec primer extension at 72°C. Each PCR product was subjected to *Hinf*I digestion. The C-to-T substitution at nucleotide 609 creates a *Hinf*I restriction site.

The PCR product (273 bp) with C allele was digested to two fragments (188 and 85 bp), whereas the PCR product with T allele was digested to three fragments (151, 85 and 37 bp). The DNA fragments were separated by electrophoresis on a 3% agarose gel at 100 volts for 40 min and detected under UV trans-illumination after ethidium bromide staining.

Detection of the mtDNA 4,977-bp deletion. The 4,977-bp deletion of mtDNA was detected using the primers L8150 (5'-CCG GGG GTA TAC TAC GGT CA-3') and H13650 (5'-GGG GAA GCG AGG TTG ACC TG-3') (22). PCR was performed in an ABI GeneAmp PCR System 9700 DNA thermal cycler. The reactions were carried out for 35 cycles in a 50 µl reaction mixture containing 200 ng DNA, 200 µM of each dNTP, 40 pmol of each primer, 1.0 U of Taq DNA polymerase and 1X reaction buffer. The PCR cycles consisted of 15-sec denaturation at 94°C, 15-sec annealing at 58°C and 40-sec primer extension at 72°C. The PCR products were separated by electrophoresis on a 1.5% agarose gel at 100 volts for 40 min and detected under UV trans-illumination after ethidium bromide staining.

DNA sequencing of the mtDNA 4,977-bp deletion. The PCR products with the primers L8150 and H13650 were sequenced with an ABI PRISM 3100 DNA analyzer (Applied Biosystems) using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis. Relationships between the mtDNA 4,977-bp deletion and NQO1 C609T polymorphisms were analyzed using the Statistical Program for Social Sciences (SPSS) program package. Fisher's exact test was used to compare the age and NQO1 genotypes. All statistical tests were two sided. The difference between groups was considered statistically significant at $P < 0.05$.

Results

We adopted the PCR-RFLP method to characterize the NQO1 C609T polymorphisms in the 60 breast cancer patients (Fig. 1). The NQO1 genotypes of these patients are summarized in Table I. The genotype frequencies of NQO1 C609T polymorphism in the 60 patients were C/C 31.7% (n=19), C/T 38.3% (n=23) and T/T 30.0% (n=18). The relationship between NQO1 609 genotypes and the clinicopathological parameters are summarized in Table II. No significant association was observed.

Moreover, mtDNA 4,977-bp deletion was detected in 29 (48.3%) non-tumor breast tissues of the 60 patients with breast cancer (Table I and Fig. 1). Only three (5%) of the breast cancer samples was found to carry the mtDNA deletion. The occurrence of the mtDNA deletion in non-tumor breast tissues was not correlated with the patient age ($P=0.448$), 5-year disease-free survival ($P=0.480$) or the 5-year overall survival ($P=0.709$). We further analyzed the DNA sequences and confirmed the 13 bp direct repeat flanking the 5'- and 3'-end breakpoints of the mtDNA deletion in three cases with NQO1 609 C/C, C/T and T/T genotypes, respectively (Fig. 2).

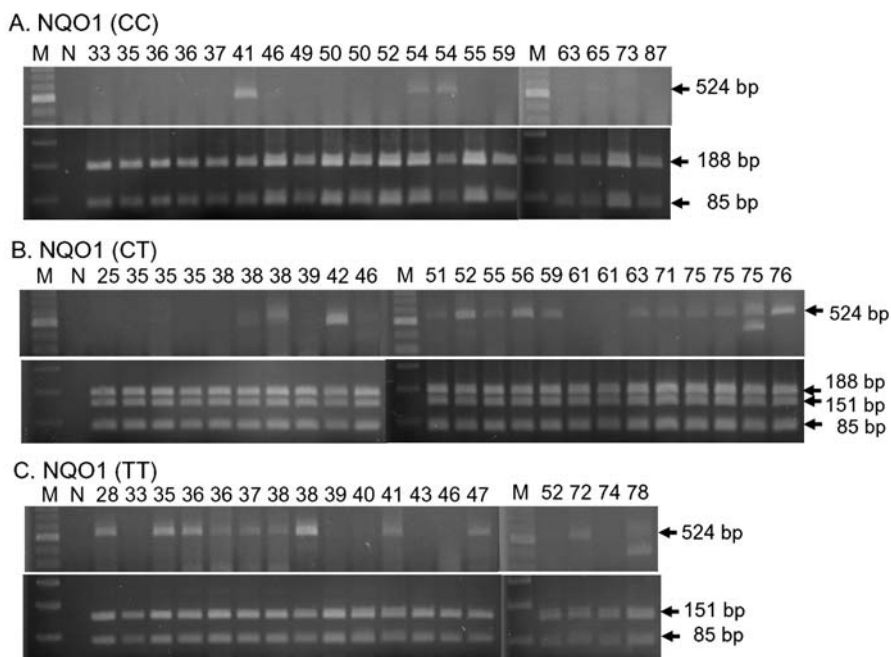


Figure 1. Mitochondrial DNA 4,977-bp deletion in patients with NQO1 C609T polymorphisms and different ages. The 4,977-bp deletion of mtDNA was detected by the PCR and the NQO1 C609T polymorphism was analyzed by the PCR-RFLP described in Materials and methods. The number at the top of each gel indicates the patient's age (years). M, DNA 100 bp ladder and N, negative control.

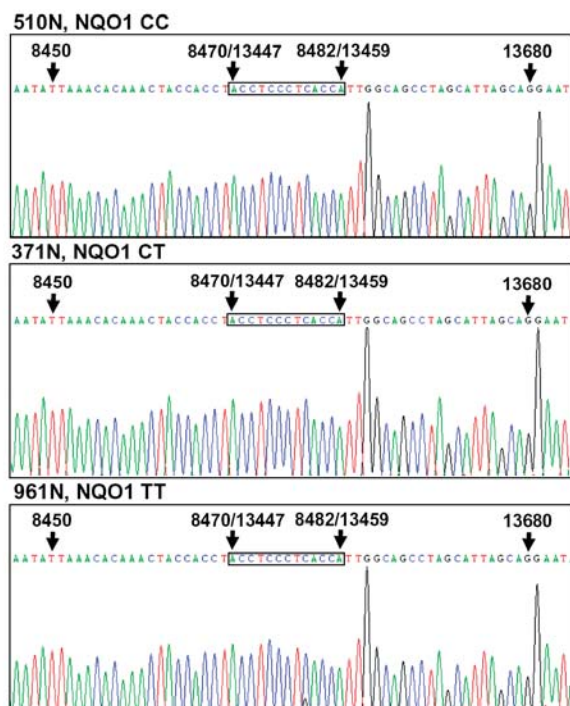


Figure 2. Mitochondrial DNA 4,977-bp deletion in breast tissues. The PCR products amplified from mtDNA with deletion in breast tissues of three patients (510, 371 and 961) is sequenced as described in Materials and methods. It was confirmed that these PCR products contain one of the 13 bp direct repeat flanking the 5'- and 3'-end breakpoints at nucleotide position (np) 8470/8482 and np 13447/13459, respectively.

Importantly, we found that 61.1% (11/18) of NQO1 T/T carriers and 65.2% (15/23) of NQO1 C/T carriers, but only 15.8% (3/19) of NQO1 C/C carriers had the mtDNA deletion

in their non-cancerous breast tissues (Table III). The mtDNA 4,977-bp deletion was more frequently detected in the breast tissues of the cancer patients with NQO1 C/T and T/T genotypes as compared with the patients with NQO1 C/C genotype ($P=0.003$). Similar results were observed in the <50 y/o group ($P=0.06$) and the ≥ 50 y/o group ($P=0.005$). Although no significant difference in the occurrence of the mtDNA deletion was observed between the two age groups ($P=0.437$), the deletion was more frequently detected in the ≥ 50 y/o group as compared with the <50 y/o group in the NQO1 C/T genotype (Fig. 2).

Discussion

The NQO1 enzyme is suggested to play a role in protection against oxidative stress and carcinogenesis. In this study, we demonstrated for the first time that NQO1 609 C/T and T/T genotypes are associated with increased frequencies of mtDNA 4,977-bp deletion in breast tissues, implicating increased oxidative stress associated with NQO1 deficiency in carcinogenesis of breast cancer.

The common mtDNA 4,977-bp deletion has been shown to accumulate with age, primarily in postmitotic tissues (13) and cancers (20-24). The different accumulation rate could result from genetic factors (such as tissue type), energy demand, intracellular oxidative stress levels, environmental insults, or cancer progression. Recently, hepatitis B virus-infected liver was observed to be correlated with the mtDNA deletion in the patients with hepatocellular carcinoma (26), suggesting increased oxidative stress may contribute to accumulation of the deleted mtDNA in the non-cancerous tissue. In the present study, we found that the incidence of the 4,977-bp deletion in the non-cancerous breast cancer is significantly higher in the patients with NQO1 609 C/T and T/T genotypes as compared

Table I. Summary of mtDNA 4,977-bp deletion and NQO1 609 genotypes in 60 patients with primary breast cancers.

Patient code	Age (y/o)	NQO1 609 genotype	4,977-bp deletion	
			N	Tu
257	37	CC	-	-
314	78	TT	+	+
324	73	CC	-	-
340	72	TT	+	-
349	51	CT	+	-
350	35	CT	-	-
371	76	CT	+	-
375	63	CT	+	-
377	46	CT	-	-
381	49	CC	-	-
383	55	CT	+	-
385	46	TT	-	-
386	59	CC	-	-
387	35	CC	-	-
416	25	CT	-	-
420	50	CC	-	-
425	87	CC	-	-
430	59	CT	+	-
432	28	TT	+	-
438	54	CC	+	-
440	38	CT	+	-
446	37	TT	+	-
447	54	CC	+	-
451	38	CT	+	-
464	41	TT	+	-
489	74	TT	-	-
498	61	CT	-	-
501	40	TT	-	-
510	41	CC	+	-
511	42	CT	+	-
529	39	CT	-	-
546	35	TT	+	-
621	35	CT	+	-
651	75	CT	+	-
692	71	CT	+	-
717	35	CT	-	-
732	65	CC	-	-
739	52	CC	-	-
760	46	CC	-	-
765	61	CT	-	-
776	52	TT	-	-
777	56	CT	+	+
779	63	CC	-	-
780	36	CC	-	-
781	43	TT	-	-
797	36	TT	+	-
801	52	CT	+	+
806	50	CC	-	-
807	47	TT	+	-
871	55	CC	-	-
900	38	TT	+	-
954	39	TT	-	-
955	75	CT	+	-

Table I. Continued.

Patient code	Age (y/o)	NQO1 609 genotype	4,977-bp deletion	
			N	Tu
958	75	CT	+	-
961	38	TT	+	-
1099	36	CC	-	-
1113	28	TT	-	-
1133	33	CC	-	-
1154	36	TT	+	-
1215	38	CT	-	-

Tu, tumor portion; N, non-tumorous portion and y/o, years old.

with the NQO1 609 C/C genotype cases. Because the NQO1 609 T/T genotype leads to a lack of NQO1 enzyme activity, our findings suggest that increased oxidative stress resulted from a lack of ROS scavenging function and/or of the capacity of maintaining antioxidant function of NQO1 thus possibly contributing to the increased incidence of the common mtDNA deletion.

We detected the common 4,977-bp deletion of mtDNA in only three breast cancers but in 47% of the non-cancerous breast tissues. The finding of lower incidence of the deletion in tumor than that in noncancerous tissue is consistent with those for different cancers (20-24) and with the evidence from a previous study in 17 breast cancer patients by microdissection of the tumor tissue and *in situ* PCR (27). The common mtDNA deletion causes a loss of 5 tRNA genes and 7 genes encoding subunits of cytochrome oxidase, complex I and ATPase, and may have a strong functional disadvantage, possibly thereby repressing the growth of cancer cells harboring the deleted mtDNA. The decrease in the incidence of the common deletion in tumor indicates that there may be a dilution effect due to rapid cytoplasmic division or an active selection mechanism by which cancer cells harboring the deleted mtDNA would be eliminated because of apoptosis.

Estrogen has been proposed to play a critical role in breast cancer initiation. According to the epidemiologic studies, prolonged exposure to estrogen, including reduced fertility rate, earlier menarche, and prolonged reproductive stimulation in the lifetimes, is significantly associated with increased risk of female breast cancer in Taiwan and in Western countries (28,29). The oxidized metabolites of estrogen, E2-3,4-semiquinones and E2-3,4-quinones, have been shown to bind to DNA to form adducts, leading to genetic damage. In addition, the generation of ROS during the conversion of E2-3,4-semiquinones to E2-3,4-quinones can also lead to oxidative DNA damage. The NQO1 may have a critical role in protecting DNA from reactive catechol estrogens through a two-electron reduction of estrogen semiquinones and/or an increase in endogenous antioxidant function, such as α -tocopherol quinines and coenzyme Q derivatives. Because the NQO1 609 T/T genotype leads to a lack of NQO1 enzyme activity, it is possible that increased oxidative stress-associated estrogen exposure in the individuals with NQO1 609 T/T genotype



	n	NQO1 609			P-value
		C/C (n=19)	C/T (n=23)	T/T (n=18)	
Stage					
I	9	5	2	2	0.216
II	25	7	7	11	
III	21	5	12	4	
IV	5	2	2	1	
Tumor size (cm)					
<2.0	7	3	2	2	0.761
2.0-5.0	47	15	19	13	
≥5.0	6	1	2	3	
Lymph node status					
0	29	10	9	10	0.533
1-3	8	2	2	4	
4-9	12	3	7	2	
≥10	11	4	5	2	
Grade					
I	6	3	2	1	0.810
II	34	10	12	12	
III	19	6	8	5	
Lymphovascular invasion					
Negative	42	15	14	13	0.432
Positive	18	4	9	5	
ER					
Negative	24	8	11	5	0.418
Positive	36	11	12	13	
PR					
Negative	32	13	10	9	0.257
Positive	28	6	13	9	
Her-2/neu					
1, 2	46	13	17	16	0.313
3	14	6	6	2	
Disease-free survival	60	72.9%	73.4%	83.3%	0.722
Overall survival	60	78.9%	68.4%	71.9%	0.736

ER, estrogen receptor and PR, progesterone receptor.

Table III. Incidence of mtDNA 4,977-bp deletion in non-cancerous breast tissue of 60 breast cancer patients with NQO1 609 genotypes.

NQO1 609 genotype		<50 years old			≥50 years old			Total		
		4,977-bp deletion			4,977-bp deletion			4,977-bp deletion		
	n	-	+	P	-	+	P	-	+	P-value
C/C	19	7	1		9	2		16	3	
C/T	23	6	4		2	11		8	15	
T/T	18	5	9		2	2		7	11	
		18	14	0.06	13	15	0.005	31	29	0.003

-, negative; +, positive.

could lead to an increased burden of mtDNA mutation. Moreover, the prevalence of the C609T polymorphism reported here (68.3%) and reported previously in Chinese (66.0-69.5%) (5,30) is higher than that of Caucasians (26%) (30). The higher prevalence of the NQO1 genetic polymorphism could contribute to the higher incidence of breast cancer in younger age women in Taiwan than their counterparts in Western countries.

In conclusion, our study provided direct evidence that the occurrence of the mtDNA 4,977-bp deletion was higher in the breast tissues of NQO1 609 C/T and T/T carriers as compared with NQO1 609 C/C patients. Our findings suggest that increased oxidative stress is associated with the lack of antioxidant function of NQO1 and may be involved in tumorigenesis of breast cancer.

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