Matrix Metalloproteinase Single-Nucleotide Polymorphisms and Haplotypes Predict Breast Cancer Progression

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Abstract

Purpose: Polymorphisms within the promoter region of several matrix metalloproteinase (MMP) genes have been linked to alterations in the level of transcription. We hypothesized that an individual's MMP genotype and haplotype will influence breast tumor progression and help predict prognosis.

Experimental Design: This study has evaluated the association between single-nucleotide polymorphisms (SNP) in the promoter regions of *MMP-1, MMP-3, MMP-7, MMP-9, MMP-12,* and *MMP-13* and metastatic spread of breast cancer in 128 lymph node – negative and 93 lymph node – positive patients. The study cohort was of mixed ethnicity, with Caucasian patients comprising 65%. Associations between genotype and lymph node status were estimated by logistic regression and with overall survival using the method of Kaplan-Meier and log-rank test. Associations between haplotype and lymph node status were also investigated.

Results: The data show a significant and independent association of the C/T genotype for *MMP-9* [mixed ethnicities odds ratio 3.6, 95% confidence interval (95% CI) 1.2-11.1; Caucasian odds ratio 9.1, 95% CI 1.7-48.4] and the 2G/2G genotype for *MMP-1* (mixed ethnicities odds ratio 3.9, 95% CI 1.7-9.4; Caucasian odds ratio 2.6, 95% CI 1.0-6.9) with lymph node – positive disease. *MMP-1* 2G/2G was associated with reduced survival (hazard ratio 3.1, 95% CI 1.1-8.7), although this is dependent on lymph node status. Two haplotypes, driven by the *MMP-1* 2G allele, were significantly associated with lymph node – positive disease and survival.

Conclusions: These results suggest that MMP single-nucleotide polymorphisms influence breast cancer behavior and that the *MMP-1* 2G/2G genotype increases the risk of lymph node metastasis and predicts poor prognosis.

Breast cancer is the most common female cancer in the Western world and constitutes ~30% of all cancers diagnosed in women. Although breast cancer – related deaths have decreased by >20% since 1995, predicting outcome still is a major challenge. Breast cancer is a very heterogeneous disease (1), and there is a continuing need to predict those patients who are likely to have more aggressive disease and to tailor treatment accordingly. As a result, intensive research is currently under way to identify the genetic determinants that may prove to be important for both classification and prognosis prediction.

Matrix metalloproteinases (MMP) are a multifunctional family, comprising >28 endoproteinases, that are involved in the degradation of extracellular matrix and basement membrane barriers (2 – 4). Members of the family have been implicated in the promotion of tumor growth, angiogenesis, invasion, and metastasis (5 – 7). Elevated levels of MMPs have been described in many tumors wherein MMPs are associated with tumor spread and poor patient prognosis (3, 8 – 10). MMP gene expression is regulated primarily at the transcriptional level and in many solid tumors, particularly breast cancer; the major source of MMPs is the host microenvironment in response to tumor cell – derived signals (11). Thus, factors which influence the host response may play a role in determining tumor behavior in an individual.

Over recent years, single-nucleotide polymorphisms (SNP), constituting insertions (e.g., *MMP-1*, G at position -1,607 bp), deletions (e.g., *MMP-3*, A at position -1,171 bp), or substitutions (e.g., *MMP-7*, A to G substitution at -181 bp; *MMP-9*, C to T substitution at -1,562 bp; *MMP-12*, A to G substitution at -82 bp; *MMP-13*, A to G substitution at -77 bp) of single bases upstream of the transcriptional start site of MMP genes have been described and shown to affect transcriptional activity,

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leading to alterations in gene expression (12). Thus, the *MMP-1* 2G allele creates a binding site for the Ets transcription factor, enhancing transcriptional activity (13), whereas the *MMP-3* 6A allele exhibits weaker transcriptional activity than the 5A allele, possibly due to enhanced binding of a transcriptional repressor (14). The *MMP-7* G allele, the *MMP-9* T allele, the *MMP-12* A allele, and the *MMP-13* G allele all have been shown and predicted to generate higher levels of gene transcription and hence enzyme activity (15–18). These polymorphisms have been variably linked to disease susceptibility (19), tumor invasive capacity (20), and patient prognosis (21) in other cancer types.

Given the role of MMPs in breast cancer progression and the association of high levels of MMPs with poor patient prognosis, we hypothesized that individuals carrying high-expressing MMP genotypes may be more susceptible to tumor spread compared with those patients lacking these alleles. This study therefore aimed to investigate the relationship between functional SNPs in *MMP-1*, *MMP-3*, *MMP-7*, *MMP-9*, *MMP-12*, and *MMP-13* and tumor behavior in a cohort of patients with breast cancer. We further hypothesized that the joint effects of these SNPs may exert a greater risk than the individual effect of each SNP; therefore, we analyzed the relationship between gene haplotypes and tumor behavior.

Materials and Methods

Patient samples and cohort selection. A total of 221 patients with breast cancer presenting between 1990 and 2006 to St. Bartholomew's Hospital, London (95% from 1999 onwards) were included in this study, following ethics approval from the North East London LREC. From each year of recruitment, all lymph node–positive cases and approximately equal numbers of grade-matched lymph node–negative cases were selected. The cases before 1999 had presented with recurrence during the main study period of 1999 to 2006, but the original diagnosis was between 1990 and 1998. The original histology was reviewed, and details regarding tumor grade, type, and lymph node status were recorded. All patients underwent segmental or total mastectomy plus an axillary node procedure. This was level 1 clearance before 2001, and sentinel node biopsy plus sample (minimum of four nodes) from 2001. All lymph nodes were assessed on single H&E alone, and micrometastasis was excluded from the study. Data were available for estrogen receptor and progesterone receptor status. Patient ethnicity details were retrieved from the clinical records, and for the purpose of this study, individuals were recorded as Caucasian or other. Patient outcome data were retrieved from the clinical records, with a median follow-up of 60 months (range, 2-180 months). MMP SNP genotype and haplotype were related to disease-free survival and overall survival. Only breast cancer–specific deaths were considered, with data from patients who died from other causes being censored.

DNA preparation. Nontumor DNA was isolated from formalin-fixed paraffin-embedded noninvolved lymph node. Eight serial sections (5 μm) were taken from selected lymph node blocks, placed into a 1.5-mL tube, and deparaffinized using xylene. DNA was extracted using the QIAamp DNA minikit (Qiagen) following manufacturer's instructions.

MMP PCR. PCR was carried out in a 25 μL volume containing 25 ng of genomic DNA, 1 μL of each primer (2 mmol/L stock), 2 μL of 2.5 mmol/L deoxynucleotide triphosphate mix (Invitrogen), 2.5 μL of 10× PCR buffer, $MgCl_2$ (1.25 μL of a 50 mmol/L stock for MMP-7, MMP-12, and MMP-13 or 2.5 μL of a 50 mmol/L stock for MMP-1 and MMP-3), 0.2 μL of Platinum Taq DNA polymerase (5 units/μL; Invitrogen), and sterile deionized H_2O up to a final volume of 25 μL.

PCR conditions were as follows: initial denaturation for 2 min at 95°C; 40 cycles of denaturing for 30 s at 94°C, annealing for 30 s at a primer-specific temperature [*MMP-1*, forward 5′ TCGTGAGAATGTCTTCCCATT 3′ (HEX tagged)/reverse 5′ TTATCACTTCAGCACCTTATGGT 3′; *MMP-7*, forward 5′ CTGAATGATACCTATGAGAGCAGT 3′/reverse 5′ GCAGGAAGCACACAATGAATT 3′; the underlined base refers to an engineered base change in the primer, which substitutes a T for an A, thus generating an *Eco*RI restriction site; *MMP-12*, forward 5′ TGCTTGAGTGATGGACTAGAT 3′/reverse 5′ TTCTAGCCTAAGTTCCTGAACTGT 3′; *MMP-13*, forward 5′ TCGCCACGTAAGCATGTTTACCT 3′/reverse 5′ GACAAATCATCTTCATCACC 3′ all at 55°C; *MMP-3*, forward 5′ GGAATTCACATCACTGCCACCACT 3′ (FAM tagged)/reverse 5′ AGTGCTAGGATTACAGACATGGGTCA 3′ at 64°C; *MMP-9*, forward 5′ TGGTCAACGTAGTGAAACCCCATCT 3′/reverse 5′ TCCAGGCCCAATTATCACACTTAT 3′ at 67°C] and elongation for 30 s at 72°C; then 10 min at 72°C.

Genotyping. Genotype information for each gene is shown in Table 1. The *MMP-1* and *MMP-3* genotypes were determined by studying PCR product length by fluorescent genotyping. The forward

Table 1. MMP polymorphism information

Gene name	Polymorphism	SNP ID	PCR product size (bp)	Screening method	Restriction enzyme	Recognition sequence*	Wild-type fragment size (bp)	Mutant fragment size (bp)
MMP-1	Insertion of guanine residue at -1,607 bp	rs1799750	132 or 133	Fluorescent genotyping			132	133
MMP-3	Deletion of an adenine residue at -1,171 bp	rs3025058	188 or 189	Fluorescent genotyping			189	188
MMP-7	A to G substitution at -181 bp	rs11568818	134	PCR-RFLP	*Eco*RI	5′ G/AATTC 3′ 3′ CTTAA/G 5′	134	102 and 32
MMP-9	C to T substitution at -1,562 bp	rs3918242	386	PCR-RFLP	*Sph*I	5′ GCATG/C 3′ 3′ C/GATCG 5′	386	320 and 66
MMP-12	A to G substitution at -82 bp	rs2276109	185	PCR-RFLP	*Hpy*CH4III	5′ ACN/GT 3′ 3′ TG/NCA 5′	163 and 22	101, 62, and 22
MMP-13	A to G substitution at -77 bp	rs2252070	233	PCR-RFLP	*Bsr*I	5′ ACTGGN/3′ 3′ TGAC/CN 5′	233	168 and 65

*Backslash (/) characters indicate the location that the restriction enzyme cuts in the sequence.

Table 2. Characteristics and pathologic factors by lymph node status and population group

Characteristics	Caucasian			Mixed ethnicity (all subjects)		
	Node-positive, *n* (%)	Node-negative, *n* (%)	*P**	Node-positive, *n* (%)	Node-negative, *n* (%)	*P**
Age (y)			0.07			0.07
≤60	31 (58)	38 (43)		46 (58)	53 (45)	
>60	22 (42)	51 (57)		33 (42)	65 (55)	
Tumor grade			0.001			0.001
1	1 (2)	18 (20)		2 (3)	25 (21)	
2	25 (49)	42 (48)		32 (42)	55 (47)	
3	24 (47)	28 (32)		42 (55)	37 (32)	
Unknown	1 (2)	0 (0)		1 (1)	0 (0)	
Estrogen receptor status			0.54			0.61
Positive	7 (13)	15 (17)		10 (13)	20 (18)	
Negative	45 (87)	71 (83)		67 (87)	94 (82)	
Progesterone receptor status			0.39			0.90
Positive	16 (33)	20 (26)		20 (27)	27 (26)	
Negative	33 (67)	58 (74)		54 (73)	76 (74)	

*Two-sided χ^2 test.

PCR primers for *MMP-1* and *MMP-3* were labeled fluorescently with HEX and FAM, respectively. Each PCR product was diluted to 40 pg/μL; 2 μL of this was then added to 8 μL of Hi-Di and Rox size standard (ratio, 50:1; Applied Biosystems). The resulting 10 μL was then loaded onto an ABI 3700 automated sequencer, and the results were analyzed using Genescan and Genotyper packages (Applied Biosystems).

The *MMP-7* (*Eco*RI), *MMP-9* (*Sph*I), *MMP-12* (*Hpy*CH4III), and *MMP-13* (*Bsr*I) genotypes were determined by PCR-RFLP assays. The restriction enzymes used for each gene are displayed in parentheses. All enzymes were obtained from New England Biosciences, and digestion was done in a 30-μL reaction volume following the manufacturer's instructions. After digestion, the products were resolved by electrophoresis on a 4% agarose gel and stained with ethidium bromide.

To determine the genotyping false call rate a 10% sample of randomly selected patients were regenotyped for each of the six MMPs (22).

Statistical analysis. Power and sample size was calculated using QUANTO version 1.2.1 (23). For the genes examined, in the Caucasian-only population, a sample size of 116 gives 80% power and a 0.05 level of significance. Differences in patients' characteristics and pathologic factors stratified by lymph node status were evaluated using χ^2 test. The associations between MMP genotype frequencies and lymph node status were estimated by computing odds ratios and 95% confidence intervals (95% CI) from both univariate and multivariate logistic regression analyses with adjustment for tumor grade. The association between genotype and disease-free and overall survival was investigated using the method of Kaplan-Meier and was estimated using the log-rank test. Cox regression models were used to adjust for potential confounders. All statistical analyses were two-sided and done using Minitab release 14.20 and STATA 9.2. We used Chaplin 1.2.2 (24, 25) and HAPSTAT (26, 27) to identify haplotypes that were associated with lymph node metastasis and Thesias (28, 29) to identify haplotypes that were associated with survival.

Results

Cohort characteristics. We analyzed MMP promoter polymorphisms in a cohort of 221 patients, comprising 128 lymph node–negative and 93 lymph node–positive patients. This cohort consisted of mixed ethnicities, with Caucasian patients comprising 65% of the study group. The remaining 35% comprised 1 Pakistani, 1 Arabic, 2 Muslim, 2 Greek, 3 Turkish, 4 Asian, 4 Jewish, 18 African, and 47 other patients for whom ethnicity was not reported. Owing to the heterogeneous nature of the group, the cohort was analyzed as two groups: Caucasian-only and mixed ethnicity, which included all patients. Patient tumors were grade matched, with the exception of the grade I tumors, which were underrepresented in the lymph node–positive cases. Patients were also matched for estrogen receptor/progesterone receptor status and age. At the time of breast cancer diagnosis, lymph node–negative patients were between ages 33 and 88 years (mean, 61 ± 12) and lymph node–positive patients were between ages 36 and 77 years (mean, 57 ± 11). No associations were observed between estrogen receptor/progesterone receptor status or age and the occurrence of lymph node metastasis. Higher grade tumors were associated with a lymph node–positive status ($P < 0.001$), and there was a significant relationship between lymph node–positive status and reduced survival ($P < 0.001$). Characteristics of the study population are summarized in Table 2.

Association between individual genotypes and tumor spread. The genotype distributions of all MMPs in the mixed ethnicity group and for the selected Caucasian group of patients are shown in Table 3. All data were adjusted for tumor grade. The presence of polymorphisms within the promoters of *MMP-1* and *MMP-3*, resulting from the insertion/deletion of a G or A, respectively, was determined by fluorescent genotyping. The SNPs for *MMP-7*, *MMP-9*, *MMP-12*, and *MMP-13*, wherein the SNP generated or eliminated a restriction enzyme recognition site, were identified by PCR-RFLP. The application of these approaches permitted the determination of an individual's genotype for each of these loci. The average genotyping success rate for the 221 patients was >90%, with a false call rate of <1%. With the exception of *MMP-13*, the distribution of genotypes did not deviate from Hardy-Weinberg equilibrium, which suggests that this patient cohort

Table 3. Analysis of association between MMPs and the risk of lymph node metastasis by population group

Genotype	Mixed ethnicity				
	No. patients	Node-negative, *n* (%)	Node-positive, *n* (%)	Crude OR [95% CI] (*P*)	Adjusted* OR [95% CI] (*P*)
MMP-1					
1G/1G	52	35 (27.8)	17 (18.5)	1.0	1.0
1G/2G	101	64 (50.8)	37 (40.2)	1.2 [0.6-2.4] (0.63)	1.2 [0.5-2.6] (0.75)
2G/2G	65	27 (21.4)	38 (41.3)	**2.9 [1.4-6.2] (0.01)**	**3.9 [1.7-9.4] (0.001)**
MMP-3					
5A/5A	49	26 (20.9)	23 (25.5)	1.0	1.0
5A/6A	114	70 (56.5)	44 (49)	0.7 [0.4-1.4] (0.32)	1.1 [0.5-2.3] (0.88)
6A/6A	51	28 (22.6)	23 (25.5)	0.9 [0.4-2.0] (0.85)	0.8 [0.3-2.0] (0.63)
MMP-7					
A/A	70	37 (31.4)	33 (37.9)	1.0	1.0
A/G	96	60 (50.8)	36 (41.4)	0.7 [0.4-1.3] (0.21)	0.6 [0.3-1.2] (0.17)
G/G	39	21 (17.8)	18 (20.7)	1.0 [0.4-2.1] (0.92)	0.7 [0.3-1.7] (0.40)
MMP-9					
C/C	167	105 (93.7)	62 (81.6)	1.0	1.0
C/T	21	7 (6.3)	14 (18.4)	**3.4 [1.3-8.9] (0.01)**	**3.6 [1.2. 11.1] (0.001)**
MMP-12					
A/A	168	98 (80.3)	70 (82.4)	1.0	1.0
A/G	39	24 (19.7)	15 (17.6)	0.9 [0.4-1.8] (71)	1.2 [0.5-2.8] (0.63)
MMP-13					
A/A	105	62 (54.9)	43 (53.8)	1.0	1.0
A/G	83	47 (41.6)	36 (45)	1.1 [0.6-2.0] (0.74)	1.2 [0.6-2.3] (0.62)
G/G	5	4 (3.5)	1 (1.2)	0.4 [0.0-3.3] (0.36)	0.6 [0.1-6.2] (0.71)

NOTE: Values in bold indicate significant results.
Abbreviation: OR, odds ratio.
*Adjusted for tumor grade.

did not deviate genetically from the general population of the United Kingdom. In the whole cohort, regression analyses showed that patients carrying the 2G/2G genotype for *MMP-1* showed a significant 3.9-fold (95% CI, 1.7-9.4; $P < 0.001$) increased risk of lymph node metastasis compared with subjects carrying the 1G/1G genotype (Table 3). Subjects carrying the C/T genotype for *MMP-9* had a significant 3.6-fold (95% CI, 1.0-11.1; $P < 0.001$) increased risk of lymph node metastasis compared with C/C carriers (Table 3). There were no patients with T/T genotype. To minimize possible confounding issues due to allele frequency variation by race, separate analyses were done on the Caucasian-only group. This strengthened the association of the *MMP-9* genotype with nodal status with the C/T genotype exhibiting a significant 9.1-fold (95% CI, 1.7-48.4; $P = 0.01$) increased risk of nodal invasion, whereas the association between the 2G/2G *MMP-1* genotype and nodal status showed a borderline-significant increased risk of metastasis (2.6-fold; 95% CI, 1.0-6.9; $P < 0.06$). The genotype frequencies for *MMP-3*, *MMP-7*, *MMP-12*, and *MMP-13* were not significantly different between node-negative and node-positive patients in either the mixed ethnicity or Caucasian-only group. Separate analyses were not done on individual ethnic groups because the numbers in each group were too small.

Association between individual genotypes and overall survival and disease-free survival. Using the Cox regression model (Table 4A), only the presence of the 2G/2G genotype for *MMP-1* compared with the presence of the 1G/1G genotype was predictive of a shorter patient survival time [crude hazard ratio (CHR) for overall survival, 3.1; 95% CI, 1.1-8.7; $P = 0.03$; Fig. 1A]. None of the other MMP polymorphisms was associated significantly with survival. After adjusting for tumor grade and lymph node status, the *MMP-1* polymorphism was no longer significantly associated with overall survival (adjusted hazard ratio for overall survival, 2.4; 95% CI, 0.8-6.6; $P = 0.1$; Fig. 1B; Table 4A). There was no significant association between individual MMP SNPs and disease-free survival (Table 4B).

Association between MMP haplotype and tumor behavior. Because *MMP-1*, *MMP-3*, *MMP-7*, *MMP-12*, and *MMP-13* genes are clustered on chromosome 11q22.3, we addressed whether there were any associations between specific haplotypes and tumor behavior. Eleven common haplotypes were identified among both node-negative and node-positive patients, with the distribution of most being similar between the two patient groups (Table 5). However, two haplotypes did exhibit a significant association with lymph node metastasis: 2G-5A-A-A-A (2% frequency in lymph node–negative patients *versus* 10% in lymph node–positive; $P = 0.02$) and 2G-5A-G-A-A (8% in lymph node–negative patients versus 20% in lymph node–positive; $P = 0.02$). In addition, these two haplotypes (2G-5A-A-A-A: CHR 2.5, 95% CI 1.2-5.2, $P = 0.02$; 2G-5A-G-A-A: CHR 4.5, 95% CI 2.3-8.8, $P = 0.00001$) also showed a significant association with overall survival. After adjusting for tumor grade and lymph node status, the 2G-5A-G-A-A haplotype (adjusted hazard ratio, 2.6; 95% CI, 1.3-5.0; $P = 0.006$) was still associated with overall survival.

Table 3. Analysis of association between MMPs and the risk of lymph node metastasis by population group (Cont'd)

Genotype	Caucasian				
	No. patients	Node-negative, *n* (%)	Node-positive, *n* (%)	Crude OR [95% CI] (*P*)	Adjusted* OR [95% CI] (*P*)
MMP-1					
1G/1G	38	26 (29.5)	12 (23)	1.0	1.0
1G/2G	63	43 (48.9)	20 (38.5)	1.01 [0.3-2.4] (0.98)	0.9 [0.4-2.3] (0.83)
2G/2G	39	19 (21.6)	20 (38.5)	2.3 [0.9-5.8] (0.08)	2.6 [1.0-6.9] (0.06)
MMP-3					
5A/5A	39	23 (27)	16 (32)	1.0	1.0
5A/6A	73	44 (51.8)	29 (58)	1.0 [0.4-2.1] (0.89)	1.1 [0.5-2.4] (0.92)
6A/6A	23	18 (21.2)	5 (10)	0.4 [0.1-1.3] (0.40)	0.3 [0.1-1.2] (0.10)
MMP-7					
A/A	47	30 (37)	17 (34.7)	1.0	1.0
A/G	59	39 (48)	20 (42.5)	0.9 [0.4-2.0] (0.81)	0.7 [0.3-1.7] (0.54)
G/G	24	12 (15)	12 (22.8)	1.8 [0.7-4.8] (0.26)	1.3 [0.4-3.7] (0.65)
MMP-9					
C/C	109	74 (97.3)	35 (81.3)	1.0	1.0
C/T	10	2 (2.7)	8 (19.7)	**8.5 [1.7-41.9] (0.01)**	**9.1 [1.7-48.4] (0.01)**
MMP-12					
A/A	107	67 (80.7)	40 (83.3)	1.0	1.0
A/G	24	16 (19.3)	8 (16.7)	0.8 [0.3-2.1] (0.71)	1.1 [0.4-2.8] (0.91)
MMP-13					
A/A	71	44 (56.4)	27 (61.4)	1.0	1.0
A/G	49	32 (41)	17 (38.6)	0.8 [0.4-1.6] (0.74)	0.9 [0.4-2.0] (0.79)
G/G	2	2 (2.6)	0 (0.0)	-	-

The haplotype 2G-5A-G-A-A was associated with disease-free survival in the unadjusted analysis (CHR, 2.7; 95% CI, 1.08-6.70; $P = 0.03$) but was no longer significant after adjusting for lymph node status and grade (CHR, 1.9; 95% CI, 0.68-5.45; $P = 0.21$).

Discussion

MMPs have been implicated in tumor progression, and high levels of expression have been related to more aggressive tumor behavior and poorer patient prognosis (20, 21). This study, therefore, investigated the association between functional SNPs in MMP genes, previously implicated in breast cancer, and breast cancer spread and patient survival. In the genotype analysis, wherein each MMP SNP was studied separately in the mixed ethnicity patient cohort, the 2G/2G genotype of *MMP-1*, which generates an increased level of gene expression (13), was more frequent in the lymph node–positive patients and conferred a 3.9-fold increased risk of lymph node metastasis. The C/T genotype of *MMP-9* was found to confer a 3.6-fold increased occurrence of lymph node metastasis. Interestingly, there is a change in the association pattern when analysis is restricted to the Caucasian-only group, with the *MMP-1* 2G/2G genotype risk decreasing to 2.6-fold, whereas the *MMP-9* C/T genotype confers a 9.1-fold increase in risk of metastasis in this group. Such differences between different ethnic groups may, in some part, explain the discrepancies reported between associations in the literature and the work described herein. Thus, although Przybylowska et al. (3) reported a similar association between *MMP-1* 2G/2G genotype and lymph node metastasis in patients with breast cancer, they showed no such association with the *MMP-9* T allele. This could be a consequence of variation in the ethnicity of the patients from the two cohorts. In our study, when the Caucasian patients were subtracted from the ethnically mixed group, and the latter analyzed, there was no association between the T allele and node status when corrected for grade ($P = 0.96$; odds ratio, 1.05; 95% CI, 0.18-5.93). Similarly, in colorectal cancer, whereas a relationship between the *MMP-1* 2G/2G genotype and poor prognosis has been identified in an Italian cohort of patients with colorectal cancer (30), no such relationship was identified in a recent large Australian study (31).

Previous studies have identified links between promoter polymorphisms in *MMP-1* and *MMP-9* (15, 32) and increased expression of enzyme levels. In addition, increased expression for both *MMP-1* and *MMP-9* has been associated with lymph node metastasis (33) and poor prognosis (3, 34, 35) in breast cancer. In keeping with this, our data and others' point to the potential prognostic role of *MMP-1* and *MMP-9* polymorphisms in identifying patients at risk of more aggressive disease.

Having identified a link between genotype and node status, the analysis was extended to determine any association between genotype, disease-free survival, and overall survival. Previous studies have identified such links for both *MMP-2* (36) and *MMP-12* (37) in non–small cell lung cancer, between *MMP-7* and gastric cancer (38) and between *MMP-1* and colorectal cancer (39). In this study, analysis was restricted to the Caucasian-only group. No association between MMP genotype and disease-free survival was identified; however, initial analysis showed a significant association between the *MMP-1* 2G/2G genotype and overall survival. However, after adjustment for node status, this association was lost. This is in keeping with our hypothesis that elevated levels of MMPs promote spread of breast cancer. Thus, the high-expressing *MMP-1* 2G/

Table 4. Multivariate analysis of overall survival and disease-free survival

A. Multivariate analysis of overall survival

Genotype	*n*	Alive, *n* (%)	Dead, *n* (%)	CHR [95% CI] (*P*)	Adjusted hazard ratio [95% CI] (*P*)*
MMP-1					
1G/1G	36	31 (29.8)	5 (15.6)	1.0	1.0
1G/2G	63	51 (49)	12 (37.5)	1.7 [0.6-4.7] (0.34)	1.3 [0.5-3.8] (0.60)
2G/2G	37	22 (21.2)	15 (46.9)	**3.1 [1.1-8.7] (0.03)**	2.4 [0.8-6.6] (0.10)
MMP-3					
5A/5A	39	26 (25.5)	13 (43.3)	1.0	1.0
5A/6A	71	58 (56.9)	13 (43.3)	0.5 [0.2-1.1] (0.07)	0.6 [0.3-1.2] (0.14)
6A/6A	22	18 (17.6)	4 (13.4)	0.4 [0.1-1.2] (0.11)	0.6 [0.2-2.0] (0.45)
MMP-7					
A/A	45	36 (36.4)	9 (28.1)	1.0	1.0
A/G	59	45 (45.5)	14 (43.8)	1.2 [0.5-2.9] (0.61)	1.1 [0.5-2.6] (0.80)
G/G	24	15 (18.1)	9 (28.1)	1.9 [0.8-4.8] (0.17)	1.4 [0.5-3.7] (0.46)
MMP-9					
C/C	105	81 (93.1)	24 (85.7)	1.0	1.0
C/T	10	6 (6.9)	4 (14.3)	1.6 [0.6-4.7] (0.37)	0.5 [0.2-1.5] (0.22)
MMP-12					
A/A	103	77 (79.4)	26 (86.7)	1.0	1.0
A/G	24	20 (20.6)	4 (13.3)	0.6 [0.2-1.7] (0.34)	0.9 [0.3-2.6] (0.80)
MMP-13					
A/A	70	51 (56.7)	19 (63.3)	1.0	1.0
A/G	48	37 (41.1)	11 (36.7)	0.8 [0.4-1.6] (0.47)	0.8 [0.4-1.7] (0.61)
G/G	2	2 (2.2)	0 (0%)	-	-

B. Multivariate analysis of disease-free survival

Genotype	*n*	Disease-free survival, *n* (%)	Recurrence *n* (%)	CHR [95% CI] (*P*)	Adjusted hazard ratio [95% CI] (*P*)*
MMP-1					
1G/1G	34	28 (28.9)	6 (20.7)	1.0	1.0
1G/2G	59	49 (50.5)	10 (34.3)	1.2 [0.4-3.2] (0.76)	1.0 [0.4-3.0] (0.89)
2G/2G	33	20 (20.6)	13 (44.8)	2.3 [0.9-6.1] (0.09)	1.7 [0.7-4.8] (0.24)
MMP-3					
5A/5A	33	23 (24.7)	9 (30)	1.0	1.0
5A/6A	69	54 (53.1)	15 (50)	0.8 [0.4-1.9] (0.63)	0.9 [0.4-2.0] (0.74)
6A/6A	22	16 (17.2)	6 (20)	0.9 [0.3-2.4] (0.76)	1.3 [0.4-3.6] (0.67)
MMP-7					
A/A	44	34 (39.1)	10 (32.3)	1.0	1.0
A/G	55	39 (44.8)	16 (51.6)	1.3 [0.6-2.8] (0.55)	1.3 [0.6-2.8] (0.54)
G/G	19	14 (16.1)	5 (16.1)	1.0 [0.3-2.8] (0.94)	0.9 [0.30-2.6] (0.83)
MMP-9					
C/C	98	74 (93.7)	24 (85.7)	1.0	1.0
C/T	9	5 (6.3)	4 (19.3)	1.6 [0.56-4.6] (0.38)	0.67 [0.22-2.07] (0.45)
MMP-12					
A/A	96	70 (78.7)	26 (83.9)	1.0	1.0
A/G	24	19 (21.3)	5 (16.1)	0.7 [0.23-1.9] (0.54)	0.8 [0.31-2.2] (0.70)
MMP-13					
A/A	62	45 (54.9)	17 (63)	1.0	1.0
A/G	45	35 (42.7)	10 (37)	0.78 [0.36-1.7] (0.54)	0.78 [0.36-1.7] (0.54)
G/G	2	2 (2.4)	0	-	-

NOTE: Values in bold indicate significant results.
*Adjusted for tumor grade and node status.

2G genotype promotes lymph node metastasis, and prognosis is dependent on this facet of tumor behavior. Interestingly, even when adjusted for lymph node status, there remains a trend for decreased overall survival in patients with the 2G/2G genotype compared with 1G/2G or 1G/1G, which suggests that the enzyme may play a role in distant tumor dissemination. There was no association between *MMP-9* T allele and overall survival despite the relationship between this allele and lymph node metastasis, which is difficult to explain, apart from acknowledging that this attests to the complexity of function of these enzymes and the process of tumor spread.

Given the likely multifaceted role of MMPs in tumor progression and the involvement of different enzymes at different stages of the disease (6, 34), it is plausible that the combined effect of multiple high-expressing MMP alleles may provide a stronger predictor of prognosis than individual SNPs.

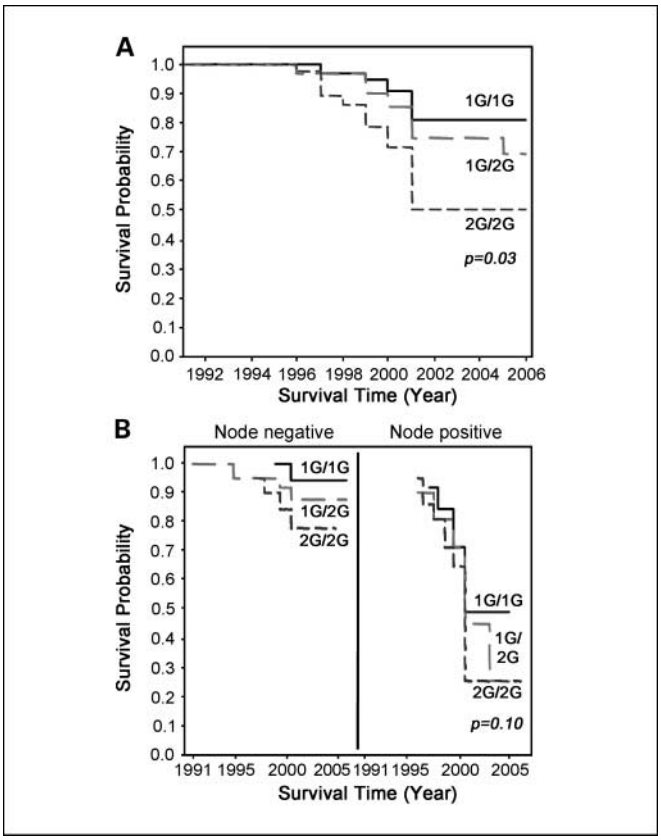

Fig. 1. Kaplan-Meier curves for *MMP-1*. *A,* displays the crude results for all Caucasian patients, the presence of the 2G/2G (15 of 37 patients with this genotype died; CHR for overall survival, 4.1; 95% CI, 1.1-8.7) genotype for *MMP-1* was predictive of a shorter survival compared with the presence of the 1G/1G (5 of 36 patients with this genotype died) or 1G/2G (12 of 63 patients with this genotype died). *B,* displays the adjusted results for all Caucasian patients. For node-negative patients, 1 of 25 patients with the 1G/1G genotype died, 4 of 43 patients with the 1G/2G genotype died, and 4 of 17 patients with the 2G/2G genotype died. For node positive patients, 4 of 11 patients with the 1G/1G genotype died, 8 of 20 patients with the 1G/2G genotype died, and 11 of 20 patients with the 2G/2G genotype died The association of genotype and overall survival was no longer significant after adjusting for tumor grade and lymph node status (adjusted hazard ratio for overall survival, 2.4; 95% CI, 0.8-6.6).

We therefore investigated disease behavior with MMP SNP haplotypes. This identified two haplotypes, 2G-5A-A-A-A and 2G-5A-G-A-A, linked to increased incidence of lymph node metastasis. The 2G-5A-A-A-A haplotype was shown to have a dominant effect (subjects with one copy of the haplotype have the same risk of lymph node metastasis as those subjects with two copies), whereas the 2G-5A-G-A-A haplotype exhibited a multiplicative effect, such that subjects with one copy of the haplotype are at an intermediate risk of lymph node metastasis, with respect to those subjects with zero or two copies. Furthermore, the 2G-5A-G-A-A haplotype was also associated with overall survival even after adjustment for lymph node status. Whereas this haplotype was associated with disease-free survival in unadjusted analysis, this relationship was lost when adjusted for lymph node status. In keeping with the genotype data, these haplotype associations are driven by the *MMP-1* 2G allele. The haplotypes also reflect the previously described linkage disequilibrium between the *MMP-1* 2G allele and the *MMP-3* 5A allele (40).

Polymorphisms in the promoters of MMPs have a functional role and have been shown to exhibit allelotypic effects on levels of gene transcript (15, 32). Although the lifelong effects of altered transcription are unclear, overexpression of MMPs caused by promoter polymorphisms may enhance cancer progression by virtue of their role in degradation of the ECM, thereby generating an environment that favors tumor cell migration. Modulation of the microenvironment is emerging as a critical factor in determining tumor cell behavior (41, 42), and changes in the stroma, such as those mediated through proteolytic remodeling, can have a major influence on tumor cell function (43). Thus, the altered expression of MMPs generated through functional SNPs and the consequent effect on tumor microenvironment may contribute to the patient-to-patient variability in breast cancer susceptibility and outcome. In patients with breast cancer, knowledge of the MMP SNP genotype and/or haplotype, as shown here, could contribute to identification of patients at higher risk and therefore influence treatment decisions.

Table 5. Analysis of the association between haplotype and node status

Haplotype*	Frequencies		Wald test (*P*) †	AIC ‡
	Node-negative	Node-positive		
1G-5A-A-A-A	19.4	13.2	1.07 (0.28)	1,095.92
1G-5A-G-A-A	9.7	11.2	0.11 (0.91)	1,097.14
1G-6A-A-A-A	6.3	4.6	1.12 (0.26)	1,095.58
1G-6A-A-G-A	1.1	2.1	0.42 (0.68)	1,096.98
2G-5A-A-A-A	2.3	9.7	2.35 (0.02)	1,090.56
2G-5A-A-A-G	2.5	4.6	1.07 (0.28)	1,094.96
2G-5A-G-A-A	8.3	19.2	2.24 (0.02)	1,092.56
2G-6A-A-A-A	16.5	13.5	0.36 (0.72)	1,097.02
2G-6A-A-A-G	1.7	1.9	0.18 (0.85)	1,097.07
2G-6A-A-G-A	1.1	2.4	0.35 (0.73)	1,097.03
2G-6A-G-A-A	6.8	2.9	0.99 (0.32)	1,095.96

NOTE: Values in bold indicate significant results.
Abbreviation: AIC, Akaike's information criteria.
*Haplotype order *MMP-1* (1G or 2G), *MMP-3* (5A or 6A), *MMP-7* (A or G), *MMP-12* (A or G), and *MMP-13* (A or G).
†Tests whether an independent variable has a statistically significant relationship with a dependent variable.
‡The AIC is a goodness-of-fit measure.

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