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Analysis of the matrix metalloproteinase family reveals that *MMP8* is often mutated in melanoma

Lavanya H Palavalli¹, Todd D Prickett¹, John R Wunderlich², Xiaomu Wei¹, Allison S Burrell¹, Patricia Porter-Gill¹, Sean Davis², Chenwei Wang¹, Julia C Cronin¹, Neena S Agrawal¹, Jimmy C Lin³, Wendy Westbroek¹, Shelley Hoogstraten-Miller¹, Alfredo A Molinolo⁴, Patricia Fetsch², Armando C Filie², Michael P O'Connell⁵, Carolyn E Banister⁶, Jason D Howard⁷, Phillip Buckhaults⁶, Ashani T Weeraratna⁵, Lawrence C Brody¹, Steven A Rosenberg², and Yardena Samuels¹

¹National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA

²National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

³The Ludwig Center for Cancer Genetics and Therapeutics, the Johns Hopkins Kimmel Cancer Center, Baltimore, Maryland, USA

⁴Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA

⁵Laboratory of Immunology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA

⁶University of South Carolina School of Medicine, Columbia, South Carolina, USA

⁷The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland, USA

Abstract

A mutational analysis of the matrix metalloproteinase (MMP) gene family in human melanoma identified somatic mutations in 23% of melanomas. Five mutations in one of the most commonly mutated genes, *MMP8*, reduced MMP enzyme activity. Expression of wild-type but not mutant *MMP8* in human melanoma cells inhibited growth on soft agar *in vitro* and tumor formation *in vivo*, suggesting that wild-type MMP-8 has the ability to inhibit melanoma progression.

MMPs are proteolytic enzymes that degrade components of extracellular matrix and basement membranes¹. MMPs have been associated with cancer metastasis^{2,3}, and small molecule inhibitors of MMPs were tested as potential anticancer agents. However, clinical trials using these inhibitors showed no effect and, occasionally, accelerated tumor growth^{4,5}. In contrast to the idea that MMP activity promotes melanoma progression, mouse models suggested that MMPs can have an antitumor role⁶⁻⁸. In particular, an increase in skin tumor incidence was seen in MMP-8-deficient mice⁶. These findings suggest that an in-depth analysis of the specific role of individual MMPs in particular cancer types is warranted. We systematically addressed

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Correspondence should be addressed to Y.S. (samuelsy@mail.nih.gov).

AUTHOR CONTRIBUTIONS

L.H.P. and Y.S. designed the study; J.R.W., P.F., A.C.F. and S.A.R. collected and analyzed the melanoma samples, A.S.B., J.C.C., N.S.A., P.B., P.P.-G., S.D., C.W., C.E.B., J.C.L. and Y.S. analyzed the genetic data; L.H.P., T.D.P., X.W., W.W., S.H.-M., A.A.M., M.P.O., J.D.H. and A.T.W. performed and analyzed the functional data. All authors contributed to the final version of the paper.

these issues by a comprehensive mutational analysis of the MMP gene superfamily in melanoma.

The human MMP family consists of 23 genes. To evaluate whether these are genetically altered in melanoma, we analyzed the coding exons of this gene superfamily in 32 individuals with melanoma (Supplementary Table 1, Supplementary Methods and Supplementary Note online). Exons were PCR-amplified from cancer genomic DNA samples (see primers listed in Supplementary Table 2 online) and sequenced with dye terminator chemistry. To determine whether a given mutation was somatic, we sequenced the gene in genomic DNA from matched normal tissue. From the ~5.5 Mb of sequence information obtained, we identified eight MMP genes containing somatic mutations (Table 1). Genes found to have one or more nonsynonymous mutations were then screened for mutations in an additional 47 melanomas. Through this approach, we identified 28 somatic mutations in eight genes, affecting 23% of the melanoma tumors analyzed (Table 1 and Supplementary Fig. 1 online).

In seven tumors, both alleles of the MMP gene were affected, a characteristic associated with tumor suppressor genes. In addition, 6 of the 28 mutations were nonsense or splice-site alterations, which were predicted to result in aberrant or truncated proteins. Most tumors with MMP gene mutations also contained mutations in *NRAS* or *BRAF*. The clinical information associated with melanoma tumors containing MMP mutations is described in Supplementary Table 3 online.

The observed somatic mutations could be either ‘driver’ mutations that have a functional role underlying neoplasia or nonfunctional ‘passenger’ changes. In the eight genes found to be mutated, 28 nonsynonymous (N) and 5 synonymous (S) somatic mutations were identified, yielding a N:S ratio of 28:5, significantly higher than the N:S ratio of 2:1 predicted for nonselected passenger mutations⁹ ($P < 0.026$), suggesting that these are driver mutations. The ratio of C>T mutations compared to other nucleotide substitutions had a significant prevalence of C>G>T:A transitions ($P < 0.0001$) (Supplementary Fig. 2 online), confirming previously reported melanoma signatures¹⁰.

MMP8 and *MMP27* were the most frequently mutated MMP genes in our samples. Notably, MMP-8 and MMP-27 have an identical protein domain structure¹ (Supplementary Fig. 3 online). We chose to focus on *MMP8* because it has been previously shown to protect against skin tumor development^{6,8}, and most of its mutations were accompanied by loss of heterozygosity, suggesting that *MMP8* is a tumor suppressor gene. To test whether *MMP8* is also inactivated through epigenetic events, we screened the sequence surrounding *MMP8* for DNA methylation using an EpiTYPER system (Sequenom). We found that local epigenetic changes are not involved in *MMP8* inactivation in our melanoma samples (data not shown), consistent with our finding that *MMP8* is expressed in melanoma (Supplementary Fig. 4 online).

To determine whether the mutations in *MMP8* affected the protein’s proteolytic activity, we expressed wild-type MMP-8 and five tumor-derived mutants (S50F, P78S, K87N, G104R and E138Q) in HEK 293T cells. A similar expression level of all MMP-8 constructs was observed. Evaluation of MMP activities in immunoprecipitated MMP-8 complexes by type I collagen substrate gel zymography showed that altered MMP-8 proteins conferred less proteolytic activity toward collagen I than the wild-type protein (Fig. 1a).

To determine whether MMP-8 has tumor suppressor activity that is inactivated by tumor-derived mutations, we created stable pooled clones of human melanocyte Mel-STR cells¹¹ expressing similar levels of wild-type or mutant *MMP8* (Supplementary Fig. 5 online). MMP-8

activity of these clones was identical to that of HEK 293T cells (Fig. 1a). These clones were used for subsequent studies.

Expression of wild-type or mutant MMP-8 did not affect the growth rate of Mel-STR cells in tissue culture (Supplementary Fig. 6 online). In contrast, expression of wild-type but not mutant MMP-8 substantially inhibited colony growth on soft agar (Fig. 1b). To test whether inhibition of *MMP8* would have the same effect on cell growth on soft agar as *MMP8* mutation, we used short hairpin RNA (shRNA) to stably knockdown MMP-8 protein levels in SK-Mel-2 cells, a melanoma cell line that expresses wild-type MMP-8. We examined the effect of two different shRNA constructs against *MMP8*: both resulted in an increase in the ability of SK-Mel-2 cells to grow on soft agar compared to control shRNA constructs (Fig. 1c).

As a previous study has reported that wild-type MMP-8 reduces cell migration⁸, we investigated whether cell migration was altered in melanoma lines expressing the various MMP-8 mutants. Cells expressing wild-type MMP-8 had a reduced migration capacity compared to cells expressing mutant MMP-8 (Supplementary Fig. 7a,b online). In contrast, when MMP-8 was knocked down in SK-Mel-2 cells, there was a significant ($P < 0.0001$, *t*-test) reduction in the ability of the cells to migrate (Supplementary Fig. 7c,d).

To determine whether the *MMP8* mutations resulted in altered growth *in vivo*, Mel-STR clones expressing empty vector, wild-type or mutant MMP-8 were administered to NOD/SCID mice by subcutaneous injection. Twenty days after injection, none of the mice with cells expressing wild-type MMP-8 had ulcerating lesions, whereas most of the tumors expressing the five mutant MMP-8 proteins presented with ulcerations (Fig. 1d). Ulceration is a measure of aggression in melanoma and is used clinically as a determinant of melanoma stage¹². To determine whether MMP-8 expression affected metastasis, we examined sections of paraffin-embedded lungs by hematoxylin/eosin (H&E) staining. No lung metastases were found in the mice injected with wild-type clones, and only one of the mice injected with clones expressing empty vector had micrometastases. In contrast, numerous perivascular, peribronchial and subpleural micrometastases with evidence of tumor invasion were observed in some of the mice receiving clones expressing MMP-8 mutants (Fig. 1e). Because of severe skin ulceration, mice had to be euthanized before macroscopic metastases were evident. It is probable that a larger number of mice injected with mutant clones would have developed metastases had they been euthanized at a later time point. Thus, a correlation between increased ulceration and micrometastasis formation was seen (Supplementary Table 4 online). Although the underlying mechanism for the metastatic phenotype seen in the mutant cells is unclear, it is consistent with murine MMP-8 conferring a reduced invasive ability⁸. In contrast to the above results, no significant difference was observed in the primary tumor growth rate. This is consistent with the similar growth rate of all the clones *in vitro*.

The combination of genetic, biochemical and cellular data presented here suggests that *MMP8* is a tumor suppressor in human melanoma. This conclusion is consistent with the previously reported antitumor properties of MMP-8 and other proteases^{5-7,13,14}. Indeed, knockdown of MMP-8 phenocopies mutant MMP-8 overexpression when growth on soft agar is evaluated. However, knockdown of MMP-8 does not phenocopy mutant overexpression when cell migration is assessed. This suggests that, in some cases, the MMP-8 mutants may have an assay-specific gain-of-function or dominant-negative effect. This scenario has precedent and has been described for p53 (ref. 15).

To our knowledge, this study represents the first systematic mutational analysis of the MMP family in any human cancer type. Despite decades of research on MMPs, this is the first study that demonstrates that a large portion of melanomas have previously uncharacterized somatic mutations in MMP genes that affect MMP activity. These findings emphasize the need to test

the role of each MMP in an individual manner and to precisely define its functional role in cancer. This may allow the development of individualized therapy on the basis of the mutant MMP present in specific tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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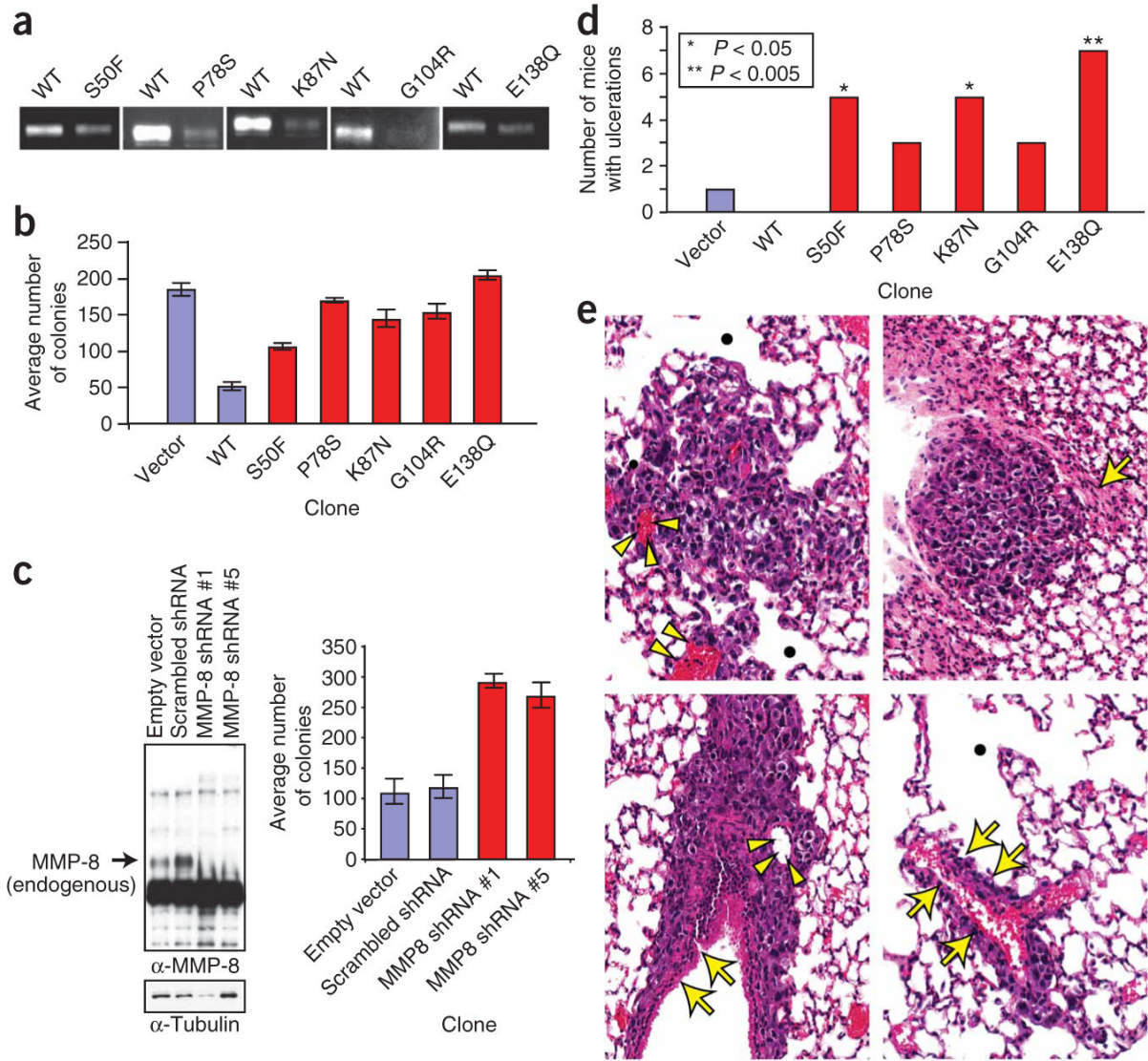


Figure 1. Effects of *MMP8* mutations on enzymic activity, growth on soft agar and growth *in vivo*. **(a)** Collagen I zymography was used to assess activity of wild-type and altered *MMP-8* proteins. Conditioned media from HEK 293T cells expressing wild-type *MMP-8* or altered *MMP-8* with the indicated amino-acid substitutions were immunoprecipitated and subjected to type I collagen substrate gel zymography. Each panel represents a separate experiment. **(b)** Anchorage-independent proliferation of Mel-STR cell clones expressing the indicated constructs was assessed by measuring colony growth in soft agar. Graph indicates number of colonies observed after two weeks of growth. **(c)** SK-Mel-2 cells were infected with either control shRNAs or two different shRNA constructs targeted against *MMP8* (#1 and #5) and selected to form stable pooled clones. Left panel: conditioned media of the clones were immunoblotted with the indicated antibodies. Right panel: anchorage-independent proliferation of infected SK-Mel-2 cell clones expressing the indicated constructs was assessed as described in **b**. **(d)** NOD/SCID mice were subcutaneously injected with Mel-STR cell clones expressing the indicated constructs and examined on a bi-weekly basis ($n=8$; * $P < 0.05$ to ** $P < 0.005$; Fisher's exact test.) **(e)** Representative hematoxylin and eosin-stained images of histopathological pulmonary sections from mice injected with *MMP-8* mutant clones. Top left:

parenchymal metastasis which probably originated in the blood vessels (arrow heads); emphysematous areas are indentified with a black dot. Top right: pleural metastasis island associated with focal parenchymal collapse or atelectasia (arrow). Bottom left: parenchymal metastasis growing along a bronchial structure (arrows). Bottom right: perivascular metastasis (arrows) with surrounding emphysematous areas. All slides are at $\times 130$. α -Tubulin, antibody to tubulin.

Table 1

Mutations identified in MMPs

Gene	No. of nonsynonymous mutations (% tumors affected) ^a	Tumor	Exon	Nucleotide ^b	Amino acid ^b	Functional domain	NRAS or BRAF mutation
MMP2	3 (3.8%)	10T	2	C251T	P84L	PGBD	BRAF
		76T	3	C384T	I128I	Hemopexin	None
		63T	9	A/T (-4)	Splice site	N/A	NRAS
		52T	12	A1865G	D622G	Hemopexin	BRAF
MMP8	6 (6.3%)	32T	2	C149T	S50F/LOH	PGBD	BRAF
		13T	2	C232T	P78S/LOH	PGBD	None
		13T	2	A261C	K87N/LOH	PGBD	None
		85T	2	G310A	G104R/LOH	Catalytic domain	BRAF
MMP11	2 (2.5%)	84T	3	G412C	E138Q/LOH	Catalytic domain	BRAF
		45T	7	G940A	E314K	Hemopexin	BRAF
		12T	2	G209T	R70L	Pro-rich	NRAS
		12T	2	G210A	R70R	Pro-rich	NRAS
MMP14	2 (2.5%)	13T	5	C704T	A235V	Catalytic domain	None
		64T	4	G549A	E183E	Peptidase M	BRAF
		71T	7	C/T (-7)	Splice site	N/A	BRAF
		17T	10	T1664C	V555A	Hemopexin	NRAS
MMP24	4 (3.8%)	55T	3	G488A	W163X	PGBD	None
		39T	4	G648A	K216K	Peptidase M	None
		55T	4	G718A	G240R	Peptidase M	None
		8T	8	G1519A	D507N	Hemopexin	BRAF
MMP26	1 (1.3%)	13T	8	C1565A	P521Q	Hemopexin	None
		17T	5	C/T (+5)	Splice site	N/A	NRAS
MMP27	7 (7.6%)	12T	1	G73A	E25K	PGBD	NRAS
		32T	2	G193A	E65K/LOH	PGBD	BRAF
		5T	2	G317A	W106X/LOH	Catalytic domain	BRAF
		76T	3	C374T	A125V/LOH	Catalytic domain	None
MMP28	2 (2.5%)	52T	8	G1047A	W349X	Hemopexin	BRAF
		81T	9	G1218A	M406I	Hemopexin	BRAF

Gene	No. of nonsynonymous mutations (% tumors affected) ^a	Tumor	Exon	Nucleotide ^b	Amino acid ^b	Functional domain	NRAS or BRAF mutation
MMP28	3 (2.5%)	81T	9	G1219A	D407N	Hemopexin	BRAF
		54T	5	G728C	G243A	Catalytic domain	BRAF
		44T	6	A936T	Q312H	Hemopexin	NRAS
		44T	6	G937A	G313R	Hemopexin	NRAS
		76T	7	G1089A	Q363Q	Hemopexin	None

X, stop codon; LOH, cases wherein the wild-type allele was lost and only the mutant allele remained. Splice site, case wherein the alteration affected ten bases flanking the exon. None, no mutation observed. PGBD, peptidoglycan binding domain. Nine tumors (12T, 13T, 17T, 32T, 44T, 52T, 55T, 76T and 81T) contained more than one somatic alteration.

^aNumber of nonsynonymous and splice-site mutations observed and percentage of tumors affected for each of the eight genes in the panel of 79 melanoma cancers.

^bNucleotide and amino acid change resulting from mutation.