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Genetic polymorphism of GSTM1 and GSTP1 in lung cancer in Egypt

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ABSTRACT

Background: Lung cancer (LC) is the most common cause of cancer-related mortality; it is one of the most important common diseases with complicate, multi-factorial etiology, including interactions between genetic makeup and environmental factors. Individuals may differ in their susceptibility to environmental risk factors. This difference of susceptibility may result from inherited polymorphisms in various genes controlling carcinogen metabolism, repair of DNA damage and cell cycle.

Objectives: Glutathione S-transferase (GST) plays a key role in detoxification of carcinogens present in tobacco smoke and consequently polymorphisms in this gene may confer susceptibility to many types of cancer such as lung cancer. In the current study the effect of GSTM1 and GSTP1 polymorphism on the development of lung cancer among Egyptian patients was investigated.

Methods: The GSTM1 was analyzed using multiplex polymerase chain reaction (PCR) while polymorphism of GSTP1 was analyzed using RFLP.

Results: It was found that there is no significant difference (p value = 0.8) in GSTM1 genotype distribution between control and lung cancer cases as it was absent in 33.3% in control and 31.25% in patients. While GSTP1 single nucleotide polymorphism (SNP) encoding A313G base change increases the susceptibility for lung cancer especially among smokers as odds ratio was 5 in case of smokers carry ile/val or val/val genotypes. Also, combination of GSTP1 and GSTM1 polymorphism increases the risk for lung cancer. Our data may provide additional information to the understanding of the molecular mechanism and individual susceptibility to lung cancer in Egypt.

Keywords: Lung cancer, Glutathione S-transferase, genetic polymorphism, smoking

Introduction

Lung cancer (LC) is the leading cause of cancer-related malignancy worldwide, accounting for one-third of all cancer-related deaths worldwide, approximately 1.2 million new cases per year [1]. Despite the fact that the cause of most lung cancer is well known, the disease has proven difficult to diagnose early and treat successfully, reflecting limited advances in our understanding of the molecular mechanisms underlying lung carcinogenesis and individual susceptibility to lung cancer [2].

Tobacco smoke is the major risk factor for lung cancer development (90% of cases diagnosed). However, not all populations are equally susceptible to tobacco-related carcinogens [3]. Exposure to other environmental respiratory carcinogens, such as asbestos, benzene, coal tar, and other industrial chemicals may interact with tobacco smoke to increase risk. Thus, the identification of genes responsible for lung carcinogenesis susceptibility may allow us to perform screening programs and chemoprevention trials in subgroups of heavy smokers [4].

A family of enzymes, the glutathione-S-transferases (GSTs), has the general function of conjugating glutathione with electrophilic substances that are capable of generating free radicals, thus leading to detoxification of their effects. Genetic polymorphisms associated with reduced activity of GSTs are therefore of interest in the study of disease susceptibility [5].

The cytosolic isoenzymes of GST are divided into at least five major classes (α, μ, π, θ, ζ) among which polymorphism have been detected in the genes encoding for GSTA1 (α class) GSTM1 (μ class), GSTP1 (π class), CSTT1 (θ class) and GSTZ1 (ζ class) [6]. Among them the GSTM1, GSTP1 and GSTT1 genotypes have been extensively studied during recent years for their potential modulating role in individual susceptibility to environmentally-induced diseases, including cancer [7]. The *GSTM1* gene on chromosome 1p13, according to the three alleles, can be grouped into two classes: *GSTM1*-null homozygote for the null allele (*GSTM1*-0), nonfunctional class and *GSTM1*-1 with at least one of the *GSTM1a* or *GSTM1b* alleles, functional class [8]. *GSTM1*-null was reported to be associated in some studies with increased susceptibility to inflammatory pathologies and increased risk of smoking related cancers [9]. The GSTM1 null polymorphism, are known to abolish enzymes activity. Individuals with GSTM1 null genotype have been reported to have higher levels of polycyclic aromatic hydrocarbon-dGMP adducts in lung tissues, which can induce genetic mutations [10].

The GSTP1 gene has been mapped to a small region of chromosome 11q [11]. GSTP1 is known to metabolize many carcinogenic compounds, among them benzo[a]pyrene diolepoxide (BPDE), which is one of the most important carcinogenic metabolites derived from tobacco smoke [12]. Given that GSTP1 is the most abundant GST isoform in the lung, it is anticipated to be of particular importance in the detoxification of inhaled carcinogens [4].

GSTP1 has two common non-synonymous single-nucleotide polymorphisms (SNPs) that result in $Ile^{105}Val$ and $Ala^{114}Val$ alterations in encoded amino acid sequence. At least two different alleles, GSTP1A and GSTP1B, have been identified encoding for GSTP1, exhibiting $A^{313}G$ base change in the latter allele which results in an $Ile^{105}Val$ amino acid replacement within the active site of the enzyme [13].

The Val^{105} substitution results in steric restriction of the H-site due to shifts in the side chains of several amino acids. Thus, the Val^{105} variant allozyme may be able to accommodate less bulky substrates than the Ile^{105} allozyme and, as a result, may display substrate specificities that differ from those of the wild-type (WT) allozyme [14]. In addition, the thermal stability of the codon 105 variant allozyme differs from that of the WT [15]. These characteristics may be responsible, in part, for the reported association between this allozyme and carcinogenesis or variation in response to antineoplastic drugs [16].

Individual differences in the susceptibility to carcinogens play an essential role in the development of sporadic cancer. The biochemical basis for the genetic susceptibility to environmental hazards is related to genetic polymorphisms that normally occur in the general population, and involves a series of

genes implicated in the metabolic activation or detoxification of environmental toxins [17].
In this study we investigate the role of GST isoenzymes polymorphism (GSTM1 and GSTP1), in lung cancer risk in Egyptian patients, considering age, gender, histopathological diagnosis and history of tobacco exposure.

Subjects and methods

Study subjects
The study population included 48 patients with lung cancer seen at bronchoscopy unit in National Cancer Institute, Cairo, Egypt. Cases were diagnosed as primary lung cancer by histological evaluation of tumor biopsies. Age, sex, self smoking, type of cancer and its grade were recorded. The control group consisted of 42 volunteers, none of them had lung cancer or any type of cancer and the same data were recorded for them.
Trans-nasal fiberoptic bronchoscopy was performed using an Olympus flexible fiberoptic bronchoscope, following the guidelines of the national institutes of health. Premedication included atropine (0.5 mg-IM) administrated 30 minutes before the procedure, with local upper airways anesthesia with 5 ml of 2% lidocaine. Multiple biopsies were obtained from the tumor mass seen and sent for histopathology.

Blood samples and DNA extraction
Blood samples of 5 ml were obtained from all subjects and collected in sterile tubes containing EDTA. Immediately after collection, whole blood was stored at -20˚C until use. Genomic DNA was extracted from whole blood using the established protocol for DNA extraction from nucleated blood cells [18].

Analysis of GSTM1 polymorphism
The genetic polymorphism analyses for the GSTM1 gene was determined by the multiplex polymerase chain reaction (PCR) using the previously described method [19]. The appropriate fragment of the GST gene for GSTM1 was amplified with specific primers from human genomic DNA. The CYP2E1 gene was co-amplified as an internal positive control. The following primers were used in PCR reaction: GSTM1 primers of (sense) 5-GAACTCCCTGAAAAGCTAAAGC-3 and (antisense) 5-GTTGGGCTCAAATATACGGTGG-3, and CYP2E1 (sense) 5-TTGGTTGACTCACTCTTTCCTTT-3 and (antisense) 5-CCATCGTTTCAAAGGCTGAT-3. PCR was performed in a total volume of 25 µl containing 200 ng genomic DNA, 5 ρM of each primer, 2.5 mM deoxyribonucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, and 1 U thermostable Taq DNA polymerase using Little Genius, Bioer PCR system (Bioer Technology Co., U.S.A.). The amplification conditions were initial denaturation at 94℃ for 5 min followed by 35 cycles of denaturation at 94℃ for 30 sec, annealing at 54 ℃ for 30 sec, extension at 72℃ for 1 min, and final extension at 72℃ for 10 min. The products of the PCR amplification (GSTM1:219 bp, CYP2E1: 495 bp) were separated electrophoretically on an ethidium bromide stained 2% agarose gel. The absence of amplified GSTM1 product (in the presence of control PCR product) indicated the respective null genotype.

Determination of the GSTP1 genotype
Each PCR reaction mixture (25 µl) contained 5 ρM of each primer sense (5-ACCCCAGGG CTCTATGGGAA-3) and antisense (5-TGA GGG CAC AAG AAG CCC CT-3), 200 ng of genomic DNA, 1.5mM $MgCl_2$, 2.5mM each dNTPs and 1 U Taq polymerase (Biron Technologies). Amplification was performed with an initial denaturation at 94℃ for 5 min

followed by 35 cycles at 94℃ for 30 sec, 56 ℃ for 30 sec, 72℃ for 1 min, and final extension at 72℃ for 10 min. After the confirmation of an amplified fragment of the expected size (176 bp) on an agarose gel, the PCR products were digested with 5 U of restriction enzyme BsmAI (New England BioLabs, UK) at 55◦C for one hour. DNA fragments were applied to electrophoresis through a 2% agarose gel and stained with ethidium bromide.

Electrophoresis of the digested PCR products showed individuals homozygous (ile/ile) for the GSTP1 BsmAI polymorphism as one band of 176 bp. Heterozygous (ile/val, val/val) for the polymorphism resulted in three bands of 176, 91 and 85. Homozygotes (val/val) showed two bands of 91 and 85 bp (which appear as one band due to close molecular size).

Statistical analysis

The Statistical Program SPSS for windows version 11 had been used in data entry and analysis. Descriptive and analytic statistics were done. Statistical significance was determined at the 95 percent confidence interval level. The lowest accepted level of significance was 0.05 or less. Risk ratios were estimated by odds ratios (OR).

The descriptive analysis was performed to compare observed and expected genotype frequencies using a chi-square test. We estimated adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between lung cancer and genetic polymorphism using logistic regression models. The lung cancer cases were separately compared to the controls using multi-category logistic models.

Results

For the demographic characteristics of the subjects, data from the control population (healthy Egyptian blood donors) and the LC patients were statistically analyzed. LC patients showed to be slightly older than controls. The mean age of the cases was 58.6 while it was 57.8 in control.

The number of male and female among cases was 32 and 16, respectively. Males were over-represented when compared to females (66.7% versus 33.3 %) showing that cases of lung cancer was significantly higher in males ($p = 0.001$). The frequency of smokers was higher in patients with LC than in controls (68.7% versus 33.3 %; $p < 0.001$) and among LC patients smokers was higher in male than females.

Among cases squamous cell carcinoma was the most hisological finding (68.75 %) followed by adenocarcinoma (18.75%) then small cell carcinoma (12.5 %), generally non-small cell lung cancer (NSCLC) is more frequent than small cell lung cancer (SCLC) and the most frequent stages were III and IV (64.6 %). Lung cancer patients compared to control group showed high distribution in old age, male and smokers (table 1).

GSTM1 gene polymorphism

The internal standard fragment amplified from GSTM1 was 219 bp. The absence of amplified product and the presence of control fragment were consistent with the null genotype (Fig. 1) The proportion of control individuals with GSTM1 null mutation was 33.3 % while in lung cancer cases it was 31.3 %. No significant differences were found between them ($p = 0.8$) (table 1) indicating that GSTM1 null mutation did not increase the risk of lung cancer incidence (odds ratio (OR) = 0.5, 95% confidence interval (CI) = (0.1-2.01), p-value = 0.33) (table 2).

GSTP1 gene polymorphism

The PCR product with G allele was digested to two fragments, whereas the PCR product with A allele remained undigested (Fig. 2). The frequency of GSTP1 ile/ile genotype was 18.7 % in patients and 66.6 % in control while the frequency of GSTP1 ile/val and val/val were 81.3 % in case of cancer and 33.4 % in control showing highly significant difference (table 1) in these polymorphism (p = 0.0001). Separately ile/val frequency was 64.6 % in cases versus 16.7 % in control (p= 0.001) while the ratio of val/val was 16.7 % in LC and 16.7 % in control.

Using the GSTP1 homozygous (ile/ile) as references group, the OR of GSTP1 heterozygous (ile/val) was 3.5 (95% CI= 1.01-12.5, P = 0.042) and the GSTP1 homozygous (val/val) was 0.4 (95% CI= 0.2-0.8, P= 0.27) indicating that GSTP1 mutant type (ile/val) represents high risk polymorphism for lung cancer (table 2).

For the association of combined gene polymorphisms with LC risk. We studied the risk of LC associated with the combination of the GSTM1 null, GSTP1 Ile/Val plus Val/Val. A statistically significant association was observed (table 2) between the combination of GSTM1 and GSTP1 and overall lung cancer, (OR= 5.06, 95% CI= 1.3-20.2, P= 0.02).

When the risks associated with GST genotypes for lung cancer were considered in relation to smoking habits, smokers with GSTM1-positive genotype (table 3) were at an approximately 2.9-fold higher risk of lung cancer than non-smokers with GSTM1-positive genotype (95% CI, 1.2-6.8) and it was more in case of smokers with GSTM1-null genotype (OR=5.8, 95% CI: 0.7-50.7).

In the evaluation of the effect of interaction of GSTP1 gene and smoking on lung cancer risk, we used GSTP1 ile/ile genotype as a reference. It was found that non-smokers with GSTP1 abnormal genotype (table 4) were at an approximately 2.3-fold higher risk of lung cancer than nonsmokers with GSTP1 ile/ile genotype (OR=2.3, 95% CI: 0.8- 6.3). While smokers with GSTP1 abnormal genotype were 5-fold high risk of lung cancer (OR= 5, 95% CI=1.8- 13.4).

Discussion

Inter–individual variability in glutathione-S-transferase (GST) enzyme activity can influence the susceptibility to cancers, especially in those with environmental determinants, such as lung cancer [20].

Nelson et al. [21] reported that the null genotype of GSTT1 was present in 64% of Chinese, 60% of Koreans, 28% of Caucasians and in 22% of African-Americans. This null genotype is more common in the Asians than Caucasians, strengthening the idea that polymorphisms in enzymes that metabolize tobacco carcinogens have a strong ethnical link. To our knowledge this is the first time to investigate the correlation between genetic polymorphism of GSTM1 and GSTP1 and lung cancer in Egyptian patients.

Recent study reported that GSTM1 null genotype was present in 52 % of lung cancer in Turkish patients [22] and in another study the percentage was 45.5% for the GSTM1 deletion in patients with LC and 48.1% in their control group among Brazilian population indicating that there is no influence for that polymorphism in the risk of LC [4].

The last finding was similar to our results as we found that there is no significant difference in GSTM1 null genotype between LC patients and control group suggesting that no association between GSTM1 polymorphism and lung cancer. Nazar-Stewart et al. [23] reported that effects of the polymorphism of the GSTM1 on lung cancer have been suspected due to loss of detoxification of carcinogens such as benzo[a]pyrene diolepoxide for 10 years of more. This is still unclear due to some reasons, e.g. low expression of GSTM1 in lung [24].

Modulation of DNA damage and mutation caused by polymorphisms in detoxification enzymes, including the GST, is a well-established risk factor for tobacco-related carcinogenesis and a similar change in cellular damage may be involved in the risk of vascular disease associated with tobacco smoking [25, 26]. In the current study, we tested interaction between GSTM1 genotype and smoking in lung cancer and control cases, it was found that smokers have high risk susceptibility for lung cancer either in GSTM1-positive or null genotype and that was similar to what is found in cases of coronary artery disease (CAD) as the smokers with GSTM1-positive genotype were an approximately 1.21-fold higher risk of CAD and it was slightly higher with GSTM1-null genotype compared to non-smokers with GSTM1-positive genotype [19].

Miller et al. [27] in a North American population showed an association between polymorphic GSTP1 gene and lung cancer. The patient' single nucleotide polymorphism (SNP) frequencies were 46, 42 and 12% for genotypes Ile/Ile, Ile/Val, Val/Val, respectively. Also, in another study who studied the effect of genetic polymorphism of six genes on lung cancer susceptibility, it was shown that among various genetic polymorphisms, only the allele distribution of the GSTP1 polymorphism was significantly different between cases and controls, i.e. the proportion of GSTP1-mutant, the Val allele was higher in cases than controls [24]. That was similar to our results as we found that there is significant overall association between GSTP1 *Ile* ^{105}*Val* polymorphism and lung cancer indicating that that GSTP1 polymorphism increases the risk of lung cancer susceptibility.

However, mechanism of how the presence of the GSTP1-Val allele increases risk for lung cancer is not clear, yet. A clear evidence of the GSTP1 involvement for lung cancer is the high localization of the GSTP1 in lung, compared to other GST-isozymes. Also GSTP1 is responsible for more than 90% of the GST activity within the adult human lung epithelial cell population [28] and the enzyme encoded by GSTP1 *Val* allele exhibits different activity, affinity and thermostability according to substrates [11].

In the evaluation of the effect of interaction of GSTP1 gene and smoking on lung cancer risk, GSTP1 ile/ile genotype was used as a reference and the genetic model (ile/val and val/val) was evaluated as a genetic polymorphism. It was found that carriers of *Val* allele have an increased risk of lung cancer in non-smokers and a higher significant risk in smokers and that may be due to genotype-phenotype interaction, as it was found by Watson et al. [13] that there is higher GST activity in lung tissue samples with GSTP1 Ile/Ile genotype than with GSTP1 Ile/Val and Val/Val genotypes.

Our results was in agreement to what found by Ritchie et al. [29] who reported that GSTP1-null mice exposed to benzo[a]pyrene, a common constituent of tobacco smoke, exhibited a marked increase in lung tumor incidence relative to wild-type mice (92% versus 29%) indicating that GSTP1 plays a key role in vivo in determining susceptibility to lung cancer following exposure to chemical carcinogens of the type commonly found in tobacco smoke.

Some studies reported the association of combined gene polymorphisms with LC risk. Jourenkova-Mironova et al. [30] reported an increased risk of LC associated with the combination of the GSTM1 null, GSTP1 Ile/Val plus Val/Val. Also, Kihara and Noda [31] reported a potential interaction between the GSTP1 and GSTM1 genes in a Japanese population of male smokers, in which a higher risk of LC was associated with the combination of the variant allele for GSTP1 and GSTM1 null genotype. Our results also showed that OR of lung cancer was particularly high for the subjects carrying

deficient genotype for both GSTM1 and GSTP1, however these results are not in agreement with other study which showed that there is no correlation of the GSTP1 polymorphism and GSTM1 null genotype and the risk of lung cancer in a Brazilian population [4]. However, combined studies that include various genetic polymorphisms in various genes/enzymes are needed to clarify genetic etiology of lung cancer.

In conclusion, our finding may be an important contribution towards the identification of the role of genetic polymorphism of glutathione S-transferase interactions for cancer-risk. Our results suggest that GSTM1 null genotype has no influence in the risk of lung cancer in Egyptian patients however presence of GSTP1 Val allele increases risk for lung cancer especially among smokers. Also, we found that combination of GSTP1 and GSTM1 polymorphism increases the risk for lung cancer. Further studies including examination of other genotypes also involved either in metabolic activation or detoxification of carcinogens are anticipated to further improve our ability to find genetic factors contributing to individual lung cancer susceptibility.

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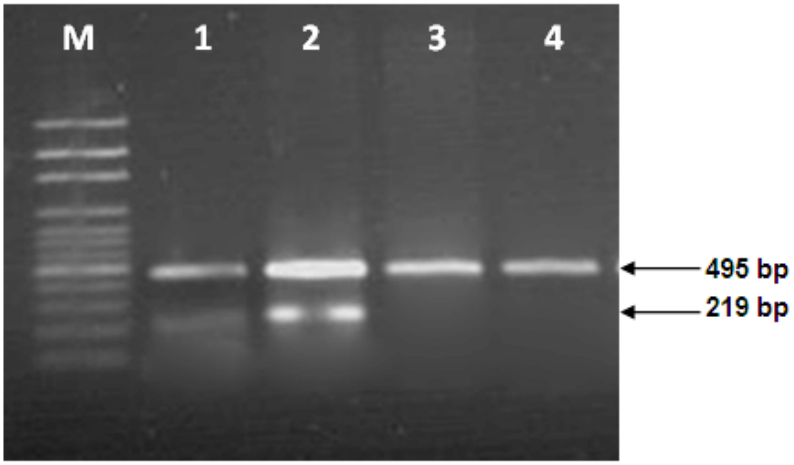

Figure 1: Polymerase chain reaction assay for GSTM1 gene polymorphism. Lanes 1, 2; GSTM1 positive genotype (219 bp), lanes 3, 4; GSTM1 null genotype, M: marker. CYP2E1 gene was used as an internal positive control (495 bp).

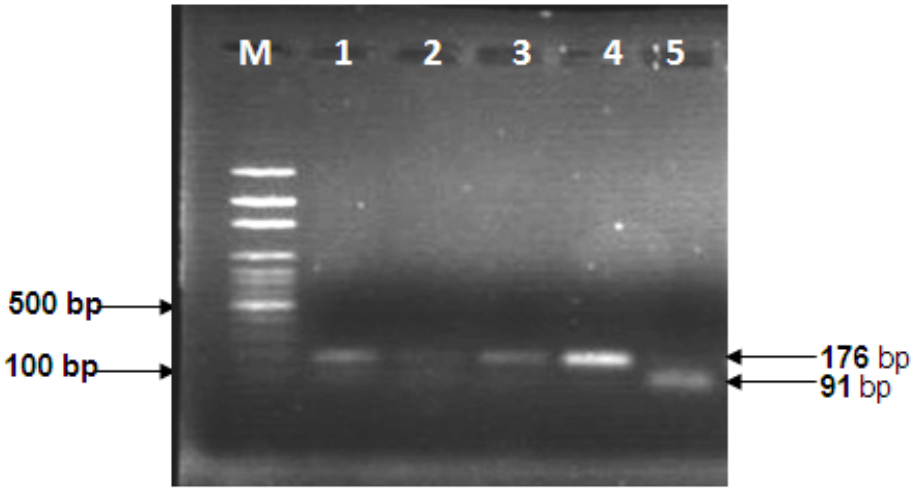

Figure 2: Electrophoresis of the digested PCR products showing individuals DNA for the GSTP1 polymorphism. Heterozygous polymorphism (lanes 1-3; ile/val heterozygous), homozygous polymorphism (lane 5; val/val homozygous), without polymorphism (lanes 4; ile/ile homozygous), and. M: DNA marker.

Table 1: Age, gender, tobacco, histology and stage characteristics of the patients and control groups

	Patients (n=48)	Controls (n=42)	X^2	P-value
Age				
<60 years	30 (62.5)	35 (83.3)	4.8	0.028
≥60 years	18 (37.5)	7 (16.7)		
Gender				
Male	32 (66.7)	27 (64.3)	0.04	0.83
Female	16 (33.3)	15 (35.7)		
Tobacco				
Yes	33 (68.75)	14 (33.3)	11.26	0. 001
No	15 (31.25)	28 (66.7)		
Histology				
Small cell carcinoma	6 (12.5)			
Squamous cell carcinoma	33 (68.75)			
adenocarcinoma	9 (18.75)			
Stage (TNM)				
I +II	17 (35.4)			
III + IV	31 (64.6)			
M 1				
+ve	33 (68.75)	28 (66.7)	0.045	0.8
-ve	15 (31.25)	14 (33.3)		
P1				
lle/lle	9 (18.7)	28 (66.6)	24.7	0.0001
lle/val	31 (64.6)	7 (16.7)		
val/val	8 (16.7)	7 (16.7)		
Combined				
Present	15 (31.25)	7 (16.7)	2.6	0.1
Absent	33 (68.75)	35 (83.3)		

- All values in brackets are in percentage.

Table 2: Association between GSTM1 and GSTP1 and lung cancer risk

	Patients n=48 (%)	Controls n=42 (%)	OR (95% CI)	p-value
Tobacco				
Yes	33 (68.75)	14 (33.3)	9.3 (2.6-33.2)	0.001
No	15 (31.25)	28 (66.7)		
M 1				
+ve	33 (68.75)	28 (66.7)	1.0 (reference)	0.33
-ve	15 (31.25)	14 (33.3)	0.5 (0.1-2.01)	
P1				
lle/lle	9 (18.7)	28 (66.6)	1.0 (reference)	
lle/val	31 (64.6)	7 (16.7)	3.5 (1.01-12.5)	0.042
val/val	8 (16.7)	7 (16.7)	0.4 (0.2-0.8)	0.27
Combined				
Present	15 (31.25)	7 (16.7)	5.06 (1.3-20.2)	0.02
Absent	33 (68.75)	35 (83.3)	1.0 (reference)	

Table 3: Interaction between GSTM1 genotype and smoking on lung cancer and control

	Case n=48 (%)	Control n=42 (%)	OR (95% CI)	P value
M1(+)/NS*	6 (12.5)	15(35.7)		
M1(-)/NS	9 (18.75)	13(30.95)	0.5 (0.2- 1.3)	0.2
M1(+)/S	27(56.25)	13(30.95)	2.9 (1.2-6.8)	0.017
M1(-)/S	6(12.5)	1(2.4)	5.8 (0.7-50.7)	0.1

***** Reference.
- M1 (+): GSTM1-positive genotype; M1 (−): GSTM1 null genotype; NS: non-smoker; S: smoker; OR: odds ratio

Table 4: Interaction between GSTP1 genotype and smoking on lung cancer and control

	Case n=48 (%)	Control n=42 (%)	OR (95% CI)	P value
P(A)/NS*	2 (4.15)	21(50)		
P (B)/NS	15(31.25)	7(16.7)	2.3 (0.8- 6.3)	0.1
P(A)/S	7(14.6)	7(16.7)	0.9 (0.3-2.6)	0.8
P (B)/S	24(50)	7(16.7)	5 (1.8- 13.4)	0.001

***** Reference.
- P (A): GSTP1-Ile/Ile genotype; P (B): GSTP1 Ile/Val plus Val/Val genotypes; NS: non-smoker; S: smoker; OR: odds ratio.