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RESEARCH

Differential Expression of Sonic Hedgehog Protein in Human Hepatocellular Carcinoma and Intrahepatic Cholangiocarcinoma

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Abstract Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (CCA) are the two most common primary liver malignancies in adult patients. The molecular mechanisms underlying the pathogenesis of HCC and CCA are still poorly understood. Sonic hedgehog (SHH) signaling plays an essential role during mammalian development, i.e., promoting organ growth, tissue differentiation, and cell polarity. The up-regulation of SHH has been observed during carcinogenesis, including colorectal carcinoma. Our aim was to investigate the expression pattern of SHH in HCC and CCA. We investigated 40 malignant tumors of the liver, including 21 HCC and 19 of intrahepatic CCA cases by immunohistochemistry (IHC) using a polyclonal antibody against SHH and Avidin-Biotin Complex method. We also investigated the co-localization of SHH and Bone morphogenetic protein 4 (BMP4) in CCA using indirect double IHC. Moreover, we examined whether SHH is expressed in two HCC cell lines HepG2 and HuH-7 and three CCA cell lines OZ, HuCCT1 and HuH28. We found that SHH was expressed in 15 out of 21 cases (71.4 %) of HCC and 100 % of CCA cases by immunohistochemistry. SHH expression showed a positive trend in liver tumors (HCC, CCA) with high grade (G2-G3). SHH localized to the epithelial cells, while BMP4 was expressed in the stromal cells in CCA by double IHC. However, both HCC and CCA cell lines showed SHH expression by Western blot analysis. In conclusion, SHH seems to be an interesting marker of de-differentiation in liver tumors and the simultaneous epithelial-mesenchymal expression may be an intriguing prompt to investigate cross-talks between SHH and BMP4.

Keywords Hepatocellular carcinoma · Cholangiocarcinoma · Sonic hedgehog · Bone morphogenetic protein 4 · Immunohistochemistry

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Introduction

Hepatocellular carcinoma (HCC) constitutes an aggressive primary liver tumor accounting for 80–90 % of primary liver cancer and represents one of the most common cancers worldwide [1]. Due to its high mortality rate, HCC is the third most fatal malignant neoplasm, killing more than 500,000 people annually [2]. The pathophysiological process detected in more than 80 % of HCC cases entails cirrhosis caused by hepatitis B or C viruses [3]. Cholangiocarcinoma (CCA) is a malignant neoplasm of the biliary tract and represents the second primary liver cancer worldwide. The incidence of CCA has been strongly linked to chronic inflammation of the biliary tract, resulting from a heterogeneous group of several risk factors. Specifically, the highest mortality is reported in East Asia, especially in Korea and Northeast Thailand, in association with liver fluke infestations such as *Clonorchis Sinensis and*

Opisthorchis viverrini, while in the Western countries primary sclerosing cholangitis (PSC) may play the major role [4, 5].

In 1978, the hedgehog (*Hh*) gene was initially identified during a *Drosophila* mutagenesis screen conducted by two *Drosophila* geneticists, Christiane Nusslein-Volhard and Eric Wieschaus [6]. Flies with a mutated *Hh* gene possessed the appearance of a hedgehog due to small projections covering the surface of the fruit fly embryo [6]. *Hh* genes play a key role during development, controlling specifically segmentation patterning of the *Drosophila*. Moreover, *Hh* genes play crucial roles during mammalian development, including development of the gastrointestinal tract [7, 8].

Sonic hedgehog (SHH) is a secreted glycoprotein member of the Hh family that regulates patterning of the brain, craniofacial structures, axial skeleton, and limbs [9, 10]. Patched-1 (PTCH-1) is a 12-transmembrane receptor that inhibits the 7-transmembrane protein Smoothened (SMO), in the absence of SHH ligand, which leads to inactivation of the SHH signaling pathway [9]. Binding of SHH ligand to its receptor PTCH-1, releases the inhibitory effect on SMO and activates SHH signaling pathway [9, 11–13]. Dysregulation of SHH signaling pathway during embryogenesis has been reported to cause embryonic developmental defect such as holoprosencephaly [14, 15]. However, the upregulation of SHH and/or mutations in the tumor suppressor PTCH-1 and the proto-oncoprotein SMO have been found to play an important role during carcinogenesis [16–18].

The transforming growth factor beta (TGF-β) superfamily regulates both human and animal embryonic development. Accordingly, the failure or dysregulation of this superfamily has been involved in different types of diseases, including carcinogenesis [19, 20]. Bone morphogenetic protein (BMP) 4 is one of the TGF-β superfamily members that regulates key developmental processes such as proliferation, differentiation, and morphogenesis. In addition, this protein performs a vital role in dorsal-ventral patterning of the neural tube during embryogenesis, as well as bone homeostasis [21, 22]. Moreover, studies have reported that BMP4 has diverse roles during carcinogenesis, potentially functioning as a promoter of cell migration or as a tumor inhibitor [23]. Recently, a study by David H. Wang et al., focusing on carcinogenesis in Barrett's esophagus, suggested that SHH becomes reactivated through acid and bile injury, inducing the stromal BMP4, which subsequently reprograms the epithelial cells of the esophagus favoring a columnar phenotype [24].

HCC and CCA are the two most common primary adult liver malignancies, yet the molecular mechanisms underlying their pathogenesis are still poorly understood. The present study seeks to understand the role of the SHH signaling pathway in carcinogenesis investigating the differential expression of SHH in HCC and CCA.

Materials and Methods

Tissue Samples

Twenty-one tissue specimens of HCC and 19 intrahepatic CCA were retrospectively retrieved from the University of Alberta Hospital archive files. The Human Research Ethics Board (HREB) of the University of Alberta has approved this research as part of two protocols (Pro00007657_Molecular Pathology and Genetics of the Abnormalities of the Intrahepatic Biliary System, and Pro00020274_Twsg-1 Expression in Cancer). All cases were deidentified (anonymous cases). The samples were fixed in 4 % buffered formaldehyde and processed for paraffin embedding according to routine protocols. Paraffin tissue blocks were sectioned at 5–6 μm and each section was stained with hematoxylin and eosin (H&E). Consecutive sections were used for immunohistochemistry.

Cell Culture

Human CCA cell lines, including OZ, HuH28, and HuCCT1 were obtained from the cell culture bank of the Japan Health Sciences Foundation. OZ and HuH28 cell lines were established from a patient with poorly differentiated tumor (G3). HuCCT1 was established from a patient with moderately differentiated tumor (G2). All cell lines were grown as monolayer cultures in their appropriate media as previously described [25]. HuCCT1 and HuH28 were grown as monolayer cultures in RPMI 1680 medium, Roswell Park Memorial Institute (Invitrogen Canada Inc. Burlington, ON, Canada). OZ was grown as monolayer culture in William E medium (Invitrogen Canada Inc. Burlington, ON, Canada). All cell lines were supplied with 10 % Fetal Bovine Serum (FBS, PAA laboratories Inc. Etobicoke, ON, Canada) and 1 ml gentamicin and incubated at 37 °C with 5 % CO_2 in the humidify chamber. Besides, a human HCC cell lines HepG2 was purchased from the American Type Cultural Collection (ATCC, Rockville, MD), and HuH-7 was purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. HepG2 and HuH-7 were established from patients with well-differentiated tumor (G1). HCC cell lines were grown as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Canada Inc. Burlington, ON, Canada) enhanced with 10 % FBS, 1 ml gentamicin and incubated at 37 °C with 5 % CO_2 in the humidify chamber. An immortalized human liver cell line (THLE-3) was purchased from ATCC. The cells were maintained in precoated flasks with a mixture of bronectin (0.01 mg/mL), bovine collagen type 1 (0.03 mg/ mL), and bovine serum albumin (0.01 mg/mL) dissolved in BEGM medium, bronchial tracheal epithelial cell growth medium, and incubated at 37 °C and 5 % CO_2.

Immunohistochemistry and Scoring

Formalin-fixed and paraffin-embedded tissue samples were used in this study. Sections were cut at 5–6 μm. For immunohistochemistry, rabbit polyclonal antibody raised against SHH (1:200) was used according to the manufacturer's recommendations (Abcam, Cambridge, MA, USA). Sections were deparaffinized in xylene and rehydrated through a series of graded alcohols, followed by incubation for 20 min in 3 % hydrogen peroxidase to block the endogenous peroxidase activity. Antigen retrieval was performed by heating in 10 mM sodium citrate buffer (pH 6.0) for 15 min. Non-immunized goat serum was used to block non-specific protein binding for 60 min and sections were incubated overnight at 4 °C with SHH primary antibodies. Tissue sections were given three washes 5 min each with TTBS and incubated with the primary antibody, rabbit anti-goat immunoglobulin (IgG) for SHH for 60 min before incubating with Avidin-Biotin Complex (ABC) (Vector Laboratories, Burlington, ON, Canada) for 30 min. The antibody complex was visualized with DAB Peroxidase Substrate (Dako, Carpinteria, CA, USA) and tissue sections were counterstained with Harris hematoxylin (Thermo Fisher Scientific Anatomical Pathology, Ottawa, ON, Canada). Negative controls (absence of primary antibody) and internal positive control using normal human fetal liver sections were used. SHH stained samples were evaluated for the extent of staining (0=none, 1=1–25 %, 2=26–50 %, 3=51–75 %, 4=76–100 % of the tumor cells) and intensity of staining (0/negative, 1/weak, 2/moderate, 3/intense) [26]. A score was assigned using a semi-quantitative method by multiplying the percentage of stained cells by the intensity of

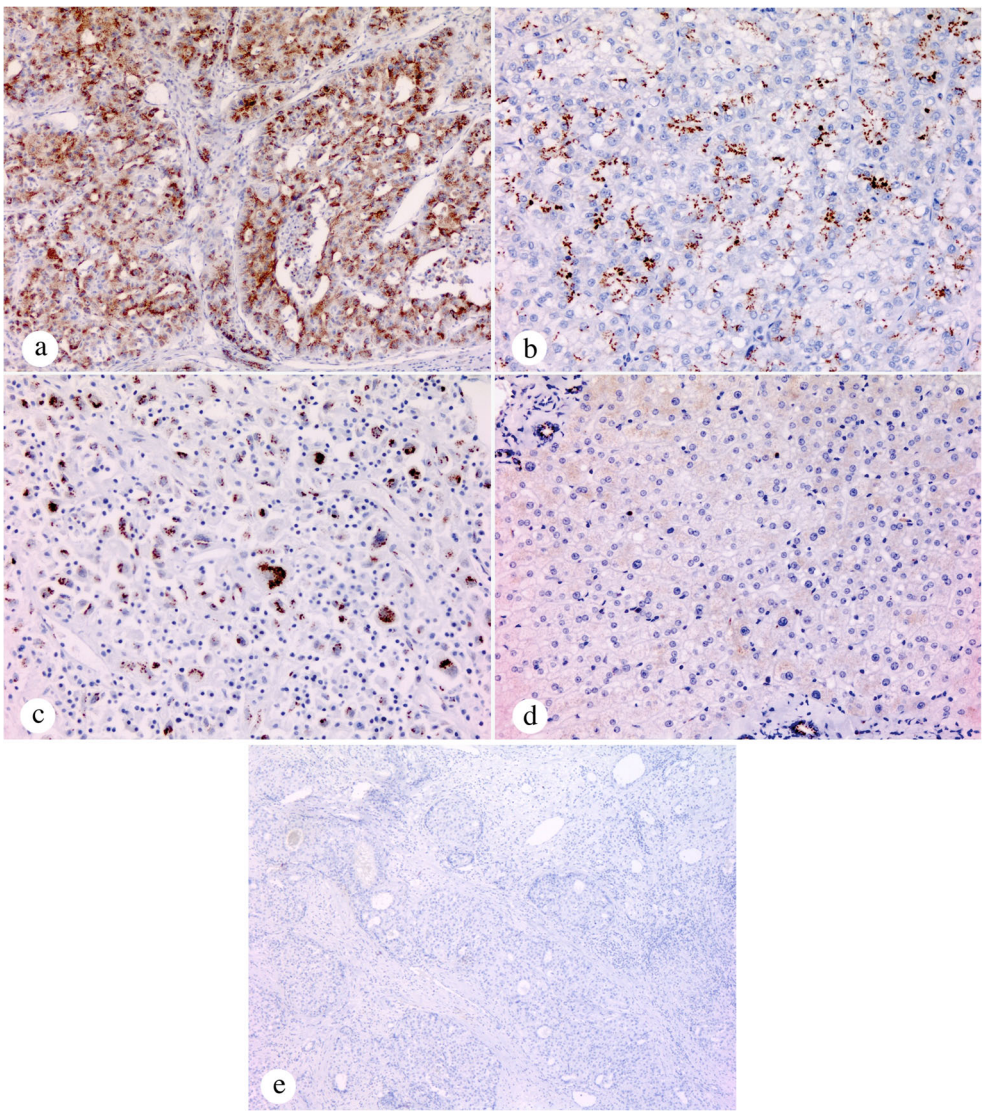

Fig. 1 Immunolocalization of sonic hedgehog (*SHH*) in hepatocellular carcinoma (*HCC*) (**a**–**d**). Strong (**a**) to slight or no expression (**b**–**d**) was seen in HCC. (**e**) Negative control. (**a**, 100X; **b**, 200X; **c**, 200X; **d**, 200X; **e**, 100X)

staining as validated previously for non-parametric evaluations [26].

For double immunostaining, the sections were first incubated with the antibody against SHH at 1:200 dilution. The sections were then reheated with antigen retrieval for 5 min and incubated with mouse monoclonal anti-BMP4 antibody at 1:100 dilution (Abcam, Cambridge, USA). The staining was visualized with the second stain V.I.P Peroxidase Substrate (Vector, CA, USA).

Western Blot Analysis

Harvested cells were lysed in RIPA lysis buffer (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Protein concentration was measured by bicinchoninic acid assay (BCA) (Fisher Scientific Company, Ottawa, ON, Canada). Western blot was performed on proteins electrophoretically transferred from sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (9 %) onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada). After blocking with 5 % non-fat dry milk, the membranes were incubated with a rabbit polyclonal primary antibody against human SHH (1:500) (Abcam, Cambridge, MA, USA), and β-actin (1:5000) (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 °C. The membranes were then incubated with secondary antibody horseradish peroxidase-labeled (HRP) rabbit anti-goat IgG for SHH and mouse anti-goat IgG for β-actin for 60 min at room temperature (GE Healthcare Inc., Baie d'Urfe, Quebec, Canada). Detection was performed with an enhanced chemiluminescent (ECL) substrate

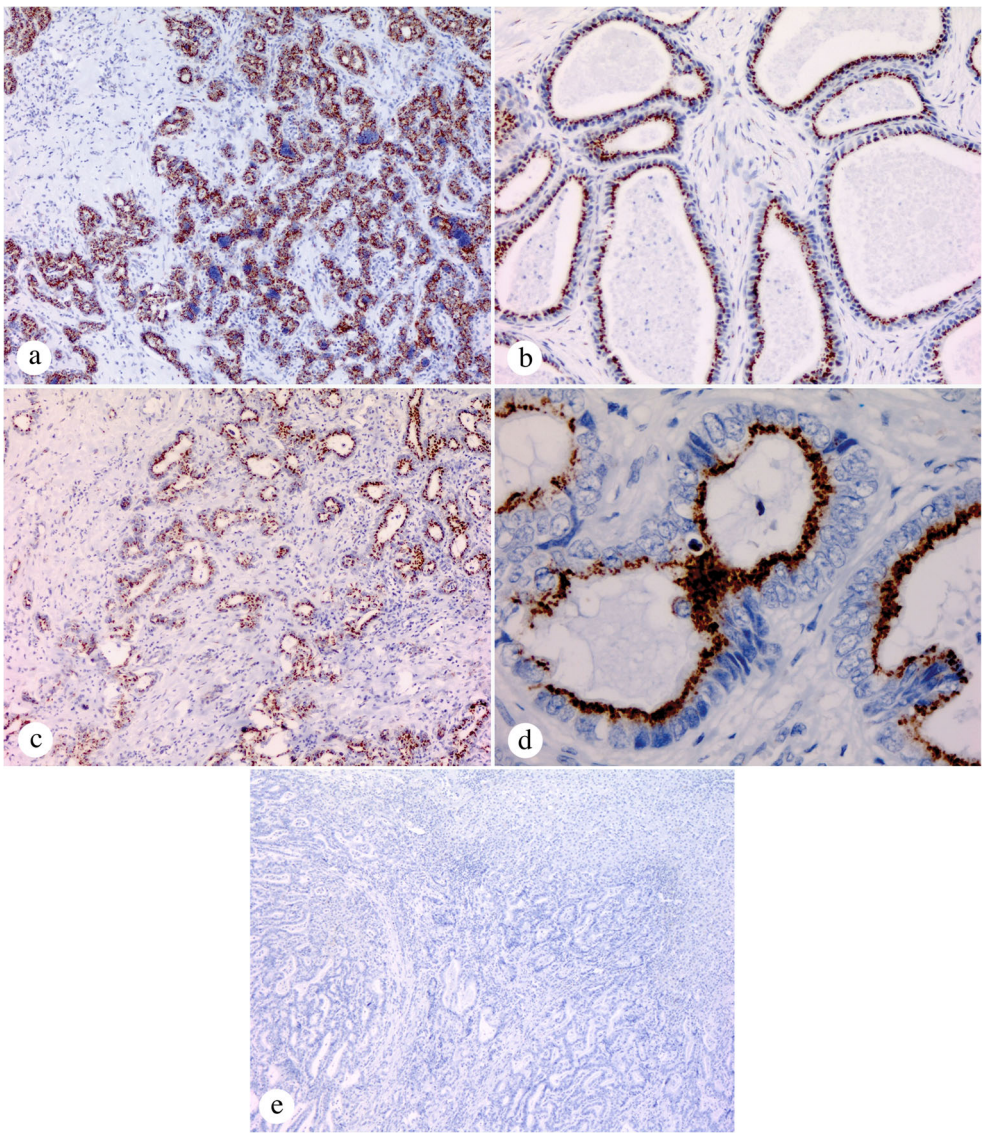

Fig. 2 Immunolocalization of sonic hedgehog (*SHH*) in cholangiocarcinoma (*CCA*). Diffuse strong (**a**–**d**) expression of SHH was seen in the subluminal epithelial cells of the bile duct tumor (*CCA*). (**e**) Negative control. (**a**, 100X; **b**, 100X; **c**, 100X; **d**, 400X; **e**, 100X)

according to manufacture's instructions (Perkin-Elmer Inc., Waltham, MA, USA) and then exposed to Kodak X-ray film (Kodak Graphic Communications Company, Burnaby, BC, Canada). All experiments were performed in triplicate.

Statistical Analysis

The tumor cells were randomly selected and counted. The total number of counted cells and the percentages of positive cells were presented as mean ± SD The Mann–Whitney test was used because of the use of two groups with paired data and working on a non-parametric platform. We also conducted Pearson bivariate correlation to determine the correlation between SHH scores and tumor grades. All p values were two-sided and p values less than 0.05 were considered to indicate statistical significance. The statistical software used was SPSS Version 20 (IBM, Armonk, NY, USA).

Results

Immunohistochemistry

We investigated SHH expression in human liver cancer tissues using immunohistochemical staining. SHH expression was either absent or barely detectable in normal (non-neoplastic) hepatocytes surrounding the nodules of liver carcinoma in the same tissue sections. In contrast, we observed a variable SHH expression, from absent to moderate (Fig. 1), in the neoplastic areas of HCC. Faint to moderate epithelial expression was found in fifteen cases out of 21 (71.4 %) of HCC, while six cases (28.6 %) showed no detectable expression of SHH. In contrast, moderate to strong expression of SHH was found in all CCA cases (100 %) in the neoplastic areas (subluminal) of the epithelial bile duct tumor (Fig. 2). Distribution of IHC SHH scores in HCC and CCA cases is shown in Table 1 and Fig. 4. In summary, the expression of SHH was found in both HCC and CCA, although the expression was higher in CCA than HCC. CCA had a mean IHC SHH score of 9.89 (±2.18 SD), while HCC had a mean score of 2.23 (±2.32 SD). This difference was statistically significant (P=0.0001, Fig. 3).

Further, the expression of SHH in HCC was strongly correlated with tumor grade, with the expression of SHH increasing from well to poorly differentiated tumor (P=0.016, Table 1). In contrast, SHH was moderately to highly expressed in all CCA tumors stage G1, G2 and G3 (Table 1; positive trend only).

To determine the co-localization of SHH and BMP4 proteins, a high specific double immunostaining was performed on CCA tumor samples. SHH was mainly expressed in the subluminal epithelial cells of bile duct tumors. In contrast, BMP4 expression was exclusively found in the stromal cells of the bile duct tumors (Fig. 4).

We detected SHH by Western blot analysis in all three CCA cell lines, although the expression was faint in two out of three CCA cell lines. Both HCC cell lines (HepG2 and

Table 1 Distribution of SHH protein immunohistochemistry scores according to tumor grade for hepatocellular carcinoma and cholangiocellular carcinoma tumor cases

IHC SHH score	Hepatocellular carcinoma N=21			Cholangiocellular carcinoma N=19		
	G1	G2	G3	G1	G2	G3
0	3	2	1			
1	3		2			
2	1	2				
3			1			
4		1				
5			2		1	
6		1	1			1
7			1		2	
8						
9					1	
10				1[a]	4	1
11					2	
12					5	1
Average score ± SD	0.71±0.75	2.3±2.33	3.5±2.61	NA	10±2.17	9.3±3.05

G1-G3, tumor stages from more to less differentiated

NA not applicable due to N=1

[a] perihilar involvement

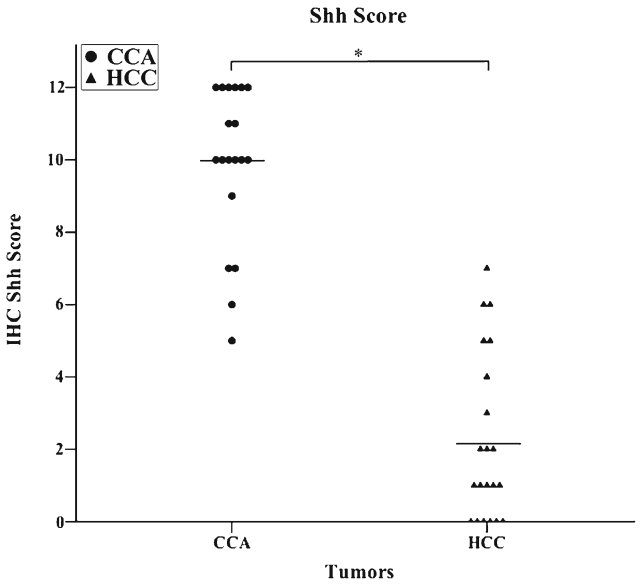

Fig. 3 Sonic hedgehog (*SHH*) IHC score in cholangiocarcinoma (*CCA*) and hepatocellular carcinoma (*HCC*), p=0.0001

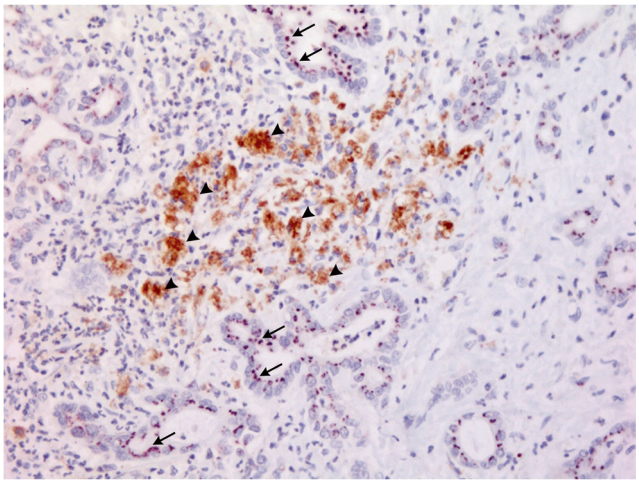

Fig. 4 Double immunolocalization of SHH and BMP4 in cholangiocarcinoma (*CCA*). Strong epithelial cell expression of SHH (*arrows*) at the subluminal region of bile duct tumors. Strong stromal cell expression of BMP4 (*head arrows*) in bile duct tumor. X200

HuH-7) had a band of a strong intensity (Fig. 5). The β-actin signal was present at comparable levels in all cell lines examined.

Discussion

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the most common primary liver malignancies in adult patients. Both have a poor prognosis and are usually difficult to diagnose until they reach an advanced stage. The molecular mechanisms underlying the development of HCC and CCA remain poorly understood. Hedgehog signaling pathway plays an important role during carcinogenesis due to its effect on cell differentiation, proliferation, and apoptosis. Previous studies have highlighted the pathogenic role of SHH in different type of malignant tumors [16, 27, 28]. However, a comprehensive investigation of the role of SHH in liver tumors has not been done yet. In the present study, the expression pattern of SHH was examined in CCA and HCC liver tumors using immunohistochemistry and Western blot analysis. Our results demonstrate that SHH pathway is activated in liver carcinomas. SHH was moderately to strongly expressed in all CCA cases but only some HCC (less differentiated). Moreover, our results demonstrate for the first time in a clear and specific way, that SHH expression strongly correlates with the tumor grade of HCC. SHH was also detected in both HCC and CCA cell lines.

SHH was intensively studied and described to have an essential role during embryogenesis and tissue repair in adult tissues [29]. Recently, a study by Pereira T de A et al. proposed that upregulation of SHH plays an important role in tissue repair in various liver diseases including liver inflammation [30]. SHH pathway has also been suggested to play an important role in carcinogenesis and tumor progression [16]. In some tumors, including nevoid basal cell carcinoma syndrome, alterations in *PTCH-1* and *SMO* genes were found, leading to activation of SHH pathway, an increase in cell proliferation and carcinogenesis [17, 18]. Overexpression of SHH was also noted in other tumors, including colorectal carcinoma, further supporting a notion that developmental genes may be reactivated during not only tissue repair, but also carcinogenesis.

Several pathways have been reported to be associated with Hh pathway during both embryogenesis and carcinogenesis. A study by Marcelle C et al. showed that SHH may antagonize Wnt signaling during patterning of the dorsal somite [31]. Moreover, Wnt signaling pathway can interact with Hh pathway during carcinogenesis where Indian hedgehog is an antagonist for Wnt signaling pathway in colon carcinoma [32]. BMP signaling pathway is another important developmental pathway [21] that has been implicated in some carcinomas. For example in breast carcinoma, BMP4 has a bi-potential function; first, BMP4 acts as tumor suppressor during the early stage of tumor development; second, BMP4 serves as a promoter for tumor cell migration/ invasion via epithelial mesenchymal transition (EMT) when the tumors become more advanced and more aggressive [23]. We have previously investigated the expression pattern of BMP4 in both HCC and CCA. There was a higher expression of BMP4 in CCA than HCC tumors [33]. In the current study we investigated the relationship between SHH and BMP4. A strong stromal expression of BMP4 and a strong epithelial expression of SHH

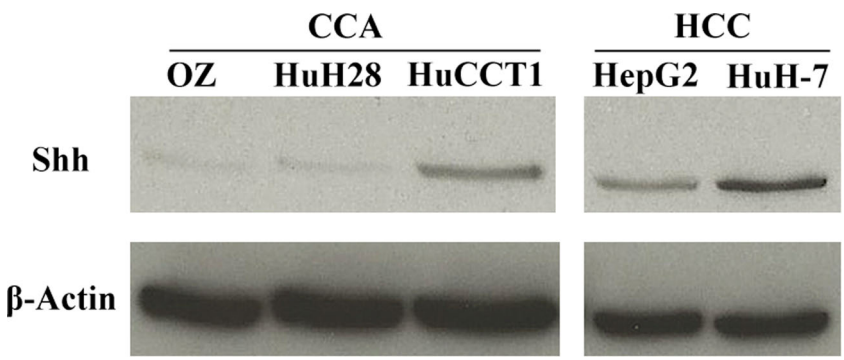

Fig. 5 Western blot analysis of SHH expression in two hepatocellular carcinoma cell lines (HepG2 and HuH-7), and three cholangiocarcinoma cell lines (OZ, HuH28, and HuCCT1). Beta-actin detection was used as loading control for constitutive protein expression to ensure consistent loading in each gel well

were detected in CCA cells. This suggests a potential role for epithelial-mesenchymal interactions in the pathogenesis of liver malignancies. Further studies should examine if, similar to Barrett's esophagus [24], SHH present in the epithelial cells can induce stromal BMP4 expression in liver cancer. There is quite a strong evidence highlighting that progression of solid tumors towards a frank malignant phenotype is not only dependent from cell-autonomous properties of the cancer cells, but there is a remarkable influence from the reactivity between tumors cells and the surrounding or intra-tumoral stroma [34]. Indeed, the liver is probably a magnificent example for the link between chronic inflammation and malignancy as it was postulated by Rudolf Virchow about 150 years ago [35].

In conclusion, our immunohistochemical analyses detected a significant expression of SHH in the epithelial liver malignancies, with a higher level of expression in intrahepatic CCA than HCC. The strong expression of stromal BMP4 may be regulated via epithelial expression of SHH signaling pathway. These results suggest that SHH may play an important role in liver carcinogenesis, thus potentially serving as a promising therapeutic target and as a diagnostic marker. SHH seems to be an interesting marker of de-differentiation in liver tumors and the simultaneous epithelial-mesenchymal expression may be an intriguing prompt to investigate cross-talks between SHH and BMP4.

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Conflict of interests No conflicts of interest were declared.

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