

A Single Nucleotide Polymorphism in the Matrix Metalloproteinase-1 Promoter Enhances Lung Cancer Susceptibility¹

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ABSTRACT

Extracellular matrix-degrading matrix metalloproteinase-1 (MMP-1) is one of the interstitial collagenases likely to be involved in tumor invasion and metastasis. *MMP-1* may also contribute to tumor initiation and development by altering the cellular microenvironment that facilitates tumor formation. Recent studies have found that overexpression of *MMP-1* is associated with the initial stages of cancer development in addition to promoting cellular invasion; however, preexisting oncogenic mutations or chemical carcinogens are required to initiate tumorigenesis as well. There is a single nucleotide polymorphism located in the promoter region of *MMP-1* that partially regulates gene expression. The 2G/2G genotype enhances transcriptional activity and may be associated with an increased lung cancer risk. Using a case-control study, we tested the hypotheses that (a) individuals with the 2G/2G genotype may be at an increased risk for lung cancer; and (b) the risk should be greatly elevated in smoking individuals. PCR-RFLP was used to determine the *MMP-1* genotypes in 456 lung-cancer cases and 451 frequency-matched controls of Caucasian ethnicity. Overall, there was a significant association between the 2G/2G genotype and lung cancer risk [odds ratio (OR), 1.76; 95% confidence interval (CI), 1.29–2.39]. In current smokers, the lung cancer risk associated with the 2G/2G genotype was significantly elevated (OR, 3.16; 95% CI, 1.87–5.35). However, this association was less evident in former smokers (OR, 1.23; 95% CI, 0.81–1.87) and absent in never smokers (OR, 1.09; 95% CI, 0.31–3.91). Similarly, this risk was more evident in heavy smokers (OR, 2.55; 95% CI, 1.61–4.03) than in light smokers (OR, 1.40; 95% CI, 0.84–2.32). Interestingly, men were observed to have a 2.15-fold increased lung cancer risk (OR, 2.15; 95% CI, 1.42–3.26) compared with women (OR, 1.34; 95% CI, 0.84–2.15). Furthermore, subjects with 2G/2G genotype developed lung cancer earlier (60.94 ± 0.64 years old) than patients with 1G/1G and 1G/2G genotypes (62.91 ± 0.59 years old; $P = 0.024$). Our data demonstrate that the 2G/2G genotype enhances lung cancer susceptibility especially in current smokers. To our knowledge, these results report the first molecular epidemiological evidence of the *MMP-1* promoter polymorphism associated with the development of lung cancer in the presence of continuing carcinogenic exposure.

INTRODUCTION

The prevailing paradigm for the development of cancer is a complex, multistage process during which a normal cell undergoes genetic changes that result in phenotypic alterations and the acquisition of the ability to invade and colonize distant sites (1, 2). Although many factors are involved in tumor development, interactions between neoplastic cells and the surrounding microenvironment are crucial to each step of tumorigenesis.

One of the proteins that plays an essential role in the dynamics of

maintaining the cellular microenvironment is the MMP³ family. The MMP family comprises >20 enzymes that are associated with degradation of the ECM, including the basement membrane, as their name implies. Disruption of basement membrane integrity, a feature of invasive tumors, allows tumors to spread locally and distantly (3, 4). Therefore, it was initially believed that the MMPs, via breakdown of the physical barrier, were primarily involved in tumor invasion, blood vessel penetration, and metastasis. However, in addition to fostering cellular invasion by disrupting ECM barriers, MMPs can also influence the microenvironment by altering cellular signals (5, 6). More importantly, most MMPs are synthesized not only by the genetically altered cancer cells but also by adjacent and intervening stromal cells (7). There is also growing evidence to support an expanded role of MMPs in creating and maintaining a microenvironment that facilitates the initial stages of tumor development (8–11).

Among the MMPs, MMP-1 is the most highly expressed interstitial collagenase degrading fibrillar collagens, the most abundant protein in the human body. Overexpression of MMP-1 has been demonstrated in tumor tissues and has been suggested to be associated with tumor invasion and metastasis (12–14). Moreover, overexpression of MMP-1 has been found to be associated with an overall poor prognosis in colorectal and esophageal cancers (14, 15). The expression of *MMP-1* is partly regulated by the upstream promoter sequences of this gene (16). A 1G/2G polymorphic site has been found to be located in a core recognition sequence of the binding sites for transcription factors that consequently modifies the level of MMP-1 expression (17). Promoters containing the 2G allele display significantly higher transcriptional activity than 1G promoters. Kanamori *et al.* (18) observed a higher frequency of 2G alleles in ovarian cancer patients than in healthy controls, which suggests an association between *MMP-1* polymorphism and cancer risk. This *MMP-1* polymorphism may provide a mechanism for more aggressive matrix degradation, thereby facilitating tumor development.

In this large molecular epidemiological study, we used DNA samples to investigate the relationship between *MMP-1* polymorphism and the risk of lung cancer. We hypothesized that individuals with 2G/2G genotype may be overrepresented in the lung cancer group.

MATERIALS AND METHODS

Study Subjects. Lung cancer cases were accrued from The University of Texas M. D. Anderson Cancer Center. All of the cases were newly diagnosed, previously untreated (chemotherapy or radiotherapy), and histologically confirmed. There were no age, gender, ethnicity, or stage restrictions. We analyzed only the Caucasian subset of cases because of inadequate numbers of other ethnic groups. Healthy controls were identified from the largest managed care practice in the Houston metropolitan area. The controls did not have a previous diagnosis of any cancer type and were frequency-matched to the cases on age (± 5 years), gender, ethnicity, and smoking status. Identification and recruitment of the control study subjects is thoroughly detailed in Hudmon *et al.* (19). Briefly, during patient registration, the clinic personnel distributed a one-page questionnaire that elicited relevant criteria for control selection and participant interest. Subjects who indicated willingness to answer more questions were

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³The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular membrane; CI, confidence interval; OR, odds ratio.

Table 1 Distributions of select characteristics by case-control status

Variable	Cases (n = 456) n (%)	Controls (n = 451) n (%)	P
Gender			
Male	249 (54.61)	248 (54.99)	0.908
Female	207 (45.39)	203 (45.01)	
Age (yrs), mean (SD)	62.00 (9.30)	62.12 (9.22)	0.909
Smoking status			
Never	46 (10.09)	42 (9.31)	0.925
Former	234 (51.32)	234 (51.89)	
Current	176 (38.60)	175 (38.80)	
No. of cigarettes/day, ^a mean (SD)	28.88 (13.05)	28.96 (14.55)	0.494
Pack-years, ^a mean (SD)	52.48 (30.29)	50.49 (30.87)	0.134

^a Cigarettes/day and pack-years are for ever-smokers.

contacted by phone at a later date for confirmation of participation and to set an appointment for the interview.

Data Collection. Epidemiological data were collected by personal interview. After written informed consent was obtained, a 45-min structured interview was conducted by trained bilingual interviewers. Institutional guidelines for human subjects were followed. Data were collected on sociodemographic characteristics, recent and prior tobacco use, other life-style habits, and family history of cancer. At the completion of the interview, blood was drawn into sodium-heparinized tubes for immediate DNA isolation and subsequent molecular analysis. Laboratory personnel were blinded to case and control status.

MMP-1 Genotyping. The PCR-RFLP assay was used to determine the MMP-1 genotypes. Genomic DNA used for the assay was extracted from peripheral blood lymphocytes. The PCR primers used for amplifying MMP-1 polymorphism were: forward, 5'-TGACTTTTAAAACATAGTCTATGT-TCA-3'; and reverse, 5'-TCTTGGATTGATTGAGATAAGTCATA GC-3'. The reverse primer was specially designed to introduce a recognition site of restriction enzyme *AluI* (AGCT) by replacing a T with a G at the second position close to the 3' end of the primer. The 1G alleles have this recognition site, whereas the 2G alleles destroy the recognition site by inserting a guanine. PCR is performed in a 25- μ l volume made up of 2 μ l (20 ng) of diluted genomic DNA, 5 μ l of primer mix (1.25 μ M), 1 μ l of 10 mM dNTP mix, 2.5 μ l of 10 \times buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), and 1% Triton X-100], 11 μ l of dH₂O, 2.5 μ l of 25 mM MgCl₂, and 1 μ l of Taq polymerase (5 units/ μ l).

The PCR cycling conditions were 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, and with a final step at 72°C for 5 min to allow for the complete extension of all PCR fragments. A 20- μ l aliquot of PCR product was digested overnight at 37°C in a 23- μ l reaction containing 10 units of *AluI* (New England BioLabs, Beverly, MA) and 1 \times reaction buffer. After overnight digestion, the products were resolved and separated for 45 min at 220 V on a 4% agarose gel stained with ethidium bromide. After electrophoresis, homozygous 2G/2G alleles were represented by a DNA band with size at 269 bp, whereas homozygous 1G/1G alleles were represented by DNA bands with sizes at 241 and 28 bp, heterozygotes displayed a combination of both alleles (269, 241, and 28 bp).

Statistical Analysis. Smoking status was defined as the following: (a) an ever smoker, smoked at least 100 cigarettes in his or her lifetime; and (b) a former smoker, had stopped smoking at least 1 year prior to diagnosis for the cases and for the controls 1 year before the interview. Pack-years was defined as the number of cigarettes/day divided by 20 and then multiplied by the number of years smoked. All of the statistical analyses were performed using

STATA statistical software. Pearson's χ^2 was used to test the differences in the distribution between cases and controls. All of the ORs with 95% confidence limits were calculated as estimates of the relative risk. The crude ORs were calculated by the method of Woolf. The adjusted ORs were calculated by logistic regression to control for age, gender, and smoking status.

The relationships between MMP-1 2G/2G polymorphism and smoking status (never, former, and current) were also modeled using the logistic regression model, by including two interaction terms between the variables (MMP-1 and former smoking status, MMP-1 and current smoking status), that tests a multiplicative model. The first interaction term was coded as 1 if the individuals had 2G/2G genotype and former smoking status and was coded as 0 for all other individuals. The second interaction term was coded as 1 if the individuals had 2G/2G genotype and current smoking status and was coded as 0 for all other individuals.

RESULTS

Table 1 displays the selected characteristics of 456 lung cancer cases and 451 controls from our Caucasian population screened for MMP-1 genotypes. By study design, there were no statistically significant differences between the cases and controls by gender, age, smoking status, or pack-years.

The MMP-1 genotype distributions for cases and controls are shown in Table 2. The cases exhibited a statistically significantly higher frequency of the 2G/2G genotype (46.05%) than the controls (31.93%; $P < 0.001$). The frequencies of the genotypes were not associated with smoking status in controls ($P = 0.23$). The genotype frequencies in the controls were not in Hardy-Weinberg equilibrium ($P = 0.007$). When the 1G/1G and 1G/2G genotypes were combined, there was a significantly elevated lung cancer risk for individuals with 2G/2G genotype (OR, 1.82; 95% CI, 1.38–2.39). After adjustment by age, gender, and smoking status, the OR was slightly reduced to 1.76 (95% CI, 1.29–2.39) for individuals with only 2G/2G genotype.

Table 3 shows a gradient of elevated risks when the data were analyzed by smoking status. There was no effect (OR, 1.09; 95% CI, 0.31–3.91) in never smokers with the 2G/2G genotype and only a slightly increased risk for former smokers (OR, 1.23; 95% CI, 0.81–1.87). Current smokers with the 2G/2G genotype exhibited a significantly 3-fold elevated risk for lung cancer (OR, 3.16; 95% CI, 1.87–5.35). We also assessed interaction between smoking status (never, former, and current) and 2G/2G genotype using a logistic regression model by including an interaction term. The interaction term between the 2G/2G genotype and current smokers was positive and borderline significant with an OR of 2.31 (95% CI, 0.88–6.05; $P = 0.088$). When we further stratified former and current smokers by their median pack-years, a significantly increased risk was observed in heavy smokers (OR, 2.55; 95% CI, 1.61–4.03) but not in light smokers (OR, 1.40; 95% CI, 0.84–2.32). We also stratified the data by years since quitting but did not detect any impact of years since quitting on lung cancer risk (data not shown).

Moreover, when stratified by gender, we observed an overall greater increase in lung cancer risk in men (OR, 2.15; 95% CI, 1.42–3.26) compared with women (OR, 1.34; 95% CI, 0.84–2.15; Table 4). When further stratified by quartiles of pack-years in smoking controls (pack-years, 24, 40, and 61.5), men exhibited a higher

Table 2 Distribution of MMP-1 genotypes and risk estimates

MMP-1 genotype	Cases (n = 456) n (%)	Controls (n = 451) n (%)	P	Crude OR [95% CI]	Adjusted ^a OR [95% CI]
1G/1G ^b	94 (20.62)	111 (24.61)	}	Reference	Reference
1G/2G	152 (33.33)	196 (43.46)			
2G/2G	210 (46.05)	144 (31.93)			

^a Adjusted by age, gender, and smoking status.

^b Genotypes 1G/1G and 1G/2G were combined in the stratified analysis.

Table 3 Stratified analysis of lung cancer risk for the *MMP-1* genotypes by smoking status

<i>MMP-1</i> genotypes	Cases	Controls	Crude OR [95% CI]	Adjusted ^a OR [95% CI]
Never smokers				
1G/1G, 1G/2G	26	26	Reference	Reference
2G/2G	20	16	1.25 [0.53–2.95]	1.09 [0.31–3.91]
Former smokers				
1G/1G, 1G/2G	136	154	Reference	Reference
2G/2G	98	80	1.39 [0.95–2.02]	1.23 [0.81–1.87]
Current smokers				
1G/1G, 1G/2G	84	127	Reference	Reference
2G/2G	92	48	2.90 [1.83–4.59]	3.16 [1.87–5.35]
Light ^b smokers				
1G/1G, 1G/2G	100	140	Reference	Reference
2G/2G	76	65	1.64 [1.07–2.50]	1.40 [0.84–2.32]
Heavy ^b smokers				
1G/1G, 1G/2G	120	141	Reference	Reference
2G/2G	114	63	2.13 [1.43–3.17]	2.55 [1.61–4.03]

^a Adjusted by age and gender.

^b Dichotomized at median value of pack-year in the controls. For former and current smokers only.

elevated lung cancer risk than women in each stratified category. This gender difference was more evident in light smokers (pack-years, ≤40). The largest difference was observed in smokers with pack-years of 24 and 40, for which the risk was 2.8-fold (OR, 2.80; 95% CI, 1.14–6.87) increased for men and only 1.31-fold (OR, 1.31; 95% CI, 0.55–3.11) increased for women. The gender difference of lung cancer risk became less evident in heavy smokers, although the risk was still elevated for both men and women with the increased pack-years. We examined the interactive effect between gender and smoking status in lung cancer risk and did not detect interaction between these two variables. When we stratified by histology, the ORs were 1.76 (CI, 1.25–2.49) and 1.98 (CI, 1.34–2.93) for adenocarcinoma and nonadenocarcinoma, respectively. Therefore, we did not observe a more profound impact of this polymorphism on nonadenocarcinoma histologies.

In addition, we also observed a significant difference in age at onset of lung cancer between patients with 2G/2G genotype (60.94 ± 0.64 years old) and patients with 1G/1G and 1G/2G genotypes (62.91 ± 0.59 years old; *P* = 0.024). However, our analysis did not

detect a relationship between genotype and stage (I to IV) of the tumor (*P* = 0.45).

DISCUSSION

The interactions between neoplastic cells and their surrounding microenvironment have been well documented (20). Such studies support the concept that carcinogenesis is a multicellular and multi-stage process in which the destruction of the microenvironment is required for the conversion of normal tissue to tumor. Molecular analysis of the microenvironment and its deregulation during neoplasia has opened a new direction of cancer research that will provide insight into the microenvironmental effectors that control cell phenotype. The role of MMPs in these interactions has been accumulating since they were first identified to be involved in melanoma invasion and metastasis in the early 1980s (4). A recent study provided further evidence demonstrating that 83% of the metastatic melanomas retained the 2G allele of *MMP-1* (21). Although initially it was assumed that the tumor cell was the origin of the MMPs, there is growing evidence that most normal cells express detectable levels of MMPs. Given the fact that the microenvironment can influence tumor formation and MMPs can alter this environment, MMPs may contribute to the initial stages of cancer development, and overexpression of MMPs may be associated with elevated risk of tumorigenesis.

Our large epidemiological study supported the above hypothesis by observing a significant association between the 2G/2G genotype of the *MMP-1* promoter and increased lung cancer risk. The hypothesis was further confirmed by age at onset analysis showing that lung cancer patients with the 2G/2G genotype tend to develop cancer earlier (an average of 2 years) than patients without it. The 2G allele possesses a binding site for the Ets transcription factor; such binding is likely to lead to a higher transcriptional rate for the *MMP-1* gene. Although a previous study found that both 1G/2G and 2G/2G genotypes were significantly more frequent in patients with ovarian cancer than they were in normal controls (18), our results demonstrated that only the 2G/2G genotype was associated with increased risk of lung carcinoma. This finding was congruent with an earlier report that showed a high frequency of 2G homozygotes in tumor cell lines (17).

Table 4 Stratified analysis of *MMP-1* genotypes by gender and smoking status

	<i>MMP-1</i> genotypes	Cases	Controls	Unadjusted OR [95% CI]	Adjusted OR [95% CI]
Overall					
Male	1G/1G, 1G/2G	129	172	Reference	Reference
2G/2G		120	76	2.11 [1.45–3.06]	2.15 [1.42–3.26] ^a
Female	1G/1G, 1G/2G	117	135	Reference	Reference
2G/2G		90	68	1.53 [1.02–2.29]	1.34 [0.84–2.15] ^a
0–24 pack-years					
Male	1G/1G, 1G/2G	18	26	Reference	Reference
2G/2G		14	12	1.69 [0.64–4.43]	1.75 [0.65–4.74] ^b
Female	1G/1G, 1G/2G	22	26	Reference	Reference
2G/2G		13	12	1.28 [0.49–3.33]	1.23 [0.45–3.33] ^b
24.1–40 pack-years					
Male	1G/1G, 1G/2G	21	37	Reference	Reference
2G/2G		21	16	2.31 [1.00–5.33]	2.80 [1.14–6.87] ^b
Female	1G/1G, 1G/2G	25	30	Reference	Reference
2G/2G		19	19	1.20 [0.53–2.73]	1.31 [0.55–3.11] ^b
41–61.5 pack-years					
Male	1G/1G, 1G/2G	39	45	Reference	Reference
2G/2G		34	20	1.96 [0.98–3.93]	1.99 [0.99–4.02] ^b
Female	1G/1G, 1G/2G	32	32	Reference	Reference
2G/2G		24	13	1.85 [0.81–4.21]	1.83 [0.80–4.23] ^b
≥ 61.5 pack-years					
Male	1G/1G, 1G/2G	43	58	Reference	Reference
2G/2G		47	26	2.43 [1.31–4.52]	2.44 [1.31–4.54] ^b
Female	1G/1G, 1G/2G	20	27	Reference	Reference
2G/2G		18	11	2.21 [0.87–5.63]	2.21 [0.86–5.69] ^b

^a Adjusted by age and smoking status.

^b Adjusted by age.

However, the distribution of *MMP1* genotypes in our control group did not meet Hardy-Weinberg equilibrium, and neither did data from two previous studies that used samples from a Japanese population and the Center d'Etude du Polymorphisme Humain (CEPH) pedigrees, respectively (17, 18). In contrast, a recent survey on British Caucasians found that the genotype frequencies were consistent with Hardy-Weinberg equilibrium distribution (22).

Moreover, we observed a higher lung cancer risk in men with the 2G/2G genotype (OR, 2.15) than in women with the same genotype (OR, 1.34). The data suggested a gender-specific effect of the *MMP-1* polymorphism. There have been some studies showing that activity of MMP-1 may be regulated by sex hormones (23, 24). The secretion and activation of several MMPs, including MMP-1, were inhibited by ovarian steroids in cultured endometrial explants (23), which suggests that the mean level of MMP-1 may be less in women than in men during their lifetime. However, in heavily exposed smokers, it is rational that the effect of MMP-1 between men and women could have less impact on cancer risk because the intake of tobacco carcinogens may make an overwhelming contribution to the development of lung cancer. This may explain why the gender difference of lung cancer risk becomes less evident in heavy smokers.

A more striking finding was the gradient of elevated risks for lung cancer when the data were analyzed by smoking status. The *MMP-1* polymorphism was not associated with lung cancer risk in individuals who never smoked. However, there was a modestly increased risk in former smokers and a significantly elevated risk in current smokers. These results suggest that MMP-1 may increase lung cancer risk only in the presence of continuing carcinogenic exposure. This gradient of elevated risk was further confirmed when we stratified former and current smokers by the median pack-years. A significantly increased risk was observed in heavy smokers (OR, 2.55) whereas the risk was less evident in light smokers (OR, 1.40). A role of MMP-1 in promoting carcinogenesis was also observed in experimental animals. For example, mice that express the *MMP-1* transgene are more sensitive to chemical carcinogens than their nontransgenic littermates (25). Furthermore, mice that lack MMP-1 form fewer and smaller carcinogen-induced tumors than wild-type mice (26). Therefore, our epidemiological data support the likelihood that MMPs profoundly influence early tumor initiation and development.

Given that gender and smoking may influence the effect of the polymorphism, it is logical to speculate that the polymorphism might be associated with tumor histology. Because a high proportion of all lung cancer in both women and former smokers is adenocarcinoma, the observed smoking and gender differences could be explained by differences in cancer histology. We found that the 2G/2G genotype is associated with a slightly higher risk in nonadenocarcinoma histologies (OR, 1.98; CI, 1.34–2.93) than in adenocarcinoma histology (OR, 1.76; CI, 1.25–2.49); however, this difference was not statistically significant. Therefore, we may conclude that the polymorphism does not have a more profound impact on nonadenocarcinoma histologies and cannot account for the observed smoking and gender differences.

Although MMPs are not oncogenic or mutagenic, there are several routes whereby they can alter the cellular microenvironment and consequently affect the process of neoplastic transformation and tumor development. By degrading extracellular matrix, MMPs can release active growth factors, angiogenic factors, and angiogenic inhibitors from the cell surface and ECM (27, 28). Some of these growth factors may influence tumor cells directly, whereas others may influence neighboring cells that are essential to support tumor growth. MMPs can also cleave a number of molecules on the cell surface, including the tumor suppressor E-cadherin (29). Moreover, MMPs may alter cell cycle checkpoint controls and conceivably promote

genomic instability by affecting cell adhesion (30). Hence, MMPs may contribute in multiple ways to all stages of cancer development, including initiation.

In summary, our data provide the first molecular epidemiological evidence of the *MMP-1* promoter polymorphism associated with the risk of lung cancer in the presence of carcinogenic exposure. Our observations provide indirect evidence of a role for MMPs in altering cellular microenvironment, thereby facilitating tumor development. These findings may ultimately help in identifying high-risk populations.

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