Effect of Vitamin Intervention on the Relationship between *GSTM1*, Smoking, and Lung Cancer Risk Among Male Smokers

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Abstract

The *GSTM1* (glutathione *S*-transferase mu-1) null genotype is suspected of increasing an individual's susceptibility to tobacco smoke carcinogens because of impaired carcinogen detoxification. We were interested in whether there were differences in lung cancer susceptibility to smoking within the *GSTM1* genotypes and the impact of antioxidant supplementation on this. For this purpose, we conducted a nested lung cancer case-control study and evaluated the role of *GSTM1* within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *GSTM1* genotype status was determined for 319 cases and 333 controls using a PCR-based approach. *GSTM1* was evaluated as an independent risk factor and as an effect modifier of smoking using logistic regression analyses. The *GSTM1* null genotype itself was unrelated to risk of lung cancer, odds ratio (OR) = 1.09 and 95% confidence interval (CI), 0.79–1.50, but it may have modified the effect of smoking. There was a suggestion for a stronger association between years of smoking and lung cancer among the *GSTM1* null genotype, but the differences between *GSTM1* null and present genotypes were not statistically significant ($P = 0.12$). Furthermore, the smoking association was strongest among those with the *GSTM1* null genotype not receiving α-tocopherol supplementation, whereas among those receiving α-tocopherol, there was no modification by *GSTM1* on the association between smoking duration and lung cancer risk. β-Carotene supplementation did not modify the relationship between *GSTM1*, smoking years, and lung cancer risk. In conclusion, *GSTM1* is not associated with lung cancer risk in male smokers but may confer a higher susceptibility to cumulative tobacco exposure. This association may be attenuated by α-tocopherol but not by β-carotene supplementation.

Introduction

Lung cancer remains one of the major causes of mortality in the United States and worldwide. Although smoking is the major risk factor for lung cancer, other factors such as nutrition or genetic predisposition may be involved. Genetic susceptibility to environmental carcinogens is thought to be attributable to genetic polymorphisms in metabolism enzymes, which have been found to substantially alter the activation and elimination of carcinogens (1, 2).

GSTM1[2] is a member in a family of enzymes that catalyze the conjugation of glutathione to activated carcinogens, facilitating their excretion. *GSTM1* is involved in the detoxification of tobacco smoke carcinogens including the PAHs such as benzo(*a*)pyrene (3). Up to 50% of Caucasians have no *GSTM1* enzyme because of the homozygous deletion of the gene (4, 5), referred to as the *GSTM1* null genotype. Individuals lacking *GSTM1* are thought to have impaired ability to eliminate carcinogens and therefore are at increased cancer risk. Although several epidemiological studies have found the null genotype to be associated with increased risk for the development of lung and other tobacco-related cancers (6–10), the findings in other studies are conflicting, and this association remains controversial (11–14).

We evaluated the relationship between *GSTM1*, smoking, and vitamin intervention in the ATBC Study, a randomized-placebo controlled trial of older male smokers in Finland. The trial showed a 16% increase in lung cancer incidence with β-carotene supplementation and no overall effect for α-tocopherol. Here, we examine the direct effect of *GSTM1* on lung cancer risk, *GSTM1* modification of the association between smoking and lung cancer risk, and whether antioxidant supplementation had an impact on this relationship. Because antioxidant supplementation may modulate glutathione levels and the oxidative state of the cell, its interaction with *GSTM1* and smoking may be particularly relevant.

Materials and Methods

Study Population. We conducted a nested case-control study within the ATBC Study conducted in Finland. This was a randomized, placebo-controlled prevention trial that tested the efficacy of 5–8 years of supplementation with α-tocopherol (50 mg/day), β-carotene (20 mg/day), or both in reducing the incidence of lung, prostate, and other cancers. The ATBC Study cohort consisted of 29,133 white male smokers who smoked at

Received 7/7/99; accepted 9/15/99.
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[2] The abbreviations used are: GSTM1, glutathione *S*-transferase *M1*; PAH, polycyclic aromatic hydrocarbon; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention; OR, odds ratio; CI, confidence interval.

least five cigarettes daily. Participants were recruited between 1985 and 1988 and followed during the active trial period until death or April 30, 1993. Men were also followed after intervention. The overall design, rationale, and objectives of this study have been published (15), as have the main trial findings (16). The trial showed a 16% increase in lung cancer incidence among subjects in the β-carotene supplemented group and a 32% reduction in prostate cancer in the α-tocopherol group (17).

General medical history, diet, smoking, and other background data along with a fasting blood sample were collected from all subjects at baseline. The dietary information was gathered using a validated, self-administered food-use questionnaire given to all participants prior to randomization. The questionnaire was linked to the food composition database of the National Public Health Institute of Finland. The ATBC Study was approved by the institutional review boards of the National Cancer Institute (United States) and the National Public Health Institute of Finland.

Selection of Cases and Controls. A nested case-control sample set was constructed based on the availability of a whole blood sample collected during the study (between April 1992 and March 1993) from 20,305 men. Incident primary cases of lung or bronchus cancer (ICD9-162) diagnosed up to December 31, 1994 were identified through the Finnish Cancer Registry and the Register of Causes of Death. The medical records of the intervention cases (up to April 30, 1993) were centrally reviewed independently by two study physicians and the later cases up to December 31, 1994 by one study physician. Histological or cytological specimens were available for 93% of the cases. The histological type was obtained from the central review of the two pathologists for the intervention period cases and from local hospital pathology review for the postintervention cases. Forty-six % of the cases were of squamous cell type, 15% were small cell type, 17% were adenocarcinomas, and 22% were of indeterminate cell type. Cases having multiple cancers were excluded ($n = 10$), leaving a total of 362 cases for DNA extraction. Controls were matched 1:1 to cases by age (±5 years), intervention group, study clinic, and date of blood draw (±45 days).

DNA Isolation and GST Genotyping Analysis. DNA was isolated from whole blood samples as described previously (18). *GSTM1* genotyping was conducted using a PCR-based approach for *GSTM1* (11). This method distinguishes homozygous null from heterozygous and homozygous wild-type. The nonpolymorphic *GSTM4* gene was amplified as an internal PCR control. Genotyping results were reviewed independently by two investigative groups. Genotyping was performed in batches containing equal numbers of cases and controls, and negative controls (PCR reagents without DNA) were included with each batch. A random sample of 10% of the study samples was repeated for quality control and showed 100% concordance. Genotyping was successful for 319 cases and 333 controls, the final sample used for this analysis.

Statistical Analyses. All statistical analysis were performed using Statistical Analysis Systems software package (SAS Corp., Cary, NC). The associations between the various risk factors and the development of lung cancer were evaluated using logistic regression. Unconditional logistic regression analyses gave essentially the same results as conditional logistic regression techniques. Thus, unconditional logistic regression was used in all analyses shown to avoid the loss of subjects because of splitting of the matched sets that fell into different genotype strata or had missing genotype information. Potential survival bias was tested by exclusion of the cases ($n = 69$ or 22% of total cases) diagnosed prior to the collection of whole blood. Because this did not change the results, they were left in the analysis. Multivariate models were developed including age, years of smoking, and number of cigarettes smoked daily as continuous terms and intervention group as an indicator variable for supplementation with α-tocopherol, β-carotene, both, or placebo (reference). Other study factors were assessed as confounders by evaluating whether their inclusion into the multivariate model changed the ORs by >15% or led to a significant change in the likelihood ratios ($P < 0.05$). The OR estimates were essentially unchanged by further adjustment for body mass index (kg/m^2), baseline dietary antioxidant intake (*i.e.*, vitamins A and C, β-carotene, α-tocopherol), or baseline serum antioxidant levels (*i.e.*, α-tocopherol, β-carotene). Smoking variables were introduced into the model as indicator variables representing tertile categories on the basis of the distribution among all control subjects. To test for linear trend, we used the continuous variables in the logistic regression models. Continuous variables were evaluated as potential confounders using Wilcoxon rank sum tests for identifying differences between cases and non-cases and between *GSTM1* genotype status. Potential confounding by categorical variables were assessed using the χ^2 test. Effect modification by *GSTM1* of the lung cancer and smoking associations overall and within intervention assignment groups were tested by including the cross-product interaction term of *GSTM1* and smoking (based on smoking tertiles scored as 0–2) in the multivariate regression models and by stratified analyses. Effect modification of smoking by *GSTM1* according to intervention assignment was analyzed with the four intervention groups separately (α-tocopherol, β-carotene, α-tocopherol and β-carotene, and placebo) and as two groups, α-tocopherol supplemented *versus* non-supplemented, and β-carotene supplemented *versus* non-supplemented groups to increase power in accordance with the trial's factorial design (15). The multivariate models for the intervention assignment analysis were the same except the intervention assignment indicator variable was changed to a single categorical indicator variable for the intervention assignment alternative to the one being tested.

Results

The associations between cigarette smoking and lung cancer risk are presented in Table 1. Even in this population of heavy chronic smokers, cigarette smoking was strongly associated with increased lung cancer risk, with the third compared with the first tertile of years smoking and daily cigarettes smoked showing ~3.5- and 2-fold risk increases, respectively. Because of the study matching, there were no case-control differences in age (the mean baseline age was 59.3 and 59.2 for the cases and controls, respectively) and intervention group distribution (data not shown).

The prevalence of the *GSTM1* null genotype was 50.2% among lung cancer cases and 48.6% among controls (Table 2). This distribution is similar to what was observed in other Finnish populations (8, 9). The risk of lung cancer associated with the *GSTM1* genotype status overall and according to histological subtype is shown in Table 2. The *GSTM1* null genotype was not associated with increased lung cancer risk, nor was there an apparent relationship with any of the histological subtypes.

Although there was no direct relationship between *GSTM1* and lung cancer risk, there was a suggestion that *GSTM1* modified the association between smoking duration and lung

Table 1 Associations between cigarette smoking and lung cancer, ATBC Study, Finnish men

	No. cases (%; n = 319)	No. controls (%; n = 333)	OR (95% CI)[a]	P-trend[b]
Years smoking				
<37	74 (38)	121 (62)	1.00 (reference)	<0.01
37–42	114 (51)	109 (49)	2.12 (1.37–3.29)	
>42	131 (56)	103 (44)	3.48 (1.98–6.12)	
Cigarettes/day				
<19	82 (38)	134 (62)	1.00 (reference)	0.08
19–22	112 (54)	95 (46)	1.77 (1.23–2.56)	
>22	125 (55)	104 (45)	1.70 (1.05–2.73)	

[a] OR and 95% CI adjusted for age, years of smoking or daily cigarettes smoked, and intervention assignment.
[b] Ps for linear trend based upon continuous variable included into the multivariate models.

Table 2 Distribution of *GSTM1* genotype by lung cases and controls and odds of lung cancer for the *GSTM1* null genotype, ATBC Study, Finnish men

	GSTM1 present n (%)	*GSTM1* null n (%)	OR (95% CI)[a]
Control (n = 333)	171 (51.4)	162 (48.6)	
Cases[b]			
Total (n = 319)	159 (49.8)	160 (50.2)	1.09 (0.79–1.50)
Squamous (n = 147)	78 (53.1)	69 (46.9)	0.95 (0.63–1.42)
Small cell (n = 47)	22 (46.8)	25 (53.2)	1.19 (0.66–2.17)
Adenocarcinoma (n = 55)	29 (54.6)	24 (45.4)	1.00 (0.55–1.80)
Other (n = 57)[c]	30 (43.9)	42 (56.1)	1.41 (0.80–2.49)

[a] OR and 95% CI for the *GSTM1* null *versus GSTM1* present genotype adjusted for baseline age, years of smoking, daily cigarettes smoked, and intervention assignment.
[b] Thirteen cases could not be classified histologically and were not included in the analysis.
[c] Other subtypes include carcinoma, metastatic (n = 5), large cell (n = 7), undifferentiated (n = 26), anaplastic (n = 9), giant cell (n = 1), and not otherwise specified (n = 9).

cancer risk (Table 3). The risk of lung cancer increased significantly with longer duration of smoking both among *GSTM1* present and null men, with a stronger association among *GSTM1* null men, although the interaction between *GSTM1* and smoking was not statistically significant (P = 0.12). There were no differences in the association between daily cigarettes smoked and lung cancer between the *GSTM1* genotypes (P for interaction = 0.78). The OR (95% CI) for the third *versus* first tertile of smoking was 1.42 (0.81–2.50) for those with the *GSTM1* present and 1.74 (0.98–3.11) for those with the *GSTM1* null genotype.

We assessed whether supplementation with α-tocopherol or β-carotene (men were supplemented between 5 and 8 years in a randomized trial) modified the effect of *GSTM1* genotype on the association between smoking and lung cancer (Table 3). With respect to α-tocopherol supplementation, longer duration of smoking was associated with lung cancer risk among men not receiving α-tocopherol supplementation within both the *GSTM1* genotypes, with a stronger association among the *GSTM1* null men. In contrast, among men receiving α-tocopherol supplementation, smoking duration was not associated with lung cancer risk for either genotype. Among those with the *GSTM1* null genotype, there was a 21-fold risk increase for lung cancer associated with the highest smoking tertile among those not receiving α-tocopherol and a 1.3-fold for those receiving α-tocopherol. The interaction between *GSTM1* genotype and smoking reached statistical significance among the group not receiving α-tocopherol (P = 0.01) but not within the supplemented group (P = 0.63). When the analysis was conducted separating the four treatment groups, the results were essentially the same to when we compared α-tocopherol alone with the placebo only group (*i.e.*, without β-carotene).

With respect to β-carotene supplementation status, there appeared to be greater risk of lung cancer associated with smoking duration for those with the *GSTM1* null genotype but essentially no differences between those supplemented and not supplemented (*i.e.*, 6- and 8-fold higher in the highest smoking category, respectively). The interaction between *GSTM1* genotype and smoking did not reach statistical significance for either the β-carotene supplemented (P = 0.24) or nonsupplemented (P = 0.27) groups. Interestingly, a significant dose-response relationship with smoking duration was observed among *GSTM1* present subjects who were receiving β-carotene but not for those with the *GSTM1* present genotype not receiving β-carotene. When the analysis was restricted to β-carotene alone and placebo, the P-trend values were essentially the same, although the magnitude of the risk estimates for the tertiles changed (were increased). This is most likely attributable to exclusion of α-tocopherol supplemented subjects and instability of the risk estimates because of the small number of observations in each stratum. However, the overall results were the same; β-carotene appeared to have no effect on *GSTM1* modification of smoking. There was no interaction between the associations between lung cancer risk and number of cigarettes smoked daily within either intervention group (data not shown).

Discussion

We studied differences in lung cancer susceptibility to tobacco smoke in terms of germ-line polymorphisms of the *GSTM1* gene and antioxidant intervention in older male smokers. We hypothesized that the *GSTM1* null subjects would have a higher lung cancer risk because of smoking and that antioxidants might alter risk differentially among individuals with different *GSTM1* genotypes. We found that the *GSTM1* null genotype was not associated with increased lung cancer risk overall or with any of the histological subtypes. There was a suggestion that lung cancer risk associated with longer smoking history may be greater among those having the *GSTM1* null genotype. Furthermore, among those of the *GSTM1* null genotype, there was a weaker association between years of smoking and lung cancer risk in the group receiving α-tocopherol than the nonsupplemented group.

Previous studies of Caucasian populations have generally observed a modest positive association between *GSTM1* null and lung cancer, with most showing ORs from 1.0 to 1.6 (6, 8, 11, 14), whereas studies among Japanese populations have shown stronger associations (7, 10). The relationship between the *GSTM1* null genotype and the different histological subtypes has been inconsistent across studies; some have reported higher associations for squamous cell (8–10, 14) and others for adenocarcinomas (6, 11), but we found no evidence of such differences. Prior reports of *GSTM1* and lung cancer were summarized in a recent meta-analysis of 1593 cases and 2135 controls (19). The authors found a combined OR of 1.4 for the null genotype, with no significant differences between histological subtype or genotype *versus* phenotype experiments, but did find significant racial differences (*i.e.*, OR were 1.2 for Caucasians and 1.6 for Japanese studies).

The *GSTM1* null genotype is thought to increase risk for tobacco-related cancers through impaired PAH detoxification. *GSTM1* deficiency has been shown to increase DNA adduct

Table 3 Lung cancer risk[a] associated with cigarette smoking by *GSTM1* genotype according to intervention group, ATBC Study, Finnish men

Group	Genotype	Years of smoking tertile			*P*-trend[b]
		<37	37–42	>42	
		OR (95% CI)[a] No. of cases/controls	OR (95% CI)[a] No. of cases/controls	OR (95% CI)[a] No. of cases/controls	
All subjects	*GSTM1* present	1.00 (reference) 41/52	1.15 (0.62–2.14) 56/65	1.85 (0.85–4.02) 54/62	0.002
	GSTM1 null	1.00 (reference) 33/69	3.77 (1.98–7.16) 53/44	6.28 (2.70–14.63) 69/49	0.0001
α-Tocopherol					
NO	*GSTM1* present	1.00 (reference) 18/31	2.41 (0.98–5.92) 29/24	2.37 (0.79–7.15) 28/29	0.003
	GSTM1 null	1.00 (reference) 13/44	6.78 (2.69–17.11) 35/29	21.15 (6.26–72.69) 45/21	0.0001
YES	*GSTM1* present	1.00 (reference) 23/21	0.60 (0.24–1.46) 27/41	1.33 (0.43–4.18) 34/25	0.29
	GSTM1 null	1.00 (reference) 20/25	2.20 (0.85–5.74) 23/15	1.34 (0.36–5.03) 24/28	0.28
β-carotene					
NO	*GSTM1* present	1.00 (reference) 18/22	1.19 (0.48–2.92) 29/28	1.19 (0.40–3.58) 26/24	0.22
	GSTM1 null	1.00 (reference) 20/39	3.38 (1.32–8.66) 22/22	8.17 (2.24–29.82) 27/21	0.004
YES	*GSTM1* present	1.00 (reference) 23/30	1.35 (0.56–3.26) 27/37	3.56 (1.14–11.12) 36/30	0.001
	GSTM1 null	1.00 (reference) 13/30	4.78 (1.91–11.92) 36/22	6.01 (1.90–19.08) 42/28	0.001

[a] OR and 95% CI after adjusting for age, daily cigarettes smoked, and intervention assignment.
[b] *P* for trend based upon the *P* (two-sided) of years smoked modeled as a continuous term.

formation (20, 21) and cytogenetic damage (22, 23). There could be several explanations for our findings of no association between the *GSTM1* null genotype and lung cancer. For example, because our population consisted of only heavy smokers, insult to the lung by tobacco smoke exposure may have been so overwhelming that any additional effect attributable to *GSTM1* genotype was negligible. Another explanation may be that interactions with other genes such as *CYP1A1* and other *GST*s such as *GSTT1* or *GSTP1* need to be taken into account. This is especially likely for exposures such as tobacco smoke, where there are hundreds of carcinogens that are detoxified by numerous distinct yet overlapping enzymes. Previous studies found a stronger association for *GSTM1* null status and lung cancer when combined with other susceptibility genes. For example, Saarikoski *et al.* (9) found that *GSTM1*, *GSTM3*, and *GSTT1* separately had no relationship with lung cancer, but *GSTM1* and *GSTT1* combined had about a 3-fold increased risk. In several Japanese studies, *CYP1A1* and *GSTM1* higher susceptibility genotypes combined had far higher lung cancer risk than either genotype alone (10, 24, 25). Studies of interactions of *GSTM1* and other susceptibility genes in the ATBC Study cohort are presently under way.

Investigations that assessed the effect of smoking on the relationship between the *GSTM1* null genotype and lung cancer have yielded conflicting results, with some reports demonstrating *GSTM1* to be a risk factor only for light smokers (10, 14) and others only for heavy smokers (6–8). Our analysis differs from these previous reports in that rather than assessing the modification of *GSTM1* by smoking status, we evaluated whether the lung cancer-smoking association differed between the *GSTM1* genotypes, an approach we felt was more biologically relevant. We found a suggestion of a stronger association for cumulative tobacco exposure for those with the *GSTM1* null genotype; however, we did not observe this same effect for present cigarette dose. This was surprising but may have been attributable, in part, to our study population being comprised of heavy smokers and the level of daily carcinogen exposure (*i.e.*, all subjects smoked >5 cigarettes/day with a mean of 20/day), possibly exceeding thresholds for carcinogenic or mutagenic effects. Alternatively, current smoking exposure may not reflect relevant lifetime exposure, and years of smoking may be a more accurate measure.

Interestingly, there was a lower lung cancer risk associated with cumulative tobacco exposure among those with the *GSTM1* null genotype who were receiving α-tocopherol supplementation. It is possible that such a finding is attributable to chance; however, there are several lines of experimental evidence to support such a finding. Studies in humans have shown reduced levels of PAH-adducts associated with high serum α-tocopherol concentrations only among subjects with the null genotype (26, 27), suggesting that high α-tocopherol is important when *GSTM1* is lacking. One explanation for this may be that α-tocopherol increases reduced glutathione levels, thus promoting the spontaneous conjugation of glutathione to carcinogens in the absence of *GSTM1* (28). It is plausible that α-tocopherol supplementation achieves an equilibrium in the redox-state of the cell, and the cells consequently maintain reduced glutathione levels. In rats, α-tocopherol supplementation increased both hepatic and gastric glutathione levels (29). Another possible mechanism is that α-tocopherol stimulated compensatory enzymes, which have redundant functions with *GSTM1*. For example, α-tocopherol supplementation induced both GST-α, and GST-μ levels in rats (29, 30). Alternatively, it is possible that α-tocopherol exerts an inhibitory effect on carcinogenesis by inhibiting oxygen radical formation, which may be particularly important in the absence of *GSTM1*. A controlled intervention trial with daily doses of vitamin C and/or α-tocopherol given to smokers showed significantly

reduced oxidative damage in lymphocyte DNA, based on the COMET assay (31).

Why our findings support a role for α-tocopherol and not β-carotene is unclear. Although both are antioxidants, they possess different antioxidant capabilities. β-Carotene functions specifically to quench oxygen radicals, whereas α-tocopherol has a strong membrane association and is more ubiquitous, functioning in a variety of oxidation pathways. α-Tocopherol may have other functions in addition to that of an antioxidant. It has been shown to exert other anticarcinogenic effects such as induction of apoptosis (32), inhibition of cell proliferation (32), and stimulation of the immune system (33). Interestingly, a significant dose-response relationship with smoking was observed among the *GSTM1* present subjects who were receiving β-carotene but not among those *GSTM1* present subjects not receiving β-carotene. This finding might be explained by an interaction between smoking and β-carotene that was described previously in the larger trial, where we observed a stronger association between β-carotene supplementation and lung cancer among the heavier smokers (16). One potential mechanism for this interaction may be the stimulation of activated carcinogens; β-carotene supplementation has been shown to induce several carcinogen-metabolizing enzymes including *CYP1A1/2* in ferrets (34).

Many previous studies that evaluated *GSTM1* polymorphisms and lung cancer used small samples and ascertained cases retrospectively, thus limiting conclusions. In the present study, however, the large number of cases provided ample power to evaluate the role of *GSTM1* in cancer development, as well as the modifying effects of *GSTM1* on other study factors. Another strength of our study is that nearly all of the whole blood samples were collected prospectively, minimizing the possibility of survival bias attributable to the *GSTM1* genotype. This may be important, especially if, as postulated by London *et al.* (14), the heterogeneity of results among *GSTM1* studies is attributed to survival bias and case selection. We further minimized survival bias by excluding cases diagnosed prior to whole blood collection, a separate analysis that gave essentially the same results. Another strength of this study, relating to our α-tocopherol findings, is that the subjects were randomly assigned to intervention groups, reducing the possibility of selection bias related to antioxidant supplementation.

A major limitation of this study is lack of a reference group of nonsmokers or light smokers that may have precluded observing an effect of *GSTM1* on lung cancer risk. Another limitation of having only smokers is that our study might be biased toward having more subjects with *GSTM1* present because those with the null genotype may have already developed cancer and thereby been ineligible for the trial. The null genotype prevalence estimate from our study, however, was nearly identical to another Finnish study (9), which used younger men and nonsmoking population controls, making this unlikely.

In summary, we showed that the *GSTM1* null genotype does not increase risk for lung cancer in male Finnish smokers. There was a suggestion that the *GSTM1* null genotype may modify lung cancer risk associated with cumulative tobacco exposure. Furthermore, α-tocopherol but not β-carotene may have attenuated lung cancer risk associated with cumulative tobacco exposure when the detoxifying activity of *GSTM1* is lacking.

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Cancer Epidemiol Biomarkers Prev 1999;8:965-970.