Novel marker in proliferative and involuting phases of infantile hemangioma
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Introduction
Infantile hemangioma (IH) is the most common benign vascular tumor occurring in infants that usually develops soon after birth owing to endothelial cell proliferation. They have characteristic clinical features consisting of progressive growth after birth followed by spontaneous involution starting from the second year and continues over a course of 3–10 years [1]. In the proliferative phase, IH demonstrates proliferating endothelial cells and pericytes that focally form lumina containing red cells [2]. However, involuting IH is characterized by apoptosis and disappearance of capillaries, with replacement by loose fibrous or fibro-fatty tissue [3].

Galectin-3 (gal-3) is the most important member in the family of β-galactoside-binding lectins, with a 31-kDa molecular weight. It is widely expressed in human tissues, including all types of immune cells (macrophages, monocytes, dendritic cells, eosinophils, mast cells, natural killer cells, and activated T and B cells), epithelial cells, and endothelial cells. It has been suggested to play a role in a variety of biological processes such as cell growth, cellular adhesion, cell cycle regulation, neoplastic transformation, and metastasis [4].

Gal-3 is a pro-angiogenic molecule that plays an important role in vascular endothelial proliferation and angiogenesis. Moreover, it is important for cell survival, owing to its interaction with certain proteins, including B cell lymphoma-2 [5].

It has also a critical role in the phagocytosis of opsonized red blood cells [6] and apoptotic neutrophils by macrophages and plays an important role in fibrogenic processes in different tissues [7–9].

Background
No single theory can explain the characteristics of infantile hemangioma (IH), but the emergence of new biomarkers will help to discover a general mechanism in its pathogenesis.

Objective
To evaluate serum level of galectin-3 (gal-3) in patients with IH and its possible role in the pathogenesis during proliferative and involuting phases.

Patients and methods
This case–control study included 60 patients with IH as group 1 (G1). They were subdivided into 30 patients with age ranged from 3 to 12 months (proliferative phase, G1A) and 30 patients with age ranged from more than 12 to 24 months (involuting phase, G1B). In addition, 20 age-matched and sex-matched healthy participants who served as a control group (G2) were included. The diagnosis was based on clinical bases and ultrasonic examination. Assessment of serum level of gal-3 was done by enzyme-linked immunosorbent assay kits in all studied groups and was correlated with clinical findings of IH.

Results
Serum gal-3 levels were significantly higher in the patient group (G1) than controls (G2) (P=0.001). Serum gal-3 levels were significantly higher in G1B compared with G1A and controls (G2B) (P<0.001 for both). Serum gal-3 level was higher in G1A than control group (G2A), but it did not reach statistical significance (P=0.67).

Conclusion
Serum gal-3 may have a role in the pathogenesis of IH possibly through its pro-angiogenic effect in the proliferative phase and induction of fibrosis in involuting phase.

Keywords:
galactin-3, infantile hemangioma, pro-angiogenic, proliferative

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Gal-3 is mainly located in the cytoplasm, but it has been also found in the nucleus, on the cell surface, and in the extracellular environment, suggesting many functions of this molecule [10]. In the cytoplasm, gal-3 is important for cell survival, owing to its interaction with certain survival-associated proteins, including B cell lymphoma-2 and activated guanosine-5′-triphosphate-bound K-Ras. In the nucleus, gal-3 promotes pre-mRNA splicing and regulates gene transcription. Extracellular gal-3 modulates cell–cell and epithelial cells–extracellular matrix interactions [11]. Previous studies have proved the role of gal-3 in liver fibrosis [12], cardiovascular diseases [13], and renal fibrosis [7]. Gal-3 has been studied in dermatologic diseases such as atopic dermatitis [14], psoriasis [15], and systemic sclerosis [16]. Yet its role in IH pathogenesis has been not studied before.

The aim of work was to evaluate the serum level of gal-3 in patients with IH and its possible role in the pathogenesis during proliferative and involuting phases.

Patients and methods
This case–control study was conducted in the period from October 2018 to May 2019. Patients were recruited from the outpatient clinic of Dermatology of Benha University Hospital. Written informed consent form was signed by parents of the patients and controls before the study. The protocol of the study was approved by the Research Ethics Committee of Faculty of Medicine, Benha University.

We included 60 children with IH [group 1 (G1)], who were diagnosed by clinical examination and were confirmed by ultrasonic examination. Patients were divided into two groups according to the age and stage of the tumor growth: G1A (30 patients their age ranged from 3 to 12 months), which represented the proliferative phase, and G1B (30 patients their age ranged from >12 to 24 months), which represented the involuting phase. A total of 20 age-matched and sex-matched patients were included as a control group [group 2 (G2)]. The latter was further subdivided into two groups according to their age: G2A (3–12 months) and G2B (>12–24 months); each subgroup included 10 patients and was matched with the patient groups.

Exclusion criteria included patients who had received treatment 1 month before taking the sample as intralesional or topical corticosteroids or systemic or topical propranolol, patients with age less than 3 months or more than 24 months, patients with syndromic hemangioma, such as PHACES syndrome or LUMBAR syndrome, and patients associated with other diseases (e.g. atopic dermatitis or bronchial asthma).

All participants in this study were subjected to detailed history taking with special reference to age, sex, history of premature delivery, history of low birth weight (LBW), and onset, course, duration, site, and size of IH. Complete general and dermatological examination for exclusion of any other diseases or syndromes (e.g. LUMBAR syndrome) was done. Detection of the type of IH (superficial, deep, and mixed) was done by ultrasound. Evaluation of serum gal-3 was done by enzyme–linked immunosorbent assay (ELISA) kits.

Blood sampling
Overall, 5 ml of venous blood sample was taken from each participant under complete aseptic condition and put in a plain tube. The tube was left at room temperature for 30 min till coagulation and then centrifuged. The resultant serum was stored in aliquot at -20°C until assay was done. The assay was done by using human gal-3 ELISA kit (Lot number: 201-12-1952; Sun Red Biotechnology Company, Shanghai, China).

Test principle
The kit used a double antibody sandwich ELISA to assay the level of human gal-3 in samples. Gal-3 was added to monoclonal antibody enzyme well which was precoated with human gal-3 monoclonal antibody. We added gal-3 antibodies labeled with biotin and combined with streptavidin–HRP to form immune complex. Incubation was carried out and washing again was done to remove the uncombined enzyme. Chromogen solutions A and B were added. The color of the liquid changed into blue, and with the effect of acid, the color finally became yellow. The chroma of the color and the concentration of human substance gal-3 of sample were positively correlated.

Statistical analysis
The collected data were computerized and statistically analyzed using IBM SPSS platform (Statistical Package for Social Science), version 24.0 (SPSS Inc., Chicago, Illinois, USA). Categorical data were presented as number and percentages, whereas quantitative data were expressed as mean±SD, median, interquartile range, and range. χ² test was used to calculate difference between qualitative variables as indicated. Student t test was used to analyze normally distributed variables among two
independent groups. However, nonparametric variables were analyzed using Mann–Whitney U test. Differences among three independent means were analyzed using Kruskal–Wallis test for nonparametric ones. Significant Kruskal–Wallis tests were followed by post-hoc multiple comparisons using Bonferroni tests to detect the significant pairs. Receiver operating characteristics (ROC) curve was constructed to determine cutoff value of gal-3 to differentiate proliferative from involuting hemangiomas. Area under the curve between 0.68 and 0.89 indicated good discrimination. All statistical comparisons were two tailed, with significance level of $P$ value less than or equal to 0.05.

**Results**

In this study, the mean age of patients and controls was 12.3±7.0 and 12.6±5.3 years, respectively. IH was more common in females (65% was females and 35% was males, and the female-to-male ratio was 1.9 : 1). Comparison between patients and controls regarding age and sex revealed nonsignificant differences (Table 1).

IH were mainly located in the head and neck (48.3%), followed by the trunk (28.3%) and then the limbs (23.3%) in both groups. Comparison between G1A and G1B regarding site of the lesion revealed nonsignificant difference ($P=0.052$). Regarding the type of IH, the superficial type of IH was the most common type in both groups (48.3%) followed by the mixed type (35%).

In this the study, the onset of the lesions in all patients starts within the first month of birth: 58.3% started within the first week, 31.7% within the second week, 6.7% within the third week, and 3.3% within the fourth week.

A total of 13 (43%) patients in GA1 had a history of premature delivery compared with seven (23%) in GA2 and none in control group. Comparison between patient groups regarding premature delivery showed nonsignificant difference ($P<0.01$). However, history of LBW was obtained from eight (26%) patients in GA1 compared with 11 (36%) in GA2 and none in control group. Comparison between patient groups regarding LBW showed nonsignificant difference ($P=0.41$).

The current study revealed significantly elevated serum gal-3 levels in patients (G1) compared with controls (G2) (Table 2). G1B had significantly higher serum gal-3 levels compared with G1A and G2B ($P<0.001$ for both). G1A had higher serum gal-3 than G2A, but without statistically significant difference ($P=0.67$) (Table 3).

ROC curve analysis showed that serum gal-3 more than or equal to 14.5 ng/ml significantly predicts involuting hemangioma with 70% sensitivity, 82% specificity, 70% positive predictive value, 82% negative predictive value, and 77.5% accuracy. The 95% confidence interval of area under the curve=0.785 indicated good discrimination (Fig. 1).

There was no significant relation between serum gal-3 level and the studied clinical variables (sex, site, type, prematurity, and LBW) among patient groups (G1A and G1B).

**Table 1** Comparison between patients and controls regarding age and sex

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (N=60) [n (%)]</th>
<th>Controls (N=20) [n (%)]</th>
<th>Test of significance</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (months)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3–12</td>
<td>30 (50.0)</td>
<td>10 (50.0)</td>
<td>$\chi^2=0.0$</td>
<td>1.0 (NS)</td>
</tr>
<tr>
<td>&gt;12–24</td>
<td>30 (50.0)</td>
<td>10 (50.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td>12.3±7.0</td>
<td>12.6±5.3</td>
<td>$t=0.17$</td>
<td>0.86 (NS)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>3–23</td>
<td>5–23</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21 (35.0)</td>
<td>9 (45.0)</td>
<td>$\chi^2=0.64$</td>
<td>0.42 (NS)</td>
</tr>
<tr>
<td>Females</td>
<td>39 (65)</td>
<td>11 (55.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\chi^2$ and Student $t$ tests. $P$ value less than or equal to 0.05 was significant.

**Table 2** Comparison between patients and controls regarding serum galectin-3 level

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (N=60)</th>
<th>Controls (N=20)</th>
<th>MWU test</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum gal-3 (ng/ml)</strong></td>
<td>Median</td>
<td>IQR</td>
<td>Range</td>
<td>Median</td>
</tr>
</tbody>
</table>

gal-3, galectin-3; IQR, interquartile range; MWU, Mann–Whitney $U$ test. *$P$ value less than or equal to 0.05 was significant.
Discussion

Although IH is a common vascular lesion, its pathogenesis remains poorly understood. Besides typical clinical presentation and histological findings, immunohistochemical cellular markers may be of interest for the characterization of each stage of IH (proliferation and involution). Many theories have been proposed for the pathogenesis of IH (e.g. placental theory and hypoxia theory) [17].

Prematurity and LBW were reported as possible risk factors for the development of IH [18]. In this study, they were detected in 23 and 31.7% patients, respectively, which match with the results of Anderson et al. [19] and Gey et al. [20].

The current study revealed significantly elevated serum gal-3 levels in patients with IH (G1) compared with controls (G2). To our knowledge, this is the first study to evaluate serum gal-3 in patients with IH.

The elevated serum gal-3 levels in patients with proliferative phase (G1A) of IH compared with controls (G2A) could be explained by its function as a pro-angiogenic molecule that plays an important role in vascular endothelial proliferation and angiogenesis [5].

Both angiogenesis and vasculogenesis have been proposed as mechanisms in the pathogenesis of hemangioma tumors. Angiogenesis is defined as the growth of new vessels from pre-existing vessels, requiring degradation of the basement membrane, migration of endothelial cells and tubulogenesis, followed by recruitment of perivascular cells. Vasculogenesis is the de novo formation of blood vessels from stem or progenitor cells [21].

The mechanism by which gal-3 induces angiogenesis has been suggested by Dos et al. [22]. Their study suggested that under hypoxic conditions, gal-3 is released by tumor cells and preferentially binds to endothelial cells and triggers angiogenic sprouting through promoting JAG1/Notch signaling activation. This could be the same mechanism in case of IH, as hypoxia theory had been proposed to explain the pathogenesis of IH [23].

The nonsignificant difference in serum gal-3 between G1A and G2A could be owing to the presence of other angiogenic factors that play a role in that stage (e.g. a fibroblast growth factor, b fibroblast growth factor, vascular endothelial growth factor and transforming growth factor β) [24].

In the current study, serum level of gal-3 in G1B (involuting phase) was significantly higher than that of G2B and G1A (proliferative phase). These results can predict the role of gal-3 as a potent fibrogenic factor in involuting IH, a finding which was confirmed by analysis of ROC curve results.

Although the role of gal-3 as a potent activator of fibroblasts has been evaluated from a broad range of tissues [8,25,26], its involvement in the pathogenesis of IH had not been well studied. However, a line of evidence suggests its role in IH in the involuting phase and formation of fibro-fatty tissue.

Henderson et al. [7] showed that the expression of gal-3 is upregulated in a mouse model of progressive renal fibrosis (unilateral ureteric obstruction), and absence of gal-3 protects against renal myofibroblast accumulation/activation and fibrosis. Furthermore, specific depletion of macrophages reduces fibrosis severity, demonstrating that macrophages are key cells in the pathogenesis of renal fibrosis.

In damaged liver cells, intracellular gal-3 induces fibrosis through Smad2/3-independent TGF-β signaling and collagen production [8]. Moreover, Jiang et al. [12] revealed that gal-3 is upregulated

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>N</th>
<th>Serum gal-3 (ng/ml)</th>
<th>KW test</th>
<th>Multiple comparisons and P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>IQR</td>
<td>Range</td>
</tr>
<tr>
<td>G1A (patients 3–12 months)</td>
<td>30</td>
<td>8.47</td>
<td>4.4–14.6</td>
<td>2.1–33.3</td>
</tr>
<tr>
<td>G1B (patients 12–24 months)</td>
<td>30</td>
<td>8.2–31.1</td>
<td>2.07–40.3</td>
<td></td>
</tr>
<tr>
<td>G2A (controls 3–12 months)</td>
<td>10</td>
<td>4.1</td>
<td>2.3–9.2</td>
<td>1.2–26.9</td>
</tr>
<tr>
<td>G2B (controls 12–24)</td>
<td>10</td>
<td>5.7</td>
<td>2.1–9.7</td>
<td>1.5–31.6</td>
</tr>
</tbody>
</table>

G1, group 1; G2, group 2; gal-3, galectin-3; IQR, interquartile range; KW, Kruskal–Wallis test. *P value less than or equal to 0.05 was significant.
in hepatic stellate cells via NF-κb-mediated pathway for the elimination of apoptotic cells during chronic liver injury and induction of fibrosis. The latter can be confirmed by finding that gal-3-deficient mice developed an attenuated fibrogenic response through decreased expression of the fibrogenic markers such as procollagen I and smooth muscle actin, TGF-β1, and tissue inhibitor of matrix metalloproteinase-1. Similar results were obtained by Kotby et al. [13]; they reported significantly elevated gal-3 serum levels and significant positive correlation between serum gal-3 levels and heart failure grades of severity. They explained that myocardial injury generates inflammatory signals, which recruit activated macrophages to the myocardium and stimulate them to secrete gal-3 [27]. Gal-3 causes cardiac fibroblasts to proliferate and produce type I collagen, leading to an accumulation of myocardial collagen and impaired diastolic and systolic functions [28].

**Conclusion**

In conclusion, the results of the present study suggested novel aspects to the role of gal-3 in the pathogenesis of IH phases. Gal-3 seems to have a role in angiogenesis during the proliferative phase and induction of fibrosis in involuting phase of IH, which needs further investigation. We recommend future studies with a larger number of patients and controls with expanding duration of involuting phase.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

**References**

Novel marker in proliferative and involuting phases


