

Substantially Reduced Risk of Cancer of the Aerodigestive Tract in Subjects with Variant –463A of the Myeloperoxidase Gene¹

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ABSTRACT

Myeloperoxidase (MPO), an enzyme that is highly expressed in neutrophil leukocytes, transforms precarcinogens such as benzo(a)pyrene and aromatic amines to highly reactive intermediates. A G/A polymorphism located 463 bp upstream of exon 1 in the promoter region strongly reduces MPO mRNA expression. In a matched case-control study, 196 lung cancer, 245 laryngeal cancer, and 255 pharyngeal cancer patients from the Berlin area were investigated for frequency of the G–463A polymorphism by PCR/RFLP, using *AciI*. They were matched by age and gender to hospital patients without known malignancies. Moreover, 270 healthy volunteers were genotyped, obtaining 61.1% of individuals with MPO genotype –463G/G, 34.8% of individuals with genotype G/A, and 4.1% of individuals with genotype A/A. In lung and laryngeal cancer patients, but not in pharyngeal cancer patients, mutant genotypes were significantly less frequent. Crude odds ratios for carriers of one or two A alleles, compared to wild-type G/G, were 0.58 [95% confidence interval (CI), 0.38–0.88; $P = 0.011$] for lung cancer patients, 0.63 (95% CI, 0.43–0.92; $P = 0.017$) for laryngeal cancer patients, and 0.82 (95% CI, 0.57–1.17; $P = 0.27$) for pharyngeal cancer patients. The relative risks, adjusted for age, gender, and extent of cigarette smoking were 0.47 (95% CI, 0.28–0.79; $P = 0.004$), 0.66 (95% CI, 0.44–1.01; $P = 0.054$), and 0.75 (95% CI, 0.51–1.12; $P = 0.16$) for lung, larynx, and pharyngeal cancer, respectively. Strikingly, relative risk for carriers of –463A among adenocarcinoma of the lung was 0.24 (95% CI, 0.10–0.58; $P = 0.002$). Two cases with larynx cancer, one case with lung cancer, and one reference subject displayed novel G/A mutations at –297 nucleotide and –296 nucleotide, destroying a constitutive *AciI* cleavage site. Our data finally suggest that the MPO –463A variant is a protective factor in the etiology of lung and larynx cancer, but possibly not of pharyngeal cancer.

INTRODUCTION

MPO³ (EC 1.11.1.7) is a lysosomal hemoprotein contained in polymorphonuclear leukocytes and monocytes. MPO catalyzes the production of hypochlorous acid in physiological situations, which leads to microbicidal activity against a wide range of organisms (1). The rare hereditary MPO deficiency is reported to be associated not only with chronic granulomatous disease, but also with some forms of leukemia (2). MPO causes DNA damage resulting from oxygen radicals produced during myeloid cell maturation and function (3, 4). Moreover, MPO has been shown to transform environmental precarcinogens such as benzo(a)pyrene and aromatic amines to highly reactive intermediates (5–8).

The MPO gene is known to be polymorphic. Amino acid exchanges R569W (9), Y173C (2), and M251T (10) were discovered in cases

with almost complete MPO deficiency. A –463G/A polymorphism was detected by Austin *et al.* (11) in the promoter region among leukemia patients. Findings of another study have suggested that this –463G/A transition strongly reduces mRNA expression: this was concluded because the polymorphic site was found to be located in an Alu hormone-responsive element, which results in the loss of a SP1 transcription factor binding site (12). On the basis of this observation and the implication of MPO in the metabolic activation of aromatic organic compounds, polymorphic MPO may also act as a susceptibility factor for those malignant diseases that are etiologically associated with exposure to precarcinogens. The genetically lower MPO activity in carriers of the A allele may represent a protecting host factor. This hypothesis was supported by a recent study by London *et al.* (13) among lung-cancer patients, in which homozygous mutant A/A carriers were shown to be significantly less frequent than in controls. The objective of our study was to investigate the prevalence of the –463G/A mutation in three large samples of patients suffering from cancer of the aerodigestive tract, namely cancers of the lung, larynx, and pharynx.

MATERIALS AND METHODS

Subjects of Investigation. A population of 196 Caucasian lung cancer patients (150 males and 46 females; median age, 63 years; age range, 35–87 years) with first tumor diagnosis within 1 year was recruited from the Department of Internal Medicine at the Lungenklinik Heckeshorn (Berlin, Germany) and from the Department of Surgery, Charité University Medical Center, Humboldt University (Berlin, Germany). In addition, 245 cases with laryngeal cancer (222 males and 23 females; median age, 61 years; age range, 37–87 years; median time since first diagnosis, 20 months) and 255 patients suffering from pharyngeal cancer (191 males and 64 females; median age, 57 years; age range, 36–86 years; median time since first diagnosis, 13 months) who had been admitted to the Department of Oto-Rhino-Laryngology, Charité University Medical Center were likewise included in the study. Each group of cases was matched by gender and age (± 5 years) to samples from the anonymous DNA pool of the Institute of Clinical Pharmacology (controls for the lung cancer group, median age = 65 years and age range = 37–85 years; controls for the larynx cancer group, median age = 61 years and age range = 37–88 years; controls for the pharyngeal cancer group, median age = 59 years and age range = 37–85 years). These samples were drawn from patients from the same catchment area who were hospitalized because of a variety of nonmalignant diseases. Main diagnoses were urological diseases like benign prostate hyperplasia (16%) or urolithiasis (11%), bronchial obstruction (13%), rheumatoid diseases (12%), abdominal surgical cases (10%), asthma (7%), various oto-rhino-laryngeological diseases (6%), trauma (6%), neurological disorders (4%), pneumonia (4%), and others (11%).

Total cigarette consumption is expressed in pack-years (1 pack-year = consumption of one pack of 20 cigarettes per day for 1 year). Alcohol consumption was recorded as low (no to occasional) or high (daily, extensive) consumption. Occupational exposure followed the consensus of risk occupations in head and neck cancer (14). An additional reference group of 270 healthy volunteers (263 males and 7 females; median age, 28 years; age range, 18–49 years) was selected for evaluation of any bias in the control groups. Patients and healthy volunteers were selected during the time period from 1994–1998; they gave their informed consent, and the study was approved by the Ethics Committee of the Charité University Medical Center. To avoid confounding by ethnicity,

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³ The abbreviations used are: MPO, myeloperoxidase; CI, confidence interval; nt, nucleotide.

only subjects of German extraction were included, as defined by their names and place of birth.

Genotyping Procedure. DNA was isolated from leukocytes by standard phenol/chloroform extraction. MPO-mutations were characterized by RFLP after PCR according to the method of London *et al.* (13). For determination of the G-463A exchange, a 350-bp DNA fragment was amplified using 1 unit of Taq polymerase (Perkin-Elmer, Weiterstadt, Germany), 10 $\mu\text{mol/liter}$ of primers MPOF (5'-CGGTATAGGCACACAATGGTGAG) and MPOR (5'-GCAATGGTTCAAGCGATTCTTC; TIB Molbiol, Berlin, Germany), 0.2 mmol/liter deoxynucleotide triphosphate (Boehringer Mannheim, Mannheim, Germany), and 1.5 mmol/liter MgCl_2 in a total volume of 25 μl . PCR conditions were 32 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. Fifteen μl of the PCR product were digested with 5 units of *AciI* (New England Biolabs, Schwalbach, Germany), which produced three fragments (168, 121, and 61 bp) for cases of the homozygous wild-type -463G/G, and two fragments (289 and 61 bp) for cases of -463A/A. Fragments were separated on a 2.5% agarose gel stained by ethidium bromide (Fig. 1).

DNA Sequencing. Fragments were processed forward and in reverse using a dye terminator sequencing kit (Perkin-Elmer) with the same primers used for genotyping, and they were analyzed with an Applied Biosystems 373A automated sequencer on a denaturing 6% polyacrylamide gel. Sequences were compared with a published MPO sequence (15).

Statistics. The study was of a case-control design, with cases matched to controls by age and gender. Expected genotype frequencies were calculated by the Hardy-Weinberg equation from the allele frequencies. Odds ratios appeared with 95% CIs and two-sided *P*, calculated by the χ^2 test. Crude odds ratios were calculated from the ratio of mutant *versus* wild-type MPO genotypes in cases compared to the ratio in controls, or other strata, respectively. Relative risks were computed by logistic regression analysis, with consideration of age, gender, MPO genotype, and smoking as confounding factors. All tests were analyzed using the SPSS 7.5 program.

RESULTS

Detection of Mutations. Sequencing of the amplified 350-bp fragment revealed complete homology with a published DNA sequence (GenBank, accession number M19508; Ref. 15). Based on this sequence, the exact position of the G/A mutation investigated was 465 bp upstream of exon 1. Owing to sequence dissimilarities, however, the polymorphic site may also be allocated to -463 nt (GenBank, accession number X15377; Ref. 16) or -461 nt (GenBank, accession number S65785; Ref. 11). To avoid confusion, we have applied the conventional terminology G-463A.

In four of the samples investigated, electrophoresis revealed a RFLP pattern, which could not be attributed to the -463G/A exchange (Fig. 1). Sequencing unveiled two sites of heterozygous G/A mutations within the constitutive *AciI* (GCGG) recognition site: (a) one site 297 bp (GCAG) upstream of exon 1; and (b) another site 296 bp (GCGA) upstream of exon 1 (Fig. 2). These samples displayed an

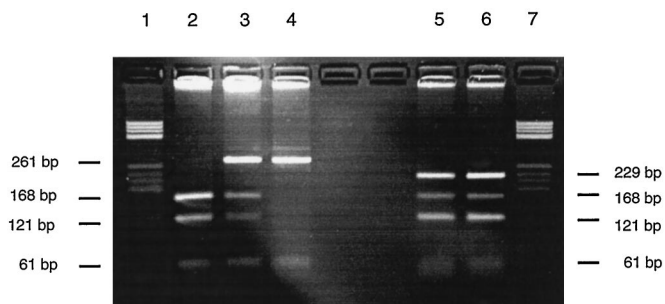


Fig. 1. 3% NuSieve-agarose gel electrophoresis of MPO-DNA fragments stained with ethidium bromide. Fragment lengths are given in bp. Lanes 1 and 7, DNA size standard; Lanes 2-4, *AciI* digestion of homozygous wild-type (-463G/G), heterozygous mutant (-463G/A), and homozygous mutant (-463A/A); Lanes 5 and 6, partial loss of the constitutive *AciI* GCGG restriction site detected in two laryngeal cancer patients due to heterozygous G/A mutations at positions -297 and -296 nt (see Fig. 2).

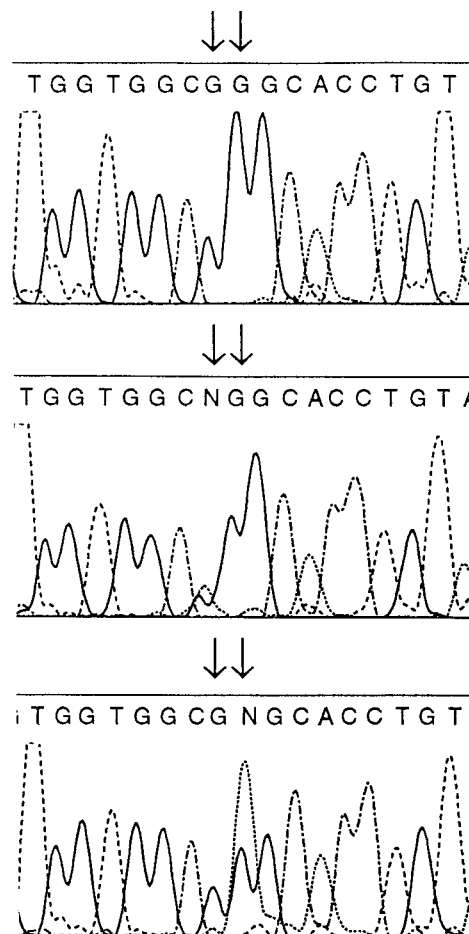


Fig. 2. DNA sequencing electropherogram of the region consisting of a second (presumably constitutive) *AciI* recognition site. The top sequence represents a wild-type sample with the GCGG motif. The middle sequence shows a laryngeal cancer case with a heterozygous G/A mutation at -297 nt (see Fig. 1, Lane 5). The bottom sequence depicts another laryngeal cancer case with a G/A mutation at -296 nt (see Fig. 1, Lane 6). Sequences were verified by reverse sequencing.

additional 229-bp fragment due to the loss of the *AciI* site. The PCR/RFLP results for the -463G/A exchange could always be confirmed in these cases by DNA sequencing. All cases were carriers of wild-type -463G/G.

Healthy Volunteers. The frequency of the MPO -463A mutant allele (21.5%) in the group of 270 young healthy volunteers was indistinguishable from the relatively older matched control groups (Table 1). The genotype distribution was in Hardy-Weinberg equilibrium: G/G, 61.1% (95% CI, 55.0-67.0%); G/A, 34.8% (95% CI, 29.1-40.8%); and A/A, 4.1% (95% CI, 2.1-7.2%). Hence, the hospital controls taken as reference for the three groups of cancer patients seemed to be a representative population.

Lung Cancer. Mutant MPO alleles were substantially less frequent in lung cancer cases. Heterozygous carriers of -463A among cancer cases were significantly underrepresented, whereas rare A/A homozygotes failed to differ from controls (Table 1). The crude odds ratio for mutant genotypes (the sum of G/A and A/A) compared to G/G was 0.58 (95% CI, 0.38-0.88; *P* = 0.011). Relative risk for carriers of mutant genotypes, adjusted for age, gender, and extent of cigarette smoking, was 0.47 (95% CI, 0.28-0.79; *P* = 0.004).

Stratification revealed no significant differences in the odds ratio of males, females, and individuals younger or older than the median age of 63 years (Fig. 3). The extent of lifelong cigarette consumption failed to modulate cancer risk as a factor of G-463A.

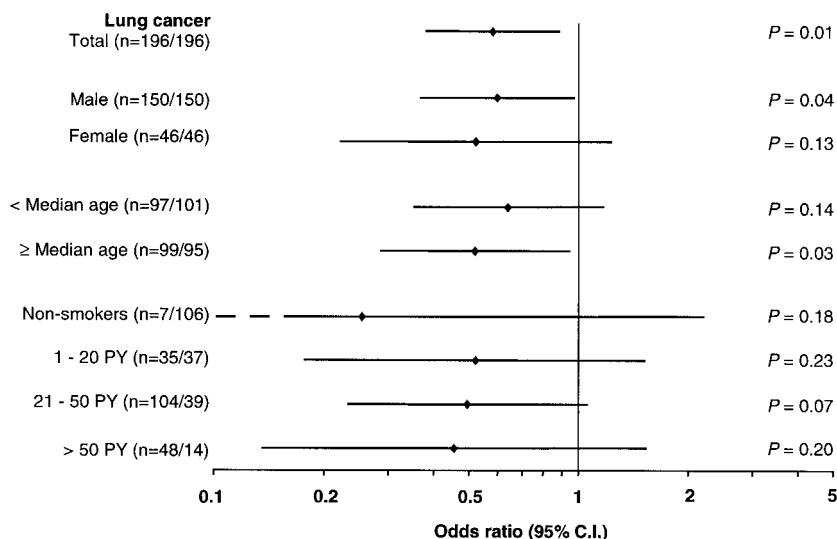


Fig. 3. Mutant MPO genotypes as a protective factor for lung cancer. Patients are stratified according to gender, age, and extent of smoking. The figure shows crude odds ratios for $-463G/A + A/A$ genotypes with 95% CIs. Numbers of cases and controls in the different strata are given in parentheses. Median age refers to cases.

Histological classification provided the following results: The $-463A$ mutation appeared with lowest frequency among 47 patients with adenocarcinoma (G/A + A/A, 14.9%); relative risk (adjusted for age, gender, and smoking and referred to the whole control group) was 0.24 (95% CI, 0.10–0.58; $P = 0.002$) for carriers of one or two copies of the $-463A$ allele relative to those with homozygous G allele. Among 66 squamous cell carcinomas, relative risk was 0.39 (95% CI, 0.18–0.82; $P = 0.014$); in 24 small cell carcinomas, relative risk was 0.39 (95% CI, 0.13–1.18; $P =$ nonsignificant). Large and mixed-cell carcinomas were too rare to allow reliable results. In 13.5% of cases, histology was not defined.

Laryngeal Cancer. The frequency of mutant MPO genotypes was significantly lower for laryngeal cancer than controls (Table 1). The crude odds ratio was 0.63 (95% CI, 0.43–0.92; $P = 0.02$). Relative risk, adjusted for age, gender, and extent of smoking and calculated by logistic regression analysis, was 0.66 (95% CI, 0.44–1.01; $P = 0.054$). Due to the small number of females, $-463A$ genotypes were significantly underrepresented only among males (Fig. 4). Considering age, significant differences were observed in patients, older than the median age. Moreover, $-463A$ genotype frequency tended to decrease in cases with increasing extent of smoking (Fig. 4). Due to

the relatively small sample size, no subgroup reached a level of statistical significance.

In addition, history of risk occupation or alcohol consumption apparently did not influence the risk with respect to MPO genotype (data not shown). MPO genotype distribution failed to differ in dependence on tumor location or on stage of development. However, a higher frequency of A alleles (33.2%) in poorly differentiated laryngeal tumors as compared to highly differentiated laryngeal tumors (25.0%) was statistically not significant.

Pharyngeal Cancer. Among pharyngeal cancer cases, mutated genotypes were not statistically different from controls. The crude odds ratio was 0.78 (95% CI, 0.54–1.13; $P = 0.19$; Table 1). Adjusted relative risk, as calculated by logistic regression analysis, was 0.75 (95% CI, 0.51–1.12; $P = 0.16$). Stratification of cases and controls according to gender, age, or smoking disclosed no differences (Fig. 5).

Further stratification provided the striking result that $-463A$ carriers were less frequent among pharyngeal cancer patients with no or low alcohol consumption than in the whole control group (adjusted odds ratio, 0.56; 95% CI, 0.35–0.90; $P = 0.017$). Regarding the location of the tumor, there was no statistically significant difference in frequency of mutant alleles for nasopharyngeal and hypopharyngeal tumors. However, highly differentiated tumors presented with a

Table 1 Frequency of MPO $-463 G/A$ mutation among patients with cancer of the lung, larynx, and pharynx compared to matched controls

MPO genotype	Cases		Controls		Exp. % ^a	Odds ratio ^b	95% CI	P
	%	95% CI	%	95% CI				
Lung cancer (n = 196)								
G/G	71.9	65.1–78.1	59.7	52.5–66.6	62.1	1.00		
G/A	25.0	19.1–31.7	38.3	31.4–45.5	33.4	0.54	0.35–0.84	0.006
A/A	3.1	1.1–6.5	2.0	0.6–5.1	4.5	1.25	0.34–4.52	0.500 ^c
G/A + A/A	28.1	21.9–34.9	40.3	33.4–47.5	37.9	0.58	0.38–0.88	0.011
Laryngeal cancer (n = 245)								
G/G	71.8	65.8–77.4	61.6	55.2–67.8	63.7	1.00		
G/A	26.1	20.7–32.1	36.3	30.3–42.7	32.2	0.62	0.42–0.91	0.014
A/A	2.0	0.7–4.7	2.0	0.7–4.7	4.1	0.86	0.24–3.02	0.81 ^c
G/A + A/A	28.2	22.6–34.2	38.4	32.3–44.8	36.3	0.63	0.43–0.92	0.017
Pharyngeal cancer (n = 255)								
G/G	65.9	59.7–71.7	61.2	54.9–67.2	63.1	1.00		
G/A	30.6	25.0–36.6	36.5	30.6–42.7	32.7	0.78	0.54–1.13	0.19
A/A	3.5	1.6–6.6	2.4	0.9–5.1	4.2	1.39	0.49–4.00	0.54
G/A + A/A	34.1	28.3–40.3	38.9	32.8–45.1	36.9	0.82	0.57–1.17	0.27

^a Expected genotype frequency, calculated from allele frequencies.

^b Crude odds ratios compared to wild-type G/G in cases versus controls.

^c Fisher's exact test.

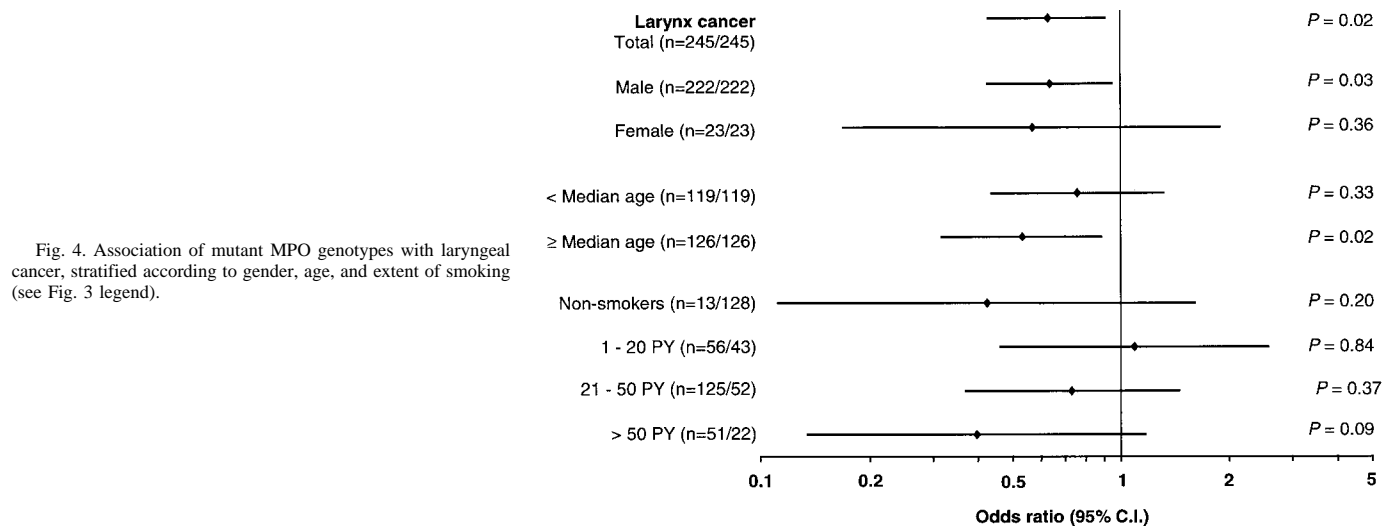


Fig. 4. Association of mutant MPO genotypes with laryngeal cancer, stratified according to gender, age, and extent of smoking (see Fig. 3 legend).

significantly lower rate of mutant genotypes than did tumors of less differentiated histology (adjusted odds ratio, 0.32; 95% CI, 0.10–0.97; $P = 0.044$).

DISCUSSION

Variants of MPO. The arylamine and arylhydrocarbon-activating and hypochlorous acid-generating MPO has been the subject of studies dealing with the etiopathogenesis of leukemia or chronic granulomatous disease (1). Rare amino acid exchanges, namely R569W (9), Y173C (2), and M251T (10), are known to lead to MPO inactivation. Initially, the G variant was assumed to be a somatic mutation because the majority of 16 patients with acute myeloid leukemia were homozygous carriers of the G variant, whereas only two displayed an adenine at –463 nt (11). These findings were supported by a study on acute promyeloid leukemia in which the –463G allele was also found to be predominant (3). Very recently, the G allele was reported to be associated with gender-specific increased risk of Alzheimer disease (17).

In contrast, the present study succeeded in demonstrating that in Caucasians, the G allele is indeed the wild-type genotype with a frequency of 78.5% in 270 healthy volunteers; however, this frequency was found to be increased in lung and laryngeal cancer

patients. This finding is in the range of 76.6% reported in American Caucasians (13). Among African Americans, G allele frequency is reportedly 70.1% (13). The –463 G/A exchange is located 645 bp upstream of the translation start. The Alu region of the MPO gene is known to be highly relevant for promoter activity (18) because it comprises a hormone-responsive element, especially the binding site of transcription factor SP1 (12). It was therefore assumed that the reportedly 25-fold activation of transcription activity of the G variant (leading to enhanced generation of toxic intermediates) would be one cause in the etiology of acute myelogenous leukemia. In comparison, the –463A variant demonstrated much lower activity.

Novel MPO Mutations. The two novel G/A mutations at –297 and –296 nt in the 5' region of the MPO gene were discovered by coincidence because they led to a loss of the constitutive *AcII* (GCGG) recognition site. These samples displayed an additional 229-bp fragment together with the wild-type 121-bp fragment, which was able to be well distinguished from the typical wild-type RFLP pattern (Fig. 2). The novel mutations did not appear together with the –463G/A mutation. This theoretical linkage would resolve in an uncut 350-bp fragment; however, this phenomenon was not observed. The functional significance of these mutations, located in an Alu sequence, is unclear.

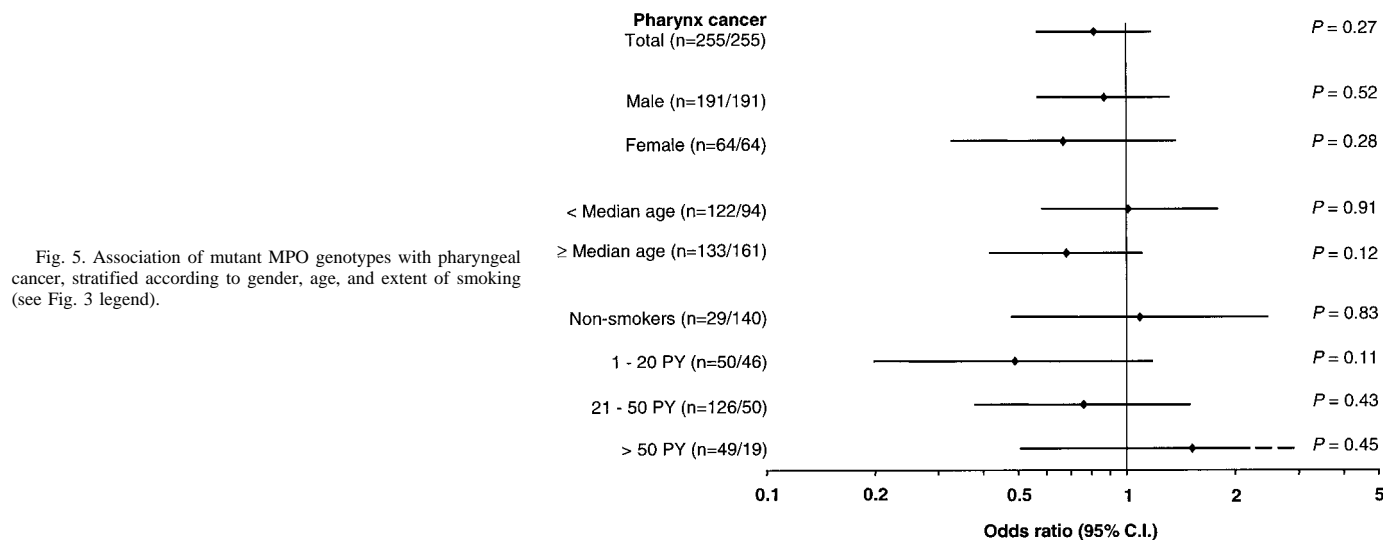


Fig. 5. Association of mutant MPO genotypes with pharyngeal cancer, stratified according to gender, age, and extent of smoking (see Fig. 3 legend).

Xenobiotics Metabolizing Enzymes. In comparison to the intriguing results from this study, polymorphisms of other arylamine-metabolizing enzymes showed partly weak associations to malignancies. Mutant genotypes of arylamine *N*-acetyltransferase (*NAT2*) are associated with increased susceptibility only among smokers or with a history of risk occupation (19). For lung and laryngeal cancer, *NAT2* has been found to be a susceptibility factor as a homozygous rapid variant (20, 21); the slow variant, however, seems to be overrepresented among older lung cancer patients (22).

As catalyzed by MPO, benzo(*a*)pyrene can be transformed to highly reactive intermediates by cytochrome P4501A1 (*CYP1A1*). A variant of *CYP1A1* has been shown to be associated with lung cancer in a Japanese sample (23); among Caucasians, results have diverged (24–28). On the other hand, a lack of detoxification enzymes such as *GSTM1*, especially in combination with polymorphisms of *CYP1A1*, was also shown to be associated with an inclination to cancer (19, 29–31). It has been possible to demonstrate this especially for benzo(*a*)pyrene DNA adduct formation in accordance with *GSTM1* and *CYP1A1* genotypes (32).

Lowered activation of aromatic compounds may therefore indeed reduce the risk of cancer with an etiology of gene-environmental interaction. London *et al.* (13) initially reported the association of the MPO polymorphism with cancer risk in Caucasians. Homozygous A carriers were significantly underrepresented among lung cancer cases in comparison to controls (odds ratio, 0.30). However, individuals heterozygous for –463A were equally distributed throughout cases and controls. In contrast, our study demonstrated that even an individual with one copy of the MPO –463A variant was at lower risk. Homozygous A carriers were rare in both groups, cases and controls, and did not differ significantly. It may therefore be assumed that there is a significant difference in MPO activity between G/G and G/A genotypes.

Impact of MPO. The present study showed that the –463G/A polymorphism is an unexpectedly strong genetic host factor, which suggests that the A variant is a protective factor in the etiology of carcinomas of lung and larynx. The modulation of pharyngeal cancer risk appears lower or even negligible, signifying that activation of aromatic compounds may play only a minor part in the etiology of pharyngeal cancer. The significance of these results was strengthened by the fact that MPO genotype distribution within the group of young healthy volunteers was similar to that of the matched control group. A population-based random sample would of course serve as an ideal control; however, the selected hospital controls of the present study evidently demonstrated a representative MPO genotype distribution.

Because MPO enhances the formation of *trans*-7,8-dihydroxy-7,8-dihydro-benzo(*a*)pyrene DNA adducts in lung tissue (5, 7), it could be expected that augmenting exposure to the precarcinogens contained in cigarette smoke would be mirrored in an increased significance of the MPO polymorphisms. Such a gene-environment interaction was indeed observed in laryngeal cancer patients (Fig. 4), but not in our group of lung cancer (Fig. 3) and pharyngeal cancer patients (Fig. 5) when compared to controls. The reason for the differing observations in lung and laryngeal cancer patients remains unclear.

In contrast to a severe deficiency in MPO activity, which leads to fatal diseases such as chronic granulomatous disease (33), normal activity as elicited by the –463G variant may increase the risk of carcinogen-mediated malignancies. An interesting observation was an increased frequency of G/G variants in chronic granulomatous disease patients with gastrointestinal complications (1). Eiserich *et al.* (34) debated a possible interaction with the formation of nitric oxide-derived inflammatory oxidants. Moreover, nitric oxide has also been reported to stimulate MPO activity (35).

In conclusion, the –463G/A polymorphism of leukocyte MPO has been proved to represent an intriguing susceptibility factor that modulates an individual's risk of lung and laryngeal cancer. Its effect on the risk of various cancers of the pharynx is either much lower or nonexistent. The role of the rare new single-nt G/A polymorphisms at –297 and –296 nt remains unclear.

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