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Activation of Hedgehog Signaling in Aggressive Hepatic Hemangioma in Newborns and Infants

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Abstract. *Background/Aim: Hepatic hemangiomas (HH) can show an aggressive course with significant complications. Prognostic markers that identify an aggressive course are entirely absent. We recently showed that Hedgehog signaling is overexpressed in aggressive hemangiomas of the skin. Here, we hypothesize that it is also altered in aggressive HH. Materials and Methods: Immunohistological staining for GLUT1 and quantitative PCR was performed in seven specimens with aggressive HH. For comparison, we included specimens of kaposiform hemangioendothelioma (KHE), skin hemangioma and normal liver tissue. Results: Overexpression of the Hedgehog signaling components SHH and GLI2 and its target gene FOXA2 in HH were similar to those found in aggressive skin hemangioma and KHE, their expression being significantly higher than in mild skin hemangioma. High expression levels of SHH and FOXA2 positively correlated with HH, but not with normal liver tissue. Conclusion: Hedgehog signaling is up-regulated in aggressive HH. This finding may lead to a biomarker allowing early intervention.*

Hepatic hemangiomas (HH) are vascular tumors typically found in the pediatric liver. Their clinical behavior is usually harmless; however, at times they show an aggressive course with significant complications including bleeding, mass effect, and shunting of blood. Prognostic clinical or biological markers that can distinguish an aggressive from a benign course are entirely absent (1, 2). With 16% of all hepatic tumors occurring during infancy, HH is the most common vascular tumor and the most common benign tumor of the liver in children (3).

The terminology of vascular lesions in the liver and especially angiomatous lesions in children is rather confusing. This uncertainty is largely due to the non-critical use of the terms hemangioma and hemangio-endothelioma, as well as the fact that the nomenclature has lately undergone several changes (4). The term hemangioendo-thelioma has previously been used to summarize a large variety of vascular neoplasms, including vascular malformations or lesions with a borderline biological behavior between benign infantile hemangiomas and highly malignant angiosarcomas (5, 6). Additionally, in the histopathology nomenclature, the terms hemangioma and hemangioendothelioma have been used synchronously to describe the same lesion. Further confusion arises from the nonchalant distinction between vascular lesions of the liver and the epithelioid hemangioendo-thelioma of the liver, a malignant tumor of childhood with metastatic potential.

According to the Boston Children's Hospital Vascular Anomalies Center, HHs now are divided into three categories: i) focal, ii) multifocal and iii) diffuse, depending on their clinical and radiological characteristics (1). Focal lesions stain GLUT1 negative on immunohistochemistry and are considered the equivalent of the rapidly involuting cutaneous hemangioma (RICH). Consequently, they are true congenital lesions (1). On the contrary, multifocal infantile hepatic hemangioma and diffuse infantile hepatic hemangioma stain positive for GLUT1 on immunohistochemistry (1, 7).

Since then, the term hemangioendothelioma is reserved for special tumor entities, for example the kaposiform hemangioendothelioma (KHE), an aggressive vascular tumor of the skin and soft tissues (8, 9). Although the term hemangioendothelioma is considered to be obsolete with respect to angiomatous lesions in the liver, some authors

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continue to use this term in an attempt to describe a subset of angiomatous vascular lesions of the liver that is believed to be associated with rapid growth as well as the potential for a more aggressive course, including the risk for Kasabach-Merritt syndrome (KMS) (9, 10). This nomenclature is unfortunate, because there is no corresponding international consensus for this usage nor are there any clinical, radiographic, histopathological or biological markers that would allow such a distinction. Moreover, it is today accepted that hepatic hemangiomas do not cause the full spectrum of the KMS (11).

Rather, these tumors show a transient thrombocytopenia and anemia, which, at times, has been mislabeled in the literature as KMS. Nevertheless, the transient thrombocytopenia seen in HH can be severe and may require urgent medical or surgical intervention (11). The only two entities that are known to have a definite association with the KMS are the tufted angioma and the KHE.

Most vascular lesions of the liver, including HH, are detected in children during the first 6 months of life and a predominance of female patients has been reported (12-14). Most lesions are harmless, however, serious symptoms including abdominal mass, hepatomegaly, high-output cardiac failure, transient thrombocytopenia and coagulopathy as well as jaundice can occur (15, 16). Furthermore, malignant sarcomas, such as angiosarcoma have been reported to arise in existing HH (17, 18).

Characteristically, like infantile hemangiomas of the skin, rapid proliferation during the first 6 months of life during phase 1 is followed by a plateau phase (phase 2) until about 12 months. Then, during phase 3, the involuting phase begins and lasts up to 4-6 years (19-21). However, in HH, these stages can be intensified and prolonged, and symptomatic patients may need early treatment due to a high risk of severe complications (16). Although medical treatment modalities like propranolol (22-27), corticosteroids and interferon-alpha (INF-α) are available, in some children it is necessary to resort to interventional therapy, such as surgical resection and liver transplantation (9, 28). A high risk of complications has been described for these surgical procedures when performed for vascular tumors of the liver (16).

Histologically, HH consist of immature and disorganized endothelial cells also containing endothelial progenitor cells (29). Traditionally, these lesions have been divided in type 1 and type 2 (30). Both types consist of a supporting fibrous stroma on which the endothelial cell layer sits. In type 1 lesions, cells consist of a single endothelial cell layer or very sporadically several layers. In type 2 lesions, endothelial cells are pleomorphic, larger and more hyperchromatic. Type 1 lesions typically show well-preserved bile ducts especially in the periphery of the lesion. In type 2 lesions, bile ducts are typically completely absent (6, 31, 32). Sometimes, the differentiation of type 2 lesions from angiosarcoma can be challenging. This similarity has led to the widely spread but ultimately unproven impression that type 2 lesions are somehow associated with a more aggressive clinical behavior.

KHE is a vascular tumor infiltrating the skin, subcutis and muscle that can be complicated by the KMS. Although it is locally very extensive and aggressive, it does not metastasize. Similarly to focal HH and in contrast to diffuse and multifocal HH, KHE does not express GLUT1 (8, 33-39). Characteristically, the lesion is composed of several solid nodules separated by connective tissue. These are composed of a mixture of small capillaries, solid lobules of endothelial cells and spindle cells (38, 40).

To date, the etiology and pathogenesis of HH is unknown (9). Further, no understanding exists as to what differentiates the large majority of cases that are harmless from the few that show aggressive growth with high morbidity. Although the hedgehog signaling pathway is implicated in embryonic development, vascularization and stem cell differentiation, its role on the pathogenesis of HH has not been investigated.

Core components of Hedgehog signaling are the ligand Sonic Hedgehog (*SHH*), its receptor PATCHED (*PTCH*), the transmembrane protein SMOOTHENED (*SMO*) and the transcription factors GLI as well as its target genes, such as *FOXA2* (41, 42). In a previous study (43), we have found an overexpression of the hedgehog signaling components *SHH, GLI2* and *FOXA2* in infantile hemangiomas of the skin with especially aggressive progression requiring early resection. These findings are highly relevant because the hedgehog signaling pathway can potentially be targeted pharmaceutically. Therefore, it was the goal of this study to investigate whether hedgehog signaling components are overexpressed in aggressive HH.

Patients and Methods

Patients. HH specimens from 7 patients aged 0 months to 4 years, who underwent corresponding liver resection at our institution from 2006-2016, were collected, snap-frozen and stored at -80˚C. Informed consent was given by the parents of the patients. Similarly, for comparison specimens from 3 children with KHE were collected. A retrospective chart review was carried out, including the analysis of pathology reports. All specimens were re-evaluated by a pathologist with specific expertise in pediatric tumors and hemangioma. Patient records were analyzed, and all data were irreversibly anonymized. Our Institutional Review Board and our Ethics committee approved the study. For further comparison with our results, previously obtained and published data on the expression levels of genes in aggressive infantile hemangioma of the skin were used (43).

Real-time reverse transcription-PCR (RT-PCR). Total RNA was extracted from fresh frozen hepatic hemangioma tissues. RNA was depleted from DNA and was subsequently purified using the RNAse free DNAse set and RNeasy Mini Kit from Qiagen (Hilden, Germany), according to the manufacturer's protocol. The concentration of RNA

Table I. *Patient clinical features*.

No.	Gender	Age at diagnosis	Symptoms	Localization	Imaging findings	Lesion size	Medical treatment	Kasabach Merritt
1	F	Prenatally	None	Liver, unifocal	Single large solid mass	7×8 cm	Cortisone, no effect	N
2	M	4 months	None	Liver, unifocal	Single mass with calcifications	7.9×5.2×7.2 cm	None	N
3	M	5 months	Recurrent vomiting	Liver, unifocal	Single homogenous mass	N/A	None	N
4	M	2 weeks	Cardiac and respiratory insufficiency postnatally, rapid growth despite of treatment with propranolol	Liver, unifocal	Single mass	7.5×3.1 cm	Propranolol, no effect	N
5	F	Postnatally	Liver mass, rapid growth at age 4 years	Liver, multifocal	Single inhomogenous mass	12.4×6.2×11.3 cm	None	N
6	M	4 months	Vomiting due to compression of the stomach	Liver, unifocal	Solid mass compressing the stomach	3.2×3.2 cm	None	N
7	F	Postnatally	Cardiac insufficiency	Liver, unifocal	Single inhomogenous mass	3.5×2.5×1.7 cm	None	N
8	F	16 months	Rapid growth, Hemolysis, acute liver failure, sepsis; death at age 23 months	Face and Neck	Inhomogenous mass	N/A	Chemotherapy Vincristin, Actinomycin D, Cyclophsphamid	Y
9	M	Postnatally	Reduced movement of right leg postnatally, hip luxation	Thigh and Leg	Inhomogenous mass	N/A	Chemotherapy Vincristin, Actinomycin D, Cyclophsphamid	Y
10	F	Postnatally	Visible tumor postnatally	Thigh and Groin	Inhomogenous mass	N/A	Propranolol + Tranexam, Vincristin, Prednisolone, Laser	Y

was measured by photometry (BioPhotometer, Eppendorf, Hamburg, Germany) and the RNA was stored at –80˚C.

Reverse transcription of total RNA was carried out using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR amplifications were carried out with 40 ng of cDNA, 500 nM forward and reverse primers and iTaq SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a Mastercycler Realplex2 cycler (Eppendorf, Hamburg, Germany) with 40 cycles consisting of a 15 second (s) denaturation at 95˚C, primer annealing for 15 s at 55-58˚C depending on the primer, and extension for 30 s at 72˚C. All experiments were performed in doublets. Amplification of the housekeeping gene *TATA-Box-binding-Protein* (*TBP*) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the ΔΔct method, which is a standard procedure (44). Forward (F) and reverse (R) primers were as follows ('5->'3 orientation):

FGF2, F': GACCTCACATAAGCTACAACTTC, R': AGACACA ACTCCTCTCTCTTCTGCT GLUT1, F': TCCACGTCCAGCT *GCCAT*, R': AGGGACCACACAGTTGCTCC
GLI, F'2: TTTGAAGCACCTACACTGGCA, R': TCTCTTCTTG TTCCTTGGACACTG
FOXA2, F': AGAAGCGCCAGAAGTGTCGT, R': GCCCCATC CTCAGACTCTGAC
SHH, F': AAGGACTTCGTGTCAGCCCTTC, R': CGGGCTAGG CACACAAGCT
TBP, F': GCCCGAAACGCCGAATAT, R': CCGTGGTTCGTG GCTCTCT

Immunohistochemistry. Immunohistochemical analysis of GLUT1 expression was performed on 2 μm sections mounted on glass slides in a Leica Bond-Max automated immunostainer (Leica Microsystems Inc., Bannockburn, Ireland). Heat-induced epitope retrieval was performed with a BOND epitope retrieval solution, pH 9 (Leica Biosystems, Nussloch, Germany) for 20 minutes. The GLUT1 polyclonal antibody (Zytomed, Berlin, Germany) was diluted 1:50 and the sections were incubated for 30 minutes, followed by the addition of the BOND Polymer Detection Kit (Leica Biosystems) (15 minutes). 3,3'Diaminobenzidine Tetrahydrochloride (Leica Biosystems) was used as a chromogen with a subsequent haematoxylin counterstain.

Statistical analysis. Data were expressed as a mean±standard deviation and were subjected to Student's unpaired *t*-test and Spearman's rank correlation. A level of $p<0.05$ was considered significant.

Results

Patients' demographics and histology. All seven patients with HH were children between the ages 0 weeks and 12 months at

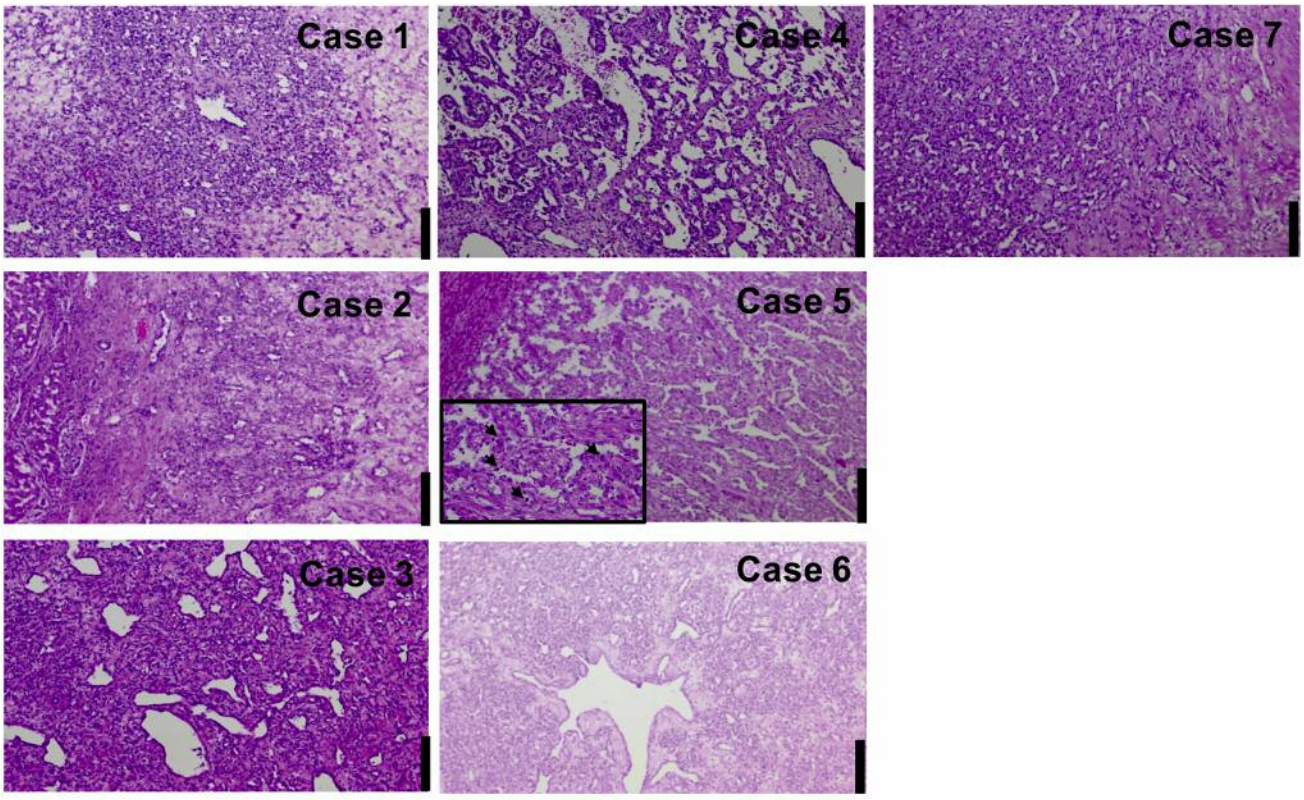

Figure 1. *Histology of hepatic hemangioma. Cases 1-4 and 6: HH with typical histologic appearance and focal regressive changes. Case 5: Hemangioma of the liver with transformation in angiosarcoma. High-power magnification (inlay) displays severe atypia which is marked by arrows. Case 7: HH with regressive changes Scale bars: 200 μm.*

the time of diagnosis (Table I). Three children were girls. Six children had unifocal lesions and one had a multifocal lesion confined to segments 2 and 3 as well as additional subcutaneous hemangiomatosis. Although one child showed transient anemia and thrombocytopenia, none of the children met the definition of KMS. All seven children with HH underwent resection for a variety of reasons (Table II). Due to the location of the HH, two children underwent formal right-sided hepatectomy, three underwent resection of the left lateral segment (segments 2 and 3), and one underwent non-anatomical liver resection for a tumor of the left lobe. At the time of operation, children were between 2 weeks and 4 years old. Six children were operated on before the age of 16 months, and one child who was operated at the age of 4 years was a child with the multifocal tumor and the subcutaneous hemangiomatosis whose tumor, after a stable growth phase and despite having no symptoms, showed new progressive growth at the age of 4 years. There were no relevant postoperative complications in any of the cases. The histology of all specimens of patients with HH is shown in Figure 1. Notably, in case 5 (Figure 1), a multifocal hepatic hemangioma with a focally aggressive area (DD: transformation in angiosarcoma) was diagnosed. The six unifocal cases stained negative for GLUT1 on immunohistochemistry, while the one multifocal case stained positive.

Three children with KHE were included in the study for comparison. All children had KMS. Two underwent biopsy to confirm diagnosis, and one underwent partial resection due to the large mass of the tumor. Six specimens with normal liver tissue were used for comparison.

HH express both FGF2 and GLUT1 on mRNA level. Initially, we tested the degree of expression of two known marker genes of angiomatous lesions. Levels of the fibroblast growth factor 2 (*FGF2*), which is a known marker of infantile hemangioma (45), and glucose transporter 1 (*GLUT1*), which is expressed in multifocal and diffuse HH, but not in unifocal HH and KHE (7, 35, 46, 47) were analyzed. Analysis of *FGF2* expression showed similar expression levels in all specimens tested, that is, in infantile hemangiomas of the skin (both mild and severe), HH (all combined), KHE and normal liver tissue (Figure 2A). Even though six of the seven children with HH analyzed had unifocal lesions that did not stain positive for GLUT1 using

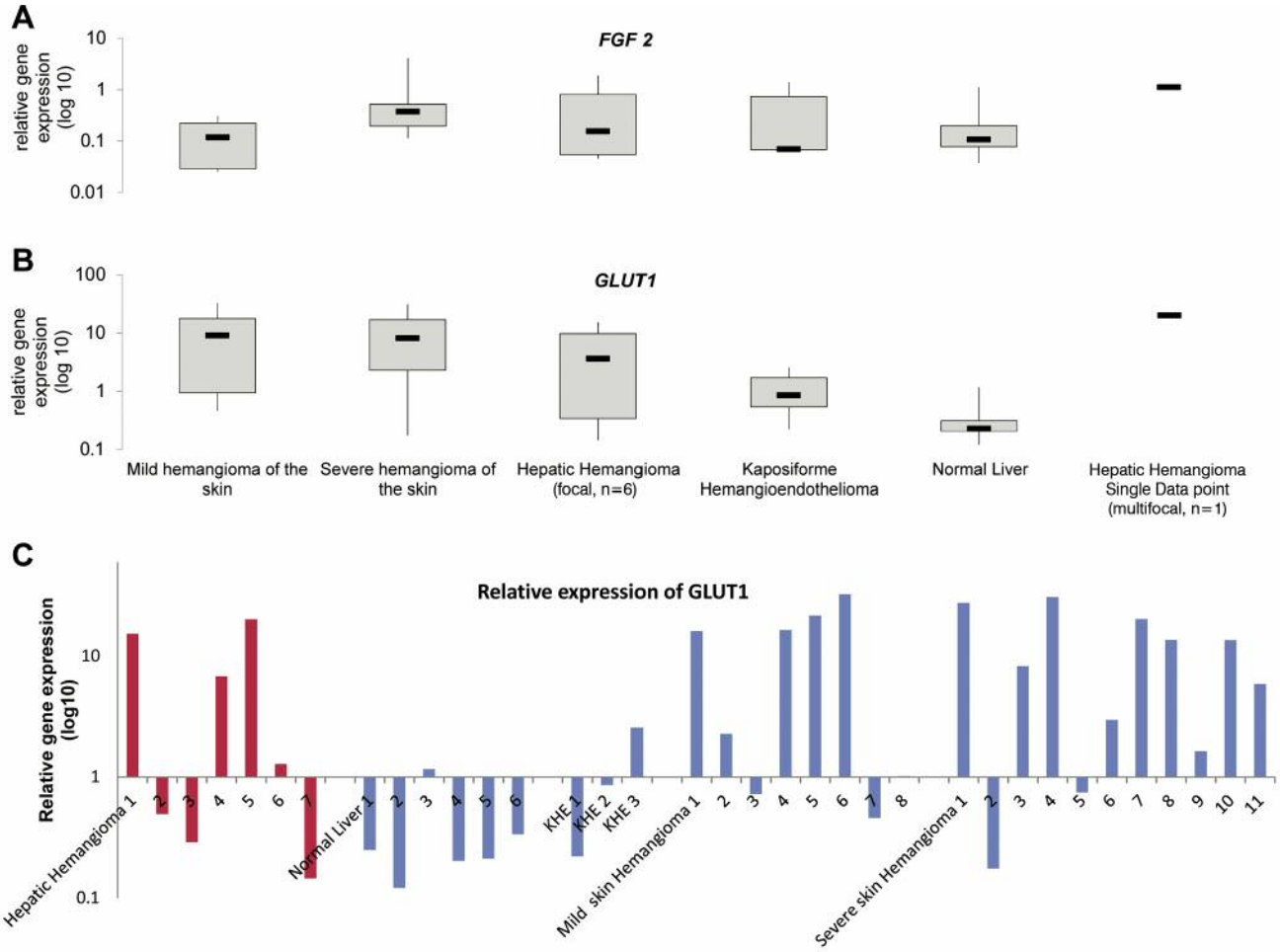

Figure 2. *FGF2 and GLUT1 are poor markers for aggressive HH. Expression analysis revealed comparable levels of FGF2 in all tumors and tissues studied (A). Remarkably, GLUT1 expression varied in different tumors (B, case numbers of the hepatic hemangiomas are given on the x-axis), but was extremely low in normal liver tissue (C).*

Table II. *Histopathological findings and details regarding the performed surgery.*

No.	Gender	Age at operation	Indication for surgery	Operation	GLUT1	Pathological features
1	F	16 months	Large Tumor mass	Laparoscopic biopsy, Right sided Hemihepatectomy	Negative	HH, infantile Dehner 1, focally type 2
2	M	4 months	Large Tumor mass	Wedge resection	Negative	HH, infantile Dehner 1
3	M	6 months	Recurrent vomiting	Right sided Hemihepatectomy	Negative	HH, infantile Dehner 1
4	M	1 month	Cardiac and Respiratory insufficiency postnatally, rapid growth despite of treatment with propranolol	Resection Segment 2 und 3	Negative	HH, infantile Dehner 1
5	F	4 years	Rapid growth at age 4 years	Resection Segment 2 und 3	Positive	Multinodular HH with focal angiosarcoma
6	M	5 months	Vomiting due to compression	Resection Segment 2 und 3	Negative	HH
7	F	12 days	Cardiac insufficiency	Resection Segment 2 und 3	Negative	HH
8	F	16 months	Tumor growth	Biopsy	Negative	KHE
9	M	4 months	Diagnostic measure	Biopsy	Negative	KHE
10	F	8 months	Tumor growth	Partial Resection	Negative	KHE

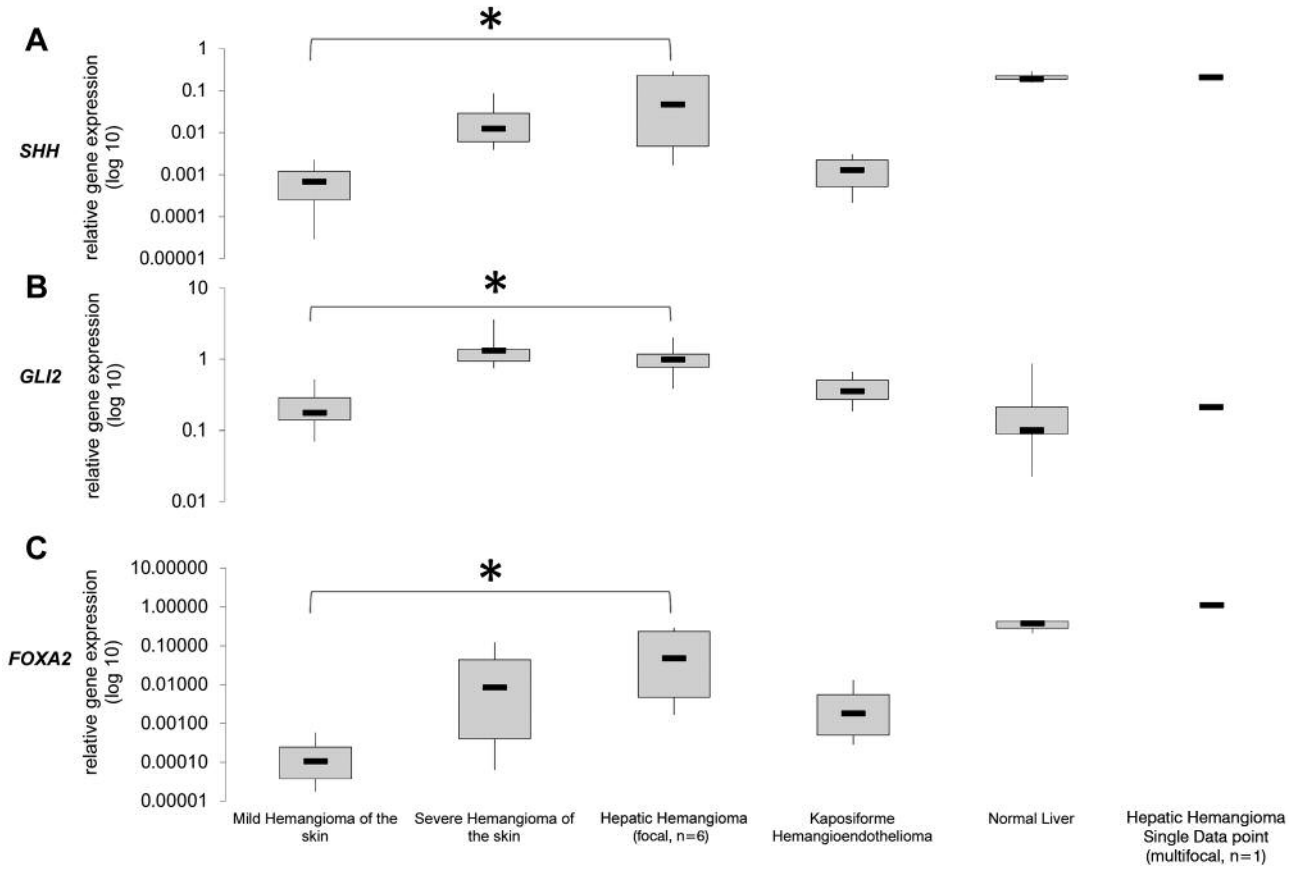

Figure 3. *Hedgehog components SHH, GLI2 and FOXA2 are significantly overexpressed in HH. Expression levels of SHH (A), GLI2 (B) and FOXA2 (C) are shown for HH and control tissues. *p<0.05.*

immunohistochemistry, we opted to analyze this marker nevertheless, since protein does not necessarily correlate with mRNA expression due to post-transcriptional regulation or other factors. Interestingly, in our analysis, two of the six unifocal cases of HH did express *GLUT1* at the mRNA level, as did the case with the multifocal tumor, although to different degrees. Analysis of *GLUT1* expression further demonstrated, that its levels in HH were comparable to the expression of hemangiomas of the skin, but it was higher compared to the expression in normal liver tissue (Figure 2B). The relative gene expression of the only specimen that showed a *GLUT1*-positive immunohistology stain is shown separately.

Hedgehog signaling is over-activated in Hepatic Hemangioma. In order to identify genes that may be involved in the proliferation of HH, we re-evaluated the gene expression profiling that we previously published for cutaneous infantile hemangioma. The study had shown that hedgehog signaling is increased in infantile hemangioma of the skin with an aggressive course (43). More specifically, expression levels of *SHH, FOXA2* and *GLI2* were upregulated in aggressive hemangiomas compared to hemangiomas with a milder course.

Therefore, we next analyzed whether hedgehog signaling is altered in HH by investigating the expression levels of the above-mentioned genes in HH specimens using quantitative RT-PCR.

As shown in Figure 3, the expression levels of *GLI2, SHH* and *FOXA2* in HH are similar to those found in aggressive hemangioma of the skin (p=0.5490 for GLI2, p=0.6451 for *SHH* and p=0.8583 for *FOXA2*) and KHE, which in turn are significantly higher than those of mild hemangioma of the skin (p=0.0010 for *GLI2*, p=0.0311 for *SHH* und p=0.0388 for *FOXA2*). We found high expression levels of hedgehog signaling components in normal liver tissue (Figure 3). Furthermore, we correlated SHH to its target genes *FOXA2* and *GLI2* using the Spearman's rank correlation coefficient (Figure 4). Here, we found a strong correlation of *SHH* with *FOXA2* for HH (Figure 4A) (r=0.82143, p=0.02345), but none for normal liver tissue (Figure 4B) (r=–0.14286, p=0.78717). In contrast, there was no correlation between

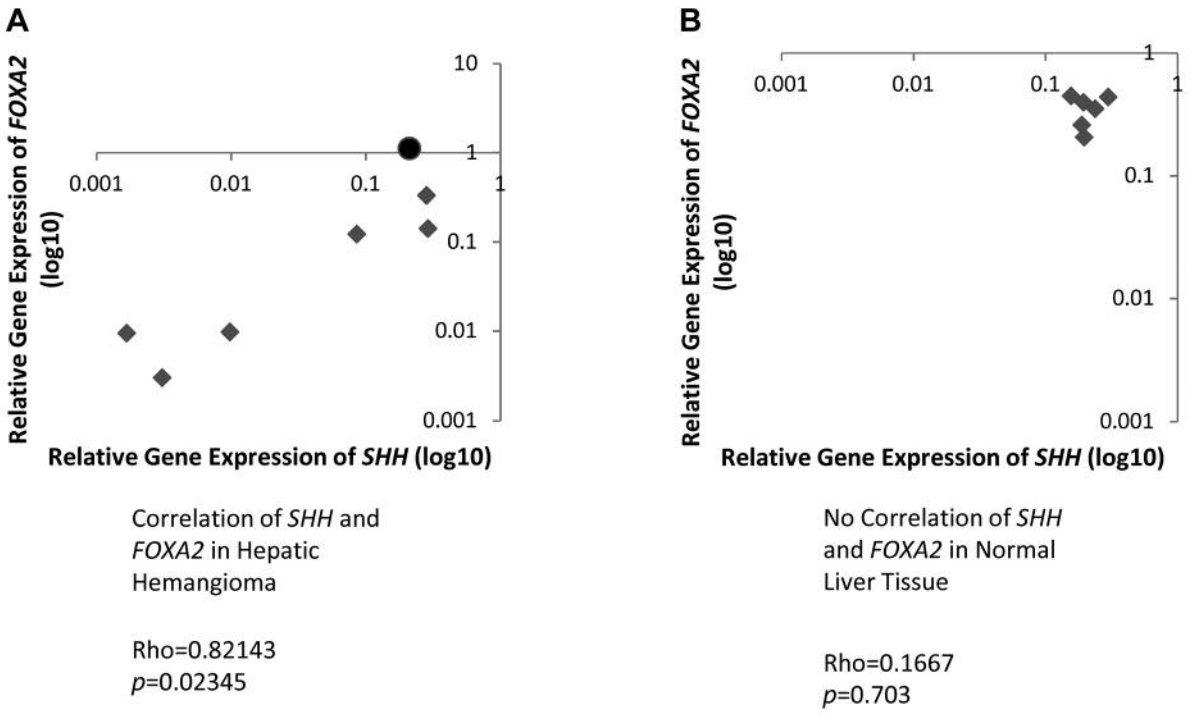

Figure 4. *SHH correlates with its target gene FOXA2 in HH. (A) Correlation analysis off SHH and its target gene FOXA2 of each individual patient with HH using the Spearman's rank correlation coefficient. The data point of the GLUT1 positive HH is marked by a circle. (B) The same analysis for normal liver tissue.*

SHH and *GLI2* (r=0.3095, p=0.4618) or between *GLI2* and *FOXA2* (r=0.1667, p=0.7033) in HH (data not shown). These findings suggest that genes involved in hedgehog signaling may be involved with progression in HH.

Discussion

HH in children are frequent and usually harmless. Nevertheless, isolated cases show enhanced growth and can lead to significant morbidity (48, 49). The understanding why some HH show a more aggressive behavior than most HH is scarce. Here, for the first time, we analyzed the hedgehog signaling pathway in aggressive HH and found significant activation similar to the one in aggressive hemangioma of the skin. Therefore, our results hint that hedgehog signaling may potentially have a critical role in the tumor development and progression of HH in children. In such a case, our findings could have significant clinical impact because hedgehog signaling can then serve not only as a potential prognostic marker but also, more importantly, as a potential drug target for hedgehog antagonists.

Several other alterations have been investigated in HH in an attempt to unravel its pathogenesis and potentially find predictors of aggressive tumor behavior. Alterations of VEGF and NOTCH-signaling amongst others have been found in HH and studies have pointed to a placental origin of endothelial cells in these lesions (50). Moreover, FGF2 has been described as a marker for hemangioma and has been discussed controversially regarding its level of expression during proliferation as compared to involution (21, 51, 52). *GLUT1* is another frequently used marker of HH (53, 54), however, etiology and pathogenesis of the HH are not fully understood. Most notably, to date no markers have been found to detect HH with especially severe progression.

In this study, we examined freshly-frozen tissue of HH and compared our findings with specimens from KHE and normal liver, as well as previously published data from cutaneous hemangioma. In a first set of experiments, we determined whether the expression level of the known hemangioma marker *FGF2* was alternated and could potentially be a marker of aggressive HH. We found that the expression level of *FGF2* in HH was similar to that of KHE and of normal liver tissue. This is in accordance with what we have previously described for the expression of *FGF2* in cutaneous hemangioma, where *FGF2* did not serve as a marker to differentiate between aggressively and mildly proliferating cutaneous hemangiomas (55).

Next, we investigated the expression level of *GLUT1*, a frequently used marker of infantile, but not of congenital hemangioma (53, 54). However, since focal lesions of HH

do not stain positive for *GLUT1* on immunohistochemistry, it was unlikely that *GLUT1* would be an appropriate prognostic tool for aggressive growth in HH. We found that two of the five lesions, despite being focal and staining negative for GLUT1 on immunohistochemistry, did express *GLUT1*. It is unclear how this finding may be interpreted at this time. One potential explanation could be a lack of translation of the expressed of mRNA for GLUT1 in these lesions. However, why only two of the lesions would express *GLUT1*, and not all, is currently not apparent to us. Nevertheless, our results indicate that neither *FGF2* nor *GLUT1* can serve as potential markers for detecting aggressive behavior in HH.

Our previous study on Hedgehog signaling in cutaneous hemangiomas (43) had led us to the analysis of Hedgehog signaling in aggressive HH. We detected an overexpression of *SHH* as well as of *GLI2* and *FOXA2* in all HH compared to mild cutaneous hemangiomas and to KME.

FOXA2, as a target gene of Hedgehog signaling, is transcriptionally activated through *GLI*-binding-sites (56-58). Notably, expression levels of *FOXA2* and *SHH* showed a positive correlation, which substantiates a potential role of Hedgehog signaling in HH. Importantly, normal liver tissue showed high levels of hedgehog components overall, but when we compared each value to the individual patient we found no correlation between SHH and its target gene FOXA2, and we, thus, identified a true activation of hedgehog signaling only in HH, but not in normal liver tissue. These findings are in accordance with previous studies that show an autocrine activation of the hedgehog pathway in a large variety of tumors, including tumors of the colon and the pancreas (7, 42, 59). In our study, we found similarly elevated hedgehog signaling and ligand overexpression in the same individual patient, hinting an autocrine activation mechanism in HH.

Our study has at least three limitations. First, the retrospective nature of our study generates a selection bias of the cases evaluated. However, it was our intent to analyze specifically lesions that displayed an aggressive behavior, which in our case were defined as those that required resection. Second, our case number is low. Although HH are frequently seen in our patient population, the number of children that ultimately require aggressive surgical treatment or even only a biopsy is miniscule. Given our current results, the future goal is to enhance national and international collaboration with other high-volume centers in order to conduct larger studies. Third, our study lacks a true control. A true control would have been tissue from a set of patients with non-aggressive HH who showed no indication for resection. Obviously, acquisition of such tissue is not possible for ethical reasons. We tried to compensate for this shortcoming by including the analysis of tissue of other hemangiomas, including cutaneous hemangiomas with mild clinical behavior, as well as tissue from both KHE and normal liver tissue.

In conclusion, in this study, for the first time, we describe a significant overexpression of the hedgehog signaling components *SHH* and *GLI2*, as well as its target gene *FOXA2* in aggressive HH, and propose these genes as potential prognostic biomarkers for aggressive growth enabling early treatment. Importantly, Hedgehog signaling could serve as a potential drug target in these lesions. More research is needed to clarify the exact role of hedgehog signaling and its importance in tumor progression in HH.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

DWK, RK and MB designed the study, performed the research, analyzed the data and wrote the paper. CV and CW performed the research. LR, RG and DvS provided clinical data, collected patients' photographs and analyzed data.

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