Eur J Med Res (2009) 14(Suppl. IV): 275-279 © I. Holzapfel Publishers 2009

Gene Polymorphisms of Biotransforming Enzymes (GSTs) and their Association with Lung Cancer in the Slovakian Population

T. Matakova[1], M. Sivonova[1], E. Halasova[2], D. Mistuna[3], A. Dzian[3], P. Berzinec[4], L. Letkova[1], D. Dobrota[1]

[1]Department of Medical Biochemistry and [2]Department of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Martin; [3]Clinic of Surgery, Jessenius Faculty of Medicine and MFH, Martin; Specialized Hospital of St. Zoerardus Zobor, Nitra, Slovakia

Abstract

Objective: The aim of present study was to present the results of a case-control study focused on genetic polymorphisms of selected Phase II metabolizing enzymes (GSTM1, T1, and P1) and to investigate the association of these polymorphisms with lung cancer risk in the Slovakian population.

Material and methods: The study encompassed 160 lung cancer cases and 220 controls. DNA was extracted from peripheral blood leukocytes, and the polymorphisms of GSTM1, GSTT1 and GSTP1 enzymes were determined by PCR-based methods. We determined the genotype distribution of all these genes and their combinations. The association between specific genotypes and the development of lung cancer were examined using logistic regression analysis to calculate odds ratios (OR) and 95% confidence intervals (CI).

Results: We found that the GSTM1 null genotype (OR=1.6; 95% CI=1.03-2.4; χ^2=4.08, and P=0.04) was associated with elevated risk. A significant correlation also was found for the combined genotypes of GSTM1 null and GSTP1 Ile/Val and Val/Val (OR=2.01; 95% CI=1.1–6.1; χ^2=3.6, and P=0.02) and GSTM1 null and GSTT1 positive (OR=2.00; 95% CI=1.2–3.2; χ^2=7.3, and P=0.006).

Conclusions: We conclude that the genotype of metabolizing enzymes and allelic combinations underscore the risk for lung cancer. Individual risk assessment may be further improved by increasing the number of polymorphisms studied and combining them with the traditional epidemiological risk factor.

Key words: glutathione S-transferase T1, M1, and P1, lung cancer, polymorphism

Introduction

It is well established that the prevalence of lung cancer have been increasing over the last decades in many countries, including Slovakia. Tobacco smoking is clearly the strongest risk factor for lung cancer. Other risk factors include air pollution, cooking, diet, and occupational exposures, such as soot and asbestoses [1]. Glutathione S-transferase (GSTs) constitutes a superfamily of ubiquitous, multifunctional enzymes that play a key role in cellular detoxification. The GSTs catalyze the conjugation of tripeptide glutathione (GSH) to a wide variety of exogenous and endogenous chemicals with an electrophilic functional group (e.g., products of oxidative stress, environmental pollutants, and carcinogens), thereby neutralizing their electrophilic sites rendering the products more water-soluble [2]. Because electrophiles can bind to DNA, forming adducts and potentially DNA mutations, GSTs play a critical role in protecting cells against the cytotoxic and mutagenic effects of these reactive compounds. GSTs are divided into two distinct superfamily members: the membrane bound microsomal and cytosolic enzymes. On the basis of sequence homology and immunological cross reactivity, human cytosolic GSTs have been grouped into eight classes, designated GST-alpha (α), mu (μ), pi (π), sigma (σ), omega (ω), theta (θ), kappa (κ), and zeta (ζ) [3]. One major reason of individual variation of GST activity is the existence of polymorphism in these genes. The most extensively studied to date are GSTM1, GSTT1 and GSTP1.

Five GST mu class genes (M1-M5) have been identified clustered on chromosome 1 [4]. The frequency of the GSTM1 null genotype varies significantly among ethnic populations, and 38-67% Caucasians do not express GSTM1 due to the GSTM1 null genotype [5]. GSTP1 appears the most widely distributed GST isoenzyme. Two polymorphisms have been described in GSTP1 gene, one in codon 105 and the other in codon 114. The codon 114 variant allele is only found in combination with the codon 105 variant allele. Codon 105 polymorphism modifies the enzyme¢s specific activity [6]. Functional polymorphism has been described for GSTP1, resulting in an I105V substitution and leading to a lower enzyme activity. Two GST theta class genes, GSTT1 and GSTT2, have been characterized and in humans, a GSTT1 null genotype may be present at frequency of ~10-20% in Caucasians [5].

Material and Methods

Study Population

Patients and control subjects signed informed consent in accordance with the requirements of the Ethics Commission for Research at the Jessenius Faculty of Medicine in Martin, Slovakia. Blood samples were obtained from 160 random lung cancer patients (cases). This group consisted of patients who attended the Surgery Clinic and Oncology Center of Martin¢s Faculty Hospital in Martin in a period of November - December 2008. The following data were retrieved from medical records: age, date of diagnosis of lung cancer, personal history, family history (number of relatives affected by lung cancer, or other malignant diseases), clinical stage, TNM classification according to UICC, tumor size, histological grade, and the type of tumor. The main criterion for inclusion of patients into the study was histologically verified lung cancer malignancy. This group of 160 patients included 43 (27%) women and 117 (73%) men. The median age was - cases 63 ± 9 and controls 63 ± 11 years. The control group comprised of 220 healthy volunteers from the same geographic region (middle Slovakia) and was matched for age, gender, and Caucasian ethnicity. Samples from the control subjects were collected during the same period as those for the cases.

Genotype Analysis

Genomic DNA was isolated using standard techniques (proteinase K digestion, the phenol/chloroform extraction and ethanol precipitation, dissolved in TE buffer (pH=7.5) from blood drawn into 4.5 ml EDTA tubes and stored at -20°C until use. The concentration of DNA was adjusted to 100 µg/ml and the DNA was stored at -20°C. All genotyping analyses were PCR-based, with a total volume of 25 µl for each reaction containing a PCR buffer (16.6 mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, pH=8.8, 1.2 µl DMSO, 1.2 µl DTT), 0.2mM deoxynucleotide triphosphates, 0.5 U *Taq* polymerase, 25 pM primers and 100ng DNA. Digests were electrophoresed on 2% agarose gel and photographed. In all reactions, positive and negative controls were included. As a quality control, 10-20% of all samples were repeated as blinded duplicates.

GSTM1 and GSTT1

The GSTM1 and GSTT1 genetic polymorphisms were determined simultaneously by multiplex PCR. Primer sequences used were: 5'-GAACTCCCTGAAAAGC TAAAGC-3' and 5'-GTTGGGCTCAAATATACGG TGG-3' for GSTM1; and 5'-TTCCTTACTGGTCCT-CACATCTC-3' and 5'-TCACCGGATCATGGCCAG CA-3' for GSTT1; and 5'-CAACTTCATCCACGT TCACC-3' and 5'-GAAGAGCCAAGGACAGGTAC-3' for β-globin. After an initial denaturation at 95°C for 1 min, the samples underwent 35 cycles of 30 s at 94°C, 30 s at 64°C, and 1 min at 72°C, followed by the final extension at 72°C for 5 min. The absence of the GSTM1- and/or GSTT1-specific PCR-product indicated the corresponding null genotype. β-globin was amplified in the same reaction as an internal positive control. PCR-reaction products were analyzed by gel electrophoresis (2% agarose) and ethidium bromide staining for the presence of a 273bp GSTM1 product, a 480bp GSTT1 product, and a 320bp β-globin product. The lack of a GSTM1 or GSTT1 amplification product in the presence of an actin amplification product is consistent with the homozygous null genotype.

GSTP1

A to G polymorphism at codon 105 was determined by PCR of 176bp fragment and restriction fragment length polymorphism (RFLP). The reported PCR-RFLP protocol [6] was modified as follows. Primer sequences for GSTP1 were 5'-GTA GTT TGC CCA AGG TCA AG-3' and 5'-AGC CAC CTG AGG GGT AAG-3'. PCR cycles started with an initial denaturation at 94°C for 1 min, five cycles were carried out at 94°C for 15 s, 64°C for 30 s, and 72°C for 1 min with an annealing temperature that decreased by 1°C for each cycle. Then, 30 cycles were carried out at 94°C for 15 s, 59°C for 30 s, and 72°C for 1 min, followed by a final extension for 5 min. An amount of 10 ml of the amplicon was digested for 4 h with 5 units of the restriction enzyme *Alw* 26I at 37°C. Three variants were identified: Ile/Ile, Ile/Val, and Val/Val.

Statistical Analysis

The Chi-square (χ^2) test was used to determine the significance of differences from the Hardy-Weinberg equilibrium and the independence of genotype frequency between cases and controls. Odds ratio (OR) and 95% confidence interval (95% CI) were obtained from an unconditional logistic regression model. A level of $P<0.05$ was accepted as statistically significant. We also analyzed the prevalence of selected combinations of genotypes as follows: *GSTM1+GSTT1*, *GSTM1+GSTP1*, and *GSTT1+GSTP1*. The selection of this combination was based on the hypothesis that the carrier of at least one variant allele in both combined genes may be at higher risk. All statistical calculations were performed using Microsoft Excel and MedCalc v.5 software for Windows.

Results

Relevant characteristics of the study cases are in Table 1. The observed frequencies and genotype distributions in our control group did not differ significantly from the data on the majority of other European Caucasian subpopulations.

The frequency of genotypes for GSTM1, GSTT1, and GSTP1 for cases and controls is shown in Table 2. The most interesting result was obtained concerning the distribution of genotypes in GSTM1. The proportion of GSTM1 null genotype in patients (59%) was higher than that in the controls (48%); this result was statistically significant (OR=1.6; 95% CI: 1.03-2.4; $\chi^2=4.08$; P=0.04).

For GSTT1, 21% of controls and 26% of cases were null. There was no difference in the frequency of the null genotype between the matched cases and controls

Table 1. Descriptive characteristics of lung cancer cases.

Sample size (n)		160
Gender n (%)	male	117 (73)
	female	43 (27)
Age (yr)	mean (SD)	63 ±9
	median	66
Smoking status n (%)	non-smokers	34 (21)
	current smokers	126 (79)
Cell type n (%)	adenocarcinoma	75 (47)
	squamous-cell carcinoma	54 (34)
	large-cell lung cancer	4 (3)
	small-cell lung cancer	13 (8)
	mixed, non classifiable lung cancer	14 (9)
Stage n (%)	IA	15 (10)
	IB	29 (18)
	IIA	2 (1)
	IIB	6 (4)
	IIIA	28 (18)
	IIIB	21 (13)
	IV	58 (36)
Site n (%)	Ca bronchogenes l.dx.	91 (57)
	Ca bronchogenes l.sin	69 (43)

(OR=0.97; 95% CI=0.64-1.48). Concerning the GSTP1 alleles, the frequencies for the Val-105 were 36% for controls and 29% for cases. In the controls, 8% of individuals were homozygous and 35% were heterozygous for Val-105, and 57% were homozygous for Ile-105. In the cases, the figures were 8, 41, and 51 %, respectively. We did not find statistical differences between the patients with lung cancer and the control group.

Analysis of Combination of Genotypes

We evaluated the association of polymorphisms with lung cancer risk (Table 3). An analysis considering two loci indicates two significant associations, both involving the GSTM1 null genotype. Individuals who presented both GSTM1 null genotype and GSTT1 positive genotype had a two times higher risk (OR=1.98; 95% CI=1.23-3.18; P=0.006) of having lung cancer. A combination of GSTP1 heterozygous for Val-105 and GSTM1 null was found in 16% of the controls and 24% of the cases; this interaction also was significant (OR=2.4; 95% CI=1.1-3.8; P=0.03; χ^2 = 4.6).

There was no difference in the distribution of the GSTP1 genotypes between the controls or cases according to the GSTT1 genotype. Examination of the segregation of null and positive genotypes for GSTT1 and GSTM1 shows that they were randomly distributed in the cases. The occurrence of the putative "worst" combination of GSTT1 null and GSTM1 null was found in 12% of all controls and 13% of cases (not significant).

Discussion

In the last two decades, and especially in recent years, a large body of medical and epidemiologic literature has described genetic variants that appear to affect susceptibility to lung cancer. Multiple genes, including several in the GST group, cytochrome P-450 1A1 (CYP1A1), and several others in cytochrome P-450 (CYP) group, microsomal epoxide hydroxylase (mEH), aryl hydrocarbon receptor (AhR), NAD(P)H quinone oxidoreductase 1 (NQO1), myeloperoxidase (MPO), and N-acetyltransferase (NAT) , have been variably associated with the disease [7-9].

Previous data on the role of GSTs markers in lung cancer are conflicting. These differences are probably

Table 2. Genotype and allele frequencies in GSTM1, GSTT1, and GSTP1 polymorphisms.

Genotype/group	Case n (%)	Control n (%)	OR	95% CI	P value	χ^2
GSTT1	n = 160	n = 220				
+	119 (74)	174 (79)	1.0	(ref.)	0.33	0.91
null	41 (26)	46 (21)	1.3	0.81-2.1		
GSTM1	n = 160	n = 220				
+	66 (41)	115 (52)	1.0	(ref.)	**0.04**	4.08
null	94 (59)	105 (48)	1.6	1.03-2.4		
GSTP1	n = 160	n = 220				
Allele						
Ile	228 (71)	328 (75)	1.0	(ref.)		
Val	92 (29)	112 (36)	1.2	0.86-1.63	0.35	0.86
Ile/Ile	81 (51)	126 (57)	1.0	(ref.)		
Ile/Val	66 (41)	76 (35)	1.4	0.88-2.08	0.20	1.57
Val/Val	13 (8)	18 (8)	1.1	0.52-2.42	0.91	0.01
Ile/Val or Val/Val	79 (49)	94 (43)	1.3	0.86-1.96	0.23	1.39
					0.32*	0.97*

*(P value, from χ^2 test for trend)

Table 3. Combination of genotype frequencies in GSTM1, GSTT1, and GSTP1 polymorphisms.

	Case n (%)	Control n (%)	OR	95% CI	P value	χ^2	P value	χ^2	*P value	*χ^2
GSTT1 + GSTM1	n = 160	n = 220								
+ / +	45 (28)	95 (43)	1.00	(ref.)						
+ / null	74 (46)	79 (36)	1.98	1.23-3.18	**0.006**	7.32	**0.02**	9.59	**0.04**	4.04
null / +	21 (13)	20 (9)	2.21	1.09-4.50	0.04	4.19				
null / null	20 (13)	26 (12)	1.62	0.82-3.21	0.22	1.49				
GSTT1+GSTP1	n = 160	n = 220								
+ / Ile/Ile	60 (38)	104 (47)	1.00	(ref.)			0.29	3.7	0.12	2.39
+ / Ile/Val	50 (31)	56 (26)	1.54	0.94-2.54	0.1	2.55				
+ / Val/Val	9 (6)	14 (6)	1.11	0.46-2.73	0.99	0.01				
+ / Ile/Val or Val/Val	59 (37)	70 (32)	1.50	0.91-2.34						
null / Ile/Ile	21(13)	22 (10)	1.65	0.84-3.26	0.19	1.66				
null / Ile/Val	16(10)	20 (9)	1.38	0.67-2.88	0.49	0.48				
null / Val/Val	4(3)	4 (2)	1.73	0.42-7.18	0.69	0.15				
null / Ile/Val or Val/Val	20(13)	24 (11)	1.44	0.74-2.83	0.14	2.14				
GSTM1+GSTP1	n = 160	n = 220								
+ / Ile/Ile	35(22)	66 (30)	1.0	(ref.)			0.1	6.19	**0.01**	6.0
+ / Ile/Val	27(17)	40 (18)	1.27	0.67-2.40	0.56	0.34				
+ / Val/Val	4(3)	9 (4)	0.83	0.24-2.92	0.97	0.001				
+ / Ile/Val or Val/Val	31(19)	49 (22)	1.19	0.65-2.19	0.68	0.17				
null / Ile/Ile	46(29)	60 (27)	1.44	0.82-2.54	0.25	1.32				
null / Ile/Val	39(24)	36 (16)	2.04	1.11-3.76	**0.03**	4.63				
null / Val/Val	9(6)	9 (4)	1.88	0.69-5.18	0.32	0.96				
null / Ile/Val or Val/Val	48(30)	45 (21)	2.01	1.13-3.58	**0.02**	5.01				

*(P value, from χ^2 test for trend)

due to ethnic and/or environmental heterogeneity as well as to gene/environment and gene/gene interactions [10]. Molecular epidemiology studies indicated that a single genetic polymorphism of GST may constitute a moderate lung cancer risk factor. However, the risk is higher when interactions with more GST polymorphisms and other risk factors occur. Previous studies show the GSTM1 null genotype to be associated with increased risk of squamous cell carcinoma [11]. A significant association of GSTM1 null genotype with lung cancer has already been observed in studies performed in large Japanese [12] and Chinese [13, 14] populations. Furthermore, studies in Caucasians reported a significant association of lung AC with the GSTM1 null genotype [15, 16]. In our study, the GSTM1 null genotype was associated with a significant 1.6-fold elevated lung cancer risk.

Ye et al. [17] undertook a large meta-analysis of 130 published studies that had examined associations between one or several of the five GST alleles (GSTM1 and GSTT1 null allele, two missense alleles in GSTP1, and one intron polymorphism in GSTM3). The risk of lung cancer is not strongly associated with the I105V and A114V polymorphisms in the GSTP1 gene or with GSTM3 intron 6 polymorphism. Given the non-significant associations in the larger studies, the relevance of the weakly positive overall associations with the GSTM1 null and the GSTT1 null polymorphisms is uncertain. As lung cancer has important environmental causes, understanding any genetic contribution to it in general populations will require the execution of particularly large and comprehensive studies.

Investigations of the association between GSTP1 polymorphisms and lung cancer have shown that the GSTP1 Val genotype is not significantly associated with lung cancer risk [18, 19]. In the present study on single GSTP1 polymorphisms, there was no association with lung cancer. However, we found a significant association for the combined null GSTM1 genotype and variant GSTP1 allele (OR=2.04). Similar results have been reported by Miller et al. [18] and Jurenkova-Mironova et al. [20].

The association of detoxification genes polymorphisms and lung cancer occurrence allow one to conclude that, in order to assess the distinct genotype as a risk factor, the knowledge of the spectrum of carcinogens influencing the organisms of a population living in a distinct territory and the living conditions of investigated individuals (unhealthy conditions of work, smoking, diet, etc.) are necessary. A degree of risk connected to the gene polymorphisms and its product may be related to the nature of chemical substances

included in polluters in a distinct territory. Moreover, risk factors observed in one condition may be protective factors in another.

In conclusion, our study shows an individual susceptibility to lung cancer estimated by the analysis of GSTs polymorphisms. Further clarification of the puzzling results brought up by this study should help identify individuals with increased cancer risk.

Acknowledgments: Authors are grateful to all patients for their collaboration and technical staff subjects for their technical assistance. This work was supported by grants no. AV4/0013/05 by the Ministry of Education of the Slovak Republic and MZ 2007/48-UK-13 by the Ministry of Health of Slovakia.

Conflicts of interest: The authors reported no conflicts of interest in relation to this article.

References

[1] Alberg AJ, Ford JG, Samet JM. Epidemiology of lung cancer: ACCP evidence-based clinical practice guidelines. 2nd ed. Chest 2007; 132 Suppl: 29S-55S.

[2] Hayes JD, Strange RC (2000) Glutathione S-transferase polymorphisms and their biological consequences. Pharmacology 61: 154-166.

[3] Landi S (2000) Mammalian class theta GST and differential susceptibility to carcinogens. A review. Mutat Res 463: 247-283.

[4] Seidegard J, Ekstrom G (1997) The role of human glutathione transferases and epoxide hydrolases in the metabolism of xenobiotics. Environ Health Perspect 105: 791-799.

[5] De Jong MM, Nolte IM, De Meerman GJ (2002) Low-penetrance genes and their involvement in colorectal cancer susceptibility. Cancer Epidemiol Biomarkers Prev 11: 1332-1352.

[6] Watson MA, Stewart R, Smith G (1998) Glutathione S-transferase P1 polymorphism. Relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 19: 275-280.

[7] Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y (2005) NQO1, MPO, and the risk of lung cancer: a HuGE review. Genet Med 7: 463-478.

[8] Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y (2006) EPHX1 polymorphisms and the risk of lung cancer: a HuGE review. Epidemiology 17: 89-99.

[9] Taioli E, Benhamou S, Bouchardy C, et al. (2007) Myeloperoxidase G-463A polymorphism and lung cancer: a HuGE genetic susceptibility to environmental carcinogens pooled analysis. Genet Med 9: 67-73.

[10] Indulski JA, Lutz W (2000) Metabolic genotype in relation to individual susceptibility to environmental carcinogens. Int Arch Occup Environ Health 2000; 73: 71-85.

[11] Ford JG, Li Y, O´Sullivan MM et al. Glutathione S-transferase M1 polymorphism and lung cancer risk in African-Americans. Carcinogenesis 21: 1971-1975.

[12] Hayashi S, Watanabe J, Kawajiri K (1992) High susceptibility to lung cancer analysed in terms of combined genotypes of P450IA1 and Mu-class glutathione S-transferase genes. Jpn J Cancer Res 83: 866-870.

[13] Lan Q, He X, Costa DJ, et al. (2000) Indoor coal combustion emissions, GSTM1 and GSTT1 genotypes, and lung cancer risk; a case control study in Xuan Wei, China. Cancer Epidemiol Biomarkers Prev 9: 605-608.

[14] Sun GF, Shimojo N, Pi JB, Lee S, Kumagai Y (1997) Gene deficiency of glutathione S-transferase mu isoform associated with susceptibility to lung cancer in a Chinese population. Cancer Lett 113: 169-172.

[15] Rish A, Wikman H, Thiel S, et al. (2001) Glutathione S-transferase M1, M3, T1 and P1 polymorphisms and susceptibility to non-small-cell lung cancer subtypes and hamartomas. Pharmacogenetics 11: 757-764.

[16] To-Figueras J, Gene M, Gomez-Catalan J, et al. (1997) Glutathione S-transferase M1 (GSTM1) and T1 (GSTT1) polymorphisms and lung cancer risk among Northwestern Mediterraneans. Carcinogenesis 18: 1529-1533.

[17] Ye Z, Song H, Higgins JPT, Pharoah P, Danesh J (2006) Five Glutathione S-transferase gene variants in 23.452 cases of lung cancer and 30.397 controls: meta-analysis of 130 studies. PLOS Med 3: e91.

[18] Miller DP, Liu G, De Vivo I, et al. (2002) Combinations of the variant genotypes of GSTP1, GSTM1, and p53 are associated with an increased lung cancer risk. Cancer Res 62: 2819-2823.

[19] Wang Y, Spitz MR, Schabath MB, Ali-Osman F, Mata H, Wu X (2003) Association between glutathione S-transferase p1 polymorphisms and lung cancer risk in Caucasians: a case-control study Lung Cancer 40: 25-32.

[20] Jourenkova-Mironova N, Wikman H, Bouchardy C, et al. (1998) Role of glutathione S-transferase GSTM1, GSTM3, GSTP1 and GSTT1 genotypes in modulating susceptibility to smoking-related lung cancer. Pharmacogenetics 8: 495-502.

Corresponding author:
Erika Halasová
Institute of Medical Biology
Comenius University in Bratislava
Jessenius Faculty of Medicine
Malá hora 4
03754 Martin, Slovakia
Phone: +421 43 4131531
halasova@jfmed.uniba.sk