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Research Article

CYP1B1 L432V Polymorphism and Lung Cancer Risk in the Iranian Population

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Background: Lung cancer is considered as one of the most frequent cancers worldwide, and has been the cause of more than one million mortalities each year. Exposure to tobacco smoke is the primary cause of most lung cancers, since it contains several thousand compounds, including more than 50 known carcinogens. However, a small fraction of individuals who are exposed to tobacco smoke develop lung cancer, therefore genetic factors may render some tobacco smokers more susceptible to cancer.

Objectives: Genetic polymorphism in genes that encode metabolizing enzymes may be related to differentiated susceptibility of malignancy. CYP1B1 protein is a member of the more significant CYP1 subfamily enzymes, involved in environmental carcinogen metabolic activation. The most studied polymorphism in CYP1B1 gene includes 4325 C→G, resulting in an amino acid change from leucine to valine amino acid.

Materials and Methods: A case-control study (included 65 lung cancer cases and 80 healthy controls) was designed based on the RFLP-PCR method to estimate the possible association of this polymorphism with lung cancer susceptibility in the Iranian population.

Results: Regarding the distribution of *CYP1B1 L432V* genotypes, there were no meaningful differences among controls and lung cancer patients, however among patients carrying the CC genotype, tobacco smokers had a considerable elevated risk for lung cancer compared to those who had the GG genotype.

Conclusions: *CYP1B1 L432V* polymorphism has an important role in lung cancer risk. Therefore, further studies are recommended for investigation of other related CYP1B1 gene polymorphisms, their association with affective genes and regulatory factors in the Iranian population.

Keywords: Case-Control Study; Genetic Polymorphism; Lung Cancer

1. Background

According to the world health organization (WHO), lung cancer has been the primary cause of cancer mortality all over the world in recent years and it is considered as the most prevalent fatal cancer. Annually lung cancer is the cause of about 20 percent of all deaths caused by cancer. Reports have demonstrated an excessive increase of lung cancer and associated mortality in the Iranian community. Lung cancer has a weak prognostic; in spite of developments related to the treatment of this disease, 5-year survival rate of lung cancer patients was less than 15 percent (1). Tobacco smoke is the most important factor in lung cancer and 85 to 90 percent of lung cancer mortality is due to tobacco smoking (2, 3). Exposure to polycyclic aromatic hydrocarbons (PAHs) which are primarily found in tobacco smoke lead to lung cancer induction (4, 5). PAHs are among various groups of primary carcinogens, which exist in tobacco smoke and perform locally (6, 7). Higher levels of PAHs and DNA-aromatic adducts exist in tobacco smokers' blood and lung tissue in comparison with non-tobacco smokers and lung cancer expansion probability in tobacco smokers is 10-20 times more (8, 9). However, according to the undeniable role of tobacco in lung cancer, only 10 percent of individuals who are exposed to tobacco smoke for a long time are afflicted to lung cancer; therefore, other factors such as lung disease, age, gender, nutritional and genetic factors have a determining role in the incidence of lung cancer (2). In fact, even after controlling tobacco smoking among relatives, lung cancer risk is influenced by genetic differences, especially polymorphisms in enzymes involved in carcinogen metabolism, thus, different individuals with similar amounts of received carcinogens contain various levels of DNA damage (10, 11). One of the metabolizing enzyme groups of xenobiotic compounds is cytochrome P450, which is

Implication for health policy/practice/research/medical education:
CYP1B1 L432V Polymorphism and Lung Cancer Risk in Iranian Population Lung cancer has been identified as one of the most prevalent and dangerous cancers. So, evaluation of important polymorphisms offers clinical advantages (early diagnostic of lung cancer).

involved in various exogenous and endogenous chemical compound metabolisms such as carcinogens and medicines.

CYP1B1 is one of the related enzymes of CYP450, which consists of a genetic polymorphism in a specified population; in addition, genetic susceptibility of malignancy affection is influenced by enzyme genotype (12-14). CYP1B1 has a prominent role in oxidation of various kinds of carcinogen compounds such as PAHs and arylamines. CYP1B1 gene is located on chromosome 2, in the 2p21-22 region. Length of this gene is 5.1 Kb and encodes a protein with 543 amino acids (15). There is usually a polymorphism in the CYP1B1 gene, which causes a C to G change in 4236 nucleotides leading to a change from Leucine to Valine amino acid, in 432 codon (*CYP1B1 L432V*). In this polymorphism, C allele leads to an increase in enzyme activity, thus increasing the risk of lung cancer (15, 16). Genotypes related to *CYP1B1 L432V* polymorphism is represented in table 1; C allele encodes Leucine amino acid and G allele encodes Valine amino acid (17).

Table 1. *Cyp1B1 L432V* Polymorphism (14)

	Homozygote Wild-Type	Heterozygote	Homozygote Mutant
Genotypes	*Cyp1B1**1/*1	*Cyp1B1**1/*3	*Cyp1B1**3/*3
Nucleotides	C/C	C/G	G/G
Amino acids	Leu/Leu	Leu/Val	Val/Val

2. Objectives

In the present study, the effect of the mentioned polymorphism on lung cancer susceptibility in the Iranian population is surveyed by the RFLP-PCR method.

3. Materials and Methods

3.1. Studied Population

In the current case control study, 65 lung cancer patients who were first diagnosed by standard histopathological procedures at the Chronic Respiratory Diseases, Research Center, Masih Daneshvari Hospital, Tehran, Iran were investigated. Blood collection was always performed under supervision of a medical oncology specialist to avoid possible error in choosing primary lung cancer cases (patients sampling takes 6 months). 80 cancer free volunteers, unrelated to the patients, randomly selected among those who had referred to clinics of Masih Daneshvari Hospital for regular health checks, formed the control group. Before blood sample collection, a structured questionnaire was completed during a brief face to face interview to obtain demographic characteristics, such as gender, age and smoking habits. Data were collected from the subject's closest relative or from the medical file, registered by physicians at admission time for patients who were unable to participate in an interview. Blood sampling was done based on patient satisfaction and an agreement was signed between the University of Isfahan and Masih Daneshvari Hospital. The blood samples were collected in tubes containing anticoagulant ethylene diamine tetraacetic acid (EDTA) and immediately stored at –20 °C until DNA isolation.

3.2. DNA Genotyping

Using a salting method, genomic DNA was extracted from peripheral white blood cells (18). To analyze *CYP1B1 L432V* polymorphism, a distinct region with 549 bp length including the variation site was amplified with the PCR technique by using forward and reverses primers (Table 2).

Table 2. Primers for PCR

Primer	Sequence
Forward	5'-AGGGACCGTCTGCCTTGTAT-3'
Reverse	5'-GCCTCTTGCTTCTTATTGGC-3'

Each 25 μl of PCR reaction mixture consisted of 2 μl of 10 mM of each forward and reverse primers, 2.5 μl of 10x solution buffer, 0.5 μl of a 10 μM of the four mixed dNTPs, 0.75 μl of 50 mM of $MgCl_2$, 0.25 μl of 5 $u.\mu l^{-1}$ Taq DNA polymerase (Cinnagene, Co., Iran), 2 μl of genomic DNA (80 $ng.\mu l^{-1}$) and 15 μl of sterile H_2O. The amplification reaction using a thermal cycler was carried out at the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles with melting at 95 °C for 30 sec, annealing at 62°C for 35 sec, and primer extension at 72 °C for 40 sec. A final extension completed the PCR reaction at 72 °C for 10 min. The amplified products were then separated by electrophoresis through 1% gel agarose and visualized with ethidium bromide staining.

PCR amplified products were digested to determine genotypes. EcoR571 restriction enzyme (New England Biolabs) was used to distinguish the *CYP1B1 L432V* polymorphism, at 37 °C overnight. This enzyme only affected the C allele sequence and it did not have any influence on the G sequence. 549bp fragment cut in the polymorphism location with EcoR571 enzyme led to the creation of fragments with 221 and 328 bp lengths; therefore, recognition and discrimination of two alleles in the final PCR products was conveniently possible; also, the created restricted and non-restricted fragments were easily recognizable and discriminated by electrophoresis.

3.3. Statistical Analyses

To compare the distribution of different genotypes and allele frequencies for the polymorphism between patient and control groups, the chi-square test was used. The same test was also used to determine possible significant associations between lung cancer risk and *CYP1B1 L432V* variant alleles. The odd ratios (ORs) and their corresponding 95% confidence intervals were calculated to estimate relative association between each *CYP1B1 L432V* variant allele and lung cancer. Analysis of all data was down with the SPSS software version 16. A P value less than 0.05 was considered statistically significant.

4. Results

The cases and controls were nearly similar regarding gender distribution, since males and females had almost the same proportions between the two groups. The mean age was computed as 53.39 ± 1.12 for the patients and 51.71 ± 1.3 for the controls. Therefore, there were no significant differences in the distribution of age between cases and controls (for age and gender distribution, P values > 0.05). However, according to smoking status a significant difference was obtained between two groups, as compared to the healthy control group; more tobacco smokers were present among cases with lung cancer (P value = 0.001) (Table 3). In addition, the average duration of smoking was much more in patients than controls, and cases smoked more intensively than healthy controls (data not shown).

Table 3. General Characteristics of the Studied Population

Characteristics	Cases, No. (%)	Control, No. (%)	P value
Gender			0.855
Men	37 (56.92)	47 (58.75)	
Women	28 (43.08)	33 (41.25)	
Age			
Mean (SD)	53.39 ± 1.12	51.71 ± 1.3	
Range	34 - 67	34 - 81	
Tobacco Smoking Status			0.001
Nonsmokers	37 (56.92)	66 (82.50)	
Smokers	28 (43.08)	14 (17.50)	
Histology			
AC[a]	22 (33.85)		
SCC[a]	9 (13.85)		
SCLC[a]	9 (13.85)		
Other/unknown types	25 (38.45)		

[a] Abbreviations: AC, Adenocarcinoma; SCC, Squamous cell carcinoma; SCLC, Small cell lung carcinoma.

Table 4 illustrates the distribution of different lung cancer histological types. Adenocarcinoma was more frequent in patients, while cases with small cell lung carcinoma and squamous cell carcinoma were much more likely to have a positive smoking history, although no noticeable difference was observed (P value > 0.05).

Table 4. Analysis of the Relationship Between Smoking and Lung Cancer Histological Types

Histological types	Tobacco Smokers	Non-tobacco smokers	P value
Adenocarcinoma	9 (40.90)	13 (59.10)	
Squamous carcinom[a]	6 (66.77)	3 (33.33)	0.193
Small cell carcinom[a]	7 (77.78)	2 (22.22)	0.062

[a] People who smoke at least two cigarettes a day

4.1. RFLP-PCR Method with EcoR751 Enzyme

Regional amplification of CYP1B1 gene, which consists of L432V polymorphism, has created a band with 549 bp length. Genotypes of controls and patients have been determined by the EcoR571 restriction enzyme on the PCR products. From each amplified sample, two equal volumes of individuals' genomic DNA was taken and digested in separated vials, where one received no enzyme and the enzyme influenced the other one. The first sample was considered as control and without digestion. Then, control and digested genomic DNA fragments were respectively loaded in adjacent wells and the produced bands were separated on 1% agarose gel. Electrophoresis

was performed with 65 mv of voltage for 90 minutes. Two bands with 221 and 328 bp lengths were achieved for CC homozygosis and one band with 549bp lengths for GG homozygosis. For GC heterozygosis all three 221, 328 and 549bp bands were observed on the gel (Figure1).

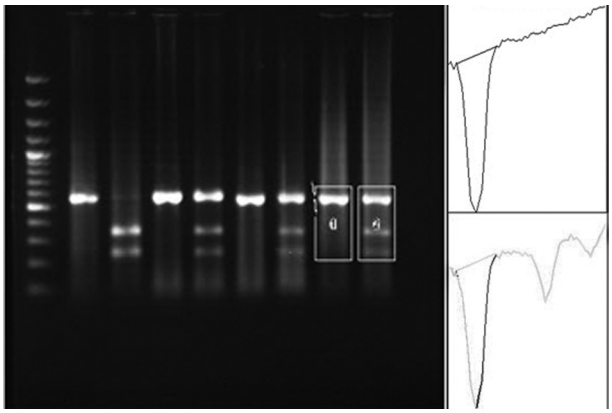

Figure 1. RFLP-PCR products electrophoresis with EcoR571 enzyme, related to two lung cancer patients and two healthy individuals that had been selected randomly and loaded on 1% agarose gel. The persons' numbers are represented above and their genotype under the figure. (A) Samples without enzyme, (B) Index related to enzyme function

4.2. RFLP Results Analysis Using Image J Software

For precise assessment of enzyme digestion, bands resulting from electrophoresis were evaluated using the Image J software. The Image J software draws two charts for each case. The area below the chart 1 shows the light emitted from non-restricted band and the area under chart 2 is the result of light from restricted bands of equal samples under the effect of enzymatic digestion. The evaluation of one of the samples obtained from the electrophoresis is shown Figure 2.

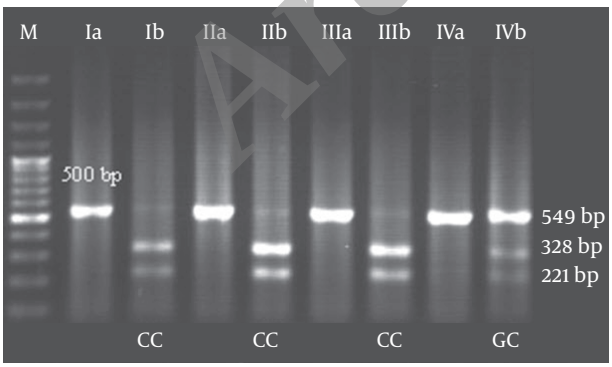

Figure 2. Comparison of the intensity of bands on agarose gel by the Image J software. Total surface under graph 2 and 3 is equal to the surface area under graph 1, so enzyme digestion was done correctly.

Measurement of the exact area under the charts gives two numbers in the results section, which show equal area under the charts. Thus, light emitted from non-restricted band and resultant emitted light of restricted bands is exactly equal and enzyme digestion was performed correctly.

4.3. CYP1B1 L432V Polymorphism Investigation of Lung Cancer Patients and Controls

In the present study, CC, CG and GG genotypes were observed respectively in 36.25, 52.25 and 12.50 percent of control samples; while these genotypes were instead present 47.70, 46.15 and 6.15 percent of all lung cancer patients. *CYP1B1 L432V* polymorphism genotype distribution for lung cancer patients and controls has been represented by table 4. The comparison of the genotype distributions of the two studied groups shows that CC genotype frequency is about 1.7 times more than GG genotype in lung cancer patients in comparison with controls; however, differences in the mentioned genotypes distributions of patients and controls were not significant and those genotype distributions are according to Hardy-Weinberg equilibrium ($P > 0.05$). In addition, significant differences have not been observed in patient and control groups ($P > 0.05$). Table 5 compares the allelic distribution between patient and control groups.

Table 5. Distribution of *Cyp1B1 L432V* Genotypes in Control and Lung Cancer Patient Groups

Genotype	Controls, No. (frequency)	Cases, No. (frequency)	P value
GG	10 (12.50)	4 (6.15)	
CG	41 (51.25)	30 (46.15)	0.340
CC	29 (36.26)	31 (47.70)	0.119
Total	80	65	

4.4. Investigation of Tobacco Smoking Relationship with CYP1B1 L432V Polymorphism Genotypes in Lung Cancer Patients

Patients were divided into two tobacco smoker and non-tobacco smoker groups; then, *CYP1B1 L432V* polymorphism genotype distribution was compared between these two groups (Table 6). The results showed that there is a significant association between CC genotype and lung cancer risk in tobacco smokers; in other words, CC tobacco smokers are exposed to higher lung cancer risk in comparison with GG genotype tobacco smokers ($P > 0.029$). There is no significant association between tobacco smoking and lung cancer risk in CG genotype ($P > 0.169$). The investigation of tobacco association with polymorphism genotype distribution showed that no tobacco smoker with GG genotype was found; therefore, OR was not calculated (Table 7).

Table 6. Distribution of *Cyp1B1 L432V* Allelic Frequencies in Control and Lung Cancer Patient Groups

Allele	Controls, No. (frequency)	Cases, No (frequency)	P value
G	61 (0.38)	38 (0.292)	
C	99 (0.62)	92 (0.708)	0.112
Total	160	130	

Table 7. Analysis of the Relationship Between Smoking and *Cyp1B1 L432V* Polymorphisms in Lung Cancer Patients

Genotype	Smokers, No.	Non-Smokers, No.	P value
GG	0	4	1
CG	10	20	0.169
CC	18	13	0.029

5. Discussion

Lung cancer has been identified as one of the most prevalent and dangerous cancers. In 2005, more than 163000 individuals died as a result of lung cancer in the U.S.A; thus, this kind of cancer has been considered as the greatest cause of cancerous mortality (19). In Iran, lung cancer is the fifth factor leading mortality, but lung cancer incidence has been improving in men and women (1). Researches and studies on the risks of lung cancer are very important due to the lack of sufficient knowledge, high mortality rate as a result of late identification (usually after metastasis) and expensive treatment costs. CYP1B1 enzyme has an important role in metabolism and activation of environmental carcinogens; also, it has a dominant role in increasing the risk of lung cancer (20, 21). On the other hand, metabolic capacity has been affected intensively by individual differences such as genetic polymorphism of metabolized enzymes. In recent decades, researches on various populations represent the association between CYP1B1 polymorphism and lung cancer risk; in addition, contradictory results have been achieved in several studies. Epidemiological studies which represent *CYP1B1 L432V* polymorphism role in lung cancer are restricted. Contradictory results have been reported about this polymorphism; for instance, leucine allele and valine allele have been associated with an increase in the risk of lung cancer, in some studies. Investigation of this polymorphism in two non-tobacco smoker populations of Caucasian and African-American races showed that there was a significant association between lung cancer susceptibility and this polymorphism (CI 95% = 1.63-5.07; OR = 2.87 for at least one valine allele) (22). In one research in Germany, no association between *CYP1B1 L432V* polymorphism and lung cancer was found; however, a significant increase in primary lung cancer risk for minor allele carrier women (G) was observed ($P < 0.0001$, OR = 1.97, CI 95% = 1.32-2.94). The effect of this allele on the risk of lung cancer is related to tobacco smoking; in other words, women that are G allele carriers, who smoke more than 20 packs per year show a significant increase in primary lung cancer risk ($P < 0.0001$, OR = 2.69, CI 95% = 1.49-4.84) (14). In this study, in spite of the high frequency of CC genotype in the patient population, there were no significant differences in two control and patient groups regarding *CYP1B1 L432V* genotype distribution; therefore, the two populations were almost the same. Generally, the most control and patient groups have contained heterozygous genotype and in spite of C allele higher frequency in patients, there was no significant differences in C and G alleles distribution between the two groups ($P > 0.05$); however, as it was pointed in previous studies, C allele existence leads to the lung cancer risk increase; in this case, CC homozygosis is more exposed to lung cancer risk in comparison with heterozygosis. The investigation of the relationship between genes and tobacco in patients represents significant differences in genotype distribution according to tobacco smoking rate; in addition, tobacco smokers with two lue432 alleles (CC genotypes) have been shown to be at a greater risk of lung cancer in comparison with tobacco smokers with two val432 alleles (GG genotypes). Thus, tobacco increases lung cancer risk in patients carrying the CC genotypes. In summary, *CYP1B1 L432V* polymorphism has an important role in lung cancer risk. Therefore, further studies are recommended for investigation of other related CYP1B1 gene polymorphisms, their association with affective genes and regulatory factors in the Iranian population.

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Authors' Contribution

All authors worked equally in the present study.

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