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Advances in Brief

Invasiveness of Cutaneous Malignant Melanoma Is Influenced by *Matrix Metalloproteinase 1* Gene Polymorphism[1]

Shu Ye,[2] Sahar Dhillon, Steven J. Turner, Adrian C. Bateman, Jeffrey M. Theaker, Ruth M. Pickering, Ian Day, and W. Martin Howell

Human Genetics Research Division [S. Y., S. D., S. J. T., I. D., W. M. H.] and Medical Statistics Group [R. M. P.], University of Southampton School of Medicine, and Department of Histopathology, Southampton University Hospitals NHS Trust [A. C. B., J. M. T.], Southampton, United Kingdom

Abstract

The matrix metalloproteinases (MMPs) are implicated in connective tissue destruction during cancer invasion and metastasis. A naturally occurring variant arising from the insertion or deletion of a guanine in the promoter of the *MMP-1* gene has recently been reported and shown to influence its transcriptional activity in melanoma cells. In this study, *MMP-1* genotype was determined in 139 Caucasian patients with cutaneous malignant melanoma. The insertion allele was associated with deep invasive, and therefore poorer-prognosis, primary tumors [(34% of patients with vertical growth phase tumor were homozygous for the insertion allele compared with 17% of patients with horizontal growth phase tumor ($P = 0.0333$; odds ratio = 2.51)]. These data suggest that the invasiveness of cutaneous malignant melanoma is influenced by variation in the *MMP-1* gene promoter that affects MMP-1 expression.

Introduction

Degradation of the extracellular matrix and basement membrane barriers is a key process in tumor invasion and metastasis. There is strong evidence indicating that the MMPs,[3] which possess proteolytic activities against extracellular matrix and basement membrane proteins, play an important role in this process and therefore facilitate tumor invasion and spread (1, 2). Recently, a naturally occurring sequence variation in the human *MMP-1* gene promoter was reported (3). This genetic variation arises from insertion or deletion of a *G* at position −1607 relative to the transcriptional start site; consequently one allele (insertion) has two 2Gs, whereas the other allele (deletion) has only 1G at this position. The insertion creates the core sequence (5′-GGA-3′) of a binding site for the Ets transcription factors, and it was demonstrated *in vitro* that the *2G* allele had a higher transcriptional activity in melanoma cells (3). CMM is the most serious cutaneous malignancy. Relatively little is known of the genetic factors underlying susceptibility to and prognosis in sporadic CMM, although polymorphisms in several genes have been implicated (4–6). In this study, we sought to determine whether this *MMP-1* genetic polymorphism is associated with susceptibility to CMM in British Caucasian subjects, and whether this polymorphism influences CMM prognosis, *i.e.*, is associated with known prognostic features of CMM, particularly those associated with tumor invasiveness, growth, recurrence, or metastasis.

Subjects and Methods

Subjects

Patients. Formalin-fixed and paraffin-embedded tissue blocks from 139 CMM patients (presenting in 1986–1993) were retrieved from the files of the Histopathology Department, Southampton General Hospital (Southampton, United Kingdom). All specimens were re-reviewed by two histopathologists (A. C. B. and J. M. T.) and the original diagnoses of CMM confirmed.

Tumor Histopathology Data. Histopathological prognostic features of each case were assessed as defined in the literature and used in previous studies (7, 8). Radial growth phase CMMs were defined as those limited in extent to the epidermis (melanoma *in situ*) or showing early invasion of the upper dermis but with dermal nests of melanocytes no larger than those at the dermoepidermal junction and containing no mitotic figures. Vertical growth phase CMM showed expansive growth within the dermis, evidenced by nests of neoplastic melanocytes that were larger than those at the dermoepidermal junction or by the presence of mitotic figures within dermally located melanocytes (8). For vertical growth phase CMM, the mitotic count/mm^2 of tumor was assessed as nil, 1–6, or >6, and the number of tumor-infiltrating lymphocytes was evaluated as absent, nonbrisk/focal, or brisk (8). The presence of tumor regression, defined as segmental tumor loss, was also recorded.

Clinical Follow-up Data. The following variables were recorded for each patient, subject to availability of clinical data: (*a*) gender; (*b*) age; (*c*) site of CMM; (*d*) length of clinical follow-up; (*e*) presence of recurrent or metastatic tumor; (*f*) disease-free survival; and (*g*) overall survival time. The clinicopathological stage of each patient at initial presentation for whom full data were available was calculated using the Tumor-Node-Metastasis system (9).

Controls. Controls consisted of stored DNA samples derived from 142 cadaveric and noncadaveric solid organ and bone marrow donors. All patients and donors were Caucasian.

Preparation of DNA Samples

DNA was extracted from Formalin-fixed, paraffin wax-embedded tissue blocks from CMM patients as described previously (7, 10). Briefly, two to five 20-μm sections were cut from each tissue block and dewaxed in xylene (Merck, Ltd., Poole, United Kingdom) and xylene-ethanol washes. DNA was extracted from the resulting cellular material by proteinase-K digestion. Control DNA samples were originally prepared from peripheral blood by standard salt-precipitation protocols (11).

Determination of Genotypes

A recently published method was used to determine the genotypes of the subjects (12). Briefly, PCR was carried out in a total volume of 25 μl containing 50 ng of genomic DNA; 10 pmol of the forward and reverse primers (5′-TCGTGAGAATGTCTTCCCATT-3′ and 5′-TCTTGGATTGATTTGAGATAAGTGAAATC-3′, respectively); 200 mM each dATP, dCTP, dGTP and dTTP; 20 mM Tris-HCl (pH 8.4); 50 mM KCl; 0.05% (v/v) W1 (Life Technologies, Inc.); 1.5 mM $MgCl_2$; and 1 unit Taq polymerase (Life Technologies, Inc.). The solution was overlaid with 25 μl of liquid paraffin and incubated for

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[2] To whom requests for reprints should be addressed, at Human Genetics, Duthie Building (Mailpoint 808), Southampton General Hospital, Tremona Road, Southampton SO16 6YD, United Kingdom. Phone: 44 (0) 23-8079-4929; Fax: 44 (0) 23-8079-4264; E-mail: Shu.Ye@soton.ac.uk.

[3] The abbreviations used are: MMP, matrix metalloproteinase; G, guanine; CMM, cutaneous malignant melanoma.

1 min at 95°C, and then by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. A 15-μl aliquot of PCR products was mixed with a 5-μl solution containing 2 μl of 10× NEBuffer 2 [50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.9)], 0.2 μl of BSA (10 mg/ml), 0.3 μl *Xmn* I (20 units/ml), and 2.5 μl of sterile deionized H_2O. The 5-μl aliquot of the digests was mixed with 2 μl of loading buffer and electrophoresed on a 10% horizontal nondenaturing polyacrylamide gel at 150 V for 2.5 h. The gel was then stained with Vistra Green (Amersham) and scanned with a fluorimager (FI595; Molecular Dynamics, Sunnyvale, CA).

Statistical Analyses

Differences in genotype and allele frequencies between the patients and the healthy British Caucasian controls were tested using χ^2 analyses. Genotype and allele frequencies were compared similarly between British Caucasian and Japanese healthy subjects (latter frequencies derived from the literature (13). Odds ratios and asymptotic confidence intervals were calculated. Genotype frequencies were also compared within patient subgroups according to tumor growth phase, Breslow depth (invasive CMM), mitotic index (vertical growth phase CMM), clinicopathological phase at presentation, and the presence of disease recurrence/metastasis. Disease-free survival according to genotype was displayed graphically among surgically treated subjects using Kaplan-Meier curves. Relative hazards between genotypes and their associated 95% confidence intervals were estimated within a Cox regression model, and differences between genotypes were tested from the log likelihood ratio. Survival analysis was carried out within Stata.

Results

MMP-1 Genotype and Susceptibility of Melanoma. *MMP-1* genotype according to insertion (*2G* allele) or deletion (*1G* allele) of a G at position −1607 (3) was determined in 139 patients and 142 healthy Caucasian subjects. No statistically significant difference in genotype and allele frequencies was detected between the melanoma patients and the healthy subjects, with the frequency of the *2G* allele being 53% in patients and 47% in healthy subjects (Table 1). The genotype frequencies in both samples were consistent with Hardy-Weinberg equilibrium distribution.

The allele frequencies in the above healthy Caucasian subjects were significantly different from reported allele frequencies in healthy Japanese (13; $P = 0.0465$; Table 1).

MMP-1 Genotype and Invasiveness of Melanoma. In the CMM patients, there was an association between the *2G* allele and deep invasive tumors; 34% of patients with vertical growth phase lesions were homozygous for the *2G* allele compared with 17% of patients with horizontal growth phase lesions ($P = 0.0333$; Table 2).

Analysis of disease-free survival was carried out in 99 subjects who had a complete recovery from surgery and were then followed up clinically. There was a nonsignificant trend ($P = 0.7364$) toward decreased disease-free survival in patients carrying the *2G* allele (Figure 1). The relative risk of a relapse at any point of follow-up among patients still disease-free increased by 52% and 12% for patients with the *2G*/*2G* and *1G*/*2G* genotypes, respectively, compared with the risk among patients homozygous for the *1G* allele (Table 3).

No significant association was detected between *MMP-1* genotype and stage at presentation (I *versus* II/III), Breslow depth (greater than or less than 1.5 mm) and mitotic count in vertical growth phase CMM, presence of tumor infiltrating lymphocytes, and presence or absence of relapse.

Table 2 *Frequency of MMP-1 genotypes in vertical and horizontal growth phase CMM*

Genotype	Vertical growth *n* (%)	Horizontal growth *n* (%)	*P*	Odds ratio (95% CI[a])
2G/*2G*	15 (34%)	14 (17%)	0.0870	2.23 (0.83, 5.95)
1G/*2G*	16 (36%)	41 (50%)		0.81 (0.34, 1.95)
1G/*1G*	13 (30%)	27 (33%)		1.00
2G/*2G*	15 (34%)	14 (17%)	0.0333	2.51 (1.08, 5.88)
1G/*2G* or *1G*/*1G*	29 (66%)	68 (83%)		1.00

[a] CI, confidence interval.

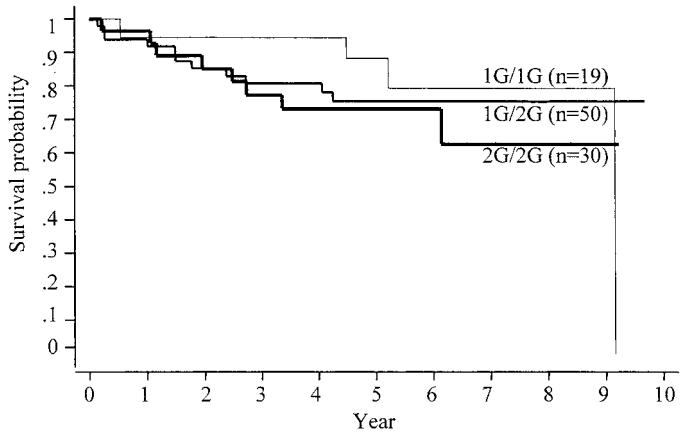

Fig. 1. Disease-free survival in CMM series according to *MMP-1* genotype. Analysis of disease-free survival was carried out in subjects who had a complete recovery from surgery and were then followed up clinically. There was a trend toward decreased disease-free survival in patients carrying the *2G* allele.

Discussion

CMM is a serious and potentially fatal condition, with the most important environmental risk factor being UV light exposure and ~5–10% of all cases being familial (14). In nonfamilial CMM cases, both susceptibility to and prognosis in this malignancy have been shown to be influenced by human leukocyte antigen DQB1 polymorphisms (7) or DQB1-associated haplotypes (15), although other nonimmunogenetic factors have also been implicated (4–6). The most important prognostic factor is stage at time of presentation, and a melanoma is most capable of being cured by surgical excision when it is at the superficial stage ("radial" growth phase CMM, as opposed to vertical growth phase CMM; Ref. 16). Radial growth phase CMMs exist solely within the epidermis or may contain small numbers of nondividing cells within the superficial dermis. Vertical growth phase CMMs contain neoplastic melanocytes actively growing within the dermis and possess the capability to metastasize to regional lymph nodes and distant organs. In vertical growth phase CMMs, prognosis is also influenced by the depth of invasion of the lesion (Breslow

Table 1 *MMP-1 genotype and allele frequencies (%) in CMM patients, British Caucasian controls, and healthy Japanese subjects*[a]

	Genotype				
	2G/*2G*	*1G*/*2G*	*1G*/*1G*	*P*	*2G* allele frequency (95% CI[b])
CMM patients (Caucasian, $n = 139$)	41 (29%)	65 (47%)	33 (24%)	0.4136	53% (47%, 59%)
Healthy subjects (Caucasian, $n = 142$)	23 (23%)	68 (48%)	41 (29%)		47% (41%, 53%)
Healthy Caucasian subjects ($n = 142$)	33 (23%)	68 (48%)	41 (29%)	0.0465	47% (41%, 53%)
Healthy Japanese subjects ($n = 150$)	30 (20%)	56 (37%)	64 (43%)		39% (33%, 44%)

[a] Genotype and allele frequencies in Japanese are those reported by Kanamori *et al.* (13).
[b] CI, confidence interval.

Table 3 *Hazard ratios for relapse in CMM series according to MMP-1 genotype*

Genotype	Hazard ratios (95% CI[a])
1G/1G ($n = 19$)	1.00
1G/2G ($n = 50$)	1.12 (0.36, 3.52)
2G/2G ($n = 30$)	1.52 (0.46, 5.06)

[a] CI, confidence interval.

depth and Clarke's level), the mitotic index and presence of tumor-infiltrating lymphocytes within the dermal component, and the presence or absence of tumor regression (8).

Several MMPs, including MMP-1, are expressed in cutaneous melanomas, and it has been demonstrated that these MMPs play a role in melanoma cell invasion with overexpression being associated with metastasis and unfavorable prognosis (17–19). As mentioned above, the *MMP-1* gene variation investigated in this study has been shown previously to have an allele-specific effect on the levels of MMP expression in cultured melanoma cells (3). It has also been reported that ovarian tumor and endometrial cancer tissues from patients carrying the *2G* allele contain higher levels of *MMP-1* transcripts compared with those from patients not carrying this allele (13, 20). In a recent Japanese study of ovarian cancer, the proportion of patients who carried the *2G* allele was significantly higher than in the control subjects, suggesting that individuals carrying the *2G* allele are genetically predisposed to the development of ovarian cancer (13). In our study, no difference in *MMP-1* genotype frequency between CMM patients and healthy subjects was detected. This may indicate a difference in genetic risk factors between melanoma and ovarian cancer or a difference in genetic risk factors for cancers between Orientals and Caucasians. It was noted that the *MMP-1* genotype frequencies within our healthy Caucasian control population differed significantly from those within the healthy Japanese subjects used in a previous study (13).

The association of the *2G* allele of the *MMP-1* gene with vertical growth phase CMM and the trend toward lower survival rate observed in this study is consistent with the hypothesis that variation in *MMP* genes can influence the potential for tumor invasion and metastasis through modulation of the expression and/or activity of these extracellular matrix-degrading enzymes. No associations were demonstrable between *MMP-1* genotype and the histopathological markers of prognosis in vertical growth phase CMM that we examined. Histological features such as Breslow depth, mitotic index, and the presence of tumor-infiltrating lymphocytes have been proven to correlate with prognosis in vertical growth phase CMM (8). Therefore, examination of a larger patient series may be required to investigate potential associations between *MMP-1* genotype and both these more detailed histological features and clinical outcome.

To our knowledge, this is the first report of a study of a naturally occurring variant in an *MMP* gene in relation to the invasiveness of carcinoma. The data generated in this study support the hypothesis that variation in the *MMP-1* gene influences the potential for invasion and metastasis of CMM through modulation of the expression of this matrix-degrading enzyme.

References

1. Birkedal-Hansen, H., Moore, W. G., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. Matrix metalloproteinases: a review. Crit. Rev. Oral Biol. Med., *4:* 197–250, 1993.
2. Curran, S., and Murray, G. I. Matrix metalloproteinases in tumour invasion and metastasis. J. Pathol., *189:* 300–308, 1999.
3. Rutter, J. L., Mitchell, T. I., Buttice, G., Meyers, J., Gusella, J. F., Ozelius, L. J., and Brinckerhoff, C. E. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter creates an Ets binding site and augments transcription. Cancer Res., *58:* 5321–5325, 1998.
4. Valverde, P., Healy, E., Sikkink, S., Haldane, F., Thody, A. J., Carothers, A., Jackson, I. J., and Rees, J. L. The Asp84Glu variant of the melanocortin 1 receptor (MC1R) is associated with melanoma. Hum. Mol. Genet., *5:* 1663–1666, 1996.
5. Hutchinson, P. E., Osborne, J. E., Lear, J. T., Smith, A. G., Bowers, P. W., Morris, P. N., Jones, P. W., York, C., Strange, R. C., and Fryer, A. A. Vitamin D receptor polymorphisms are associated with altered prognosis in patients with malignant melanoma. Clin. Cancer Res., *6:* 498–504, 2000.
6. Strange, R. C., Ellison, T., Ichii-Jones, F., Bath, J., Hoban, P., Lear, J. T., Smith, A. G., Hutchinson, P. E., Osborne, J., Bowers, B., Jones, P. W., and Fryer, A. A. *Cytochrome P450 CYP2D6* genotypes: association with hair colour, Breslow thickness and melanocyte stimulating hormone receptor alleles in patients with malignant melanoma. Pharmacogenetics, *9:* 269–276, 1999.
7. Bateman, A. C., Turner, S. J., Theaker, J. M., and Howell, W. M. *HLA-DQB1*0303* and **0301* alleles influence susceptibility to and prognosis in cutaneous malignant melanoma in the British Caucasian population. Tissue Antigens, *52:* 67–73, 1998.
8. Clark, W. H., Jr., Elder, D. E., Guerry, D., Braitman, L. E., Trock, B. J., Schultz, D., Synnestvedt, M., and Halpern, A. C. Model predicting survival in stage I melanoma based on tumor progression. J. Natl. Cancer Inst., *81:* 1893–1904, 1989.
9. Beahrs, O. H., and Myers, M. H. (eds.) Manual for Staging of Cancer. American Joint Committee on Cancer, Ed. 2, pp. 117–122. Philadelphia: J. B. Lippincott Co., 1983.
10. Howell, W. M., Leung, S. T., Jones, D. B., Nakshabendi, I., Hall, M. A., Lanchbury, J. S., Ciclitira, P. J., and Wright, D. H. HLA-DRB, -DQA, and -DQB polymorphism in celiac disease and enteropathy-associated T-cell lymphoma. Common features and additional risk factors for malignancy. Hum. Immunol., *43:* 29–37, 1995.
11. Miller, S. A., Dykes, D. D., and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res., *16:* 1215, 1988.
12. Dunleavey, L., Beyzade, S., and Ye, S. Rapid genotype analysis of the *matrix metalloproteinase-1* gene *1G/2G* polymorphism that is associated with risk of cancer. Matrix Biol., *19:* 175–177, 2000.
13. Kanamori, Y., Matsushima, M., Minaguchi, T., Kobayashi, K., Sagae, S., Kudo, R., Terakawa, N., and Nakamura, Y. Correlation between expression of the *matrix metalloproteinase-1* gene in ovarian cancers and an insertion/deletion polymorphism in its promoter region. Cancer Res., *59:* 4225–4227, 1999.
14. Greene, M. H. The genetics of hereditary melanoma and nevi. 1998 update. Cancer (Phila.), *86:* 2464–2477, 1999.
15. Lulli, P., Grammatico, P., Brioli, G., Catricala, C., Morellini, M., Roccella, M., Mariani, B., Pennesi, G., Roccella, F., Cappellacci, S., and Trabace, S. *HLA-DR*, and *-DQ* alleles in Italian patients with melanoma. Tissue Antigens, *51:* 276–280, 1998.
16. Sober, A. J., and Koh, H. K. Melanoma and other pigmented skin lesions. *In:* K. J. Isselbacher, E. Braunwald, J. D. Wilson, J. B. Martin, A. S. Fauci, and D. S. Kasper (eds.), Harrison's Principles of Internal Medicine, Ed. 13, pp. 1867–1871. New York: McGraw-Hill, Inc., 2000.
17. Ray, J. M., and Stetler-Stevenson, W. G. Gelatinase A activity directly modulates melanoma cell adhesion and spreading. EMBO J., *14:* 908–917, 1995.
18. Walker, R. A., and Woolley, D. E. Immunolocalisation studies of *matrix metalloproteinases-1, -2* and *-3* in human melanoma. Virchows Arch., *435:* 574–579, 1999.
19. Vaisanen, A., Kallioinen, M., von Dickhoff, K., Laatikainen, L., Hoyhtya, M., and Turpeenniemi-Hujanen, T. Matrix metalloproteinase-2 (MMP-2) immunoreactive protein: a new prognostic marker in uveal melanoma? J. Pathol., *188:* 56–62, 1999.
20. Nishioka, Y., Kobayashi, K., Sagae, S., Ishioka, S., Nishikawa, A., Matsushima, M., Kanamori, Y., Minaguchi, T., Nakamura, Y., Tokino, T., and Kudo, R. A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter in endometrial carcinomas. Jpn. J. Cancer Res., *91:* 612–615, 2000.