European Review for Medical and Pharmacological Sciences 2018; 22: 4806-4811

Expression and correlation of MMP-9, VEGF, and p16 in infantile hemangioma

R.-R. YIN, D. HAO, P. CHEN

Department of Dermatology, Xuzhou Children's Hospital, Xuzhou, Jiangsu, P.R. China

Abstract. – **OBJECTIVE: The incidence and progression of infantile hemangioma depend on several factors. The objective of the present study was to analyze the expression of MMP-9, VEGF, and p16 in hemangioma, and evaluate the clinical significance of the correlation between the gene and protein expression of MMP-9 and VEGF.**

PATIENTS AND METHODS: 60 pathological tissue samples from patients with hemangioma (30 cases of proliferative hemangioma and 30 cases of degenerative hemangioma) and 30 normal epidermal tissue samples were collected. Tissues were examined by immunohistochemistry. Additionally, MMP-9 and VEGF gene expression were evaluated by qRT-PCR to determine the correlation between gene and protein level.

RESULTS: Immunohistochemistry showed that the expression of MMP-9 and VEGF was higher in proliferative hemangioma, while the expression of p16 was lower, and the differences were statistically significant (p<0.05). According to qRT-PCR, MMP-9 and VEGF gene expression were positively correlated with their protein expression (p<0.05).

CONCLUSIONS: The tumor suppressor gene p16 is negatively correlated with the occurrence and degradation of hemangioma. Furthermore, the gene and protein expression of MMP-9 and VEGF in infantile hemangioma are positively correlated. The levels of MMP-9 and VEGF are higher in the proliferative stage of hemangioma than other stages. Both MMP-9 and VEGF facilitate angiogenesis and tumorigenesis, and are likely to serve as markers for the development of hemangioma in the future.

Key Words

Infantile Hemangioma, MMP-9, VEGF, P16.

Introduction

Infantile hemangioma is a common benign tumor. The pathogenesis of the disease mainly involves angiogenic factors, angiogenesis inhibitor factors, and the extracellular matrix[1-5]. Hemangiomas are caused by the accumulation of endothelial cells and pericyte from excessive proliferation. They are composed of capillaries with numerous branches that cause abnormalities in their morphology. Factors involved in the growth and apoptosis of hemangiomas have emerged as markers for analyzing and identifying the evolution of the disease. Matrix metalloproteinase-9 (MMP-9) participates in the formation and degradation of extracellular matrix components[6-9]. MMP-9 can participate in angiogenesis by regulating the release of vascular endothelial growth factor (VEGF). Measuring the levels of MMP-9 and VEGF may have clinical significance for evaluating the proliferation of vascular endothelial cells, angiogenesis, and tumorigenesis in infants[10-13]. The p16 protein is the product of the p16 tumor suppressor gene. Deficiency of p16 is closely associated with the occurrence and progression of many tumors[14-17]. The aim of the present study was to analyze the relationship between the expression of MMP-9, VEGF, and p16 with hyperplasia and degradation of hemangioma. In addition, we evaluated the clinical significance of the correlation between gene level and protein expression of MMP-9 and VEGF in infantile hemangioma.

Patients and Methods

Patients

Pathological tissue samples were selected from 60 infantile patients with hemangioma admitted to Xuzhou Children's Hospital from March 2014 to August 2016. The samples were from 35 males and 25 females. Thirty normal epidermal tissue samples were selected as controls. All tissues were proven as reliable and traceable after detection by the hospital. Infants did not suffer from severe complications, genetic diseases, or factors that may have interfered with the experimental results. According to the Mulliken staging criteria, there were 30 cases of proliferative hemangioma

Corresponding Author: Ping Chen, MD; e-mail: pj2j47@163.com

and 30 cases of degenerative hemangioma. Approval for the study was obtained from the Ethics Committee of the Xuzhou Children's Hospital. Written informed consent was obtained from the legal guardians.

Reagents

The rabbit anti-human monoclonal antibodies against VEGF and MMP-9 were from Abcam, USA; the rabbit anti-human p16 (Clone No. 6H12) monoclonal antibody was from Leica Novocastra. The reagents for manual immunohistochemistry were from Beijing Zhongshan Biology. The RNA extraction kits for paraffin-embedded tissue were from Omega (Bio-Tek, Norcross, GA, USA), and the 2× Taq Mix was from CWBIO. Real-time fluorescence quantitative PCR kits and pipettes were from Thermo, Darmstadt, Germany. The microscope was from ABI, (Vernon, CA, USA) and the vials were from Corning (Corning, NY, USA.

Immunohistochemistry

Hemangioma tissue was fixed with formalin, embedded in paraffin, and sectioned into 3 μm for preparation of slides. After baking at 65°C in an incubator for 2 h, the tissue slices were cooled and stored at 4°C. The samples were then processed as follows: 1. Dewaxing: slides with tissue sections were removed from the 4°C freezer and placed at room temperature for over 30 min. Sections were then separately soaked twice in xylene. 2. Hydration: sections were treated with anhydrous alcohol twice for 2 min each, and then successively soaked in 95%, 80%, 70%, and 50% ethanol solutions. Sections were then washed twice with phosphate-buffered saline (PBS) for 3 min. 3. Hydrogen peroxide block: tissue sections were blocked with 3% H_2O_2 in deionized water, incubated at room temperature, and washed with PBS. 4. Antigen retrieval: tissue sections were boiled in 0.01 M citric acid buffered solution (pH 9.0) for 20 min, allowed to cool, and washed with PBS. 5. Blocking: sections were blocked with goat serum and incubated at 37°C for 60 min. 6. Incubation with primary antibodies: a total of 50 μl of primary antibody against MMP-9, VEGF, or p16 was added dropwise to tissue sections. The sections were then incubated at 37°C for 1 h and washed with PBS. 7. Incubation with secondary antibodies: biotin-labeled secondary antibodies were added dropwise, sections were incubated at 37°C for 30 min, and washed with PBS. 8. The avidin-conjugated horseradish peroxidase was added, and incubated at 37°C for 30 min. 9. Signals were developed with diaminobenzidine (DAB). 10. Samples were counterstained with hematoxylin for 5-8 min and washed with tap water. 11. Samples were then dehydrated and mounted for observation. Scoring method: < 5% positive cells was scored 0 points, 5-25% was 1 point, 25-50% was 2 points, 50-75% was 3 points, and > 75% was 4 points. The staining intensity value was multiplied by the percentage of positive cells to arrive at the final score as follows: "negative" was 0-1 point, "+" was 2-4 points, "++" was 5-8 points, and "+++" was 9-12 points.

Design of MMP-9 and VEGF Primers for Real-time Fluorescence Quantitative PCR

The formalin fixed, paraffin embedded (FFPE) RNA kits produced by Omega (Bio-Tek, Norcross, GA, USA) were used to extract total RNA from paraffin-embedded tissue. After extraction, RNA was reverse transcribed into cDNA using the kit by CWBIO by incubation at 95°C for 5 min, 37°C for 60 min, and 95°C for 5 min with oligo (dT)[15]. The cDNA was stored at −80°C until use. The MMP-9 and VEGF gene sequences were acquired from the NCBI gene bank. Primer sequences were designed with reference to previously published studies (Table I).

Table I. Primer sequences and lengths.

Primer	Primer sequence	Sequence length
Human GAPDH	upstream 5'-GGTGGTCTCCTCTGACTTCAAC-3' downstream 5'-ACAACGAATTTGGCTACAGCA-3'	127
Human VEGF	upstream 5'-TGCTGTCTTGGGTGCATTGG-3' downstream 5'-AGGTCTCGATTGGATGGCAG-3'	163
Human MMP-9	upstream 5'-CGACGTCTTCCAGTACCGAG-3' downstream 5'-TTGTATCCGGCAAACTGGCT-3'	220

Primers were synthesized by Sangon Biotech (Shanghai, China). Primers were reconstituted in deionized water to concentrations of 10 µm. The 20 µl reaction system for qRT-PCR included 8 µl of ddH_2O, 0.5 µl of the upstream and downstream primers, 1 µl of template cDNA, and 10 µl of 2× Taq Mix. The thermal profile for PCR was 95°C for 7 min, 95°C for 30 s, and 58°C for 60 s, for 35 cycles.

Standard Curve Construction

To construct standard curves, 1:4 dilutions of cDNA samples with known concentration were prepared. The curves contained five data points. The standards were amplified by Real-time fluorescence quantitative PCR to determine if the relative transcript levels of the target genes were accurate.

Calculation of Relative Transcript Levels

As the concentration of sample deceased, the cycle threshold (Ct) increased. The Ct values of samples and the internal control were obtained according to the aforementioned methods. Transcript levels were calculated according to the $2^{-\Delta\Delta Ct}$ method. On account of the relatively large sample size and large amount of data from sample replicates, it was only necessary to analyze the relative expression of genes. The relative expression levels were calculated according to the RQ values.

Interpretation and Evaluation of Results

The solubility curves of the three sets of primers were unimodal, demonstrating primer specificity. Additionally, the standard curves for MMP-9, VEGF, and GAPDH were required to be linear, with R^2 value or slope approaching 1, and gene amplification efficiency (Eff %) of over 90%.

Statistical Analysis

SPSS 19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for data analysis. The χ^2-test and Fisher's exact probability test were used to analyze immunohistochemistry data. Real-time fluorescence quantitative PCR data are presented as x ± s. The correlations between MMP-9 and VEGF gene and protein expression were evaluated with Spearman's correlation. $p<0.05$ was considered statistically significant.

Results

Electrophoresis After Total RNA Extraction

Total RNA was extracted from tissues using a paraffin tissue extraction kit and identified by nucleic acid gel electrophoresis. Figure 1 shows the total RNA nucleic acid electrophoresis map in parts of the paraffin tissues, in which there are clear bands shown at the 28s RNA and 18s RNA positions. The quality of extracted RNA was satisfactory and could be used for future fluorescence quantitative PCR experiments.

MMP-9, VEGF, and p16 Protein Expression in Hemangioma Tissue

The immunohistochemical results of MMP-9, VEGF, and p16 expression in hemangioma tissue are shown in Figure 2.

Correlations Between the Positive Expressions of MMP-9, VEGF, and p16 in Infantile Hemangioma

The positive rates of MMP-9 and VEGF expression were relatively high and were important for the corresponding neoangiogenesis and occurrence of hemangioma. As shown in Table II, the positive rates of expression of these two proteins

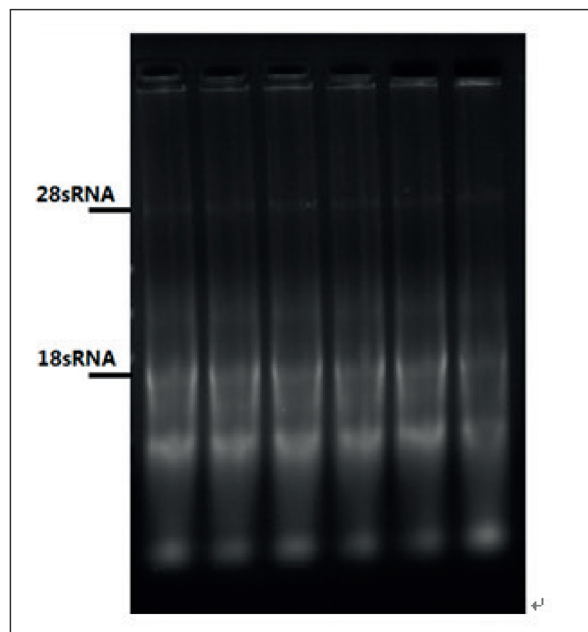

Figure 1. Total RNA tested by semi-quantitative PCR. Electrophoresis showed that the bands representing 28sRNA and 18sRNA were clear, indicating that RNA was of good quality and could be used for future experiments.

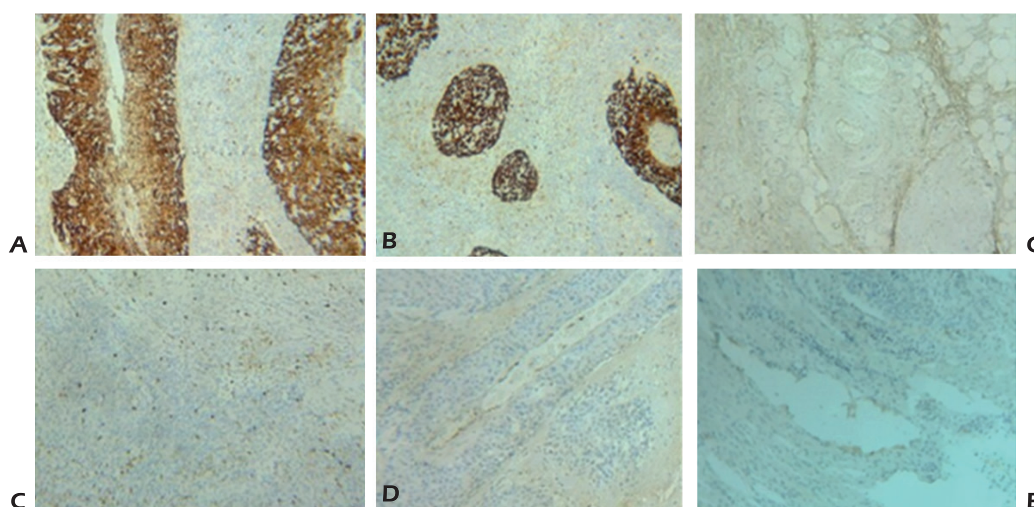

Figure 2. The protein expression of MMP-9, VEGF, and P16 in hemangioma detected by immunohistochemistry. **A**, Positive result of immunohistochemical staining of VEGF. **B**, Positive result of immunohistochemical staining of MMP-9. **C**, Positive result of immunohistochemical staining of p16. **D**, Negative result of immunohistochemical staining of VEGF. **E**, Negative result of immunohistochemical staining of MMP-9. **F**, Negative result of immunohistochemical staining of p16. Microscope was set at 100×.

were positively correlated with the occurrence of hemangioma. Furthermore, the expression of p16 protein was lower in the proliferative phase of hemangioma compared with the involuting phase. In the involuting phase of hemangioma, the protein expression of p16 in endothelial cells was lower than that in normal tissue.

Evaluation of the GAPDH, MMP-9, and VEGF Standard Curves

The five GAPDH, MMP-9, and VEGF standards were mostly linear, with the R^2 values of the three curves approaching 1 (the smallest was 0.989) and Eff% in the range of 90-110%. All solubility curves were unimodal. Primer design in this study met the necessary requirements and was acceptable for accurately measuring the expression levels of target genes.

Analysis of MMP-9 and VEGF mRNA Expression in Tissue by Real-time Fluorescence Quantitative PCR

Evaluation of the levels of MMP-9 and VEGF mRNA in hemangioma tissue is shown in Table III and Figure 3. The amount of MMP-9 in proliferative and degenerative hemangioma were both higher than that in normal epithelial tissues ($p<0.05$). However, the amount of MMP-9 in proliferative hemangioma was significantly higher than in degenerative hemangioma tissue ($p<0.05$). The levels of VEGF mRNA in both proliferative and degenerative hemangioma were higher than that in normal epithelial tissues, but the levels of VEGF mRNA were significantly higher in proliferative than in degenerative hemangioma tissues ($p<0.05$). It indicated that MMP-9 and VEGF mRNA were overexpressed in the proliferative

Table II. Correlations between the positive expressions of MMP-9, VEGF, and p16.

Index	Proliferative hemangioma	Regenerative hemangioma	Normal epithelial tissue	χ^2	*p*
MMP-9 (9–12)	25	8	0		
MMP-9 (5–8)	5	22	2	22.354	<0.05
MMP-9 (0–4)	0	0	28		
VEGF (9–12)	25	7	0		
VEGF (5–8)	5	23	3	26.251	<0.05
VEGF (0–4)	0	0	27		
P16 (9–12)	0	6	19		
P16 (5–8)	0	20	11	23.582	<0.05
P16 (0–4)	30	4	0		

Table III. Analysis of MMP-9 and VEGF mRNA expressions and their relationship.

Index	Proliferative hemangioma	Regenerative hemangioma	Normal epithelial tissue	*F*-value	*p*-value
MMP-9	0.533±0.114	0.225±0.084	0.064±0.031	41.354	<0.05
VEGF	0.732±0.113	0.323±0.063	0.125±0.022	73.854	<0.05

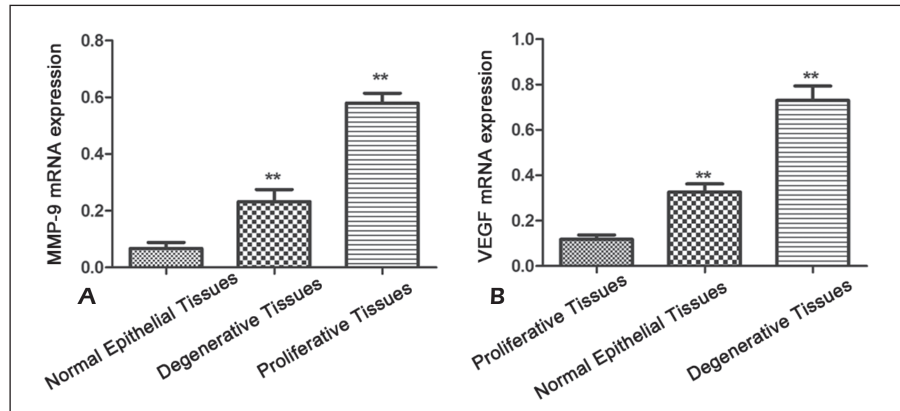

Figure 3. Analysis of MMP-9 and VEGF mRNA expression in tissue by real-time fluorescence quantitative PCR. **A**, Compared with normal epithelial tissues and degenerative tissues, the expression of MMP-9 was higher in proliferative tissues. **B**, Compared with normal epithelial tissues and degenerative tissues, the expression of VEGF was higher in proliferative tissues.

phase of hemangioma, which facilitated tumor progression to some extent.

The Correlation Between MMP-9 and VEGF in Hemangioma

MMP-9 and VEGF were compared in three different sample tissues, and Spearman's correlation was used for analysis. The positive rate of expression of MMP-9 and VEGF protein was positively correlated with their mRNA expression (p<0.05) (Table IV).

Discussion

Hemangioma is a common benign congenital tumor that occurs in children. Mulliken et al[18] determined that hemangioma was significantly different from vascular malformation in pathogenesis, histology, and prognosis. The rate of morbidity of hemangioma among newborns was shown to be 1.1-3.8%, and this number can reach 10-12% around 1 year of age. In the later phase of hemangioma, there is a slow recession of the disease. The essence of the disease is the abnormal proliferation of vascular endothelial cells. A study[19] focusing on the factors that regulate the proliferation of vascular endothelial cells are likely to identify potential therapeutic options for the occurrence and development of hemangioma related diseases. Presently, ultrasound is used for diagnostic procedures related to hemangioma to distinguish it from lymphangioma, lipomyoma, and venous malformations[20,21]. MMP-9 is a metal ion-dependent protease. Its main function is degradation of the various proteins components of the basement membrane and extracellular matrix. The primary function of VEGF is the regulation of angiogenesis. During the development and progression of cancer, new blood vessels form to facilitate the acquisition of nutrients by cancer cells. Furthermore, MMP-9 can accelerate the regeneration and development of vessels by regulating the release of VEGF. Research on the relation between MMP-9 and VEGF should have positive effects on the study of hemangioma. p16 is a tumor suppressor gene. The levels of p16 can indirectly reflect the developmental stage of some cancers.

Table IV. The correlation between mRNA levels of the MMP-9 and VEGF genes-

	Category 1	Category 2	*R*-value	*p*
Proliferative hemangioma	MMP-9	VEGF	0.897	<0.05
Degenerative hemangioma	MMP-9	VEGF	0.935	<0.05
Normal epithelial tissue	MMP-9	VEGF	0.983	<0.05

Conclusions

Through the study of the gene and protein expression of MMP-9 and VEGF, we showed that they were highly expressed in the proliferative phase of hemangioma. MMP-9 and VEGF can serve as markers of the proliferative and involuting phases of hemangioma. The expression of p16 in proliferative and degenerative hemangioma objectively reflected the change in the course of the disease. The potential roles of factors involved in changes in the degenerative process of hemangioma will be evaluated in future studies.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

References

1) Marehuk DA. Pathogenesis of hemangioma. J Clin Invest 2001; 107: 665-666.
2) Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells form hemangiomas. J Clin Invest 2001; 107: 745-752.
3) Mulliken JB, Zetter BR, Folkman J. In vitro characteristics of endothelium from hemangiomas and vascular malformations. Surgery 1982; 92: 348-353.
4) Storch CH, Hoeger PH. Propranolol for infantile haemangiomas: insights into the molecular mechanisms of action. Br J Dermatol 2010; 163: 269-274.
5) Kaselas C,Tsikopoulos G, Papouis G, Kaselas V. Intralesional administration of interferon A for the management of severe haemangiomas. Pediatr Surg Int 2007; 3: 215-218.
6) Maatta M, Soini Y, Liakka A, Autio-Harmainen H. Differential expression of matrix metalloproteinase (MMP)-2,MMP-9, and membrane type 1-MMP in hepatocellular and pancreatic adenocarcinoma: implications for tumor progression and clinical prognosis. Clin Cancer Res 2000; 6: 2726-2734.
7) Sier CF, Kubben FJ, Ganesh S, Heerding MM, Griffioen G, Hanemaaijer R, van Krieken JH, Lamers CB, Verspaget HW. Tissue levels of matrix metalloproteinase MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma. Br J Cancer 1996; 74: 413-417.
8) Li Q, Zhang L, Han Y, Jiang Z, Wang Q. Propofol reduces MMPs expression by inhibiting NF-κB activity in human MDA-MB-231 cells. Biomed Pharmacother 2012; 66:52-56.
9) Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol 2007; 26: 587-596.
10) Roskoski R Jr. Vascular endothelial growth factor (VEGF) signaling in tumor progression. Crit Rev Oncol Hematol 2007; 62: 179-213.
11) Gardner RM, Verner G, Kirkland JL, Stancel GM. Regulation of uterine epidermal growth factor (EGF) receptors by estrogen in the mature rat and during the estrous cycle. J Steroid Biochem 1989; 32: 339-343.
12) Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 1993; 91: 2235-2243.
13) Ishikawa M, Kitayama J, Kazama S, Nagawa H. Expression of vascular endothelial growth factor (VEGF)-C in preoperative biosy speciments and metastatic foci of regional lymph nodes in submucosal gastric carcinoma. World J Surg Oncol 2005; 3: 2-8.
14) Kostopoulou E, Samara M, Kollia P, Koukoulis G. Different patterns of p16 immunoreactivity in cervical biopsies: correlation to lesion grade and HPV detection, with a review of the literature. Eur J Gynaecol Oncol 2011; 32: 54-61.
15) Kim SK, Wang KC, Cho BK, Lim SY, Kim YY, Oh CW, Chung YN, Kim CY, Lee CT, Kim HJ. Adenoviral p16/CDKN2 gene transfer to malignant glioma: role of p16 in growth, invasion, and senescence. Oncol Rep 2003; 10: 1121-1126.
16) Jaffrain RMI, Ferretti E, Toniato E. P16 gene polymer Phism and methylation status in human pituitary tumors. Clin Endocrinol 1999; 51: 317-325.
17) Rocc JW, Sidrandky D. p16(MTS-l/cdkn2/ink4a) in cancer progression. Epx Cell Res 2001; 64: 42-45.
18) Mulliken JB, Glowacki J. Hemangiomas and vascular malformations in infants and children: A classification based on endothelial characteristics. Plast Reconstr Surg 1982; 69: 412-422.
19) Fu Y, Yang ZG, Zhao LY. Angiogenesis characteristics of infantile hemangioma and feasibility observation of transplantation model of human hemangioma on mice. Eur Rev Med Pharmacol Sci 2017; 21: 1276-1280.
20) Kutz AM, Aranibar L, Lobos N, Wortsman X. Color Doppler Ultrasound Follow-up of infantile hemangiomas and periphetal vascularity in patients treated with propranole. Pediatr Dermatol 2015; 32: 468-475.
21) Pan FS, Xu M, Wang W, Zhou LY, Xie XY. Infantile hepatic hemangioendothelioma in comparison with hepatoblastoma in children: clinical and ultrasound features. Hepat Mon 2013; 13: e11103-e11103.