

Vasculogenic mimicry is a major feature and novel predictor of poor prognosis in patients with orbital rhabdomyosarcoma

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Abstract. Vasculogenic mimicry (VM) is a key developmental program, frequently activated during cancer invasion and metastasis. The aim of the present study was to evaluate the role of VM in orbital rhabdomyosarcoma (RMS), the correlation between VM and tumor differentiation, recurrence and survival duration, as well as the contribution of epithelial cell kinase (EphA2) and matrix metalloproteinase-2 (MMP-2) in VM initiation. A total of 32 patients were enrolled to investigate the associations between VM in orbital RMS tumors and clinical characteristics, as well as its impact on overall survival. VM was identified and confirmed by CD31/periodic acid-Schiff double staining, while the presence of EphA2 and MMP-2 were examined by immunohistochemical analysis. VM was identified in eleven patients, particularly those with poorly differentiated orbital RMS ($P=0.001$). Patients with VM exhibited significantly worse survival rates ($P=0.001$, log-rank test), a significantly increased risk of mortality ($P=0.008$) and EphA2 and MMP-2 expression levels were enhanced ($P=0.005$ and 0.001 , respectively). The VM and mitotic rate were independent predictors of poor prognosis ($P=0.001$ and 0.004 , respectively), indicated by multivariate Cox proportional hazards models. These results demonstrated that VM is present in orbital RMS and represents an independent prognostic factor for overall

survival. In addition, overexpression of EphA2 and MMP-2 may promote VM formation in orbital RMS.

Introduction

Orbital rhabdomyosarcoma (RMS) as a prevalent malignancy, and is the most common malignant tumor of the orbit amongst children (1,2). Malignant tumors require a sufficient blood supply in order to support their growth. It was previously believed that blood vessels were only able to be formed by endothelial cells. However, the rigid cellular identity of the blood vessel has since been questioned, following the observation that tumor cells are also capable of forming extravascular networks in high-grade malignancies (3). The process of vasculogenic mimicry (VM), describes the mechanism by which highly aggressive tumor cells are able to form vessel-like structures, as a result of their high plasticity. It has therefore been suggested that tumors may develop vascularization via angiogenesis, and that this process is associated with poor prognosis (4-6).

Orbital RMS is a solid tumor with the morphological characteristic of bidirectional differentiation, which is the cellular basis of the formation of VM (7). However, to the best of our knowledge, no specific study regarding the novel blood-supplying pattern in orbital RMS has previously been published. Furthermore, the precise molecular mechanism of VM formation remains to be elucidated. Epithelial cell kinase (EphA2) and matrix metalloproteinase-2 (MMP-2) have been found to be a crucial inducers of VM (8,9), therefore it is of significant interest to examine the role of EphA2 and MMP-2 in VM formation in orbital RMS. The present pilot study was designed to investigate whether VM occurs in orbital RMS and if so, to evaluate the correlation between VM and clinicopathological disease features. Furthermore, the present study aimed to elucidate the potential mechanisms underlying VM formation.

Materials and methods

Ethics statement. The present study adhered to the conditions of the Declaration of Helsinki. Ethical approval was obtained from the institutional Medical Research Ethics Committee, and informed consent was obtained from all patients prior to

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treatment. The study was performed following approval by the ethics committee of the Second Affiliated Hospital of Tianjin Medical University (Tianjin, China).

Patients and samples. In the present cross-sectional study, tissue specimens were obtained from 55 patients who underwent surgery for orbital RMS between 1997 and 2009 through the Tumor Tissue Bank of the Second Affiliated Hospital of Tianjin University (Tianjin, China). The diagnoses of these orbital RMS samples were verified by pathologists. Thirteen cases were excluded due to poor fixation or processing. Detailed pathological and clinical data of the remaining 32 cases were collected, including age, gender, tumor location and size, mitotic rate, histological type, recurrence and survival duration. The paraffin-embedded tumor tissue samples were obtained from patients who had not been subjected to therapy prior to surgery.

Definition of VM and patient grouping. VM was defined as tumor-cell-surrounded channels, within which red blood cells (RBCs) were detectable. In hematoxylin and eosin-stained slides, VM was observed to be formed by tumor cells, but not endothelial cells, without hemorrhage, necrosis or inflammatory cells infiltrating proximal to these structures. In CD31/periodic acid Schiff (PAS) double-stained slides, VM was defined as tumor cells (rather than endothelial cells) forming channels with PAS-positive material and RBCs. No VM-positive cells were detected in CD31-stained slides. The cells forming VM were confirmed to be orbital RMS cells by actin staining. Laminin-stained slides confirmed the topography of the basement membrane of the vessels and vessel-like structures, which were positively stained. The 32 patients evaluated possessed sufficient follow-up data for evaluation and were divided into 2 groups according to VM-positivity.

Immunohistochemical analysis

Reagents. The primary antibodies used were as follows: Monoclonal mouse antiserum against human Actin (dilution, 1:500; cat. no. M087401, clone Alpha-Sr-1; Dako Cytomation, Glostrup, Denmark); monoclonal mouse anti-human CD31 (dilution, 1:100; cat. no. M082301, clone JC70A; Dako Cytomation); polyclonal rabbit anti-mouse IgG against EphA2 (dilution, 1:100; cat. no. sc-924; clone no. C-20; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and monoclonal mouse anti-human MMP-2 (dilution, 1:100; cat. no. MAB9554; clone no. 17B11; Abnova, Tapei City, Taiwan). All slides were subjected to heat-induced epitope retrieval at 92°C in citrate buffer (0.01 mol/l; pH 6.0) prior to immunohistochemical staining. The 0.5% PAS solutions were produced in the Pathology Department of Tianjin Eye Hospital (Tianjin, China) and confirmed to be effective in previous experiments (10).

Immunohistochemical assay. Staining with primary antibodies against Actin, CD31, EphA2 and MMP-2 was performed on formalin-fixed, paraffin-embedded tissues using the SP-9000 kit (Zhongshan Chemical Co.). Briefly, the tumor tissues were formalin-fixed and embedded in paraffin. The tissues were cut into 4-μm sections and mounted onto slides, dried overnight at 65°C and deparaffinized in xylene.

Next, the sections were rehydrated through graded alcohol into water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 50% methanol for 10 min at room temperature. The sections were washed with phosphate-buffered saline (PBS; Zhongshan Chemical Co.) and pretreated with citrate buffer (0.01 M citric acid; pH 6.0) for 20 min at 100°C in a microwave oven. After rinsing with PBS, the slides were incubated overnight at 4°C with primary polyclonal antibodies against EphA2 (dilution, 1:100), MMP-2 (dilution, 1:100) and MMP-9 (dilution, 1:100). The sections were then washed with PBS and incubated with polyclonal HRP-conjugated goat anti-rabbit IgG (dilution, 1:1,000; cat. no. ab6721; Abcam, Cambridge, UK) secondary antibody for 30 min at 37°C. Visualization was performed using a 2-Solution DAB Kit (cat. no. 88-2014; Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The sections were then incubated in 95% alcohol with agitation for 30 min to remove formalin granules. After washing with tap water, the sections were incubated with potassium permanganate for 3 h and bleached with 2% oxalic acid for 5 min. The nuclei were counterstained with hematoxylin, followed by dehydration and cover-slip mounting. The slides were visualized using a light microscope (Leica DM4000 B LED; Leica Microsystems GmbH, Wetzlar, Germany). Paraffin-embedded gastric mucosa samples obtained from gastric carcinoma patients at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China), and PBS were used as the positive and negative controls, respectively.

CD31 staining and PAS double-staining. CD31/PAS double-staining of the samples was performed as previously described by Sun *et al* (11). Following CD31 immunostaining, the sections were treated with 0.5% periodic acid solution for 10 min at room temperature, prior to rinsing in distilled water for 3 min. These sections were then treated with periodic acid solution for 15-30 min in the dark at room temperature. After rinsing in distilled water, sections were counterstained with hematoxylin (Zhongshan Chemical Co.). Finally, the sections were counterstained with hematoxylin or PAS. PBS replaced the primary antibodies for the negative control, while normal gastric mucosa tissue, obtained from the Department of Pathology, Tianjin Medical University Cancer Institute and Hospital, was used as a positive control. VM channels and mosaic vessels (MVs; in which both endothelial cells and tumor cells form the luminal surface) were counted. The microvessel density (MVD) was established by light microscopic examination (Leica DM4000 B LED; Leica Microsystems GmbH) of CD31-stained sections at the location with the highest quantity of capillaries and small venules. The mean vessel count of three fields (magnification, x400) with the greatest neovascularization was considered to indicate the MVD. Counts were conducted blindly in a minimum of three randomly selected sections from each group. The mean value from 10 fields observed in each section for each type of microvessel comprised the final outcome.

Assessment methods. The staining index was evaluated to investigate differences in the expression of EphA2 and MMP-2 between the VM-positive and -negative lesions. Tumor cells with brown cytoplasmic staining were considered to be positive. Ten randomly selected visual fields were examined, and 100 cells were counted within each of these fields. The mean

Table I. Association between vasculogenic mimicry and clinicopathological data.

Factor	Patients, n	VM, n (%)	Non-VM, n (%)	χ^2	P-value
Gender				1.948	0.163
Male	21	9 (42.9)	12 (57.1)		
Female	11	2 (18.2)	9 (81.8)		
Age, years				0.204	0.652
<12	22	7 (31.8)	15 (68.2)		
≥12	10	4 (40.0)	6 (60.0)		
Tumor location				2.586	0.108
Right orbit	17	8 (47.1)	9 (52.9)		
Left orbit	15	3 (20.0)	12 (80)		
Recurrence				10.891	0.008 ^a
Yes	17	13 (76.5)	4 (23.5)		
No	15	7 (46.7)	8 (53.3)		
Survival				6.362	0.012 ^a
Dead	11	7 (63.6)	4 (36.4)		
Alive	21	4 (19.0)	17 (81.0)		
Cell type				7.469	0.024 ^a
Embryonal	19	6 (31.6)	13 (68.4)		
Alveolar	7	5 (71.4)	2 (28.6)		
Multiformity	6	0 (0)	6 (100)		
Mitotic rate/50 HPF				11.23	0.001 ^a
≥5	10	7 (70.0)	3 (30.0)		
<5	22	3 (13.63)	19 (86.37)		

^aSignificantly different (P<0.05); HPF, high-power fields.

Table II. Multivariate analysis of factors influencing survival.

Factor	Cox proportional hazards analysis		
	Hazard ratio	95% CI	P-value
VM, positive	6.521	3.136-12.800	0.001 ^a
Mitotic rate	2.910	1.632-6.116	0.001 ^a

^aSignificantly different (P<0.05). CI, confidence interval; VM, vasculogenic mimicry.

percentage of positive cells was considered to indicate the expression of the two proteins within each section. The results were quantified as previously described (12).

Statistical analysis. All data in the study were evaluated using SPSS software version 11.5 (SPSS Inc., Chicago, IL, USA). Survival rates were calculated using the Kaplan-Meier method, statistically significant differences were identified using the log-rank test and potential prognostic factors were analyzed using the Cox proportional hazards model. The Wilcoxon-Mann-Whitney test was performed to analyze differences in EphA2 and MMP-2 expression between the VM and non-VM groups. P<0.05 was considered to indicate a statistically significant difference.

Table III. Comparison of the expression of EphA2 and MMP-2 in the VM and non-VM groups.

Factor	VM, mean rank		Z	P-value
	Positive, % (±SD)	Negative, % (±SD)		
EphA2	49.3±5.02	34.20±3.21	2.600	0.005 ^a
MMP-2	69.02±2.22	45.36±1.28	3.416	0.001 ^a

^aSignificantly different (P<0.05). Mean rank was calculated using SPSS statistical software. EphA2, epithelial cell kinase; MMP-2, matrix metalloproteinase-2; VM, vasculogenic mimicry; SD, standard deviation; Z, Wilcoxon-Mann-Whitney test statistic.

Results

Evidence of VM in orbital RMS. VM structures were identified in a subset of orbital RMS specimens. VM was identified by the formation of a tubular, fracture-like structure by tumor cells (not endothelial cells), with RBCs within the cavities, observed with structural integrity, no incidence of hemorrhage and an absence of necrosis or inflammatory cells infiltrating the channels. Such structures were detected in 11 orbital RMS samples (34%; Fig. 1). Clinical data are presented in Table I.

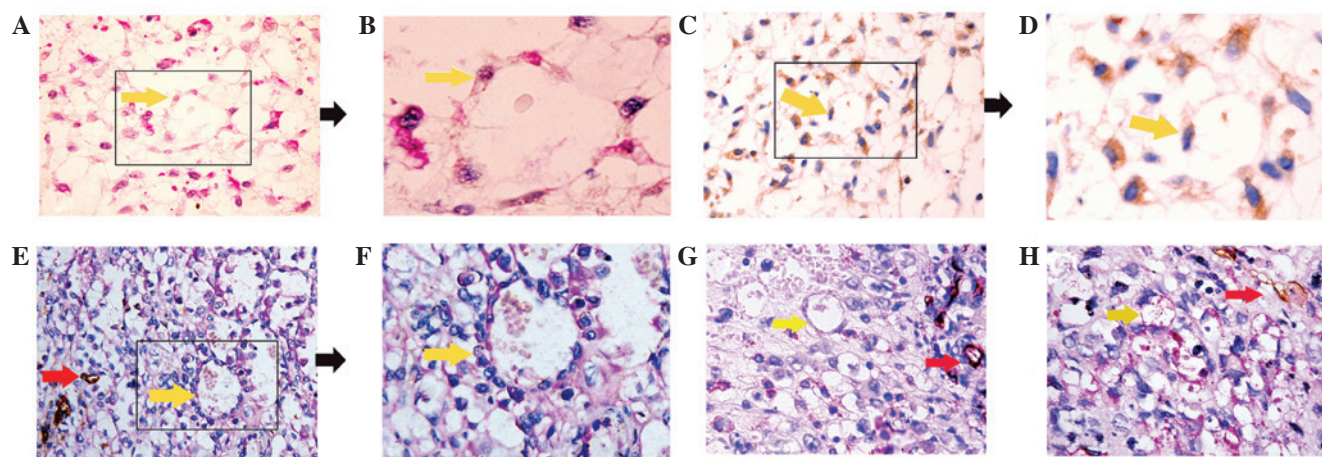


Figure 1. Evidence of VM in orbital RMS. (A) Morphological observation with hematoxylin and eosin staining. The channels (yellow arrow) lined with tumor cells contained red blood cells, without shuttle-like endothelial cells in the VM wall (magnification, x400). (B) Enlargement of box outlined in A (magnification, x1,000). (C) Orbital RMS tumor cells arranged in tubular structure, indicated by actin immunohistochemical staining. Actin positivity was observed as brown-colored cytoplasmic and membrane staining in the specimen. Serial sections from one block, as in the panel, indicated that the cells lining the VM were orbital RMS tumor cells (magnification, x200). (D) Enlargement of box outlined in C (magnification, x400). (E) CD31/PAS double-staining of VM. The wall of the VM channel was positive for PAS staining, while tumor cells lining the external wall were negative for CD31 staining (yellow arrow). The endothelial-dependent microvessels were positive for CD31 and PAS (red arrows) (magnification, x400). (F) Enlargement of box outlined in E (magnification, x1,000). (G and H) VM was identified in various cases of orbital RMS tissue by CD31/PAS double-staining (magnification, x400). Yellow arrows, VM; red arrows, microvessel co-existence in the same field. VM, vasculogenic mimicry; RMS, rhabdomyosarcoma; PAS, periodic acid Schiff.

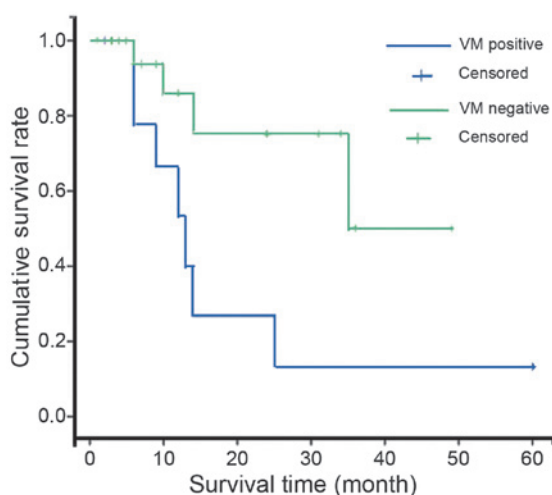


Figure 2. Kaplan-Meier survival analysis of patients with VM-positive and VM-negative orbital rhabdomyosarcoma. The VM-negative group exhibited a higher percentage of 5-year survivors than that of the VM-positive group ($P=0.001$). VM, vasculogenic mimicry; Cum, cumulative.

Association between VM and clinicopathological data. The clinicopathological data regarding the 32 patients are summarized in Table I. The incidence of VM was significantly greater in lesions with a mitotic rate of $\geq 5/50$ high-power fields (HPF) (7/10; 70.0%), than in lesions with a lower mitotic rate (3/22; 13.6%). The incidence of VM was also significantly greater in lesions associated with necrosis (2/8, 25.0%), than in those without necrosis (7/24, 29.2%). The VM positive rate was higher for alveolar cell types (5/7; 71.4%) than for embryonal cell types (6/19; 31.6%), which exhibited greater incidence than that of the multi-formity cell type (0/6; 0%). The VM-positive group also had a lower survival rate (7/11; 63.6%) than that of

the VM-negative group (4/21; 19.0%; $P=0.012$). The incidence of VM did not differ with respect to patient gender, age or tumor location.

VM is associated with poor prognosis. VM-positive patients exhibited a lower survival rate (36.4%) and shorter survival period than that of patients without VM (Table II). Kaplan-Meier survival analysis indicated that the survival rate for patients with tumors exhibiting VM was significantly poorer than that of patients without VM ($P=0.001$; Fig. 2). Cox proportional hazards model analysis was performed according to VM and mitotic rate (Table II). This multi-variate analysis revealed that the presence of VM and mitotic rate were independent indicators of poor prognosis ($P=0.001$ and $P=0.001$, respectively).

VM is associated with enhanced expression of EphA2 and MMP-2. EphA2 was expressed in the cytoplasm of orbital RMS cells (Fig. 3); and expression was markedly greater in the VM-positive group than that of the VM-negative group ($P=0.005$). MMP-2 was also expressed in the cytoplasm of orbital RMS cells, and the expression levels of MMP-2 were greater in VM-positive tumors than those in the VM-negative group ($P=0.001$; Table III).

Discussion

RMS is a highly malignant tumor, and orbital RMS is one of the few life-threatening diseases initially presented to ophthalmologists. However there are certain differences between the orbital RMS and RMS in other locations; for example, the majority of orbital RMS tumors (60.6%) present with localized disease and are consequently more favorable when compared with such tumors at sites in other parts of the

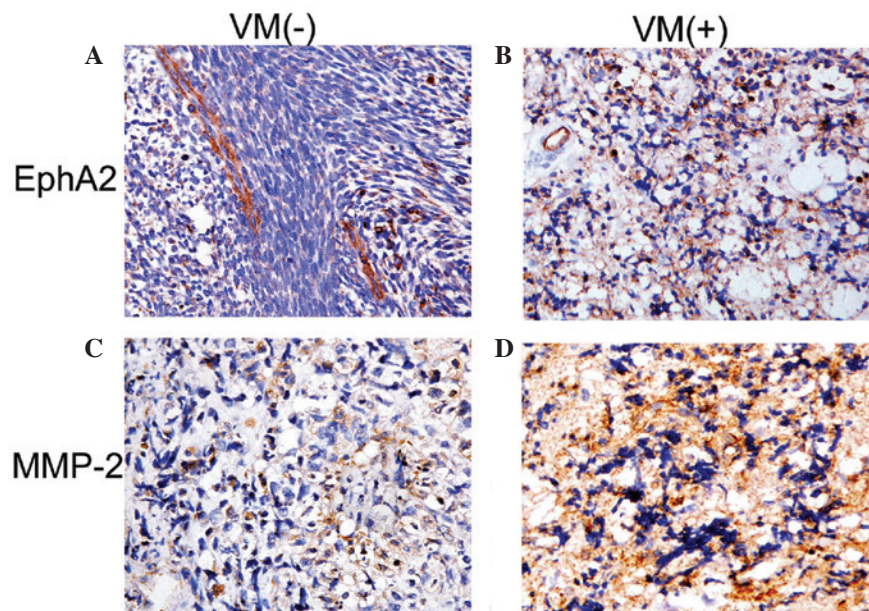


Figure 3. EphA2 and MMP-2 expression in VM-negative and VM-positive specimens of orbital RMS. Brown or yellow staining observed in the cytoplasm indicated a positive result. Immunostaining of EphA2 cytoplasmic expression in (A) VM-negative and (B) VM-positive orbital RMS samples (magnification, x400). Expression of MMP-2 in the cytoplasm of tumor cells from the (C) VM-negative and (D) VM-positive groups (magnification, x400). EphA2, epithelial cell kinase; MMP-2, matrix metalloproteinase-2; VM, vasculogenic mimicry; RMS, rhabdomyosarcoma.

body. Orbital RMS is associated with an excellent survival rate of >85%, whereas the survival rate for RMS at other sites is 63-76.9% (2). Furthermore, orbital RMS patients have a 5-year survival rate of ~84.3%, which is associated with low-grade malignancy (13-15). Tumor cells must acquire an adequate blood supply in order to facilitate their survival, proliferation and metastasis. Angiogenesis describes the process of the formation of novel vessels from the existing vasculature. The identification of non-endothelialized vessel-like channels in malignant tumors, termed VM, has provided insight into underlying tumor behaviors and presents potential targets for drug therapy (10,16). The formation of VM by tumor cells requires a genetic reversion of cells to a pluripotent embryonic-like genotype, a change known as 'cancer plasticity' (6,17,18). Orbital RMS is a solid tumor with the capacity for bidirectional differentiation, which encompasses features of the endothelial cells lining the vessels, as well as the mesenchymal cells that secrete extracellular matrix (ECM) proteins, associated with the remodeling of the matrix for the cellular basis of the formation of VM (19).

To the best of our knowledge, the present study provides the first evidence of the pattern of VM in human orbital RMS. This hypothesis is based on the principle that the walls of VM channels are similar to those of blood vessels, possessing an ECM and glycosaminoglycans that make them PAS positive. However, by definition, these VM channels have no endothelial lining and are not stained by endothelial markers, including CD31 (20,21). In 2004, Sun *et al* confirmed that VM channels were an indicator of poor prognosis in alveolar RMS (12), although their study was principally based on virtual patient samples. In the present study, CD31/PAS double-staining combined with actin immunohistochemical staining were performed, to validate the existence of VM in orbital RMS for the first time. Serial

sections were obtained for each VM-positive lesion, and VM channels were identified at random in each section. Based on CD31 and PAS staining, VM was detected in 11 patients out of 32 (34.4%). These 11 VM-positive lesions confirmed that the VM channels were not generated accidentally. Based on the results discussed above, it was concluded that VM tissues were present in orbital RMS. VM was indicated by matrix-associated channels, which contained RBCs and were not lined by endothelium.

VM in orbital RMS, which generates lumens containing RBCs, is similar to the pattern of VM observed in alveolar RMS (12), but not identical to that seen in uveal melanomas, in which a higher number of lumens are observed (3). A potential reason for this is that uveal melanoma cells are rich in reticular fibers, while orbital RMS cells are less reticular and collagen fiber-rich. This point is determined by the enhanced reticular fiber content in tumor cells, rather than the distribution of tumor cells.

In the present retrospective study of 32 orbital RMS patients, it was demonstrated that VM was correlated with tumor cell type, high mitosis-risk, recurrence and short survival time. These results indicated that VM may be a potential indicator of poor prognosis for orbital RMS. The VM-positive rate was also found to be significantly greater in high mitosis-risk patients than that in the low mitosis-risk group. This likely reflects the more complex genotype and higher growth rate of high proliferation-risk orbital RMS tumors. Further analysis suggested that the survival rate was shorter in the VM group than that in the non-VM group. Kaplan-Meier and multivariate analyses revealed that VM negatively affected patient prognosis; and therefore VM may be used for the assessment of orbital RMS patient prognosis. Due to the specialized structure of VM, namely the lack of a barrier formed by endothelial cells to prevent tumor cells

from accessing the microcirculation (3,22), tumor cells are able to migrate easily into the blood flow. Patients with orbital RMS with VM are therefore particularly vulnerable to metastasis, explaining why VM is associated with shorter survival in many malignancies. Another valuable finding from the present study is that VM may be used as a predictor of decreased response to clinical therapy. This is due to the fact that certain chemotherapeutic strategies may only target traditional angiogenesis-associated factors, hence would be ineffective for VM-positive cases. Therefore, identification of the molecular markers of VM may provide an improved approach for the selection of appropriate cancer therapeutic strategies.

VM describes the ability of highly aggressive tumor cells to express endothelial cell-associated genes, including EphA2 and VE-cadherin, enabling them to form ECM-rich, tubular networks when cultured on a three-dimensional (3D) matrix (9). However, the exact mechanism underlying VM remains to be elucidated. To date, several molecules have been identified which have functional roles in VM. Hess *et al* (23) demonstrated that the expression of epithelial cell marker EphA2 by highly aggressive melanoma tumor cells, facilitated their ability to mimic endothelial cells, generating VM in 3D culture. Furthermore, transient knockout of EphA2 attenuated the ability of these tumor cells to generate tubular structures. These results indicated that EphA2 may be involved in the formation of tubular networks by orbital RMS tumor cells, and therefore may represent a novel therapeutic target for the treatment of orbital RMS, requiring further clinical investigation.

The present study and previous studies have suggested that EphA2 expression occurs with respect to MMP-2 upregulation. The MMP-2 protein is considered to have a significant role in VM formation in melanomas (24). EphA2 and VE-cadherin may activate MMP-2 via the PI3K pathway, and the activated MMP-2 may subsequently promote VM formation (25). In the present study, a difference in the staining index of MMP-2 expression was identified; with higher expression in the VM group than that of the non-VM group. It was hypothesized that orbital RMS cells secrete and activate MMP-2, and then assist the formation of VM. In the tissue samples evaluated in the present study, MMP2 expression was markedly higher in the VM-positive group than that of the VM-negative group, and was correlated with EphA2 expression. Notably, the results revealed that EphA2 and the associated molecular pathways may represent novel therapeutic targets for the inhibition of orbital RMS recurrence and angiogenesis.

VM is a predictor of poor prognosis for orbital RMS. In the present pilot study, VM was identified in orbital RMS, and was found to be indicative of poor prognosis. VM was observed in RMS at the protein levels, significantly influencing progression, metastasis and targeting therapy. Furthermore, EphA2 and MMP-2 may have critical roles in VM formation. These two proteins form a complex *in vivo* which results in the development of VM and tumor promotion (26,27). However, the present study was retrospective in nature and comprised a relatively small sample size. Further research regarding the underlying molecular mechanisms of VM may identify novel therapeutic strategies for orbital RMS.

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