Pathol. Oncol. Res. (2011) 17:705–711
DOI 10.1007/s12253-011-9373-z

RESEARCH

Extracellular Matrix Metalloproteinase Inducer is a Negative Prognostic Factor of Pediatric Medulloblastoma

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Received: 6 January 2010 / Accepted: 25 January 2011 / Published online: 31 March 2011

Abstract Medulloblastoma (MB) is the most common embryonal CNS tumor of childhood. Its survival rates have significantly improved over the years due to developments in diagnostic techniques and therapeutic strategies. However, it is still an important cause of cancer-related deaths in children. Extracellular matrix metalloproteinase inducer (EMMPRIN) is a member of the immunoglobulin family and a glycoprotein enriched on the surface of many types of tumor cells. Therefore, the aim of this study was to investigate whether the expression patterns of EMMPRIN may predict the clinical prognosis in MB. EMMPRIN expression in a series of 56 MB with various grades and pathological types was analyzed by immunohistochemical staining on paraffin-embedded sections. Then, the correlation of EMMPRIN expression patterns with clinical-pathological features of patients and its prognostic relevance were determined. Immunohistochemistry revealed that the positive expression rate of EMMPRIN in MB (75.0%, 42/56) was significantly higher than that in normal cerebellums (6.7%, 2/30, $p<0.001$). In addition, EMMPRIN expression in MB was up-regulated in higher metastatic stage ($p<0.01$), aggressive histopathological type ($p<0.005$), necrosis ($p<0.01$), as well as with undifferentiated tumor ($p<0.01$). Furthermore, over-expression of EMMPRIN correlated significantly with poor prognosis ($0.01<p<0.05$) and represented an independent prognostic marker of overall survival on multivariate analysis ($p=0.01$). Our study suggests that EMMPRIN expression was associated with the progression of MB and its over-activation may be an important predictor of poor survival in this patient cohort. Therefore, EMMPRIN may be regarded both as a prognostic factor and a therapeutic target for MB.

Keywords Medulloblastoma · Extracellular matrix metalloproteinase inducer · Immunohistochemistry · Prognosis

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Background

Medulloblastoma (MB) is the most common embryonal CNS tumor of childhood and is likely com of biologically different subsets of tumors arising from stem and/or progenitor cells of the cerebellum. There are four major histologic subtypes of MB including classic, desmoplastic, extremely nodular, and large-cell-anaplastic [1]. Each subtype has unique histologic features and prognosis. The large-cell-anaplastic variant is clinically the most aggressive subtype, and represents between 4 and 25% of all MB cases

[2]. The survival rates of this disease have significantly improved over the years due to developments in diagnostic techniques, neurosurgery, chemotherapy, radiotherapy, and supportive care. However, only 60% of children with MB will be cured, and most of these will suffer long-term side effects [3, 4]. Therefore, it is necessary to identify patients who can be cured with less intensive therapy and also to develop more effective treatments for children with resistant disease.

Extracellular matrix metalloproteinase inducer (EMMPRIN), a highly glycosylated cell surface transmembrane protein, belongs to the immunoglobulin superfamily, was discovered twenty five years ago [5, 6]. EMMPRIN has been demonstrated to promote tumor invasion and metastasis via stimulating matrix metalloproteinase synthesis in neighboring fibroblasts, to enhance angiogenesis via vascular endothelial growth factor, to induce chemoresistant tumor cells via the production of hyaluronan, and to confer resistance of cancer cells to anoikis through inhibition of Bim [7–10]. EMMPRIN has also been shown to interact with the $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins which interact with laminins. Integrins serve as the major receptors for cellular attachment to the ECM [11]. Therefore, it is not surprising that their interaction with laminin, the major component of the basement membrane, serves as a key event in the metastatic process.

According to the molecular functions of EMMPRIN, we speculated that this protein may play a role in the progression of MB. The goal of the present study was to investigate whether the expression patterns of EMMPRIN may predict the clinical prognosis in MB.

Materials and Methods

Patients and Tissue Samples

Our study was approved by the Ethics Committee of Chongqing Medical University, Chongqing, P.R.China. Tumour samples obtained from 56 paediatric patients with a diagnosis of MB were selected from the pathology database of Chongqing Medical University based on diagnosis and availability of paraffin-embedded tumour material. All patients were treated at Children's Hospital of Chongqing Medical University between 1994 and 2004. Patients without documented follow-up were excluded from case selection. Treatment was based on diagnosis and extent of surgical resection. Patients received a combination of surgery, radiation and/or chemotherapy. The demographic data and clinical parameters assessed are outlined in Table 1.

All patients were given a follow-up investigation. Follow-up data was obtained from these records and from the tumor registry records. Follow-up was terminated August 2nd, 2009.

Table 1 Clinical and histological parameters of patients

Parameters	No. of patients	Percent (%)
All cases	56	100.0
Gender		
Female	23	47.1
Male	33	52.9
Age (years)		
<3	15	26.8
≥3 to <8	30	53.6
≥8	11	19.6
Metastatic stage[a]		
M0	10	17.9
M1~M2	28	50.0
M3~M4	18	32.1
Tumor location		
Fourth ventricle	22	39.3
Outside of Fourth ventricle	34	60.7
Tumor size		
<50 cm^3	30	53.6
≥50 cm^3	26	46. 4
Histopathological subtype		
Classic	10	17.9
Desmoplastic	12	21.4
Nodular	16	28.6
Large-cell anaplastic	18	32.1
Necrosis		
Yes	19	33.9
No	37	66.1
Differentiation level		
Undifferentiated	20	35.7
Differentiated	36	64.3
Differentiation direction		
Differentiated to nerve cell	25	44.6
Differentiated to gliocyte	31	55.4

[a] Metastatic stage was classified according to North American stratification (Children's Cancer Group; CCG) [25]

Methods

Immunohistochemical Staining

The expression patterns of EMMPRIN in MB tissues were analyzed by immunohistochemical staining. Tissues were fixed in 10% buffered formalin and embedded in paraffin. Commercially available antibody against EMMPRIN (Santa Cruz Biotech, CA, USA) was used. Immunohisto-

chemical staining was carried out using the avidin-biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Deparaffinized sections were treated with methanol containing 3% hydrogen peroxide for 10 min before conducting antigen retrieval using a microwave oven at 95°C for 5 min and cooling at 25°C for 2 h. After washing with PBS, blocking serum was applied for 10 min. The sections were incubated with primary antibody against EMMPRIN (1: 500) overnight at 4°C. Negative control sections were incubated with PBS instead of the primary antibody. Then, the section was incubated with a secondary biotinylated antibody (Dako Cytomation Inc.) followed by incubation with a streptavidin-peroxidase complex (Dako Cytomation Inc.) for 10 min at room temperature. Reaction products were developed using diaminobenzidine containing 0.3% H_2O_2 as a substrate for peroxidase and nuclei were counterstained with diluted hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by reimmunostaining via the same method in multiple, randomly selected specimens.

The immunostaining was assessed for staining intensity (grades 0~2) using light microscopy. The criteria used for assessment were as previously reported [12], where: 0 (negative, <5%); 1 (weak~moderate, 6–50%); 2 (strong, >51%) of the tumor cells stained. Each section was independently analyzed in a blind study by two independent observers.

Statistical Analysis

The software of SPSS version13.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. Continuous variables were expressed as $\overline{X} \pm s$. Statistical analysis were performed with Fisher's exact test for any 2×2 tables,Pearson χ^2 test for non- 2×2 tables, chi-square trend test for ordinal datum, Kaplan-Meier and Cox Regression analysis for the question of survival analysis. A difference between means was considered significant if the p value was less than 0.05.

Results

Expression Patterns of EMMPRIN in Pediatric MB Tissue

The expression and localization of EMMPRIN in 56 patients of MB were examined using immunostaining analysis. EMMPRIN was strongly expressed on the membrane and partly in the cytoplasm of MB cells. Scattered areas of weak heterogeneous EMMPRIN immunostaining were observed in 2/30 (6.7%) normal cere-

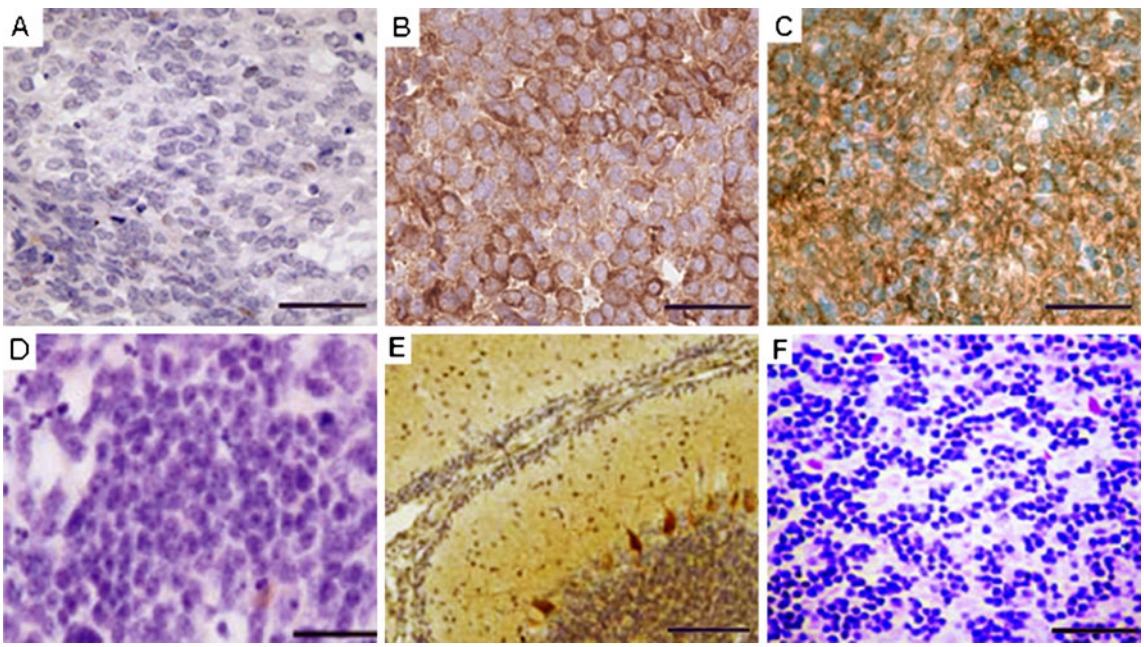

Fig. 1 Immunohistochemical staining for EMMPRIN in children MB (Original magnification×200). **a** EMMPRIN negative staining (score 0) was found in MB cells; **b** EMMPRIN weak and moderate positive staining (score 1) was found in the membrane and partly in the cytoplasm of MB cells; **c** EMMPRIN strong positive staining (score 2) was found in the membrane and partly in the cytoplasm of MB cells; **d** Photomicrograph from MB tissues with H&E staining; **e** EMMPRIN weak positive staining was found in the cell membrane and partly in the cytoplasm of normal cerebellums; **f** Photomicrograph from normal cerebellums with H&E staining

bellums whilst 42/56 (75.0%) MB tissues were positive for EMMPRIN (score 1~2). The difference in EMMPRIN positive expression rates between normal cerebellum and MB tissues had statistic significance ($p<0.001$). For EMMPRIN positive MB tissues, heterogeneous weak~moderate (score 1, Fig. 1B) and strong staining (score 2, Fig. 1C) were found in 26/56 (46.4%) and 16/56 (28.6%), respectively.

Correlation of EMMPRIN Expression with the Clinical Features of Pediatric MB

Table 2 summarized the substantial differences in EMMPRIN expression between tumors with different clinical features. EMMPRIN expression in MB was up-regulated in higher metastatic stage ($p<0.01$), aggressive histopathological type ($p<0.005$), necrosis ($p<0.01$), as well as with undifferentiated tumor ($p<0.01$).

Prognostic Relevance of EMMPRIN Expression in Pediatric MB

Univariate analyses of each factor with Cox log-rank analysis (Table 3) show that metastatic stage, histopathological subtype, necrosis status, differentiation level and EMMPRIN expression patterns were significantly associated with prognosis. Among them, metastatic stage and histopathological subtype were the most significant ($p<0.01$). The median survival of patients with strong positive expression rates of EMMPRIN was significantly shorter than those with low positive expression rates and without expression ($0.01<p<0.05$, Table 3, and Fig. 2). In multivariate analysis, the metastatic stage ($p< 0.009$), histopathological subtype ($p=0.008$), necrosis status ($p=0.03$), differentiation level ($p=0.02$) and EMMPRIN expression patterns ($p=0.01$) were significant predictors of survival (Table 4).

Table 2 Association between EMMPRIN expression and clinicopathological features

Clinicopathological features	No. of patients	EMMPRIN expression (n)			X^2	p
		0	1	2		
Gender						
Female	23	6	11	6	0.12	>0.05
Male	33	8	15	10		
Age (years)						
<3	15	4	6	5	1.40	>0.05
≥3 to <8	30	7	16	7		
≥8	11	3	4	4		
Metastatic stage[a]						
M0	10	5	5	0	13.63	<0.01
M1~M2	28	8	14	6		
M3~M4	18	1	7	10		
Tumor location						
Fourth ventricle	22	5	10	7	0.22	>0.05
Outside of Fourth ventricle	34	9	16	9		
Tumor size						
<50 cm^3	30	8	15	7	0.87	>0.05
≥50 cm^3	26	6	11	9		
Histopathological subtype						
Classic	10	7	3	0	30.38	<0.005
Desmoplastic	12	3	8	1		
Nodular	16	2	11	3		
Large-cell anaplastic	18	2	4	12		
Necrosis						
No	37	12	21	4	16.96	<0.01
Yes	19	2	5	12		
Differentiation level						
Differentiated	36	13	18	5	12.86	<0.01
Undifferentiated	20	1	8	11		
Differentiation direction						
Differentiated to nerve cell	25	6	12	7	0.05	>0.05
Differentiated to gliocyte	31	8	14	9		

[a] Metastatic stage was classified according to North American stratification (Children's Cancer Group; CCG) [25]

Table 3 Univariate analyses with the Cox log-rank test of the effect on 5-year survival rate

Parameters		No. patients	5-year survival rate (*n*, %)	*P*
Gender	Female	23	11 (47.8)	>0.05
	Male	33	16 (48.5)	
Age (years)	<3	15	7(46.7)	>0.05
	≥3 to <8	30	15(50.0)	
	≥8	11	5(45.5)	
Metastatic stage[a]	M0	10	7(70.0)	<0.01
	M1~M2	28	14(50.0)	
	M3~M4	18	6(33.3)	
Tumor location	Fourth ventricle	22	11(50.0)	>0.05
	Outside of Fourth ventricle	34	16(47.1)	
Tumor size	<50 cm^3	30	15(50.0)	>0.05
	≥50 cm^3	26	12(46.2)	
Histopathological subtype	Classic	10	7(70.0)	<0.01
	Desmoplastic	12	7(58.3)	
	Nodular	16	8(50.0)	
	Large-cell anaplastic	18	5(27.8)	
Necrosis	No	37	22(59.5)	$0.01<p<0.05$
	Yes	19	5(26.3)	
Differentiation level	differentiated	36	21(58.3)	$0.01<p<0.05$
	Undifferentiated	20	6(30.0)	
Differentiation direction	differentiated to nerve cell	25	11(44.0)	>0.05
	differentiated to gliocyte	31	16(51.6)	
EMMPRIN	0	14	10(71.4)	$0.01<p<0.05$
	1	26	13(50.0)	
	2	16	4(25.0)	

[a] Metastatic stage was classified according to North American stratification (Children's Cancer Group; CCG) [25]

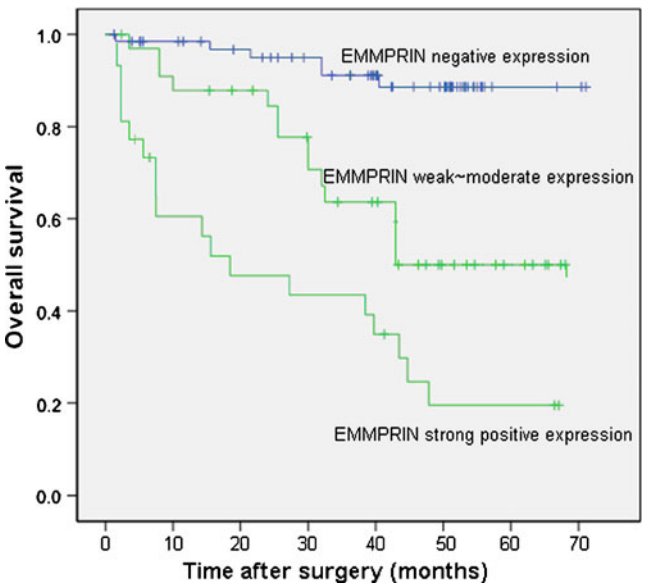

Fig. 2 Kaplan-Meier survival curves for EMMPRIN expression in pediatric MBs. Survival was significantly poorest for patients with EMMPRIN strong positive expression ($0.01<p<0.05$)

Discussion

MB is a primitive neuroectodermal tumor arising within the cerebellum. The morbidity of the current therapy and the small but significant failure rate are significant shortcomings that require improvement. A more detailed understanding of the biology underlying MB offers the possibilities of more effective, less toxic treatments. Identifying tumor subtypes with distinct prognoses may allow the provision of less intensive treatment to low-risk patients and the direction of more intensive treatment to patients less likely to be cured by standard therapy. In addition, new targeted therapies, rationally designed from an insight into the biology of MB, may offer greater efficacy and decreased treatment-related toxicity. Biologic investigation of MB has moved forward through both molecular analysis of tumor samples, and through a series of striking mouse models. In this study, by immunohistochemical staining in clinical samples, we have demonstrated that EMMPRIN were expressed at much higher levels in the MB tissues when compared with normal human cerebellum tissues. This finding suggests that EMMPRIN plays an active role in tumourigenesis in MB. Moreover,

Table 4 Multivariate Cox regression analysis for MB patients

	Wald [1]	df [2]	*p*	Exp(B)	95.0 CI for Exp(B)	
					Lower	Upper
Metastatic stage[a]	16.8	2	0.009	3.2	1.5	4.6
Histopathological subtype	13.2	3	0.008	2.8	1.1	3.9
Necrosis	8.9	1	0.03	2.7	1.3	3.5
Differentiation level	10.1	1	0.02	3.1	1.2	4.3
EMMPRIN	11.5	2	0.01	3.5	1.6	5.1

[a] Metastatic stage was classified according to North American stratification (Children's Cancer Group; CCG) [25]. [1] 'Wald' refers to Chi-square value; [2] 'df' refers to degree of freedom

the level of EMMPRIN protein expression was variable, but it was higher in MB samples from patients with more aggressive disease [13, 14]. Furthermore, we also found a statistically significant relationship between the percent of EMMPRIN-positive cells in the tumors and clinical outcome, with higher levels of EMMPRIN correlating with a worse prognosis.

EMMPRIN is expressed in neoplastic and some normal epithelial cells, although heterogeneity was observed within and between individual tumor biopsies. In the present study, EMMPRIN expression was associated with metastatic stage, histopathological type, necrosis, as well as with differentiation level, but not with other clinicopathologic factors. Previous literatures have reported that higher EMMPRIN immunostaining scores in hepatocellular carcinomas and cervical cancer correlate significantly with tumor grading and tumor-node-metastasis stages [15, 16]. Maria et al. [17] indicated that the expression of CD44, EMMPRIN, and BCRP/ABCG2 was shown in pediatric intramedullary spinal astrocytoma and diffuse pontine glioma and these proteins were interacted with hyaluronan, which was further demonstrated to have potential therapeutic value in malignant central nervous system tumors. EMMPRIN expression in breast carcinomas is associated with risk factors such as poor histologic grade, negative hormone status, themitotic index, and tumor size [18]. In gastric carcinoma, EMMPRIN expression was positively correlated with tumor size, depth of invasion, and lymphatic invasion, but not with lymph node metastasis, staging, or differentiation [19]. However, EMMPRIN protein expression patterns within esophageal squamous cell carcinoma and dysplastic lesions were not associated with any of these clinicopathologic factors [12]. These discrepancies suggest that there are different regulatory mechanisms of EMMPRIN expression in cells of different origin. In addition, recent studies also have demonstrated that EMMPRIN expression may be correlated with poor prognosis in breast cancer, esophageal squamous cell carcinoma [12], prostate cancer [20] and ovarian serous cancers [21] and can be viewed as an independent prognostic factor, which was consistent with the results of our study. It has been shown that EMMPRIN is associated with tumor infiltration and invasion into vessels via up-regulation of matrix metalloproteinases and vascular endothelial growth factor [22], suggesting that blockade of these molecules may prolong disease recurrence–free survival or overall survival by interfering with tumor infiltration and invasion.

In addition to the investigation of its role as a prognostic indicator, the research on the potential therapeutic value of EMMPRIN for neoplasms is now underway. For example, it has been demonstrated that silencing CD147 by RNA interference approach could inhibit tumor progression and increase chemosensitivity in murine lymphoid neoplasm P388D1 cells [23]. Kuang et al. [24] also indicated that down-regulation of CD147 by transfection with CD147 siRNA resulted in decreased X-linked inhibitor of apoptosis (XIAP) expression and had an anti-tumor effect through enhancing the susceptibility of cancer cells to apoptosis.

In conclusion, our study suggests for the first time that EMMPRIN expression was associated with the progression of MB and its over-activation may be an important predictor of poor survival in this patient cohort. EMMPRIN may be regarded both as a prognostic factor and a therapeutic target for MB. A potential limitation of this study is the small study population and relatively short term follow-up. Therefore, this study is hypothesis generating, and that further prospective analysis would be worth doing.

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