doi:10.4149/neo_2012_021

Lung Adenocarcinoma and Squamous Cell Carcinoma in association with Genetic Polymorphisms of *GST*s in Slovak Population

A. DZIAN[1,*], E. HALASOVA[2], T. MATAKOVA[3], E. KAVCOVA[4], M. SMOLAR[1], D. DOBROTA[3], J. HAMZIK[1], D. MISTUNA[1]

[1]Clinic of Surgery, Jessenius Faculty of Medicine and University Hospital in Martin; [2]Institute of Medical Biology, Jessenius Faculty of Medicine in Martin; [3]Institute of Medical Biochemisty, Jessenius Faculty of Medicine in Martin; [4]Clinic of Pneumology and Phthisiology, Jessenius Faculty of Medicine in Martin

**Correspondence: anton.dzian@gmail.com*

Received July 20, 2011 / Accepted September 30, 2011

Slovak Republic belongs to the countries with high incidence of lung cancer. Gene polymorphisms of the glutathione S-transferases (*GSTs*) may play a role in individual lung cancer susceptibility. In presented case-control study we investigate the incidence of polymorphism of GSTT1, *GSTM1, GSTP1* genes and their combinations as possible predictive factors for identification of individuals with increased risk of formation and development of adenocarcinoma (AC) and squamous cell carcinoma (SCC) of lung in Slovak population. The study was conducted on 520 individuals consisting of 118 patients with adenocarcinoma, 112 patients with squamous cell carcinoma and 290 control individuals. GSTT1, *GSTM1, GSTP1* gene polymorphisms were assayed by standard PCR and PCR-RFLP technique. The results of this study indicate that the *GSTT1null*-genotype and combination *GSTT1 null* and *Ile/Val* or *Val/Val* are associated with increased risk of lung adenocarcinoma. A significant association with 2.13 – fold increased risk was observed between lung adenocarcinoma and *GSTT1 null* genotype (95% CI = 1.29 – 3.51; p= 0.004). Also it was proved 2.83 times statistically higher risk for development of this histological type of lung cancer (95% CI = 1.34 – 6.01; P = 0.005) in combination of *GSTT1null* and *Ile/Val* or *Val/Val* genotypes. *GSTT1, GSTM1, GSTP1* polymorphism did not show any significant association with SCC. Our study suggests that genetic make-up in metabolizing genes may increase susceptibility towards lung cancer development.

Key words: lung cancer, polymorphism, glutathione S-transferase, adenocarcinoma, squamous cell carcinoma

Lung cancer is a worldwide problem because of its high mortality and morbidity. Slovak Republic belongs to the countries with increasing tendency of its incidence in both sexes. In 2008 the incidence of this illness was in men and women 49.2/100 000 and 10.6/100 000 respectively, while its mortality was 43.4/100 000 and 7.7/100 000 respectively (1).

Approximately 80-90% of lung carcinomas are associated with the carcinogenetic effect of smoking (2). However, only about 10-15% of all smokers fall ill on lung cancer. On the other side 10-15% of all lung carcinomas occur in non-smokers (3). These facts indicate that there is individual susceptibility to lung carcinogens as well as to development of disorders. Genetic and environmental factors essentially influence the risk of lung cancer genesis, while molecular pathophysiology of genetic-environmental interaction is complex and only partly identified up to now. That is why it is so important to explore, whether individual difference in susceptibility to lung cancer genesis has a genetic background.

Many studies considering the role of biotransformation processes in detoxification or activation of carcinogens show significant interindividual difference in activities of particular biotransformation enzymes, which are both environmental and genetic origin. Despite of that, the particular polymorphism has a relative small influence on the risk of malignant disease genesis. The accumulation and combination of polymorphisms significantly increases this risk of particular individual. Within biotransformation enzymes, the most studied are the polymorphisms of genes of Glutation-S-transferases (*GSTs*).

Two isoforms of GSTT (GSTT1, GSTT2) metabolize epoxy butane, ethylene oxide, halogen derivatives of methane, methyl bromide, methyl chloride, dichloromethane and diepoxybutane. Deletion in *GSTT1* gene leads to genesis of null genotype

that causes the loss of the enzyme's activity (4). The incidence of null genotype is associated with higher frequentation of malignant diseases (5 – 8).

GSTM1 catalyzes the conversion of reactive electrophilic intermediates derived from cigarette smoking, such as benzo(a)pyrene- 7,8-diol-9,10-epoxides (BPDE) to less reactive and easier excreted glutathione conjugates (9). Deletion of the gene very often influences both alleles and leads to so called null genotype, which leads to the loss of activity of this izoenzyme. Deletion of *GSTM1* is associated with higher sensibility to chemical mutagens (10, 11) and with higher frequency of malignant diseases (12 – 15).

The importance of GSTP enzyme in lung cancer lies in metabolizing of bezo(a)pyrene products and carcinogenetic substances contained in cigarette smoke as bezo(a)pyrene diol epoxide. *GSTP1 Ile105Val* polymorphism modifies the kinetic properties of coded enzyme (16, 17) and some other studies demonstrated higher sensibility for carcinomas in individuals bearing the variant valine allele (18).

In presented case-control study we evaluated the incidence of polymorphism of genes of biotransformation as *GSTT1, GSTM1, GSTP1* and their combinations as possible predictive factors of formation and development of adenocarcinoma (AC) and squamous cell carcinoma (SCC) of lung in studied group of Slovak population.

Material and methods

Studied group. In the case-control study 230 patients with histologically verified adenocarcinoma or squamous cell carcinoma of lungs were included. According to the clinical state, advanced stages of cancer prevailed, what refers on late diagnosis, long silent stage of the disease and non-existing screening of this severe carcinoma. 19.6% of patients (n 45) were in the time of diagnosis in first stage, 7 % (n 16) were in second clinical stage, 31.2% (n 72) in third stage. 42.2 % of patients (n 97) were in forth clinical stage. We created two groups from the patients according to the histological type of carcinoma: patients with adenocarcinoma in number of 118 and patients with squamous cell carcinoma in number of 112. In control group 290 healthy volunteers and patients hospitalized for non-cancer disease were included. The controls were recruited in parallel with the cancer cases. More accurate demographic characteristics of the file including smoking status are displayed in table 1. All included objects were from Slovak population only Caucasians.

Determination of GSTT1, GSTM1 and GSTP1 genotypes. Genomic DNAs were extracted from leukocytes in peripheral venous blood by conventional procedures (19). Genetic polymorphisms of *GSTM1* and *GSTT1* are determined at the same time by application of polymerase chain reaction (PCR) in one reaction with gene for β-globin, which has the function of inner positive control. For determination of *GSTM1* primer according to Chen et al. 5′-GAA CTC CCT GAA AAG CTA AAG C-3′ and 5′-GTT GGG CTC AAA TAT ACG GTC G-3′ is used (20), for *GSTT1* primer described by To-Figueras et al. 5′-TTC CTT ACT GGT CCT CAG ATC TC-3′ and 5′-TCA CCG GAT CAT GGC CAG CA-3′ (21). β-actin fragment is amplified parallely as control of amplification (primer 5′-CAA CTT CAT CCA CGT TCA CC-3′ and 5′-GAA GAG CCA AGG ACA GGT AC-3′). Polymorphism of *GSTP1* gene in codon 105, where adenine is changed for guanine (what leads to the change of amino acid isoleucine – Ile for amino acid valine – Val), was detected by PCR-RFLP (Restriction Fragment Length Polymorphism) method. GSTP1 was analyzed using the primers 5′-ACC CCA GGG CTC TAT GGG AA-3′ and 5′-TGA GGG CAC AAG AAG CCC CT-3′ and the PCR products were digested by the restriction endonuclease *ALW26I* to cut the mutated allele.

Statistical analysis. Associations between the *GSTM1*, *GSTT1* and *GSTP1* polymorphisms and the risk of lung AC and SCC were estimated using odds ratios (ORs) and 95% confidence intervals (95% CIs). Differences in genotype distributions in cancer cases and the controls were compared

Table 1. Demographic characteristics of the lung AC and SCC cases and controls

Characteristics	Cases Adenocarcinoma (n=118)	Cases Squamous cell carcinoma (n=112)	Controls (n=290)	p value AC	p value SCC
Gender					
Male (n)	88	95	224	0.66 [a]	0.07 [a]
Female (n)	30	17	66		
Age					
Median (years)	62	63	59		
Range (years)	48 – 85	47 – 88	51 – 78		
Mean (SD)	62.5 ± 7.6	63.7 ± 9	62.2 ± 7	0.7 [b]	0.07 [b]
Smoking status					
Non-smokers	40 (33.9%)	11 (9.8%)	96 (33.1%)		
Smokers	78 (66.1%)	101 (90.2%)	194 (66.9%)	0.9692 [a]	< 0.0001 [a]

a Chi^2-test
b t-test

for statistical significance using the Chi^2-test. Student's t-test was used to determine differences in means of age between cancer cases and controls. Bonferroni correction was taken into consideration between different combinations of GST genopytes and the risk of lung cancer because of the multiple comparisons carried out in this study. According to this correction p-value <0.017 (0.05/3) for combinations of two polymorphism and p-value <0.007 (0.05/7) for combinations of three polymorphism was considered statistically significant. Gene-smoking interactions adjusted for age, gender and relevant genotypes were not analyzed due to the small number of patients.

Results

The distributions for gender and age among cases (AC and SCC separately) and controls were not statistically different (tab. 1). The distribution of smokers was not significantly different between cases with AC (66.1%) and controls (66.9%). The distribution of smokers was significantly different between cases with SCC (90.2%) and controls (66.9%), what indicates on the main etiological factor in genesis of this histological type of carcinoma. The distribution of *GSTT1*, *GSTM1* and *GSTP1* genotypes and their respective ORs for lung AC and SCC are presented in table 2 and 3 separately.

The distribution of GST genotypes among controls in this study was found to be similar to those reported for Caucasians.

The frequencies of the *GSTT1 null* genotype were significantly elevated in cases with AC (29.6%) when compared with the controls (16.6%) (χ^2=8.1; p= 0.004). The adjusted ORs of lung AC for the *GSTT1 null* genotype was 2.13 (95% CI = 1.29 – 3.51) (tab. 2). This association was not observed in SCC. Elevated frequencies of the *GSTT1 null* in cases with SCC (21.5%) compared with the controls (16.6%) were not significant. The adjusted ORs of lung SCC for the *GSTT1 null* genotype was minimally elevated 1.38 (tab. 4).

The GSTM1 distributions did not show significant differences between the controls (55.2%) and AC (54.2%) and SCC (58.9%) patients (tab. 2, 4).

The frequencies of three *GSTP1* genotypes among controls (Ile/Ile, 52.8; Ile/Val, 39.7; and Val/Val, 7.5%) were not different from those expected from the Hardy-Weinberg equilibrium. Frequencies of the *GSTP1* homozygote (Val/Val) were elevated in cases with AC and SCC (11.0% and 9.8%, respectively) when compared to the controls (7.5%), but the difference was not significant.

We did not find statistically significant associations between the risk of AC or SCC and any of the *GSTM1* and *GSTP1* polymorphisms.

The ORs for combination of *GSTM1*, *GSTT1* and *GSTP1* genotypes for lung AC and SCC are shown in table 3, 5 respectively. In AC it was found 2.83 times statistically higher risk for development of this histological type of lung cancer (95% CI = 1.34 – 6.01; P = 0.005) in combination of *GSTT1null* and *Ile/Val* or *Val/Val* genotypes. The other combined genotypes were not significantly associated with higher risk than the separate genotypes. We did not observe any significant interactions between combinations of genetic polymorphisms of *GSTT1*, *GSTM1*, *GSTP1* and the SCC cases.

Discussion

Molecular – epidemiologic studies focus on investigation of enzymes of the first and second phase of xenobiotic metabolism. The extent of association between human *GSTM1*, *GSTT1* or *GSTP1* genetic polymorphisms and lung cancer risk is discussed controversially (22). It is most likely because it is about genes with low penetration ability and moreover in observed control groups composed from healthy individuals there was also found difference in frequency of *GST* polymorphisms among races and also a smaller heterogenity was found in Caucasian population from different countries (23). The distribution of *GST* genotypes among controls

Table 2. Distribution of genotype and frequency of alleles in polymorphisms of GSTP1, GSTT1 and GSTP1 in patients with AC.

Genotypes	Cases n(%)	Controls n(%)	ORs	95% CI	p value	χ
GSTT1						
present	83 (70.4%)	242 (83.4%)	ref.			
null	35 (29.6%)	48 (16.6%)	**2.13**	1.29 – 3.51	**0.004**	8.10
GSTM1						
positive	54 (45.8%)	130 (44.8%)	ref.			
null	64 (54.2%)	160 (55.2%)	0.96	0.63 – 1.48	0.95	0.004
GSTP1						
Ile/Ile	59 (50%)	153 (52.8%)	ref.			
Ile/Val	46 (39%)	115 (39.7%)	1.04	0.66 – 1.64	0.97	0.002
Val/Val	13 (11%)	22 (7.5%)	1.53	0.72 – 3.24	0.36	0.85
* *Val*	59 (50%)	137 (47.2%)	1.12	0.73 – 1.71	0.69	0.16

* Ile/Val or Val/Val

Statistically significant differences between cases and controls are shown in bold.

Table 3. Mutual combinations of genotypes of GSTP1, GSTT1 and GSTP1 and their association with adenocarcinoma.

Combined genotypes			Cases n(%)	Controls n(%)	ORs	95% CI	p value [a]	χ
GSTT1	***GSTM1***							
positive	*positive*		35 (29.7%)	104 (35.9%)	ref.			
positive	*null*		48 (40.7%)	138 (47.6%)	1,03	0.62 – 1.71	0,898	0,016
null	*positive*		19 (16.1%)	26 (8.9%)	2,17	1.07 – 4.39	0,029	4,762
null	*null*		16 (13.5%)	22 (7.6%)	2,16	1.02 – 4.57	0,041	4,168
GSTT1	***GSTP1***							
positive	*Ile/Ile*		41 (34.7%)	123 (42.4%)	ref.			
positive	** Val*		42 (35.6%)	119 (41.1%)	1,06	0.64 – 1.74	0,822	0,050
null	*Ile/Ile*		18 (15.3%)	30 (10.3%)	1,8	0.91 – 3.56	0,089	2,889
null	** Val*		17 (14.4%)	18 (6.2%)	**2,83**	1.34 – 6.01	**0,005**[b]	7,760
GSTM1	***GSTP1***							
positive	*Ile/Ile*		28 (23.7%)	69 (23.8%)	ref.			
positive	** Val*		26 (22%)	61 (21%)	1,05	0.56 – 1.98	0,880	0,023
null	*Ile/Ile*		31 (26.3%)	84 (29%)	0,9	0.50 – 1.66	0,757	0,096
null	** Val*		33 (28%)	76 (26.2%)	1,07	0.59 – 1.95	0,825	0,049
GSTT1	***GSTM1***	***GSTP1***						
positive	*positive*	*Ile/Ile*	16 (13.6%)	50 (17.2%)	ref.			
positive	*positive*	** Val*	19 (16.1%)	54 (18.6%)	1,1	0.51 – 2.37	0,809	0,059
positive	*null*	*Ile/Ile*	25 (21.2%)	73 (25.2%)	1,07	0.52 – 2.21	0,854	0,034
positive	*null*	** Val*	23 (19.4%)	65 (22.5%)	1,11	0.53 – 2.31	0,789	0,072
null	*positive*	*Ile/Ile*	12 (10.2%)	19 (6.5%)	1,97	0.79 – 4.93	0,143	2,150
null	*positive*	** Val*	7 (5.9%)	7 (2.4%)	3,13	0.95 – 10.27	0,053	3,741
null	*null*	*Ile/Ile*	6 (5.1%)	11 (3.8%)	1,7	0,54 – 5.35	0,357	0,848
null	*null*	** Val*	10 (8.5%)	11 (3.8%)	2,84	1.02 – 7.92	0,042	4,155

* Ile/Val or Val/Val

Statistically significant differences between cases and controls are shown in bold.

a Corrected for multiple testing.

b Differences statistically significant after Bonferroni correction as p-values are < 0.017.

Table 4. Distribution of genotypes and frequency of alleles in polymorphisms of GSTP1, GSTT1 and GSTP1 in patients with SCC.

Genotypes	Cases n(%)	Controls n(%)	ORs	95% CI	p value	χ
GSTT1						
positive	88 (78.5%)	242 (83.4%)	ref.			
null	24 (21.5%)	48 (16.6%)	1.38	0.80 – 2.38	0.32	0.99
GSTM1						
positive	46 (41.1%)	130 (44.8%)	ref.			
null	66 (58.9%)	160 (55.2%)	1.17	0.75 – 1.81	0.57	0.32
GSTP1						
Ile/Ile	56 (50%)	153 (52.8%)	ref.			
Ile/Val	45 (40.2%)	115 (39.7%)	1.07	0.67 – 1.7	0.87	0.03
Val/Val	11 (9.8%)	22 (7.5%)	1.37	0.62 – 3.0	0.57	0.33
** Val*	56 (50%)	137 (47.2%)	1.12	0.72 – 1.73	0.7	0.15

** Ile/Val or Val/Val*

in this study was found to be similar to those reported for Caucasians.

Different histological subtypes of lung cancer particularly may also be related to respective exposures or factors and that is why they need to be analyzed separately (24). Therefore we studied lung cancer patients and controls to estimate the association of the *GSTM1*, *GSTT1* or *GSTP1*-polymorphisms in relation to two most frequent histological types of lung cancer.

Table 5. Mutual combinations of genotypes of GSTP1, GSTT1 and GSTP1 and their association with SCC.

Combined genotypes			Cases n(%)	Controls n(%)	ORs	95% CI	p value [a]	χ
GSTT1	***GSTM1***							
positive	*positive*		34 (30.4%)	104 (35.9%)	ref.			
positive	*null*		54 (48.2%)	138 (47.6%)	1,17	0.73 – 1.97	0,480	0,499
null	*positive*		12 (10.7%)	26 (8.9%)	1,41	0.64 – 3.1	0,389	0,744
null	*null*		12 (10.7%)	22 (7.6%)	1,67	0.75 – 3.72	0,209	1,581
GSTT1	***GSTP1***							
positive	*Ile/Ile*		44 (39.3%)	123 (42.4%)	ref.			
positive	** Val*		44 (39.3%)	119 (41.1%)	1,04	0.63 – 1.68	0,894	0,018
null	*Ile/Ile*		12 (10.7%)	30 (10.3%)	1,12	0.53 – 2.37	0,771	0,085
null	** Val*		12 (10.7%)	18 (6.2%)	1,86	0.83 – 4.18	0,127	2,330
GSTM1	***GSTP1***							
positive	*Ile/Ile*		23 (20.5%)	69 (23.8%)	ref.			
positive	** Val*		23 (20.5%)	61 (21%)	1,13	0.58 – 2.22	0,720	0,129
null	*Ile/Ile*		33 (29.5%)	84 (29%)	1,18	0.63 – 2.19	0,604	0,270
null	** Val*		33 (29.5%)	76 (26.2%)	1,3	0.7 – 2.43	0,406	0,691
GSTT1	***GSTM1***	***GSTP1***						
positive	*positive*	*Ile/Ile*	17 (15.1%)	50 (17.2%)	ref.			
positive	*positive*	** Val*	17 (15.1%)	54 (18.6%)	0,93	0.43 – 2.01	0,846	0,038
positive	*null*	*Ile/Ile*	27 (24.1%)	73 (25.2%)	1,09	0.54 – 2.2	0,815	0,055
positive	*null*	** Val*	27 (24.1%)	65 (22.5%)	1,22	0.6 – 2.49	0,580	0,306
null	*positive*	*Ile/Ile*	6 (5.4%)	19 (6.5%)	0,93	0.32 – 2.71	0,892	0,018
null	*positive*	** Val*	6 (5.4%)	7 (2.4%)	2,52	0.74 – 8.55	0,130	2,295
null	*null*	*Ile/Ile*	6 (5.4%)	11 (3.8%)	1,6	0.51 – 5	0,413	0,671
null	*null*	** Val*	6 (5.4%)	11 (3.8%)	1,6	0.51 – 5	0,413	0,671

* Ile/Val or Val/Val

a Corrected for multiple testing.

There was a change in occurrence of histological types of lung carcinomas in last two decades and adenocarcinoma became the most frequent (30 – 40 % of all). In past squamous cell carcinoma dominated. Percentual proportion of adenocarcinomas is higher in Asian population than in North America or Europe. Adenocarcinoma dominates in young people, women of every age, non-smokers and ex-smokers who didn't smoke for longer time. There is no satisfying explanation for this change of histological type. A possible cause can be the change of compounds of inhaled carcinogens and filtration of tobacco smoke, increasing production of volatile nitrosamines and their accumulation in terminal bronchioles and alveols when deeper inhalation (25). The studies describe various genetic and epigenetic alterations in tumor cells of lung cancer, which differ according to various histological types and thus suggest different pathogenic ways of each type of lung cancer development (26). That is why some of the polymorphisms of single nucleotide could be associated with different types of lung cancer.

Previous studies did not find any significant effect of the *GSTT1* null genotype on lung cancer (27-33). Two of the studies by Saarikoski et al. and Stucker et al. found that the *GSTT1* null genotype did not elevate the risk with neither lung AC or SCC (33, 34). So far Spitz et al. have shown statistically significant risk with OR= 1.41 for *GSTT1* null genotype (35). Also the study on Chinese (31) and Indian study (36) confirm the association between *GSTT1 null* genotype and lung cancer. A recent study in Japan provided evidence that the *GSTT1 null* genotype is a risk factor for lung AC development and that the genetic factors for susceptibility to AC differ from those for SCC (37). Sorensen et al. found a strong effect of the *GSTT1* polymorphism for SCC, suggesting that the GSTT1 enzyme may be important in preventing the development of this type of lung cancer (38). We found that in the Slovak population the *GSTT1 null*-genotype was significantly associated only with lung AC cancer risk (OR = 2.13), but not with SCC.

According to the published data the association of *GSTM1* polymorphism with the lung cancer formation is controversial. A study by Woodson et al. in Caucasians reported a significant association of lung AC with the GSTM1 null genotype (39). Mc. Williams et al. in their meta-analysis mention that *GSTM1* null genotype is a moderate risk factor for lung cancer formation with OR=1.41 (95%CI 1.23-1.61), while this association is more significant in Japanese population (OR= 1.6; 95% CI= 1.2-2.1) than in Caucasian population (OR= 1.2; 95% CI=

1.0-1.4). According to this study 59.1% of patients with lung AC are carriers of *GSTM1* null genotype and it was provided non-significantly increased risk in patients with AC, OR= 1.449 (40). Next meta-analysis by Houlston et al. of genotype studies has shown 1.13 times higher risk of lung cancer formation (95%CI= 1.04-1.25) (41). Other meta-analysis published by Benhamou et al. did not confirm statistically significant relationship between carriers of *GSTM1 null* genotype and susceptibility to lung cancer (22). The analysis of 14 case-control studies on Caucasian population did not find increased risk of *GSTM1 null* genotype for lung cancer (42). Another meta-analysis found the strongest effect of the *GSTM1* polymorphism for squamous and small cell carcinomas. The last meta-analysis from Carlsten et al. observed modest effect of the *GSTM1* null variant on lung cancer risk (43). In our population we did not find increased risk for AC or SCC formation in patients with *GSTM1* null genotype.

The most of the studies have been monitoring the association of *GSTP1* polymorphism in codon 105 and lung cancer did not prove significant risk of *GSTP1 Val* genotype (28, 33, 44-50). Not even in our study was found significant association between polymorphism of *GSTP1* and AC or SCC formation.

Some studies have suggested that subjects with a defect genotype in 2 *GST* genes are at higher risk of lung cancer formation than subjects with no or only 1 defect of GST gene (33). More studies evaluate the combined effects of *GSTM1* and *GSTT1*, *GSTP1*. When following the combinations of *GSTT1* and *GSTM1* genotypes, the most of the studies did not find any association (21, 30, 51). Other studies have shown: significant association of combination of both null genotypes of *GSTM1* and *GSTT1* with the occurrence of SCC (33); significantly increased risk for formation of lung cancer in presence of both null polymorphisms of *GSTM1* and *GSTT1* (28, 31). Increased risk of lung cancer development was found in Japanese men smokers at the age of 50-69 years with combination of genotypes *GSTP1* and *GSTM1 null* (47); increased risk in individuals from Caucasian population with the combination of genotype *GSTP1 Val/Val* and *GSTM1 null* (48) and combination of genotypes *GSTP1 Val/Val* and *GSTM1 null* represents 6.9 times higher risk for lung cancer development (34). *GSTT1 null* genotype has in our study shown to be a strong factor increasing the risk of AC development. For AC we have not find the risk combination of both null *GSTT1* and *GSTM1* genotypes. Statistically significant risk was presented by the combination *GSTT1 null* with *GSTP1Ile/Val* or *Val/Val (*OR = 2.83). No significant association of combinations of single genotypes of *GST* and lung SCC was observed.

In conclusion, the results of this study indicate that the *GSTT1* null-genotype and combination *GSTT1* null and *GSTP1 Ile/Val* or *Val/Val* are associated with the elevated risk of lung adenocarcinoma in Slovak population. *GSTT1*, *GSTM1*, *GSTP1* polymorphism are not associated with SCC. Our study suggests that genetic make-up in metabolizing genes may increase susceptibility towards lung cancer development. They should be studied in association with other factors as race, ethnic, age, smoking, exposition to xenobiotics and histological type.

Acknowledgements: This work was supported by project "CENTER OF TRANSLATIONAL MEDICINE" co-financed from EU sources and European Regional Development Fund ; by grants of the Ministry of Education AV 4/0013/06, VEGA 1/3397/06; Grant of Commenius University UK/48/2010 and Europian Social Found.

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