

## Evaluation of EMMPRIN and MMP-2 in the prognosis of primary cutaneous malignant melanoma

Tiefu Chen · Jie Zhu

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**Abstract** The aim of the study was to investigate whether the presence of matrix metalloproteinase-2 (MMP-2) and its inducer, extracellular matrix metalloproteinase inducer (EMMPRIN), in primary cutaneous malignant melanoma (PCMM) might help to predict patient prognosis. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections of PCMM from 150 patients. Association of clinical variables (gender, age, tumor location, thickness, Clark level and AJCC stage) with EMMPRIN and MMP-2 expression were analyzed by Fisher's exact test. Survival rates were subsequently estimated using the Kaplan–Meier method and compared using the log-rank test. The expression of EMMPRIN and MMP-2 was detected in 117/150 (78.0%) and 115/150 (76.7%) of patients with PCMM, respectively. Higher positive rates of both EMMPRIN and MMP-2 expression were significantly correlated with increased tumor thickness (both  $P = 0.004$ ), higher Clark level ( $P = 0.02$  and  $0.03$ ) and higher AJCC stage (both  $P = 0.006$ ). A significant correlation was found between the expression of EMMPRIN and MMP-2 in PCMM ( $r = 0.89$ ,  $P = 0.01$ ). Kaplan–Meier analysis demonstrated that patients who had EMMPRIN+/MMP-2+ expression had a significantly decreased 3-year disease-free survival ( $P = 0.005$ ) and 5-year overall survival ( $P = 0.006$ ). In multivariate analyses, tumor thickness and EMMPRIN+/MMP-2+ co-expression were the significant predictors of clinical outcome. EMMPRIN and MMP-2 may be independent biomarkers for disease recurrence and overall survival in patients with PCMM. A

combined detection of EMMPRIN/MMP-2 co-expression may benefit us in prediction of a poor survival of PCMM.

**Keywords** EMMPRIN · MMP-2 · Primary cutaneous malignant melanoma · Survival

### Abbreviations

PCMM	Primary cutaneous malignant melanoma
EMMPRIN	Extracellular matrix metalloproteinase inducer
MMPs	Matrix metalloproteinases
ECM	Extracellular matrix

### Introduction

The incidence of primary cutaneous malignant melanoma (PCMM) is increasing more rapidly than any other cancers in China. Surgical excision of localized PCMM (American Joint Committee on Cancer [AJCC] stages I and II; approximately 75% of diagnoses) may lead to cure in many patients. The overall 5-year survival rate for these patients (AJCC stages I and II) is approximately 80%, suggesting that approximately 20% of stage I and II patients may have micrometastatic disease at the time of diagnosis [1, 2]. Several histopathological parameters, including tumor thickness, ulceration, satellites and lymph node status, have been reported to have prognostic significance in PCMM [3]. However, the assessment of molecular factors, more strictly related to tumor cell biology and influencing the development and progression of PCMM, could help to identify high-risk patients for disseminated disease and could improve overall clinical management of PCMM.

T. Chen · J. Zhu (✉)  
Department of Burns and Plastic, The 3rd Hospital of Central South University, 410013 Changsha, Hunan, China  
e-mail: zhujie5078@163.com

The metastatic potential of tumor cells is dependent upon their ability to disengage themselves from environmental cues that serve to regulate and constrain them. Typically, successful metastasis requires alterations in cytoskeletal architecture, expression of surface adhesion molecules and penetration of the basement membrane. Results of investigations into tumor biology can contribute to the development of novel promising therapies, such as protease inhibitors for myeloma treatment [4, 5]. Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as CD147 or basigin, is highly expressed on the outer surface of carcinoma cells, but not on normal mucosal cells [6]. EMMPRIN was initially identified following the observation that interstitial collagenase (MMP-1) production was induced during the co-culturing of tumor cells and fibroblasts. Further studies revealed that EMMPRIN is capable of inducing the expression of several MMPs other than MMP-1, including MMP-2, MMP-3, MMP-9 and MMP-11 [7, 8]. Thus, tumor cells can interact with adjacent normal cells to produce MMPs via EMMPRIN on their surface and, in turn, invade lymphatic tissue and blood vessels and penetrate through the ECM to adjacent organs with the help of MMPs. The roles of EMMPRIN and MMPs in tumor invasiveness have been confirmed immunohistochemically in several types of cancer cells and surrounding tissue, including gliomas [9], hepatocellular carcinoma [10], prostate cancer [11] and thyroid carcinoma [12]. Moreover, the expression of MMPs is reported to correlate with the clinical prognosis of patients with breast carcinoma and other types of cancers. Yoshino et al. [13] demonstrated that the interaction and/or balance between MMP-2 and tissue inhibitor of metalloproteinase (TIMP)-2 *in vivo* may play important roles in the process of tumor growth, invasion and metastasis of malignant melanoma. However, the expression of EMMPRIN is unclear in clinical samples of PCMM. In the present study, we have investigated for the first time the immunohistochemical expression of EMMPRIN and MMP-2 in a large series of patients with PCMM, analyzed the correlation with clinicopathological parameters and elucidated the prognostic role.

## Materials and methods

### Patients and tissue samples

The study was approved by the Research Ethics Committee of Ministry of Public Health of China. Informed consent was obtained from all of the patients. From the files, we retrieved 150 patients with PCMM consecutively diagnosed and treated in our institution between January 2000 and December 2005. Treatment consisted of wide surgical

excision of the primary lesion, and regional lymph node dissections were performed in all patients with suspected regional metastases. The clinical records of the patients were reviewed, and the relevant information was recorded in standardized forms. Follow-up data was obtained from these records and from the tumor registry records. Follow-up was terminated in May 22, 2009. Patients without documented follow-up were excluded from case selection. Lymph node status and the presence of metastasis were verified by both clinical and pathological examination. The demographic data and clinical parameters assessed are outlined in Table 1.

Each tumor, after surgical resection, was fixed in formalin and embedded in multiple paraffin blocks. Prognostic parameters were evaluated in sections taken from the block with the largest tumor thickness. Tumor was identified on hematoxylin and eosin-stained sections and on adjacent sections stained immunohistochemically for melanoma-associated antigens, including S100 protein, melan A and HMB-45. Independent histopathological reviews were performed by two pathologists on separate occasions.

### Immunohistochemistry analysis

Immunostaining was performed using the alkaline phosphatase-anti-alkaline phosphatase method (Dako, Glostrup, Denmark). After dewaxing, the sections were digested with 0.1% trypsin in TRIS-buffered saline for 15 min at 37°C.

**Table 1** Clinical and histological parameters of patients

Parameters	No. of patients	Percent
All cases	150	100
Gender		
Female	90	60.0
Male	60	40.0
Age (years)		
<58	75	50.0
≥58	75	50.0
Tumor location		
Head and neck	42	28.0
Trunk	42	28.0
Extremities	66	44.0
Thickness [14]		
T1–T2	93	62.0
T3–T4	57	38.0
Clark level [15]		
II–III	87	58.0
IV–V	63	42.0
AJCC stage [16]		
IA–IB	90	60.0
IIA–IIC	60	40.0

They were then processed in a 600 W microwave oven, at maximum power, 5 times for 2 min each time in citrate buffer (pH = 6). After removal of excess normal serum, incubation of the primary antibody (mouse anti-human EMMPRIN monoclonal antibody (#sc-71038, 200 µg/ml, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) diluted 1:100, mouse anti-human MMP-2 polyclonal antibody (#sc-53630, 200 µg/ml, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) diluted 1:150 against respective target proteins was performed overnight at 4°C. After washing, peroxidase-labeled polymer and substrate-chromogen were then employed to visualize the staining of the interested proteins.

The quality (number, intensity and pattern) of every staining procedure for EMMPRIN and MMP-2 has been comparatively evaluated using consecutive control sections, and the immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological data and clinical outcomes of the patients. The scores of the two pathologists were compared, and any discrepant scores were trained by re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells in ten representative microscopic fields was counted, and the percentage of positive cells was calculated. Given the homogeneity of the staining of the target proteins, tumor specimens were scored in a semiquantitative manner based on the percentage of tumor cells that showed immunoreactivity. The staining results of MMP-2 were classified into negative (0, staining of  $\leq 10\%$  of cells) or positive (1, staining of  $>10\%$  of cells); the staining results of EMMPRIN staining were classified into three patterns: negative (0, less than 10% of cells stained), moderate (1, 10–50% of cells stained) and strong (2, more than 50% of cells stained) [17].

#### Statistical analysis

The software of SPSS version 13.0 for Windows (SPSS Inc., IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as  $\bar{X} \pm s$ . Association between EMMPRIN and MMP-2 expression and various clinicopathological characteristics were analyzed using the  $\chi^2$  test. Correlation between EMMPRIN and MMP-2 expression was analyzed using Spearman's rank correlation analysis. Two endpoints were examined for survival analyses: disease-free survival (DFS) and overall survival (OS). DFS and OS curves were plotted according to the Kaplan–Meier method, the log-rank test being used to determine the significance of differences between clinicopathological parameters. The Cox proportional hazards model was used for multivariate analysis. Differences were considered statistically significant when  $P$  was less than 0.05.

## Results

### Expression and location of EMMPRIN and MMP-2 in PCMM

The expression of EMMPRIN and MMP-2 was detected in 117/150 (78.0%) and 115/150 (76.7%) of patients with PCMM, respectively. Their signals concentrated primarily within the cytoplasm and membrane (EMMPRIN) and cytoplasm (MMP-2) of the tumor cells. In contrast, non-cancerous adjacent tissues showed no EMMPRIN and MMP-2 immunoreactivity.

The correlation of EMMPRIN and MMP-2 expression in all patients was assessed. The percentage of positive MMP-2 expression in EMMPRIN-negative, moderate and strong tumor cells were 72.7, 86.6 and 92.0%, respectively. MMP-2 expression was significantly correlated with EMMPRIN expression by Spearman's rank correlation analysis ( $r = 0.89$ ,  $P = 0.01$ ).

### Correlation of EMMPRIN and MMP-2 expression with the clinicopathological features of PCMM

The correlation of EMMPRIN and MMP-2 expression with the clinicopathological features of patients with PCMM is summarized in Table 2. Higher positive rates of both EMMPRIN and MMP-2 expression were significantly correlated with increased tumor thickness (both  $P = 0.004$ ), higher Clark level ( $P = 0.02$  and  $0.03$ ) and higher AJCC stage (both  $P = 0.006$ ). However, among clinical factors, EMMPRIN and MMP-2 expression did not correlate with location of the primary tumor, gender or age of the patients ( $P = 0.82$ ,  $0.56$  and  $0.60$ , respectively).

### Prognostic implications of EMMPRIN and MMP-2 expression in PCMM

Kaplan–Meier analysis demonstrated that patients who had EMMPRIN+/MMP-2+ expression had a significantly decreased 3-year DFS ( $P = 0.005$ ; Fig. 1a, Table 3) compared with those with EMMPRIN-/MMP-2-, EMMPRIN-/MMP-2+ and EMMPRIN+/MMP-2-. The predictive value of EMMPRIN/MMP-2 co-expression ( $P = 0.003$ ,  $0.01$ ,  $0.009$ ) for 3-year recurrence-free survival maintained significance after the model had been adjusted for independent prognostic factors such as thickness, Clark level and AJCC stage, respectively. In addition, patients with EMMPRIN/MMP-2 co-expression had significantly decreased 5-year OS ( $P = 0.006$ ; Fig. 1b, Table 3) compared with those with EMMPRIN-/MMP-2-, EMMPRIN-/MMP-2+ and EMMPRIN+/MMP-2-. Multivariate analysis was also conducted using a Cox proportional hazards model. Results are shown in Table 4.

**Table 2** Correlation of EMMPRIN and MMP-2 expression with clinical parameters in PCMM

Clinical parameters	EMMPRIN (n, %)			<i>P</i> <sup>c</sup>	MMP-2 (n, %)		<i>P</i> <sup>c</sup>
	0 <sup>a</sup>	1 <sup>a</sup>	2 <sup>a</sup>		0 <sup>b</sup>	1 <sup>b</sup>	
<b>Thickness [14]</b>							
T1–T2	23 (24.7)	47 (50.5)	23 (24.8)	0.004	26 (28.0)	67 (72.0)	0.004
T3–T4	10 (17.5)	20 (35.1)	27 (47.4)		9 (15.8)	48 (84.2)	
<b>Clark level [15]</b>							
II–III	21 (24.1)	42 (48.3)	24 (27.6)	0.02	22 (25.3)	65 (74.7)	0.03
IV–V	12 (19.0)	25 (39.7)	26 (41.3)		13 (20.6)	50 (79.4)	
<b>AJCC stage [16]</b>							
IA–IB	22 (24.4)	45 (50.0)	23 (25.6)	0.006	7 (30.43)	16 (69.57)	0.006
IIA–IIC	11 (18.3)	22 (36.7)	27 (45.0)		8 (26.67)	22 (73.33)	

<sup>a</sup> Immunostaining grades observed using light microscopy. The criteria used for assessment were as follows: 0 (negative, <10%); 1 (weak–moderate, 10–50%); 2 (strong, >50%) of the tumor cells stained

<sup>b</sup> Immunostaining grades observed using light microscopy. The criteria used for assessment were as follows: 0 (negative, ≤10%); 1 (positive, >10%) of the tumor cells stained

<sup>c</sup> Pearson  $\chi^2$  test for non-2 × 2 tables was used

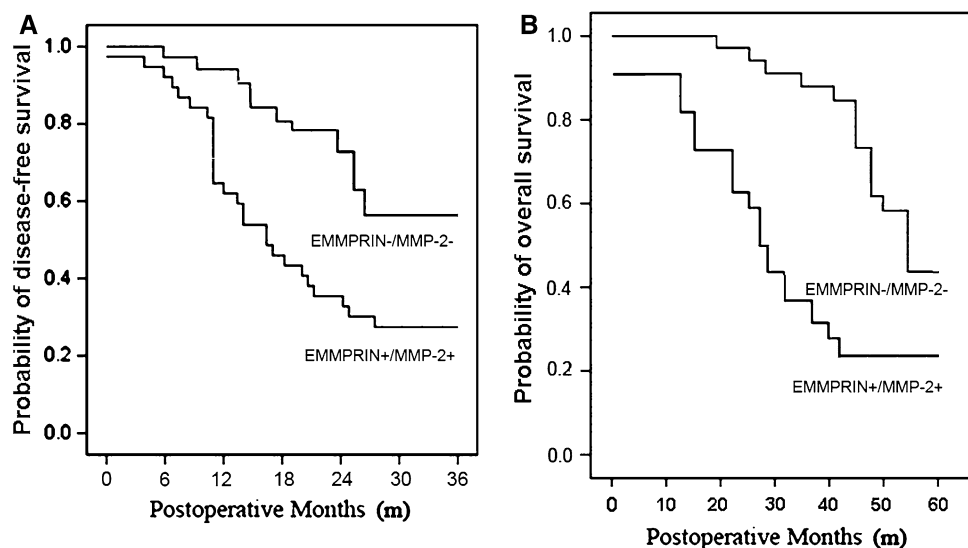
The tumor thickness (T1–T2 versus T3–T4) and EMMPRIN/MMP-2 co-expression (EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2–) were significant for 3-year DFS (*P* = 0.02 and 0.01) and for 5-year OS (*P* = 0.02 and 0.008), respectively.

## Discussion

The primary objective of this study was to evaluate EMMPRIN and MMP-2 staining in a large series of PCMM. Our findings indicate that the presence of EMMPRIN and MMP-2 in melanoma cell cytoplasm and plasma membrane, demonstrated by immunohistochemical staining, is significantly correlated with shorter survival, and a significant

difference was observed in 5-year survival between patients with EMMPRIN+/MMP-2+ and EMMPRIN–/MMP-2– expression. Moreover, the recurrence rate was increased, especially in the first three postoperative years, in patients with EMMPRIN+/MMP-2+ co-expression compared with patients with EMMPRIN–/MMP-2–, EMMPRIN–/MMP-2+ and EMMPRIN+/MMP-2– staining. Cox's proportional hazard model results indicate that EMMPRIN/MMP-2 co-expression is an independent predictor for the 3-year risk of disease recurrence. The results of this study suggest that EMMPRIN/MMP-2 co-expression is an important marker for recurrence and survival, independent of other factors. This finding adds further weight to the current evidence that EMMPRIN and MMP-2 expression is potentially relevant to tumor progression.

**Fig. 1** Kaplan–Meier survival curves for EMMPRIN and MMP-2 expression in PCMM. **a** 3-year DFS of 150 patients with PCMM, as a function of EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2– expression (*P* = 0.005); **b** 5-year OS of 150 patients with PCMM, as a function of EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2– expression (*P* = 0.006)



**Table 3** Univariate survival analysis (log-rank tests) of clinical parameters in PCMM

Clinical parameters	3-year DFS		5-year OS	
	% (95% CI)	<i>P</i>	% (95% CI)	<i>P</i>
<b>Thickness [14]</b>				
T1–T2	87.1 (75.2, 99.6)	0.03	83.9 (70.8, 97.2)	0.03
T3–T4	68.0 (47.1, 88.9)		67.1 (46.0, 88.3)	
<b>Clark level [15]</b>				
II–III	89.7 (77.8, 99.7)	0.01	86.2 (73.2, 97.8)	0.01
IV–V	66.3 (46.1, 87.9)		66.9 (45.1, 86.0)	
<b>AJCC stage [16]</b>				
IA–IB	90 (78.9, 99.6)	0.008	86.7 (75.3, 99.2)	0.01
IIA–IIC	64.7 (42.7, 86.3)		64.1 (42.3, 85.9)	
<b>EMMPRIN/MMP-2 co-expression</b>				
EMMPRIN+/MMP-2+	52.3 (32.0, 76.3)	0.005*	53.3 (30.2, 76.5)	0.006*
EMMPRIN–/MMP-2–	70.9 (53.2, 88.6)		71.3 (53.9, 92.7)	
EMMPRIN–/MMP-2+	72.1 (56.7, 89.9)		72.8 (55.9, 90.7)	
EMMPRIN+/MMP-2–	92.7 (85.6, 99.9)		92.4 (80.1, 99.9)	

\* EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2–; differences in other pairs (EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2+, EMMPRIN+/MMP-2+ versus EMMPRIN+/MMP-2–, EMMPRIN–/MMP-2+ versus EMMPRIN–/MMP-2– and EMMPRIN+/MMP-2– versus EMMPRIN–/MMP-2–) had no statistic significance

**Table 4** Prognostic value of clinical parameters in PCMM in multivariate analysis by Cox regression

Clinical parameters	3-year DFS		5-year OS	
	<i>P</i>	Relative risk (95% confidence interval)	<i>P</i>	Relative risk (95% confidence interval)
<b>Thickness [14]</b>				
T1–T2 versus T3–T4	0.02	4.337 (1.362–16.385)	0.02	4.536 (1.601–13.359)
<b>Clark level [15]</b>				
II–III versus IV–V	0.07	0.179 (0.053–0.677)	0.08	0.251 (0.040–0.883)
<b>AJCC stage [16]</b>				
IA–IB versus IIA–IIC	0.11	0.136 (0.076–0.538)	0.09	0.152 (0.027–0.693)
<b>EMMPRIN/MMP-2 co-expression</b>				
EMMPRIN+/MMP-2+ versus EMMPRIN–/MMP-2–	0.01	5.261 (1.28–19.379)	0.008	7.320 (1.19–20.285)

EMMPRIN is a transmembrane glycoprotein with two immunoglobulin-like domains and over-expressed on the surface of malignant tumor cells [18]. Depending on the cell system, EMMPRIN can stimulate production of MMP-1 (interstitial collagenase), MMP-2 (gelatinase A) and MMP-3 (stromelysin 1) but has no effect on their physiological inhibitors TIMP-1 or TIMP-2, hence modifying the collagenolytic balance toward MMP production and activation [19, 20]. Our results show that increased EMMPRIN expression in PCMM tissues has a trend toward the positive correlation with MMP-2 expression. Besides, there is a distinct positive correlation between EMMPRIN expression and clinicopathological features, which are related to tumor progression. These findings may reflect the multifunctional role of the EMMPRIN protein in PCMM, not

only as a cell–cell adhesion molecule involved in cell–cell and cell–ECM interactions, but also as a mediator of tumor invasion via stimulating MMPs. These results are consistent with recent reports concerning the role of EMMPRIN, from an MMP stimulator to an angiogenic promoter [7, 21, 22].

MMPs, particularly MMP-2, are a group of zinc-dependent endopeptidases capable of degrading the extracellular matrix of the parenchymal and vascular basement membranes [23]. MMP-2 expression was found to be correlated with activation of extracellular signal-regulated kinase (ERK), with which EMMPRIN expression strongly correlates [24]. While fibroblasts normally constitutively express MMPs at low levels, dysregulated MMP expression and activation aids in cancer cell migration. It has

been widely documented that tumorigenic cells expressing EMMPRIN induce MMP expression by neighboring fibroblasts [25, 26]. Of the MMPs induced by EMMPRIN, malignant progression has been most closely correlated with the expression of MMP-2 in several forms of cancer, and these increased levels are typically indicative of poor prognostic outcome [27]. Antibodies to EMMPRIN have potential anti-cancer therapeutic utility and have been shown to decrease MMP expression leading to an inhibition of tumor cell invasion [28]. In our series of patients with PCMM, statistic analysis shows that positive expression of EMMPRIN and MMP-2 in tumor cells has significant correlation with poor prognosis. These findings further confirm previous reports that EMMPRIN and MMP-2 immunoreactivities are most intense in the cells of the tumorous tissues and their positive expression shows the invasive potential and significant correlation with poor survival [29].

The present findings show that the co-expression of EMMPRIN and MMP-2, independent of other factors, is significantly associated with worse outcome in patients with PCMM. EMMPRIN/MMP-2 may be considered an important early prognostic marker, which may aid patient selection for adjuvant therapies and/or represent a valid target for treatment, as it appears to be related to the pathogenesis and progression of PCMM.

**Conflict of interest statement** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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