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Role of Detoxifing Enzyme Nicotinamide Adenine Dinucleotide (Phosphate) H: Quinone Oxidoreductase-1 C609T Gene Polymorphism in Bronchogenic Carcinoma

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Abstract- Lung cancer is currently one of the most common cancers and a major cause of cancer-related death in the world. Eighty-five percent of lung cancers are non-small cell lung cancers (NSCLCs), and 15% are small cell lung cancers (SCLCs). The most important risk factor for lung cancer is tobacco smoking. Polycyclic aromatic hydrocarbons (PAHs) are abundant in tobacco smoke and constitute a major etiological factor in lung cancer. NAD (P) H: quinone oxidore - ductase (NQO1) is a cytosolic flavoprotein that catalyzes the two-electron reduction of quinoid compounds into less toxic hydroquinones. A single base substitution (C→T) polym - orphism at 609 in the NQO1 gene reduces quinone redu - ctase activity. Published data on the association between NQO1609 C>T polymorphism and lung cancer risk are conflicting. In this study, we investigated NQO1genotype in relation to lung cancer risk. The cases were patients attending Chest diseases unit in the Alexandria Main University Hospital with bronchogenic carcinoma in different stages. The control group consisted of age-matched male adults from the same socioeconomic class. DNA extraction from EDTA blood samples and genotyping was successfully carried out for 100 cases and 100 controls by PCR-RFLP and PCR-CTPP. Pat - ients carrying at least one variant allele for the NQO1 609 SNP (CT/TT genotype) were found to have almost a 2.2 -fold increased lung cancer risk than those with CC genotype, 4.3-fold increased risk of developing SCLC and 3.8- fold increased risk for lung cancer with other histological type. Furthermore, the heavy smokers (>21 p-y) patients with one or two copies of the T variant allele had 3.6-fold increased lung cancer risk compared to those with CC genotype, while, the risk for squamous cell carcinoma was 2.4-fold and 17.9-fold for SCLC. These results suggest that individuals with reduced enzyme activity, due to NQO1 609 C>T polymorphism, may therefore have an increased risk of lung cancer.

I. Introduction

Lung cancer is currently one of the most common cancers and a major cause of cancer-related death in the world. Among males, the highest lung can - cer incidence rates are in Central and Eastern Europe 53.5 (per 100,000) and 50.4 in Eastern Asia. Among females, the highest lung cancer incidence rates are 33.8 in Northern America and 23.7 Northern Europe. [(1)]

Incidence data (ASR) for the Arab countries; for males, lung cancer incidence estimated as; 31.1 (per 100,000) in Tunisia followed by Lebanon, Libya, Jordan and in Egypt 11.21 (per 100,000). For females, estim - ated as; 11.0 in Lebanon followed by Bahrain, Syrian Ar -ab Rebuplic, United Arab Emirates, and in Egypt it reaches 3.76 (per 100,000).[(1)]

Eighty-five percent of lung cancers are non-small cell lung cancers (NSCLCs), and 15% are small cell lung cancers (SCLCs).[(2)]

The most important risk factor for lung cancer is tobacco smoking. International variations in lung cancer rates and trends largely reflect differences in the stage and degree of the tobacco epidemic because smoking accounts for about 80% of global lung cancer deaths in men and 50% of the deaths in women.[(3)]

Many of the compounds in tobacco smoke are oxidized by phase I enzymes into reactive metabolites, which are detoxified by phase II enzymes. Polycyclic aromatic hydrocarbons (PAH) are abundant in tobacco smoke and constitute a major etiological factor in lung cancer.[(4)]

NQO1 [NAD (P): H-(quinone acceptor) oxidore ductase; EC 1.6.99.2] [(5)] enzyme is a homodimeric flavin adenine dinucleotide (FAD) containing cytosolic protein catalyzing the two-electron reduction of quinone subs - trates.[(6)] Quinine compounds are mainly derived from endogenous quinones, such as vitamin E quinine, and exogenous quinones, such as exhaust gas, tobacoo smoke.[(7)]

NQO1 prevents the generation of free radicals and reactive oxygen, thus protecting the cells from oxidative damage. This pathway is thought to be the major mechanism responsible for modifying the toxicity of quinones, including those arising from the formation of DNA adducts induced by benzo(a)pyrene 3,6-quin - one, one of the most potent polycyclic aromatic hydro - carbons present in tobacco smoke.[(8)]

The NQO1gene is located on chromosome 16q22.1, spanning 17.2 kb and consisting of 6 exons

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and 5 introns.[9] The polymorphic variant is a C→T transition at nucleotide position 609 (amino acid codon 187) that results in a proline-to-serine amino acid subs - titution in the protein. The reference number of this SNP in the database of the National Center for Biotechnology Information (NCBI) is rs1800566.[10]

Three genotypes of NQO1 are known to be associated with different enzymatic activities: C/C is the homozygous wild-type with normal activity, C/T is heter - ozygous with reduced activity, and T/T is the homoz - ygous variant with only 2–4% of the enzyme activity of the wild-type.[11]

Several studies have examined the relationship between the NQO1 genetic polymorphism and lung cancer risk, but the conclusions have been inconsistent.[9,12,13]

The study of the role of SNPs and in particular the NQO1 609C>T polymorphism as a risk factor for the development of bronchogenic carcinoma among Egy - ptian male patients is of utmost importance for the unmasking of the risk factors underlying bronchogenic carcinoma in Egypt.

II. Patients and Methods

The current study was conducted as a hospital-based case-control study of 200 subjects; 100 male patients presenting to the Chest diseases unit in the Alexandria Main University Hospital with bronchogenic carcinoma in different stages of the disease between 2011 and 2013. Lung cancer cases were newly diagnosed cases aged above 40 years old with absence of previous precancerous conditions and any known primary cancerous lesion elsewhere. Diagnosis of primary lung cancer was confirmed through a review of each patient's pathology report by the Alexandria University Hospital pathology department. One hundred age-matched male adults from the same socioe - conomic class were recruited. All patients and controls provided informed consent.

a) DNA extraction and genotyping analysis

DNA was extracted from EDTA whole blood using DNA purification kit (QIAamp DNA blood Mini Kit, Qiagen, Hilden, Germany). The extraction was done according to the manufacturer's instructions using the Spin Protocol. The quantity and purity of DNA for each sample was assayed using Nanodrop 2000 spectrop - hotometer (Thermo scientific, USA). The final conce - ntration of target DNA was adjusted to 50-100 ng in the following amplification reaction to exclude variability in DNA concentration.

DNA amplification was conducted by polym - erase chain reaction with confronting two-pair primers (PCRCTPP) [14] , using four primers; F1: 5'-CCT TAT CAGAGT GTC TTA CTG AGA-3' (54.4°C) and R1: 5'-CAA TGCTAT ATG TCA GTT GAG G-3' (54.7°C), for C allele amplifying a 165-bp band, as well as F2: 5'-GTG GCT TCC AAG TCTTAG AAT-3' (54.9°C) and R2: 5'-TTT CTA GCT TTG ATC TGGTTG-5' (54.5°C) for T allele amplifying a 283-bp band. A common 406-bp band was designed to be amplified between primers F1 and R2.

The results of PCR-CTPP genotyping were confirmed by PCR-RFLP with HinF1 enzyme, which produces 188-bp and 85-bp bands for C allele and 151-bp and 85-bp bands for T allele using primers F: 5'-AGT GGCATT CTG CAT TTC TGT G-3', and R: 5'-GAT GGA CTT GCCCAA GTG ATG-3'. (14) Two hundred samples were successfully genotyped;

100 lung cancer patients (62 were CC, 31 were CT, and 7 were TT) and 100 healthy individuals as a control group (73 were CC, 26 were CT, and 1 was TT), The results of PCR-CTPP were consistent with those of the gold standard method PCR-RFLP.

b) Statistical analysis

All statistical analyses were performed using the SPSS20.0 program. An effect was considered statis - tically significant at $P<0.05$. X2 test was cond -ucted for examining the Hardy-Weinberg equilibrium and indepe - ndence of genotype frequency between cases and controls. Logistic regression analysis was then perfo - rmed to compute odds ratios and 95% confidence inte - rvals, after adjustment for age, family history and smoking index pack-years (number of years smo - ked×number of packs smoked per day).

III. Results

The study population was Egyptians, they consisted of 100 male lung cancer patients and 100 male of age- matched healthy controls. The mean age was 54.2 ± 8.2 years for cases and 52.7 ± 8.4 years for controls. There was a statistically significant difference between the cases and controls concerning the smo - king status and smoking index ($p<0.001$).Cases were more likely than controls to be current smokers. The mean Pack-Years were 43.9 ± 20.8 among cases and 19.5 ± 14.3 among controls. Twenty three (23%) of the patients had positive family history of cancer compared to 4% of the control group with a statistically significant difference ($p<0.001$).

In the group of lung cancer patients, the most prevalent histological subtype of lung cancer was Aden - ocarcinoma with a frequency of 37%, followed by 31% squamous cell carcinoma, 19% SCLC (small cell lung cancer) and 13% with other histological subtypes (7 cases were large cell anaplastic carcinoma, 4 cases were undifferentiated NSCLC and 2 cases were carci - noid tumor). As regards the tumour staging, 5 out of 100 patients were stage I, 27 patients were stage II, 32 patients were stage III and 36 patients were stage IV.

All included subjects were analyzed for the NQO1 609C>T (rs1800566) SNP genotype. The distrib - utions of the NQO1 genotypes among lung cancer patients and controls were in agreement with Hardy-

Weinberg equilibrium (p= 0.267, 0.425, respectively). The frequency of NQO1 CC, CT, TT genotypes was 62, 31 and 7%, respectively in patients and 73, 26 and 1% in controls. The relative frequencies of the wild (C) allele and the variant (T) allele of NQO1 609C>T (rs1800566) SNP were 77.5% and 22.5%, among the patients respe - ctively, and were 86% and 14% among the controls, respectively. There was a significant difference among the two groups as regards their allele frequencies (p =0.028).

Table 1 : Comparison between patients and controls according to genotypes and allele frequencies

	patients (n=100)		Controls (n=100)		χ^2	p
	No.	%	No.	%		
Genotype						
CC	62	62.0	73	73.0		
CT	31	31.0	26	26.0	5.835*	$^{MC}p = 0.047$*
TT	7	7.0	1	1.0		
Allele						
C	155	77.5	172	86.0	4.843*	0.028*
T	45	22.5	28	14.0		

*χ^2: value for Chi square *: Statistically significant at $p \leq 0.05$*

Patients with one or two copies of the T variant allele had 2.2 -fold increased lung cancer risk than those with CC genotype (adjusted OR=2.2; 95%CI: 0.63-7.9). To assess whether the NQO1 variant T allele may impart different risks for the various lung cancer cell types, we computed the adjusted ORs for lung cancer for each histological type. Patients with one or two copies of the T variant allele had 4.3- fold increased SCLC risk than those with CC genotype (adjusted OR=4.3; 95%CI: 0.57-33.4), while, they had 3.8- fold increased risk for lung cancer with other histological type (adjusted OR=3.8; 95%CI:0.49-30.7), 2.1-fold increased adeno - carcinoma risk (adjusted OR=2.1; 95%CI: 0.43-10.3), and 1.2-fold increased squamous cell carcinoma risk more than those with CC genotype (adjusted OR=1.2; 95%CI: 0.21-7.0).

Table 2 : Stratified analysis for NQO1 609C>T (rs1800566) SNP by histological type of the tumor among cases and controls

	Genotype						OR (95% CI)[a]	
	CC		CT		TT		Crude	Adjusted#
	No	%	No	%	No	%		
Controls	73	54.1%	26	45.6%	1	12.5%	1	1
All cases	62	45.9%	31	54.4%	7	87.5%	1.7 (0.91-3.0)	2.2 (0.63-7.9)
SCC	19	20.7%	8	23.5%	4	80.0%	1.7 (0.73-3.9)	1.2 (0.21-7.0)
SCLC	9	11.0%	10	27.8%	0	0.0%	3.0 (1.1-8.2)*	4.3 (0.57-33.4)
Adeno-carcinoma	25	25.5%	10	27.8%	2	66.7%	1.3 (0.57-2.9)	2.1 (0.43-10.3)
Others	9	11.0%	3	10.3%	1	50.0%	1.2 (0.34-4.2)	3.8 (0.49-30.7)

[a]Odds ratios are for CT and TT versus CC.
 # OR adjusted for age, family history, smoking index OR significant at 0.05*

The study subjects were stratified by age and smoking status. As regards study subject's age, elevated lung cancer risk associated with TT genotype was evident in younger individuals (age <60) (adjusted OR=13.6; 95%CI: 0.55-58.7). On the contrary, elevated lung cancer risk associated with CT genotype individ - ually and with one or two copies of the T variant allele (CT and TT combined) were evident in older individuals (age >60) ((adjusted OR=9.1; 95%CI: 0.90-91.7), (adjusted OR=10.6; 95%CI: 1.1-58.7), respectively). The only significant OR was for the combined CT and TT genotype group above 60 years.

When adjustment was made for smoking index pack-years (light smokers and heavy smokers), among light smokers (<21 p-y), the patients with one or two copies of the T variant allele had 1.9-fold increased lung

cancer risk compared to those with CC genotype (adjusted OR=1.9; 95%CI: 0.29-13.2), while, the heavy smoker (>21 p-y) patients with one or two copies of the T variant allele had 3.6-fold increased lung cancer risk compared to those with CC genotype (adjusted OR=3.6; 95%CI: 0.41-30.7). The patients with one or two copies of the T variant allele had 3.6-fold increased lung cancer risk compared to those with CC genotype (adjusted OR=3.6; 95%CI: 0.41-30.7), while, the risk for squamous cell carcinoma was 2.4-fold (adjusted OR=2.4; 95%CI: 0.21-27.9), 17.9-fold for SCLC (adju - sted OR=17.9; 95%CI: 1.5-40.6), 2.4-fold for adenoca - rcinoma (adjusted OR=2.4; 95%CI: 0.25-23.8), and 2.5-fold for lung cancer other histological type (adjusted OR=2.5; 95%CI: 0.18-34.4). The odds ratio of the combined SCLC heavy smokers group was statistically significant.

Table 3 : Comparison between NQO1 609C>T genotypes as regards smoking among patients

	Genotype						Test of sig.	p
	CC (n = 62)		CT (n = 31)		TT (n = 7)			
	No	%	No	%	No	%		
Smoking								
Non smoker	0	0.0	2	6.5	2	28.6		
Smoker	62	100.0	27	87.1	5	71.4	18.737*	^{MC}p = 0.001*
EX smoker	0	0.0	2	6.5	0	0.0		
p_1			0.012*		^{FE}p = 0.009*			
p_2			^{MC}p = 0.173					
Smoking intensity								
Min. – Max.	0.50 - 4.0		0.50 - 3.0		1.50 - 2.50		^{KW}	
Mean ± SD.	1.58 ± 0.63		1.39 ± 0.51		1.90 ± 0.42		5.436	0.066
Median	1.50		1.50		2.0			
$^{MW}p_1$			0.187		0.091			
$^{MW}p_2$			0.025*					
Duration of smoking (years)								
Min. – Max.	15.0 - 50.0		5.0 - 55.0		20.0 - 40.0			
Mean ± SD.	28.60 ± 9.99		27.93 ± 13.0		31.0 ± 7.42		^{KW} 0.367	0.832
Median	30.0		30.0		30.0			
$^{MW}p_1$			0.935		0.499			
$^{MW}p_2$			0.711					
Smoking Index (p/year)								
Min. – Max.	7.0 - 100.0		2.50 - 110.0		30.0 - 100.0			
Mean ± SD.	43.37 ± 17.28		41.91 ± 26.20		60.50 ± 25.27		^{KW} 2.873	0.238
Median	42.50		40.0		60.0			
$^{MW}p_1$			0.680		0.105			
$^{MW}p_2$			0.117					

p: p value for comparing between different genotype
p_1 : p value for comparing between CC with CT and TT
p_2 : p value for comparing between CT and TT
χ^2: value of Chi square
MC: Monte Carlo test *FE: Fisher Exact test*
$^{KW}\square^{\square}$: Kruskal Wallis test *MW: Mann Whitney test*
**: Statistically significant at $p \leq 0.05$*

Table 4 : Stratified analysis for NQO1 609C>T (rs1800566) SNP by smoking index among cases and controls

	Smoking index <21				OR (95% CI)		Smoking index >21				OR (95% CI)	
	CC		CT+TT		Crude	Adjusted #	CC		CT+TT		Crude	Adjusted #
	No	%	No	%			No	%	No	%		
Controls	13	76.5%	5	45.5%	1	1	8	12.1%	1	3.7%	1	1
All cases	4	23.5%	6	54.5%	3.9 (0.76-19.9)	1.9 (0.29-13.2)	58	87.9%	26	96.3%	3.6 (0.43-30.1)	3.6 (0.41-30.7)
SCC	3	18.8%	2	28.6%	1.7 (0.22-13.7)	1.2 (0.05-18.6)	16	66.7%	6	85.7%	3.0 (0.31-29.4)	2.4 (0.21-27.9)
SCLC	1	7.1%	0	0.0%	2.4 (0.25-14.7)	2.1 (0.21-25.9)	8	50.0%	9	90.0%	9.0 (1.0-88.5)*	17.9 (1.5-40.6)*
Adenocarcinoma	0	0.0%	2	28.6%	3.7 (0.16-10.6)	1.3 (0.07-16.8)	25	75.8%	9	90.0%	2.9 (0.32-26.4)	2.4 (0.25-23.8)
Others	0	0.0%	2	28.6%	3.7 (0.16-10.6)	1.4 (0.05-17.5)	9	52.9%	2	66.7%	1.8 (0.13-23.5)	2.5 (0.18-34.4)

 # OR adjusted for age and family history
** OR significant at 0.05*

Assessing the efficiency of CTPP-PCR considering allelic discrimination through genotyping analysis of NQO1 609C>T (rs1800566) SNP, comparing CTPP-PCR results with those of RFLP-PCR as a gold-standard method. The efficiency of CTPP-PCR was assessed in 200 samples; 100 lung cancer patients and 100 healthy individuals as a control group. There was no discrepancy between the CTPP-PCR and RFLP-PCR results in all our study subject samples. The previous performance reflects a sensitivity of 100% and specificity of 100%. The positive predictive value (PPV) and the negative predictive value (NPV) were 100%.

IV. Discussion

Cancer lung is one of the leading causes of cancer related mortalities worldwide. The rapidly evolving field of cancer genetics has opened up new possibilities for the discovery of susceptibility genes for numerous cancers including lung cancer.

Lung cancer has been the most common cancer worldwide since 1985, both in terms of incidence and mortality. The 5-year survival rate in the United States for lung cancer is 15.6 %. [15]

Cigarette smoking is the main risk factor for lung cancer, accounting for about 90% of the cases in men and 70% of the cases in women. [16] The pathogenesis of lung cancer had a genetic component, whether it relates to host susceptibility to lung cancer, with or without exposure to cigarette smoke to the development of certain types of lung cancer. [15]

NAD (P) H: quinone oxidoreductase 1 (NQO1) is a two-electron reductase, which reduces reactive quinones to less reactive and less toxic hydroquinones, resulting in protection of the cells. [13] The quinones are mainly derived from endogenous quinones, such as vitamin E quinine and exogenous quinones, such as tobacoo smoke. [17] The T/T (Ser/Ser) variable allele of NQO1 lacks enzymatic activity and fails to detoxify quinone metabolites into the reduced form. [13] It was thus hypothesized that individuals lacking NQO1activity would be at high risk of malignancies (lung cancer) because of the exposure to procarcinogens, that are included in cigarette smoke, which are oxidized to quinine metabolites. [6]

In this study, we analyzed the relationship between NQO1genetic polymorphisms and lung cancer, comparing lung cancer patients and healthy individuals in Egypt. Our results showed, the frequencies of the wild (C) allele and the variant (T) allele of NQO1 609C>T (rs1800566) SNP for the patients were 77.5% and 22.5%, respectively, and for the controls were 86% and 14%, respectively. The allele frequencies in both cases and controls were in Hardy–Weinberg equilibrium. The frequency of the variant allele in Egyptians was similar to the frequencies reported in another study on Arab population. [18]The NQO1 609C>T polymorphism exhibits ethnic variation (4–22%) with the highest prevalence of the T allele occurring in Asian populations and the lowest in Caucasians (4%), while, in Arabs TT genotype frequency was 6.4% (Middle Eastern Arab origin (95% Saudi Arabians and 5% from other Arab countries such as Jordan, Syria, Lebanon, Yemen) and data in this study are consistent with this frequency.[18, 19]

We found that, Egyptian Patients with one or two copies of the T variant allele had 2.2 -fold increased lung cancer risk than those with CC genotype. However, the patient with one or two copies of the T variant allele had a 4.3-fold increased risk for developing small cell lung cancer, 3.8-fold increased risk to lung cancer other than squamous cell carcinoma or adinocarcinoma and 2.1-fold increased risk of adenocarcinoma more than those with CC genotype.

Table 5 : Log-rank test comparing survival distributions among the three groups of the patients as regards NQO1 609C>T genotype

Overall Comparisons			
	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	5.010	2	.057
Test of equality of survival distributions for the different levels of Genotype.			

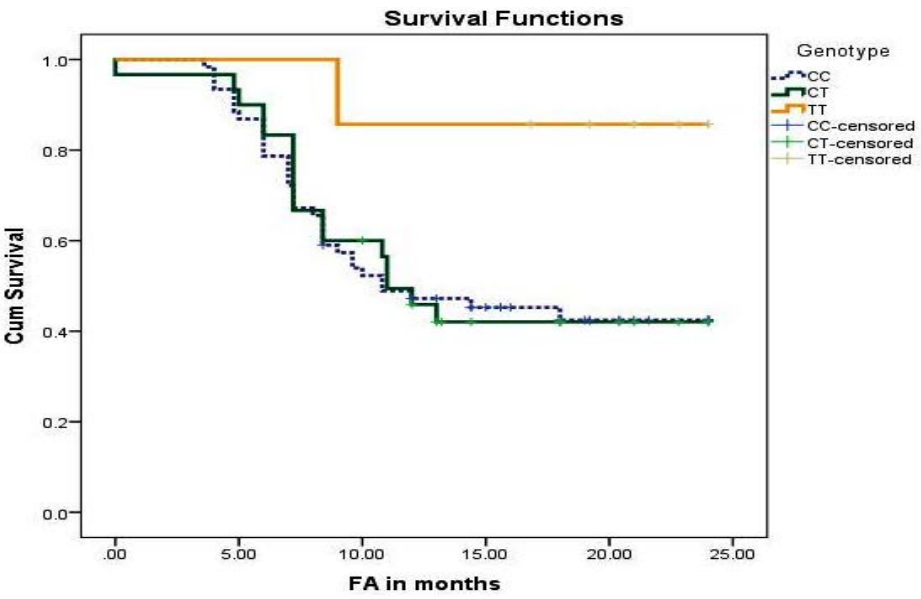

Figure I : Kaplan-Meier curve comparing estimated survival among the study lung cancer patients with CC, CT and TT genotype

Our results are supported by Lewis et al., [13] who reported the increased risk associated with genotypes containing at least one variant T allele seems to be restricted to SCLC only among Caucasians. In studies performed in Asians, the NQO1 CC genotype was found to be associated with lung cancer, particularly adenocarcinoma, [20] whereas the variant NQO1 variant T allele has been suggested to be a risk factor for lung cancer in Caucasians.[9,13,21] The only previous study on Caucasians with sufficient number of cases that performed an analysis of histologic subtypes was in agreement with our study that the variant NQO1 genotypes were overrepresented in squamous cell carcinoma. [21]

On the other side, in Asian population, the wild-type C allele was found with higher incidence among subjects with adenocarcinoma. [12, 20] Furthermore, the variant allele was found protective against adenoc - arcinoma in this population, while no such effect was observed in case of SCLC. [22]

To explore the connection between NQO1 609C>T (rs1800566) polymorphism and lung cancer risks, we stratified the data by age, smoking and family history among our study subjects. We observed a statistically significant 10.6-fold increased risk among older individuals (age >60) with even one variant T allele compared to 1.2-fold among younger individuals (age<60). However, the association between NQO1poly -morphism and lung cancer risk might differ depending on subject's age. Studies demonstrating the pro-carcinogenic effect of NQO1variant T allele in young Caucasians but rather protective effect in older ones can be found (age <50 years: OR = 1.28; age ≥50 years: OR = 0.46). [23]

In our study, the patients who were heavy smokers (>21 pack-years) and with one or two copies of the T variant allele had 17.9- fold increased risk for SCLC lung cancer than light smoker patients with 2.1-fold increased risk. However, the overall lung cancer risk among heavy smokers (>21 pack-years) 3.6-fold incre - ased risk compared to 1.9-fold among light smokers (<21 pack-years).

Xu et al. [21] found that both the C/T and T/T genotype produced a higher risk of lung cancer comp - ared with the wild-type genotype in those who smoked

more intensely over a shorter period of time in former smokers.

Polymerase chain reaction with confronting two-pair primers (CTPP-PCR) is an effective genotyping method for single nucleotide polymorphisms (SNPs) in aspects of reducing time and costs for analysis. In the present study, the study subject's genotypes of NQO1 by CTPP-PCR method were the same as those genotyped with a RFLP-PCR.

Triplex PCR-RFLP for CYP1A1, GSTM1and GSTT1 polymorphisms has been reported by Bailey et al., [24] compared with PCR-RFLP, PCR-CTPP has the advantage of low cost and rapidity, because it allows genotyping of SNPs without incubation with a restriction enzyme for PCR product digestion. Multiplex PCR-CTPP is applicable; there is no doubt that it is superior to multiplex PCR-RFLP. PCR-CTPP needs less material input and time than PCR-RFLP, even for single polymorphism genotyping. [25]

However, technical problems should be noted for PCR-CTPP. The strength of bands is dependent on the balance in melting temperature of each primer. The balance is also sensitive to annealing temperature of PCR. General speaking, a similar melting temperature for all primers provides the best chance to find an optimal primer set, so primers with a similar melting temperature have to be used. If a suitable primer set cannot be found, this method may not be applicable. This is a common problem to usual PCR with one pair of primers. (14) Kawase et al., (25) reported these conditi - ons after several unsuccessful combinations were tried.

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