Indian J Med Res 148, December 2018, pp 728-733
DOI: 10.4103/ijmr.IJMR_500_17

Quick Response Code:

Clinical significance of galectin-3 expression in malformed hepatic venous tissue

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Received March 22, 2017

***Background & objectives*: Hepatic venous malformation gradually develops over time and exhibits the malignant biological behaviours of being locally invasive, causing morphological and functional damage to local tissue, and may even cause systemic coagulopathy. Studies show that galectin-3(Gal-3) expression is closely associated with local invasion of malignant tumours. In this study an attempt was made to assess the clinical significance of Gal-3 in local invasion during hepatic venous malformation in patients.**

***Methods*: Gal-3 protein and its mRNA expression were examined using immunohistochemistry and *in situ* hybridization in a total of 126 patients with hepatic venous malformation. For control tissue, 20 cases of normal tissue distal to surgical margins were also examined. In addition, the association between Gal-3 expression and pathological parameters was analyzed in hepatic venous malformation patients.**

***Results*: Gal-3 mRNA positivity was observed in 65.08 per cent (82/126) of hepatic venous malformation tissue samples, which was higher than the rate of 20 per cent (4/20) ($P<0.05$) seen in control tissues. Gal-3 protein positivity was observed in 58.73 per cent (74/126) of hepatic venous malformation tissue samples, which was higher than the rate of 15 per cent (3/20) ($P<0.05$) seen in the normal tissue. Gal-3 expression was not significantly associated with age or gender. However, there was a significant association between Gal-3 positivity and lesion size, local invasion depth, and involvement with the hepatic vein and the portal system.**

***Interpretation & conclusions*: Local tissue invasion and destruction by hepatic venous malformation may be related to the upregulation of Gal-3. Gal-3 expression and the development of venous malformation may be related and needs to be studied further.**

Key words Galectin-3 - invasion - liver - venous malformation

Among congenital vascular disorders, venous malformation is a common disease[1-3]. The liver is the most common organ for its occurrence. The severity of disease in patients with hepatic venous malformation often varies. In mild cases the deformity is limited to a small area, whereas in severe cases it

can be diffusely infiltrative. Although it is a benign disease, yet it has the potential to be locally invasive and destructive to surrounding normal tissue. Venous malformation can cause bloating, pain, and other persistent symptoms affecting normal life. Given that most such malformations develop gradually over time, these can cause systemic coagulopathy due to mass consumption of coagulation factors and reduction in serum fibrinogen[4,5]. There is also the life-threatening risk of mass haemorrhage from rupture[6]. If early treatment is delayed, treatment at the later stages becomes difficult. Molecular treatment options must be advanced to solve the clinical limitations of this disease. Apart from the study done by Vikkula *et al*[7], where they attempted to study genetic abnormalities of tyrosine kinase-associated receptor recognition in venous malformation without conclusive results, there have been no other in-depth studies conducted on this disease. It has been shown that galectin-3 (Gal-3) plays an important role in angiogenesis[8]. We have studied Gal-3 expression in venous malformations of the limbs and eyes[9], and found that there is a close association of Gal-3 expression with disease severity. Thus, there may also be a close association between Gal-3 expression and venous malformations of the liver. Thus, this study was undertaken to investigate if there was differential expression of Gal-3 protein and mRNA in hepatic venous malformation tissues versus normal tissue by applying immunohistochemistry and *in situ* hybridization to patient tissue samples.

Material & Methods

Biopsy samples were collected from surgically resected hepatic haemangiomas (hepatic venous malformation) of 126 patients with hepatic venous malformation, who were treated between January 2013 and September 2015 at both the Hemangioma and Vascular Malformation Treatment Center of the People's Hospital of Zhengzhou University (Henan Province People's Hospital) and the Hepatobiliary Surgery department of the People's Liberation Army 301 General Hospital, Beijing, PR China. Pathology specimens were collected from 54 males and 72 females. Among the 126 patients, 20 specimens of normal tissue distal to surgical margins, which were confirmed by pathology examination, were also analyzed as control tissues. The study was approved by the Ethics Committee of Zhengzhou University and written informed consent was obtained from all patients.

Methods: Hepatic venous malformation resection was performed on all patients. Surgical tissue samples were embedded in paraffin, and then made into serial sections 3 and 6 μm thick. One section was stained with haematoxylin-eosin for diagnostic review. The rest of the sections were used for immunohistochemistry[10] and *in situ* hybridization.

Immunohistochemical detection of Gal-3 expression: Paraffin sections were deparaffinized and rehydrated, washed with distilled water three times for three minutes each, then washed with phosphate-buffered saline (PBS) three times for three minutes each. Citrate antigen retrieval buffer (*p*H 6) was used for heat-induced epitope retrieval. Samples were incubated for 10 min with three per cent (v/v) H_2O_2 at 18-25°C to eliminate endogenous peroxidase activity, washed three times with PBS for two minutes each, and then blocked with 10 per cent (v/v) normal goat serum for 10 min. Serum was poured off, and ready-to-use murine anti-human Gal-3 monoclonal antibody was added (working concentration 1:100, clone number: 9C4) (US Oncogene Company, USA). The samples were incubated overnight at 4°C, and washed with PBS three times for two minutes each. A biotin-labelled secondary antibody (Wuhan Boster Bio-Engineering Co., Ltd., PR China) working solution was added (working concentration 1:100), and the samples were incubated for 20 min at 37°C, then washed with PBS three times for two minutes each. HRP-streptavidin working solution was added (working concentration 1:500). A steptavidin-peroxidase kit was obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., PR China. 3,3'-Diaminobenzidine (DAB) was used to develop colour. Paraffin sections of human thyroid carcinoma tissue were used as positive controls for each batch of staining, and sections incubated with PBS instead of primary antibody were used as negative controls. Scoring was conducted for five randomly selected microscopy fields under ×200. Scoring was based on the intensity of staining: 0 for no colour, 1 point for minimal or weakly yellow staining, 2 points for yellow or dark yellow staining of medium intensity, and 3 points for brownish yellow staining of strong intensity. Scoring was also conducted based on the number of stained cells: 0 for <25 per cent stained cells, 1 point for 25-<50 per cent stained cells, 2 points for 50-<75 per cent stained cells, 3 points for ≥75 per cent stained cells. Stain intensity score was added to per cent stained cell score. A score >3 was considered positive expression; a score <3 was considered negative

expression; a score=3 was considered indeterminate and re-staining was conducted for determination.

In situ hybridization for detecting relative Gal-3 mRNA expression

Gal-3 probe sequences were as follows: 5'-CTTCCACTTTAACCCACGCTTCAATGAGA A-3'; 5'-AATAACTGGGGAAGGGAAGAAAGACA GTCG-3', and 5'-AAACCATTCAAAATACAA GTACTGGTTGA A-3'. Sections were deparaffinized and rehydrated in the usual manner and treated for 12 min with three per cent H_2O_2 at room temperature to eliminate endogenous peroxidase activity. The samples were digested for 15 min at room temperature with three per cent (v/v) fresh citrate prepped pepsin (10:1 dilution) to remove DNA-binding protein, then 20 µl of pre-hybridization solution without probe was added and incubated for 4 h at 42°C. Hybridization solution containing the probe, obtained from the Gal-3 *in situ* hybridization detection kit (Wuhan Boster Bio-engineering Co., Ltd., PR China), was added and incubated in a wet box at 42°C overnight. After washing with ×2 sodium citrate solution at 37°C, blocking solution was added and the samples were blocked for 30 min. Biotinylated mouse anti-digoxin was added to the samples and incubated for 1 h at 37°C. Samples were washed three times with PBS dedicated for *in situ* hybridization use for five minutes each. DAB was used to develop colour. Using Gal-3 probe-free samples as a negative control, samples were observed under light microscopy. The positive signal presented as brown granular matter localized in the cytoplasm. Very few nuclei had positive staining. Based on the standards in the literature[11], a two-level scoring method was used for semi-quantitative analysis. Scoring was conducted for five randomly selected microscopy fields under ×200 magnification, according to the percentage of positive cells: 0 for <5 per cent positive cells, 1 point for 5-<25 per cent positive cells, 2 points for 25-<50 per cent positive cells, 3 points for 50-<75 per cent positive cells and 4 points for ≥75 per cent positive cells. Scoring was also conducted based on the intensity of staining: 1 point for light yellow, 2 points for yellow or dark yellow and 3 points for tan or brown. The two scores were multiplied together and scores >1 were considered positive expression.

Statistical analysis: SPSS 13.0 statistical software (IBM-SPSS, Chicago, IL, USA) was used for statistical analysis. The data for each measured index in this study were presented as frequency and rate. Chi-square test was used for inter-group comparison.

Results

Clinical and pathological features of hepatic venous malformations are shown in Table I.

Hepatic venous malformation intraoperative histopathology: Venous masses were pale reddish-blue in colour, soft, spongy, significantly congested, largely without clear demarcation, non-pulsatile and bled easily. These were dispersed in the liver parenchyma, distinct from one another. Under light microscopy, numerous medium-to-large diameter vessel compartments lined with flat endothelial cells were observed in the tissue. Pericytes and fibroblasts were seen in the background (Figure A and B).

Galectin-3 immunohistochemistry and in situ hybridization staining

Galectin-3 protein and mRNA expression in hepatic venous malformation tissue: Gal-3 protein-positive granules were mainly expressed within the cytoplasm and the nucleus (Figure C); Gal-3 mRNA-positive granules were mainly expressed within the cytoplasm and little in the nucleus (Figure D). Gal-3 protein positivity in hepatic venous malformation tissue was 58.73 per cent (74/126), which was significantly higher (P<0.05) than that (15%, 3/20) seen in normal tissue (control group) (Figure E). Galactin-3 mRNA positive rate was 65.08 per cent (82/126), which was

Table I. Clinical and pathological features of hepatic venous malformations

Clinical and pathologic features	Number of cases (%)
Age (yr)	
<35	60 (47.62)
≥35	66 (52.38)
Gender	
Male	54 (42.86)
Female	72 (57.14)
Size [lesion diameter (D) in cm]	
5 ≤ D < 7	56 (44.44)
D ≥7	70 (55.56)
Local invasion depth (cm)	
From liver surface <2	69 (54.76)
From liver surface ≥2	57 (45.24)
Portal or hepatic vein involvement	
Yes	85 (67.46)
No	41 (32.54)

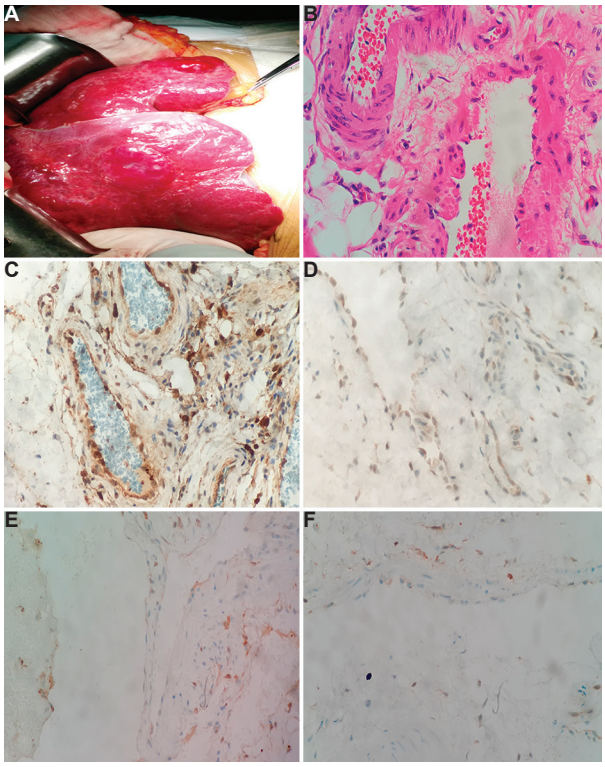

Figure. Gross picture of hepatic venous malformation tissue (**A**), and its histopathology (H & E, ×400), where numerous medium- to large-diameter vessel compartments can be seen lined with flat endothelium (**B**). Positive galectin-3 protein (**C**) and mRNA staining (**D**), respectively, in hepatic venous malformation tissue (×400); negative galectin-3 protein (**E**) and mRNA staining (**F**), respectively, in hepatic venous malformation tissue (×400).

significantly higher (P<0.05) than the rate of 20 per cent (4/20) in the control group (Figure F).

Association between galectin-3 expression and clinical pathology features: There was no significant association between the age and gender (54/72) of the patients and positive Gal-3 mRNA or protein expression. However, there was an association between Gal-3 mRNA positivity and lesion size, depth of local invasion, and hepatic artery and portal system involvement (P<0.001). An association was also seen between Gal-3 protein positivity and lesion size, depth of local invasion and hepatic artery and portal system involvement (P<0.001) (Table II).

Discussion

Hepatic haemangioma is formed by the grouping of deformed varices originating from hepatic veins[12-15]. It is a deformity in the veins that occurs congenitally during liver development. Hepatic venous malformation does not account for a high percentage of all venous malformations occurring in the body, for example, head, face and limbs, but it is the most commonly seen venous malformation occurring in visceral organs. Venous malformations can be further divided into dilated versus non-dilated types[16], but essentially, these are all congenital diseases. The disease often progresses gradually over a lifetime. Based on the timing of diagnosis, the severity of the disease varies. Hepatic venous malformation patients with mild disease[17] mostly have involvement of the tissue on the liver surface, limited to one hepatic segment. Those with severe disease can have involvement of the intrahepatic portal system, the hepatic vein and multiple hepatic segments. Both the left and the right hepatic lobes can be involved in severe disease[18]. Due to the significant congestion caused by this disease, local blood circulation is slow and can even be static. Local intravascular coagulation[19] can occur, consuming large quantities of coagulation factors, leading to coagulopathy, which can be incurable. Clinically, the patient can have significant localized pain in the hepatic area, which can affect the patient's sleep, work and quality of life. Therefore, in principle, early surgical resection is the ideal treatment method. However, surgical resection is not possible in severe cases. This shows that there is an urgent need to resolve the development and progression of this disease. Given this locally invasive biological behaviour, it is particularly important to uncover the nature of occurrence and development of this disease. Studying the function of Gal-3 in local invasion of hepatic venous malformation may be useful for making treatment decisions and for developing novel therapies.

The involvement of Gal-3 protein and RNA in the mechanism of invasion and metastasis in malignancies has been studied in colorectal and gastric cancer. Due to the relatively unique anatomical structure of the liver, obvious symptoms can present when the lesion involves the portal vein, hepatic veins or the biliary system.Once venous malformation invades and destroys the surrounding normal tissue, the sequelae can be relatively serious. This biological behaviour of this disease is similar to the behaviour of malignant tumours[8].

Studies have reported interactions of Gal-3 with intracellular glycoproteins, cell surface molecules, and the extracellular matrix, where it may be involved in cell adhesion, proliferation, apoptosis and mRNA splicing[20,21]. Gal-3 is especially important in promoting angiogenesis[22,23] and may be associated with the proliferation, invasion, and the malignant behaviour

Table II. Correlation of galectin-3 (Gal-3) protein and mRNA expression with clinical pathology features in patients with hepatic venous malformation

Clinical pathology feature	Number of cases	Gal-3 protein		*P*	Gal-3 mRNA		*P*
		-	+		-	+	
Age (yr)							
<35	60	25	35	0.007	21	39	0.001
≥35	66	27	39	0.931	23	43	0.986
Gender							
Male	54	21	33	0.221	19	35	0.003
Female	72	31	41	0.638	25	47	0.957
Size [lesion diameter (D) in cm]							
5 ≤ D < 7	56	33	23	12.968	29	27	12.616
D ≥ 7	70	19	51	0.001	15	55	0.001
Local invasion depth (cm)							
From liver surface <2	69	40	29	17.553	37	32	23.476
From liver surface ≥2	57	12	45	0.001	7	50	0.001
Portal or hepatic vein involvement							
No	85	50	35	33.208	41	44	20.377
Yes	41	2	39	0.001	3	38	0.001

-, galectin-3 protein and mRNA expression is negative; +, galectin-3 protein and mRNA expression is positive

of a variety of tumours[24-26]. If it is a good marker for reflecting the degree of liver damage due to liver cirrhosis and toxic hepatitis[27], it may also have a close association with the development of angiofibrosis. However, there have not been any studies conducted on the association between Gal-3 and hepatic haemangioma (venous malformation).

There have been studies on the surgical treatment of hepatic haemangioma and the intra-tumour injection of sclerosing agents[28]. Sclerosing agents can only cure lesions that are small, superficial, and far from the portal and hepatic veins and the biliary system. Surgery is still the best method for a possible cure.

This study showed no association between Gal-3 expression in the endothelial cells of hepatic venous malformation tissue with either age or gender. However, there was a significant difference in the expression of Gal-3 protein and mRNA when the lesions were larger, especially when those were >7 cm, whereas smaller lesions were more likely to be negative for Gal-3. Negative expression was more likely when the lesion was more superficial to the liver surface. When the lesion involves deeper tissues, especially when it is close to or involves the portal system or the hepatic veins, the positive expression rates for both Gal-3 protein and mRNA are higher, and their expressions are stronger[27]. Clinically, the occurrence rate of localized pain may be related to local pressure caused by the lesion on the surrounding tissue. There may be a positive association between Gal-3 expression and the size of the lesion and the depth of involvement. This may indicate that Gal-3 can participate in lesion evolution by furthering the ability of venous malformations to be locally invasive and progressive.

In addition, tissue ischaemic injury studies have shown that Gal-3 can inhibit cellular apoptosis[21], promote vascular endothelial cell proliferation, induce angiogenesis, and reduce the damage caused by ischaemic retinopathy[29]. Therefore, local tissue invasion and destruction by hepatic venous malformation may be related to the upregulation of Gal-3. Gal-3 may have an intimate association with the occurrence and development of blood vessels. This would support the hypothesis of this study. Additional studies are required to provide the foundation for future research on the clinical prediction of venous malformations.

***Financial support & sponsorship*:** The study was supported by Research Project of Henan Science and Technology Agency, Zhengzhou, Henan, China (Project no. 162102310282) and the Medical Science and Technology Research Project of Henan Province (201601015).

***Conflicts of Interest*:** None.

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