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Pulmonary Expression of Glutathione *S*-Transferase M3 in Lung Cancer Patients: Association with *GSTM1* Polymorphism, Smoking, and Asbestos Exposure[1]

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ABSTRACT

To characterize the relative roles of glutathione *S*-transferases (GST) M1 and M3 in the susceptibility to lung cancer, the pulmonary expression of GSTM3 was quantified immunochemically and related to the *GSTM1* genotype in 100 lung cancer patients. Among active smokers and recent ex-smokers (for 6 years or less), parenchymal GSTM3 expression was lower in patients with a homozygous *GSTM1* null genotype than in those who were *GSTM1* positive and had similar smoking habits ($P < 0.001$ and $P = 0.004$, respectively). However, in long-term ex-smokers (for 15 years or longer) GSTM3 was not affected by the *GSTM1* genotype. Among active smokers and recent ex-smokers who were homozygous *GSTM1* null, those with a definite or probable exposure to asbestos expressed GSTM3 at significantly higher levels than those for whom it was unlikely ($P = 0.04$). A similar effect of the homozygous *GSTM1* null genotype on GSTM3 expression was not detected in the bronchial epithelium when GSTM3 was visualized immunohistochemically. Different mechanisms may result in an increased risk of either squamous cell or adenocarcinomas in patients with the homozygous *GSTM1* null genotype. Low expression of GSTM3 due to smoking in the parenchymal lung of *GSTM1* null individuals can theoretically favor the development of adenocarcinoma. Our data indicated a predominance of this tumor type in patients with low expression of GSTM3.

INTRODUCTION

GSTs[3] detoxify a number of endogenous and exogenous electrophilic lipophiles (1). Class mu GSTs [for nomenclature see Mannervik *et al.* (2)] are interesting in pulmonary carcinogenesis because they detoxify carcinogenic diol-epoxide derivatives of PAHs present in tobacco smoke (3). They are active also toward reactive oxygen species and a number of products of free radical-initiated lipid peroxidation (3–6). Since the effects of two important pulmonary carcinogens, tobacco smoke and asbestos, may partly be mediated by the oxygen radical damage during the multistage process of carcinogenesis (7, 8), GSTM enzymes may offer protection against DNA damage induced by free radicals as well as electrophilic metabolites of PAH.

In the mu family a gene deletion occurs in *GSTM1* giving rise to a polymorphism so common that 40–60% of the human population are homozygous *GSTM1* null (9, 10). This deletion is associated with an increased risk of lung cancer, mostly squamous cell carcinoma (11–14), but in a few other studies the increased risk has concerned adenocarcinoma only (15, 16).

The effect of the *GSTM1* gene in pulmonary carcinogenesis appears to be indirect, since little or no GSTM1 is detectable in the lung (3, 17, 18). GSTM1 is rich in the liver of those with an expressing allele (namely, GSTM1–1 or GSTM1–0), and this is assumed to be the origin of the effect of *GSTM1* polymorphism on lung cancer (3, 14). However, the lung, albeit at much lower concentrations, expresses another mu class enzyme, namely GSTM3–3 (3, 18).

In this study we have determined the expression of GSTM3 in the lung tissue from 100 patients exposed to tobacco smoke and asbestos, and relate it to *GSTM1* polymorphism, to assess the relative roles of GSTM1–1 and GSTM3–3 in governing susceptibility to lung cancer.

PATIENTS AND METHODS

Sixty active smoker and 40 ex-smoker lung cancer patients were selected from among 170 patients who had undergone pulmonectomy or lobectomy for a tumorous lung lesion at the Helsinki University Hospital during a 5-year study period. Smoking habits and occupational history, and informed consent to the investigation, were obtained by personal interview. All of the active smokers (47 men and 13 women) who had either smoked until the date of operation or given up smoking up to 7 days previously and the ex-smokers who had given up smoking 1 year previously or earlier were included. Ex-smokers fell into two groups, recent ex-smokers (20 men and 2 women) who had given up smoking between 1 and 6 years prior to surgery and long-term ex-smokers (18 men) who had given up smoking for 15 years or longer. The age of the patients ranged from 35 to 80 (median, 65) years.

Assessment of the Exposure to Asbestos and Mineral Dust. The probability of past occupational exposure to asbestos was evaluated by two occupational hygienists on the basis of questionnaires. The definite exposure group included employees in the manufacture of asbestos products, asbestos insulation, or the demolition of old buildings. The probable exposure group included employees in shipyards, the construction industry, or in metal workshops. An exposure time of 1 month was considered as minimum. Employees in various trades with exposure to dust were considered as possibly exposed and those in occupations with no known exposure to asbestos as unlikely exposed (19). Pulmonary asbestos fiber concentration was assessed by reducing a sample from normal lung tissue to ash according to Karjalainen *et al.* (20). Asbestos fibers longer than 1 μm were counted by scanning electron microscopy using a JEOL 100CX-ASID4D electron microscope equipped with an energy-dispersive spectrometer for identification of fiber types.

The exposure to mineral dust other than asbestos was evaluated separately and classified into three categories on the basis of questionnaires. The intense exposure group included employees in foundry work, mining, and stone breaking and the moderate exposure group employees in the construction and demolition of buildings and masonry. The remaining individuals were designated unlikely exposed.

Pathological Examination of Lung Specimens. The histological type of lung tumor was categorized according to the 1981 WHO classification. The site of the origin of the tumor in a cartilaginous bronchus or in a more peripheral airway was assessed macroscopically and by histological examination, taking into account intrabronchial tumor growth and an association of the tumor with bronchial epithelium.

Western Blotting. Samples for Western blotting were taken from the part of the surgical specimen that appeared macroscopically closest to normal. This material, which excluded both tumor tissue and areas behind bronchial obstruction, was frozen at −70°C within 1 h after resection.

A one-fourth homogenate of lung tissue in 0.15 M KCl-50 mM sodium-potassium phosphate buffer (pH 7.4) was obtained using a Thoma homogenizer. The homogenate was centrifuged and the 105,000 × *g* for 60 min supernatant, which contains the soluble GSTs, was collected and stored at −70°C until use.

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[3] The abreviations used are: GST, glutathione *S*-transferase; PAH, polycyclic aromatic hydrocarbon; CI, confidence interval.

SDS-PAGE was performed according to Laemmli (21), and blotting onto nitrocellulose was conducted according to Hayes and Mantle (22). Unspecific binding sites were blocked by soaking the blot overnight in 3% gelatin in 20 mM Tris/HCl buffer (pH 7.5). The blot was then incubated for 3 h with rabbit polyclonal anti-GSTM3 antibody in the same buffer containing 1% gelatin. The anti-GSTM3 displayed no cross-reactivity with other GST mu class protein (4).

GSTM3 was detected with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) using 4-chloro-1-naphthol as the chromogen. The intensity of the immunostaining was quantified by using an EDC scanning densitometer with EZ-Scan software (Helena Laboratories, Beaumont, TX), enabling the determination of peak area integrals. One of the most intensely positive samples was used as a control in each series, and integrals from other samples were adjusted to the control. The peak area integral of the control sample varied 11% (SD) between series. The integrals were converted to protein concentrations by immunostaining a series of dilutions of a standard GSTM3 protein sample.

Immunohistochemistry. The immunohistochemistry for GSTM3 was performed on paraffin-embedded lung tissue from active smokers, as described previously (18). A blind assessment of the intensity of the immunostaining of bronchial epithelium was made and graded into ambiguous, weak, moderate, and intense cytoplasmic staining according to the criteria described earlier (18).

Determination of the *GSTM1* Genotype. *GSTM1* polymorphism was determined in peripheral blood cell DNA using a PCR-based method described in detail elsewhere (23).

Statistical Analyses. The crude evaluation of the relations between GSTM3 and the *GSTM1* genotype, smoking status, and asbestos exposure was done by analyzing the ranks of GSTM3 using the Wilcoxon test (24). Finally, we analyzed the numeric variable GSTM3 by fitting ordinary least-squares multiple regression. The independent variables were dichotomized, or they were entered into the models with the use of category indicators, except for age, which was used as a numerical variable. Calculations were made using the SAS software system (25, 26).

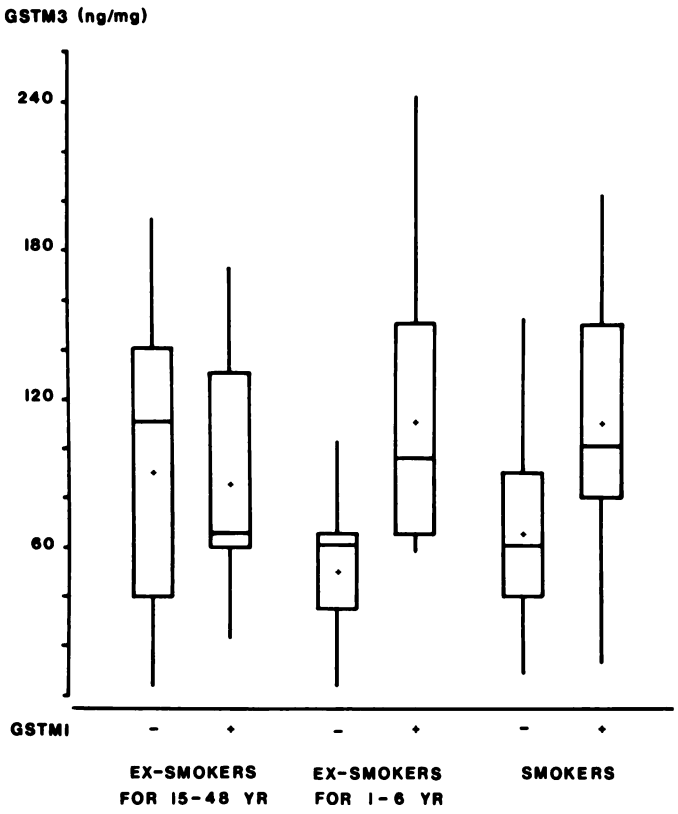

Fig. 1. Distribution of pulmonary parenchymal expression of GSTM3 in *GSTM1*-nulled (−) and *GSTM1*-positive (+) patients according to smoking. *Lower and upper ends of vertical lines*, minimum and maximum values; *horizontal lines from the bottom*, 25th, 50th, and 75th percentiles; and +, mean. Number of patients in groups from *left* to *right*, 9, 9, 12, 10, 35, and 25.

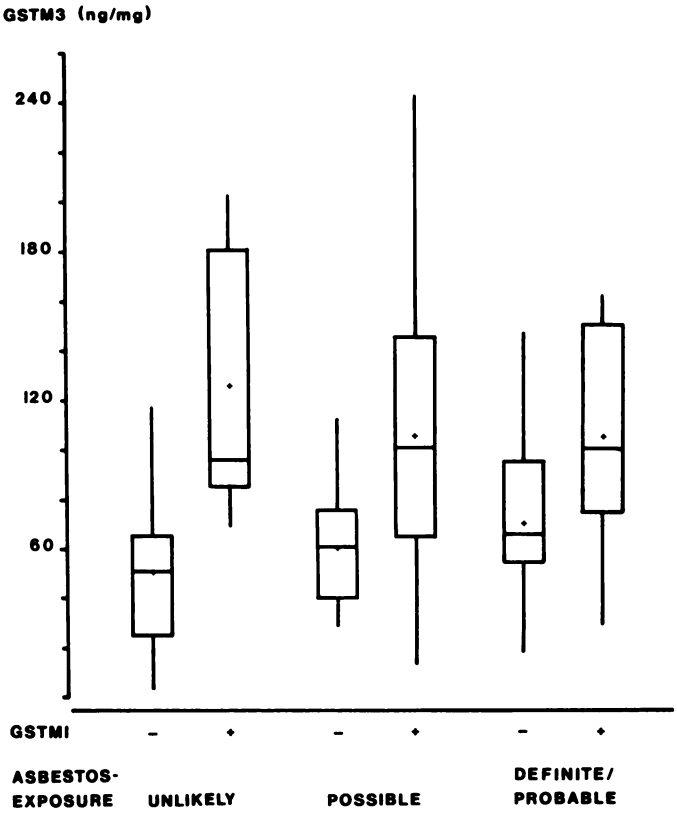

Fig. 2. Distribution of pulmonary parenchymal expression of GSTM3 in *GSTM1*-nulled (−) and *GSTM1*-positive (+) patients (smokers and ex-smokers for 1 to 6 years) according to asbestos exposure classified on the basis of exposure history. Numbers of patients in groups from *left* to *right*, 17, 8, 12, 17, 18, and 10.

RESULTS

Expression of GSTM3, *GSTM1* Polymorphism, and Smoking. The expression of GSTM3 in lung parenchyma determined by quantitative Western blotting varied between individuals from less than 10 to 240 ng/mg cytosolic protein (Figs. 1 and 2). The level of GSTM3 was significantly higher in patients with a *GSTM1*-positive allele (median, 97 ng/mg) as compared with homozygous *GSTM1* null individuals (median, 58 ng/mg; $P < 0.001$).

The association of the expression of GSTM3 with *GSTM1* polymorphism was dependent on smoking habit. The expression of GSTM3 was higher in the *GSTM1*-positive individuals, who were active smokers and recent ex-smokers, than in homozygous *GSTM1* null individuals with similar smoking habits ($P < 0.001$ for active smokers and $P = 0.004$ for recent ex-smokers), whereas no difference by *GSTM1* genotype was observed in long term ex-smokers (Fig. 1 and Table 1). The association of GSTM3 with *GSTM1* polymorphism and smoking was retained in the multiple regression analysis that adjusted the effect of asbestos exposure, age, and gender (Table 1). The expression of GSTM3 increased with age (regression coefficient, 1.4; 95% CI, 0.3, 2.6; $P = 0.01$), whereas gender did not influence on GSTM3.

The data indicate that smoking decreases the expression of pulmonary GSTM3 in patients with a homozygous *GSTM1* null genotype and increases GSTM3 in patients with a *GSTM1*-positive allele (Fig. 1). In homozygous *GSTM1* null individuals, the difference of the expression of GSTM3 (regression coefficient adjusted for the history of asbestos exposure, age, and gender) was −34 ng/mg (95% CI, −73, 5) for recent ex-smokers and −12 ng/mg cytosolic protein (95% CI, −46, 23) for active smokers as compared to long-term ex-smokers. The corresponding regression coefficients showing the increase of

GSTM3 with smoking in *GSTM1*-positive individuals were 33 ng/mg (95% CI, −9, 75) and 35 ng/mg cytosolic protein (95% CI, −2, 72) in recent ex-smokers and active smokers, respectively.

Expression of GSTM3, *GSTM1* Polymorphism, and Asbestos Exposure. The past occupational exposure to asbestos was found to influence the pulmonary parenchymal expression of GSTM3 in active smokers and recent ex-smokers with a homozygous *GSTM1* null genotype. The expression of GSTM3 was higher in patients with a definite or probable occupational exposure to asbestos than in those individuals where exposure was considered to have been either possible or unlikely (Fig. 2, $P = 0.04$ between definitely/probably exposed and unlikely exposed). Similar results were obtained in multiple regression analysis adjusting age and gender both when asbestos exposure was determined by exposure history and by fiber analysis of tissue ash by electron microscopy (Table 2), although differences did not reach statistical significance.

The proportion of homozygous *GSTM1* null individuals was 51% (36/71) of the asbestos-exposed (definite/probable or possible exposure) and 69% (20/29) of patients unlikely to have been exposed to asbestos (Table 2).

In the analysis of the association of GSTM3 with the past exposure to inorganic dust other than asbestos, only patients whose pulmonary asbestos fiber count was less than 1 million fibers/g dry weight were included. The exposure to mineral dust other than asbestos did not influence the expression of GSTM3.

Expression of GSTM3 in the Bronchial Epithelium. The immunohistochemical analysis for bronchial epithelial expression of GSTM3 showed moderate or intense staining in 76% of the active smokers with a *GSTM1*-positive allele and in 91% of those with a homozygous *GSTM1* null genotype.

Table 1 *Mean expression of GSTM3 and regression coefficients (adjusted GSTM3 differences) in GSTM1 nulled (−) and GSTM1 gene-positive (+) patients according to smoking*

Smoking status[a]	No.	GSTM3[b]	Regression coefficient[c]	95% CI
Smokers				
GSTM1$^-$	35	64	Referent	
GSTM1$^+$	25	110	47	22, 72
Ex-smokers for 1–6 yr				
GSTM1$^-$	12	52	Referent	
GSTM1$^+$	10	110	67	28, 107
Ex-smokers for 15–48 yr				
GSTM1$^-$	9	89	Referent	
GSTM1$^+$	9	86	0.3	−42, 43

[a] −, homozygous gene deletion; +, expressing gene.
[b] Unadjusted mean expression of GSTM3 in ng/mg lung cytosolic protein.
[c] Adjusted for history of asbestos exposure (unlikely, possible, definite/probable), age (years), and gender.

Table 3 *Histological type and location of lung cancer according to GSTM1 polymorphism and pulmonary expression of GSTM3*

	GSTM3[a]							
	GSTM$^-$ [b]				GSTM$^+$			
	Low		Medium-high		Low		Medium-high	
	No.	%	No.	%	No.	%	No.	%
Histological type								
Squamous cell	10	50	20	56			17	43
Small cell	2	10	2	5			1	2
Large cell	1	5	4	11			5	12
Adenosquamous			1	3			2	5
Adenocarcinoma	7	35	9	25	4	100	15	38
Location								
Bronchial	9	45	17	55	1	25	20	53
Peripheral	11	55	14	45	3	75	18	47
ND[c]			5				2	
All	20		36		4		40	

[a] GSTM3 expression: low, <48 ng/mg cytosolic protein (25th percentile); medium-high, ≥25th percentile.
[b] −, homozygous gene deletion; +, expressing gene.
[c] ND, not determined.

Expression of GSTM3 and Different Types of Lung Cancer. The distribution of histological types and location of tumors are shown in Table 3 according to *GSTM1* polymorphism and the expression of GSTM3. The proportion of squamous cell carcinomas was slightly higher (53.6%) among the patients with a homozygous *GSTM1* null genotype than in those with a *GSTM1*-positive genotype (38.6%). When the patients were classified according to the expression level of GSTM3, a slight predominance of peripheral tumors (58.3%) and adenocarcinomas (45.8%) was noted in patients with low expression of GSTM3 (less than the 25th percentile or 48 ng/mg cytosolic protein) as compared with patients with moderate or high expression of GSTM3 (46.4% and 31.6%, respectively).

DISCUSSION

To determine whether or not there is a role for GSTM3 in pulmonary carcinogenesis, interindividual variation in the pulmonary expression of GSTM3 and its relation to homozygous *GSTM1* null genotype were investigated. We observed that in active smokers and recent ex-smokers, the expression of GSTM3 was significantly higher in patients with a *GSTM1*-positive allele than in patients with a homozygous *GSTM1* null genotype; an effect not seen in long-term ex-smokers. The association of the homozygous null genotype with susceptibility to lung cancer has already been proposed to originate in hepatic metabolism of tobacco smoke carcinogens (3, 14, 18). How-

Table 2 *Mean expression of GSTM3 and regression coefficients (adjusted GSTM3 differences) according to asbestos exposure in active smokers and recent ex-smokers[a] (for 1 to 6 years) with a different GSTM1 genotype*

	GSTM1$^-$[b]						*GSTM1*$^+$					
	Asbestos exposure			Asbestos fibers[c]			Asbestos exposure			Asbestos fibers		
	Unlikely	Possible	Definite/probable	≤0.5	0.5–2	≥2	Unlikely	Possible	Definite/probable	≤0.5	0.5–2	≥2
No.	17	12	18	25	15	7	8	17	10	20	7	8
GSTM3[d]	48	62	72	55	62	77	124	107	104	105	105	128
Regression coefficient[e]	Referent	11	23	Referent	8	23	Referent	−15	−18	Referent	−5	25
95% CI		−24, 46	−9, 55		−22, 37	−16, 61		−58, 27	−68, 32		−48, 37	−16, 65

[a] For long-term ex-smokers (for 15 to 48 years) regression coefficients obtained from the model are not given because of numerical limitations. Numbers of patients are as follows: *GSTM1*$^-$, asbestos exposure unlikely, 3; possible, 2; definite/probable, 4; *GSTM1*$^+$, asbestos exposure unlikely, 1; possible, 2; definite/probable; 6; *GSTM1*$^-$, asbestos fibers ≤0.5, 2; 0.5–2, 5; ≥2, 2; *GSTM1*$^+$, asbestos fibers ≤0.5, 2; 0.5–2, 4; ≥2, 3.
[b] −, homozygous gene deletion; +, expressing gene.
[c] Asbestos fibers in millions/g dry weight lung tissue.
[d] Unadjusted mean expression of GSTM3 in ng/mg lung cytosolic protein.
[e] Adjusted for age (years) and gender.

ever, the polymorphic expression of GSTM3 that is dependent on the *GSTM1* genotype raises the possibility that the association of the homozygous *GSTM1* null genotype with the susceptibility to lung cancer might be contributed by GSTM3 in the lung itself.

A possible explanation for the polymorphic expression of GSTM3, which is dependent on the *GSTM1* genotype, is that GSTM3 expression is regulated directly by primary or secondary components of tobacco smoke, and that this regulation results from *cis*-acting sequences absent from the *GSTM1* null genotype which has been shown by Seidegård *et al.* (10) to result from a gene deletion. This is feasible because *GSTM1* and *GSTM3* are present in the class mu gene cluster near 1p-13.3 (27, 28), and *cis*-acting sequences in these genes may act cooperatively (29). The persistency of this effect for some years after the cessation of smoking is to be expected since islands of tar sequestered by macrophages are present in lung for some years after the cessation of smoking. The PAH they contain have been shown to be available for metabolism and adduct formation (30). It is assumed that they also contain the regulators referred to above.

The present study indicates that exposure to asbestos induces the expression of GSTM3 in patients with a homozygous *GSTM1* null genotype, a finding that was not observed in patients with a *GSTM1*-positive allele. Inhaled asbestos fibers not rapidly cleared by the mucociliary escalator may penetrate the epithelium to the pulmonary interstitium or be ingested or coated by pulmonary alveolar macrophages and be retained in the lungs for years. Thus, asbestos fibers can induce radical reactions in the lung long after exposure to inhalation of asbestos. Organic hydroperoxides serve as substrates for GSTM1a-1a and GSTM3–3, and GSTM3–3 is also active with hydrogen peroxide (6), suggesting that these enzymes may play a role in protection against highly reactive products of oxygen metabolism including those induced by asbestos. Induction of GSTM3 was not found to result from the exposure to mineral dust other than asbestos, although various minerals can give rise to reactive oxygen species *in vitro*. Additional studies are warranted concerning the effect of asbestos fibers on the expression of GSTs in different cell types of the lung.

It is surprising that no association between the *GSTM1* genotype and GSTM3 expression in the bronchial epithelium was observed in our previous study (18) or in the present study on active smokers. Immunostaining for GSTM3 in the bronchial epithelium was moderate or intense in most patients. Adaptation to xenobiotics in the bronchial lung may differ from that of the more peripheral parts. A higher xenobiotic load normally falls on the bronchial lung, since this compartment is exposed to a larger amount of inhaled substances per surface area than the peripheral part. Also, the bronchial lung is supplied by systemic circulation via the bronchial artery, whereas the nutrition of the peripheral lung is less dependent of the systemic vasculature. These factors may account for the greater accessibility of the bronchial epithelium to inhaled and circulating xenobiotics.

We observed earlier that the expressing *GSTM1* gene can protect the bronchial lung from cancer in patients with inducible PAH-activating enzyme CYP1A1 in the lung (14). Given that the release of electrophilic metabolites from the liver may be higher in the homozygous *GSTM1* null than in gene-positive individuals and that the level of GSTM1 is very low in the lung (3, 18), the attack of circulating electrophiles on the bronchial lung could result in the finding of an increased risk of bronchial squamous cell carcinomas among the individuals with homozygous *GSTM1* null genotype (11, 13).

A few other investigations have shown, however, an increased risk of adenocarcinomas among the *GSTM1* nulled (15, 16). The low expression of GSTM3 in the parenchymal lung of smokers with homozygous *GSTM1* null genotype that was observed in the present study could in theory favor the development of peripheral cancers, which usually are adenocarcinomas. We indeed noted more peripheral tumors and adenocarcinomas in patients with low expression of GSTM3 as compared to those with moderate or high expression. There may be at least two competing mechanisms for the increased susceptibility to lung cancer by homozygous *GSTM1* null genotype: one regulated by the presence or absence of GSTM1 in the liver and another mediated by the pulmonary expression of other GSTM enzymes. The weight of competing mechanisms may vary in different populations depending on other factors such as other metabolic phenotypes and exposure to carcinogens other than tobacco smoke. Our results suggest that exposure to asbestos has an effect the opposite of that caused by smoking on the expression of GSTM3 in individuals with the homozygous *GSTM1* null genotype.

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