Original Article

Pingyangmycin Pretreatment Influences the Biological Behavior of Ocular Venous Malformation and Relates with Galectin-3 Expression

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Abstract

Background: Galectin-3 (Gal-3) plays a role in the mechanisms underlying ocular venous malformation. We conducted this study to investigate the effect of pingyangmycin pretreatment on the Gal-3 expressions and biological behavior of ocular venous malformation.

Methods: Tissue samples were collected from 136 patients with ocular venous malformation. Patients were randomly divided into pingyangmycin ($n = 69$) and nonpingyangmycin group ($n = 67$). Patients in the pingyangmycin group received a local injection of 0.02% pingyangmycin once every 2 days for 2 weeks (7 doses) before removal surgery, whereas patients in the nonpingyangmycin group underwent removal surgery without local injection. The protein and messenger RNA (mRNA) expression of Gal-3 were detected by using immunohistochemistry and *in situ* hybridization.

Results: Gal-3 protein was expressed in 35 (52%) of 67 samples in the nonpingyangmycin group and in 19 (28%) of 69 samples in the pingyangmycin group ($P < 0.05$). Gal-3 mRNA expression was detected in 39 (58%) of 67 samples in the nonpingyangmycin group and 22 (32%) of 69 samples in the pingyangmycin group ($P < 0.05$). The higher Gal-3 expressions were detected in samples with deeper invasiveness than those with superficial invasiveness before ($\chi^2 = 12.720$ and 13.369, respectively, both $P < 0.05$) and after pingyangmycin treatment ($\chi^2 = 8.429$ and 4.590, respectively, both $P < 0.05$). It was more frequently detected in mesh-like lesions with unclear boundary than round lesions with clear boundary before ($\chi^2 = 30.291$ and 41.466, respectively, both $P < 0.05$) and after pingyangmycin treatment ($\chi^2 = 14.619$ and 15.130, respectively, both $P < 0.05$). Pingyangmycin treatment led to a significant difference in Gal-3 expressions at both protein and mRNA levels ($\chi^2 = 8.664$ and 9.524, respectively, both $P < 0.05$).

Conclusions: Gal-3 expression may be involved in the development and invasiveness of ocular venous malformation, and pingyangmycin can inhibit Gal-3 expression, indicating a role of pingyangmycin treatment before the removal of ocular venous malformation.

Key words: Galectin-3; Invasiveness; Pingyangmycin; Venous Malformation

Introduction

Venous malformation is a congenital vascular abnormality that can affect any part of the body and is clinically manifested in childhood and adulthood.[1-3] Ocular venous malformation is a rare disease. If only the eyelid is involved, the symptoms are commonly subtle. However, if the ocular muscle or even the optic nerve is invaded, the symptoms can be serious.[4,5] In rare cases, ocular venous malformation can affect the orbital bone.[6] In general, ocular venous malformation is believed to be benign, but its invasive behavior can also lead to ocular dysfunction and even blindness. Thus, identifying the underlying

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mechanisms of this disease is important for more effective treatment.

Galectin-3 (Gal-3), a member of the lectin family, is involved in several cellular processes such as cell adhesion, proliferation, apoptosis, and messenger RNA (mRNA) splicing.[7] However, it remains unclear whether Gal-3 is involved in the pathogenesis of ocular venous malformation.

Pingyangmycin was first used as an antitumor agent[8] and later it was also used as a sclerosing agent in the treatment of hemangioma and venous malformation.[9,10] In this study, we compared the expression levels of Gal-3 in patients with ocular venous malformation who were given pingyangmycin injection before surgery and those who did not receive pingyangmycin pretreatment. One purpose of this study was to investigate the expression levels of Gal-3 in patients with ocular venous malformation. The other purpose was to investigate whether the effect of pingyangmycin on ocular venous malformation is mediated through the inhibition of Gal-3.

Methods

Ethical approval

This prospective, randomized study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Ethics Committee of People's Hospital of Zhengzhou University. Informed written consent was obtained from all patients prior to their enrollment in this study.

Patients

Patients with ocular venous malformation scheduled for elective removal surgeries between June 2010 and June 2015 were recruited for this study. Patients with congenital and progressive aggravation of the upper eyelid or lower eyelid with a soft spongy mass, no pulsation were included. A mass of abnormal venous blood vessels was further confirmed by color Doppler ultrasound and MRI, and the surgical treatment was performed if the eyeball, retrobulbar tissue and nerve have not been involved. Eligible patients who agreed to participate in this study were randomly divided into two groups, based on a random number generated by a simple randomization method. Patients with number 0 after the random number was divided by 2 were assigned to the nonpingyangmycin group (67 cases), whereas patients with number 1 after the random number was divided by 2 were assigned to the pingyangmycin group (69 cases). Patients in the pingyangmycin group received a local injection of 0.02% pingyangmycin once every 2 days for a total of 7 doses before the surgery. Patients in the nonpingyangmycin group underwent the removal surgery directly without pingyangmycin pretreatment. The dose of pingyangmycin is based on the need of each patient's condition and individual choice, and we determined the dose of 0.5–3.0 ml according to the texture and swelling degree of the lesions. In addition, we obtained thirty negative control samples from the tissues adjacent to the surgical margin of the deformed veins (2–5 mm) in patients with ocular venous malformation, and we made the cutting edge clean to prevent postoperative recurrence. These negative control samples were from the nonpingyangmycin group samples.

Sample preparation

Tissue samples were obtained by surgical removal of the deformed vein in the eye. The samples were embedded in paraffin to make tissue sections (3 or 6 μm thick). One section was stained with hematoxylin-eosin (HE) staining for diagnosis, whereas the rest was used for analysis of immunohistochemistry and *in situ* hybridization.

Immunohistochemistry

Tissue sections were washed in xylene to remove the paraffin, rehydrated with serial dilutions of alcohol, and washed with phosphate-buffered saline (PBS) solutions. The sections were incubated with primary antibodies against Gal-3 (monoclonal mouse anti-human Gal-3, clone 9c4; Oncogene, USA) overnight at 4°C. Sections of human thyroid cancer were used as positive controls, whereas sections incubated with PBS instead of primary antibodies were used as negative controls. Sections were then incubated with goat anti-mouse biotin-conjugated secondary antibodies for 1 h at 37°C, followed by incubation with streptavidin horseradish peroxidase for 30 min and 3,3-diaminobenzidine (DAB) substrate for 5 min at room temperature. The sections were then counterstained with hematoxylin.

The immunostaining was examined under a light microscope at a high-power filed (original magnification ×200). For each section, five fields were randomly selected for immunohistochemical score. The immunostaining was evaluated according to the percentage of stained cells and the intensity of the immunoreactivity as previously reported by Willihnganz-Lawson *et al*.[11] The intensity of immunoreactivity was scored as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percentage of stained cells was scored as follows: 0 for <25% of stained cells, 1 for 25–49% of stained cells, 2 for 50–74% of stained cells, and 3 for ≥75% of stained cells. The final immunoreactive score was determined by the sum of both the intensity score and the score for the percentage of positively stained cells. The negative and positive staining were defined by a final score of <3 and ≥3, respectively.

In situ messenger RNA hybridization

The procedure of *in situ* hybridization was performed using Gal-3 *in situ* hybridization kit (Wuhan Boster, Bio-engineering Co., Ltd., Wuhan, China), according to the manufacturer's instruction. The digoxigenin-labeled oligonucleotide probes for Gal-3 were synthetized by Wuhan Boster (Bio-engineering Co., Ltd., Wuhan, China), and the sequences were as follows: 5'-CTTCCACTTTAACCCACGCTTCAATGAGAA-3', 5'-AATAACTG GGGAAGGGAAGAAAGACAGTCG-3', and 5'-AAACCA TTCAAAATACAAGTACTGGTT GAA-3'. Tissue sections were deparaffinized and treated with 3% H_2O_2 at room temperature for 12 min to block

endogenous peroxidase activity. Sections were then treated with pepsin (1:10 dilution in fresh citrate solution) for 15 min at room temperature to remove DNA-binding proteins. The sections were then incubated in hybridization solution at 42°C for 4 h, followed by incubation with oligonucleotide probes in a humidified hybridization chamber at 42°C overnight. Sections in which oligonucleotide probes were omitted were used as a negative control. After washing with saline sodium citrate twice, the sections were incubated in the blocking solution at 37°C for 30 min. The sections were then incubated with biotin-conjugated mouse anti-digoxigenin antibodies for 1 h at 37°C. After washing with PBS, sections were incubated with streptavidin biotin peroxidase complex at 37°C for 30 min, followed by incubation with DAB substrate. Sections were examined under a light microscope.

The staining was evaluated under a light microscope at a high-power field (original magnification ×200). For each section, five fields were randomly selected. The staining was semi-quantified as previously reported by Miyazaki *et al*.[12] The intensity of staining was scored as follows: 1 for light yellowish, 2 for dark yellowish, and 3 for brownish. The percentage of stained cells was scored as follows: 0 for <5% of stained cells, 1 for 5–24% of stained cells, 2 for 25–49% of stained cells, 3 for 50–74% of stained cells, and 4 for ≥75% of stained cells. The final score was determined by the multiplication of the intensity score and the score from the percentage of positively stained cells. The negative and positive staining were defined as a final score of ≤1 and >1, respectively.

Statistical analysis

All data were shown as *n* or *n* (%). Statistical analyses were performed using SPSS software version 21.0 (IBM Inc., Chicago, IL, USA). Chi-square tests were used to compare differences between groups. A $P < 0.05$ was considered statistically significant.

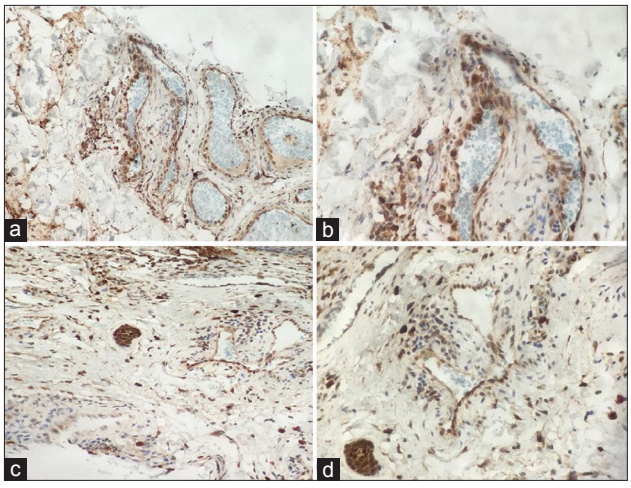

Figure 1: Representative micrographs showing immunohistochemical staining of galectin-3 in tissue samples from patients with ocular venous malformation in the nonpingyangmycin group (a and b) and the pingyangmycin group (c and d) (a and c: original magnification ×200, and b and d: original magnification ×400).

Results

A total of 136 patients (101 females and 35 males) with ocular venous malformation scheduled for removal surgery were finally included in this study. Sixty-nine patients were assigned in the pingyangmycin group and 67 were in the nonpingyangmycin group.

Intraoperative resection specimen in the nonpingyangmycin group presented as light reddish or blue cavernous malformed veins with unclear boundary. The veins were not pulsatile and easy to bleed. When the ocular muscles were involved, the malformed veins showed a diffuse distribution with starry appearance and no signs of interconnection. Samples in the pingyangmycin group displayed smaller malformed veins relative to those in the nonpingyangmycin group and rarely distributed in a diffuse manner. Unlike the nonpingyangmycin group, the malformed veins in the pingyangmycin group formed a hard mass with clear boundary. Moreover, coagulation, increased thrombosis, and cavity closure were found in most of the malformed vein cavities. Meanwhile, congestion was reduced in malformed vein.

Pingyangmycin pretreatment decreased the messenger RNA and protein expressions of galectin-3 in ocular malformed veins

As shown in Figures 1 and 2, the positive Gal-3 immunopositive staining was mainly observed in the cytoplasm and nuclei. The positive Gal-3 immunoreactivity was detected in 35 (52%) of 67 malformed vein samples in the nonpingyangmycin group, while it was detected in three (10%) of the thirty negative control samples. This indicated that Gal-3 immunoreactivity occurs at significantly higher rate in the malformed veins than in the normal veins ($P < 0.05$). In the pingyangmycin group, Gal-3 immunoreactivity was observed in 19 (28%) of 69 malformed vein samples. Compared with the positive detection rate in three (10%) of the thirty negative control samples, the detection rate of Gal-3 immunoreactivity in the pingyangmycin group was not significantly different

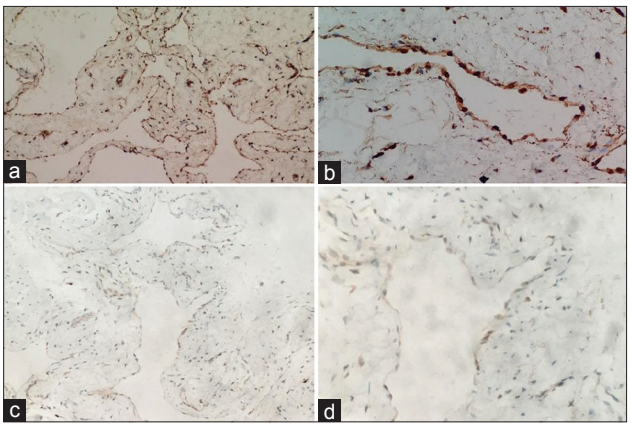

Figure 2: Representative micrographs of *in situ* hybridization showing the mRNA expression of galectin-3 in tissue samples from patients with ocular venous malformation in the nonpingyangmycin group (a and b) and the pingyangmycin group (c and d) (a and c: original magnification ×200, and b and d: original magnification ×400).

Table 1: Influence of clinicopathological parameters of patients with ocular venous malformation on Gal-3 protein expression in the nonpingyangmycin group

Variables	Gal-3 protein, *n*		χ^2	*P*
	Negative	Positive		
Age				
<12 years (*n* = 18)	12	6	3.526	0.060
≥12 years (*n* = 49)	20	29		
Sex				
Male (*n* = 19)	9	10	0.002	0.968
Female (*n* = 48)	23	25		
Depth of lesion				
Eyelid, skin, and fat (*n* = 35)	24	11	12.720	0.000
Within the muscular layer (*n* = 32)	8	24		
Appearance and boundary				
Round with clear boundary (*n* = 29)	25	4	30.291	0.000
Mesh like with unclear boundary (*n* = 38)	7	31		

Gal-3: Galectin-3.

Table 2: Influence of clinicopathological parameters of patients with ocular venous malformation on Gal-3 mRNA expression in the nonpingyangmycin group

Variables	Gal-3 mRNA, *n*		χ^2	*P*
	Negative	Positive		
Age				
<12 years (*n* = 18)	11	7	3.777	0.052
≥12 years (*n* = 49)	17	32		
Sex				
Male (*n* = 19)	11	8	0.260	0.610
Female (*n* = 48)	31	17		
Depth of lesion				
Eyelid, skin, and fat (*n* = 35)	22	13	13.369	0.000
Within the muscular layer (*n* = 32)	6	26		
Appearance and boundary				
Round with clear boundary (*n* = 29)	25	4	41.466	0.000
Mesh like with unclear boundary (*n* = 38)	3	35		

Gal-3: Galectin-3; mRNA: Messenger RNA.

Table 3: Influence of clinicopathological parameters of patients with ocular venous malformation on Gal-3 protein expression in the pingyangmycin group

Variables	Gal-3 protein, *n*		χ^2	*P*
	Negative	Positive		
Age				
<12 years (*n* = 22)	19	3	3.127	0.077
≥12 years (*n* = 47)	31	16		
Sex				
Male (*n* = 16)	14	2	2.360	0.124
Female (*n* = 53)	36	17		
Depth of lesion				
Eyelid, skin, and fat (*n* = 41)	35	6	8.429	0.004
Within the muscular layer (*n* = 28)	15	13		
Appearance and boundary				
Round with clear boundary (*n* = 33)	31	2	14.619	0.000
Mesh like with unclear boundary (*n* = 36)	19	17		

Gal-3: Galectin-3.

Table 4: Influence of clinicopathological parameters of patients with ocular venous malformation on Gal-3 mRNA expression in the pingyangmycin group

Variables	Gal-3 mRNA, *n*		χ^2	*P*
	Negative	Positive		
Age				
<12 years (*n* = 22)	18	4	2.792	0.095
≥12 years (*n* = 47)	29	18		
Sex				
Male (*n* = 16)	14	2	3.604	0.058
Female (*n* = 53)	33	20		
Depth of lesion				
Eyelid, skin, and fat (*n* = 41)	32	9	4.590	0.032
Within the muscular layer (*n* = 28)	15	13		
Appearance and boundary				
Round with clear boundary (*n* = 33)	30	3	15.130	0.000
Mesh like with unclear boundary (*n* = 36)	17	19		

Gal-3: Galectin-3; mRNA: Messenger RNA.

between the pingyangmycin pretreated-malformed veins and the normal veins ($P > 0.05$). These findings indicated that pingyangmycin pretreatment significantly reduced Gal-3 protein expression.

Results of *in situ* hybridization showed that Gal-3 mRNA expression was mainly in the cytoplasm and only a few were expressed in the nuclei [Figure 2]. Gal-3-positive staining was detected in 39 (58%) of 67 malformed vein samples in the nonpingyangmycin group and 22 (32%) of 69 samples in the pingyangmycin group. In the negative control group, Gal-3-positive staining was detected in four (13%) of the thirty normal vein samples. Gal-3 mRNA expression in ocular malformed vein was significantly decreased after pretreatment with pingyangmycin ($P < 0.05$). There was no significant difference in the Gal-3 mRNA expression between the pingyangmycin group and the negative control ($\chi^2 = 3.716$, $P > 0.05$).

Association of galectin-3 expression with clinicopathological characteristics of patients with venous malformation

Tables 1-4 summarize the influence of clinicopathological

Table 5: Influence of pingyangmycin pretreatment on expression of Gal-3 protein and mRNA in the tissues of ocular venous malformation

Groups	Gal-3 protein, *n*		χ^2	*P*	Gal-3 mRNA, *n*		χ^2	*P*
	Negative	Positive			Negative	Positive		
Nonpingyangmycin (*n* = 67)	32	35	8.664	0.003	28	39	9.524	0.002
Pingyangmycin (*n* = 69)	50	19			47	22		

Gal-3: Galectin-3; mRNA: Messenger RNA.

characteristics of patients with ocular venous malformation on Gal-3 protein and mRNA expression in all patients. Protein and mRNA expressions of Gal-3 were not significantly associated with patients' age and sex in both groups. However, Gal-3 expression was significantly higher in venous malformation with deeper invasiveness (in the muscular layer) than in superficial invasiveness (in the eyelid, skin, and fat). Furthermore, Gal-3 was more frequently detected in mesh-like lesions with unclear boundary than round lesions with clear boundary ($P < 0.05$).

Differential expression of galectin-3 in ocular malformed veins with or without pingyangmycin pretreatment

Table 5 shows that the pretreatment of 0.02% pingyangmycin led to a higher negative rate of Gal-3 expressions in protein and mRNA levels, respectively, in ocular malformed veins ($\chi^2 = 8.664$ and 9.524, respectively, both $P < 0.05$).

Discussion

Gal-3 has been reported to interact with intracellular glycoproteins, cell surface receptors, and extracellular matrix.[13,14] Moreover, Gal-3 plays an important role in regulating angiogenesis, invasion, and metastasis of tumors.[15] Recently, Gal-3 has been found to promote hypoperfusion-induced retinal degeneration.[16] In the present study, we investigated the Gal-3 expression in patients with ocular venous malformation. Our results show that the mRNA and protein expression of Gal-3 were significantly increased in ocular venous malformation. Pingyangmycin can delay the development of venous malformation in the trunk and extremities and presents encouraging treatment efficiency in the treatment of hemangioma.[17] Pingyangmycin treatment can result in lesion shrinkage and reduction of congestion, and thus reduces patient's symptom and facilitates surgical removal of the lesion. Moreover, pingyangmycin shows less toxicity and side effects in the treatment of venous malformation.[9,10] As a result, it has been used as an effective standard therapy for severe venous malformation which cannot be removed by surgery.[18] Furthermore, pingyangmycin treatment reduced the lesions of ocular venous malformation, accompanied by a decrease in Gal-3 expression. This finding suggests that Gal-3 expression is associated with the prognosis of ocular venous malformation.

Ocular venous malformation can invade the local tissues leading to serious consequences such as blindness. Therefore, it is important to identify a biomarker for the invasiveness of ocular venous malformation. Gal-3 has been reported as a biomarker for invasiveness in several malignant tumors such as colon and gastric cancers.[19] It is upregulated in tumors that are highly invasive compared to those that are less invasive. The invasiveness feature of ocular venous malformation is similar to invasive tumors, so there are reasons to suspect whether Gal-3 expression can be used as a biomarker for the invasiveness of ocular venous malformation.

In this study, more female patients (75%) were enrolled compared to male patients. The high incidence of ocular venous malformation in women may be associated with high estrogen level in women.[20] We found that the protein and mRNA expressions of Gal-3 were not significantly associated with patient's age and sex, suggesting that age and sex have no major impact on Gal-3 upregulation in patients with ocular venous malformation. In addition, we found that Gal-3 expression was significantly associated with the depth of the lesion in those patients. Venous malformation with deeper invasiveness (in the muscular layer) showed higher expression levels of Gal-3 than those with superficial invasiveness (in the eyelid, skin, and fat). Furthermore, Gal-3 was more frequently detected in mesh-like lesions with unclear boundary than round lesions with clear boundary, suggesting that Gal-3 may contribute to the development of ocular venous malformation.

We found that the lesion size was reduced and congestion was decreased in patients with pingyangmycin pretreatment. Blood was coagulated in the malformed veins and more thrombi were formed which resulted in narrowing the cavity of the malformed veins. Upon treating with pingyangmycin, the malformed veins were found to be more likely to have clear boundary, which may be attributed to the fibrous tissue hyperplasia induced by local aseptic inflammation after pingyangmycin treatment. This change will facilitate the surgical removal of the lesion. In addition, since pingyangmycin treatment effectively reduced Gal-3 expression, which is associated with the invasiveness of malformed veins, it is possible that pingyangmycin may be effective in limiting the development and invasiveness of venous malformation. Therefore, pingyangmycin can be used as a standard preoperative therapy to facilitate the surgical removal of the venous malformation.

In summary, we found that Gal-3 expression was upregulated in ocular venous malformation and was significantly associated with the invasiveness of venous malformation, suggesting that Gal-3 protein may contribute to the

development of ocular venous malformation. Pingyangmycin treatment inhibited Gal-3 expression and reduced the lesion in patients with ocular venous malformation, suggesting that pingyangmycin is an effective therapy that may control the invasiveness of venous malformation.

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Conflicts of interest

There are no conflicts of interest.

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