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Original article

Hepatic villin expression in biliary atresia

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Background: Villin is a protein that severs, binds, and bundles actin filaments, necessary for maintenance of the structural integrity of the microvilli in the absorptive epithelia, which include enterocytes, kidney proximal tubule cells, and bile canaliculi. Biliary atresia (BA) is a progressive, sclerosing, inflammatory process that results in complete obliteration of the extrahepatic bile ducts. Liver histopathology in BA might be similar to idiopathic neonatal hepatitis (INH).

Objective: We compared the hepatic expression of villin in children with BA and INH.

Methods: This study was conducted on stored liver specimens of 28 children with BA (age 2.78±1.33 months) and 10 children with INH (age 2.40±1.26 months). Villin was investigated on liver tissue by immunohistochemistry. Villin mRNA analyzes were undertaken on liver homogenates using real-time PCR technique, with primers from coding regions of the human villin gene. Amounts of villin PCR product (villin mRNA relative to the concentrations of GAPDH product) were expressed as median (minimum, maximum).

Results: Villin mRNA expression in patients with BA and INH were 15.98 (0.02, 11880.47) and 0.09 (0, 111.95), respectively. Villin mRNA expression in BA patients was significantly higher than in patients with INH ($p = 0.01$). Villin staining and mRNA expression were undetectable in two INH.

Conclusions: The higher villin expression in BA might have been due to pronounced bile duct proliferation in comparison with INH. Hepatic villin expression might be helpful in discrimination BA from INH and hence BA patients can be timely diagnosed.

Keywords: Actin, biliary atresia, cholestatic liver disease, idiopathic neonatal hepatitis, villin

Cholestasis is a clinical syndrome that results from the impairment in the formation of bile. The cause of neonatal cholestasis is either extrahepatic or hepatic disorder. The extrahepatic cause includes biliary atresia, choledochal cyst, and spontaneous perforation of the bile duct. The hepatic cause includes idiopathic neonatal hepatitis (INH), infection, administration of certain drugs or parenteral nutrition, paucity of interlobular bile ducts, metabolic diseases, and rare genetic diseases. Whatever is the nature of the cause, cholestasis is manifested by hepatic retention of the products normally excreted in the bile such as bile salts and cholesterol. Progressive cholestasis leads to hepatic fibrosis, cirrhosis, and finally the clinical signs of liver failure develop.

Biliary atresia (BA) is a progressive inflammatory sclerosing cholangiopathy that leads to a complete obliteration of the extrahepatic bile ducts and cholestatic jaundice. It is the most common indication for liver transplantation in children. The etiology of BA remains unknown. Several hypotheses include infection, congenital, toxic, and dysregulation of immunity leading to destruction of the extrahepatic biliary tree [1-3]. Early diagnosis and timely Kasai portoenterostomy may prevent the infants to develop persistent cholestasis and eventually cirrhosis. Liver biopsy obtained percutaneously has a high diagnostic accuracy for BA if reviewed by an experienced pathologist and is diagnostic for INH. Liver histology in BA often reveals varying degrees of portal tract fibrosis, edema, ductular proliferation, and cholestasis with the appearance of bile plugs. Evidence of giant cell transformation might be present, making differentiation from INH difficult.

Actin-binding proteins have been reported to play a role in the formation of the microvillous core bundle [4]. Actin-binding proteins regulate the polymerization and depolymerization of actin, connect actin-based

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structures to membranes and to other cytoskeletal elements, power the movement of actin filaments, and cross-link actin filaments into bundles. Villin is one of the actin-binding proteins that sever, bind, and bundle actin filaments, which is necessary for maintenance of the structural integrity of the microvilli in the absorptive epithelia, which includes enterocytes, kidney proximal tubule cells, and bile canaliculi. Villin is a monomeric 92.5-kD protein, composed of two domains repeated in tandem forming the core, and a COOH-terminal domain as the headpiece [4]. Villin expression is detected in the epithelial cells forming the intrahepatic and extrahepatic ductal structures during the development of the liver and persists in mature duct cells [5].

This study aimed to compare the expression of villin in children with BA and INH, and to correlate the expression of villin with prognostic outcomes of BA.

Methods

This retrospective study was conducted on stored liver specimens of 28 children with BA and 10 children with INH. Biliary atresia patients were classified in term of bile flow establishment into favorable and unfavorable outcomes (total bilirubin <2 mg/dL, and ≥2 mg/dL at 1-year post Kasai portoenterostomy, respectively). The study was carried out in King Chulalongkorn Memorial Hospital, which is a university hospital in Bangkok. The study protocol has been approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University. Permission for the use of the specimens has been granted by the Director of King Chulalongkorn Memorial Hospital. The parents of every participating child also gave their informed consent for the subject to join the study.

Liver tissue was obtained by either percutaneous needle biopsy or wedge resection during Kasai portoenterostomy for BA. The tissue was stored in liquid nitrogen until tested for mRNA expression.

Immunohistochemistry

Villin monoclonal antibodies (Cell Marque, Hot Springs, AR) were stained on formalin-fixed, paraffin-embedded liver tissue. The second antibody used was peroxidase-conjugated swine anti-mouse antibodies. Diaminobenzidine tetrahydrochloride was used to visualize the antibody reaction.

RNA extraction and cDNA synthesis

RNA was extracted from frozen liver tissues by available test kit (RNeasy mini kit, Qiagen, German). All RNA samples were reversed to cDNA. Reverse transcription was performed using random primers and M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Promega, Medison, WI) following the manufacturer's recommendation.

House keeping gene detection using real-time PCR

The housekeeping gene GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was selected to serve as an internal control for RNA extraction and cDNA synthesis step. GAPDH forward and reverse primer had sequence as follows: GAPDH-F85 5'-GTGAAGGTCGGAGTCAACGG-3' and GAPDH-R191 5'-TCAATGAAGGGGTCATTGATGG-3', respectively. The reaction mixture consisted of 2 μl of cDNA, 0.5 μM of each GAPDH forward primer and GAPDH reverse primer, 10 μl of 2.5X MasterMix (Eppendorf, Hamburg, Germany), 0.4 μl of ROX reference dye and 0.2 μl of 10X SYBR Green I and nuclease-free water to a final volume of 25 μl. The amplification reaction was performed in a real-time PCR roter gene 3000 (Corbett Research) under the following conditions: denaturation at 95°C for 15 seconds, followed by 40 amplification cycles consisting of denaturation at 94°C for 15 seconds, primer annealing and extension at 60°C for 30 seconds.

Villin gene detection using real-time PCR

The primers from coding regions of the human villin gene were used for real-time PCR amplification. Villin forward and reverse primer had sequence as follows: Exon 2 forward primer 5'-TGTTCCTTCCAG CACCTTTG-3' and Exon 5 reverse primer 5'-CCT GAGTCTCTCCATACGGG-3', respectively. The reaction mixture consisted of 2 μl of cDNA, 0.5 μM of each villin forward and reverse primer, 10 μl of 2.5X MasterMix (Eppendorf, Hamburg, Germany), 0.4 μl of ROX reference dye and 0.2 μl of 10X SYBR Green I and nuclease-free water to a final volume of 25 μl. The amplification reaction was performed in a real-time PCR roter gene 3000 (Corbett Research) under the following conditions: denaturation at 95°C for 15 seconds, followed by 40 amplification cycles consisting of denaturation at 95°C for 15 seconds, primer annealing at 58°C for 15 seconds, and extension at 72°C for 20 seconds.

Preparation of positive controls

The villin primers were amplified to be used as positive samples by conventional PCR. The reaction mixture consisted of 3 µl of cDNA, 0.5 µM of each villin forward and reverse primer, 10 µl of 2.5X MasterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 µl. The amplification reaction was performed in a thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: denaturation at 94°C for 3 minutes, followed by 40 amplification cycles consisting of denaturation at 94°C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72°C for 30 seconds and concluded by a final extension at 72°C for 7 minutes. The PCR amplification products of 269 bp in length, were separated by 2% agarose gel electrophoresis and purified using Perfectprep Gel Cleanup kit (Eppendorf, Humburg, Germany). The amplification products were confirmed by direct sequencing. The resulting purified DNA served as templates for DNA sequencing using Big Dye Terminator V.3.0 Cycle Sequencing Ready Reaction kit (ABI, Foster City, CA) in the ABI PRISM® 310 automated DNA sequencer. Subsequently, the purified products were inserted into pGEM-T Easy Vector System (Promega, Medison, WI, USA) by TA-cloning strategy and positive colonies were selected by blue/white colony assortment. The plasmids were extracted and purified using FastPlasmid Mini kit (Eppendorf, Hamburg, Germany). These products were used as positive controls and for the sensitivity test.

Sensitivity test

DNA cloned as described above served as positive controls for villin detection and determination of test sensitivity. DNA concentration was determined by measuring OD_{260}. DNA samples with known concentrations were 10-fold serially diluted from 10^6 to 10 copies/µl and then used as templates for sensitivity tests. The lowest amount of GAPDH and villin mRNA expression which can be detected by quantitative PCR was 10 copies/µL.

Statistical analysis

The amounts of villin PCR product (villin mRNA relative to concentrations of GAPDH product) were expressed as median (interquartile range). Mann-Whitney test was used to compare villin expression between groups. p <0.05 based on a two-tailed test was considered statistically significant. All the statistical analyses were performed using a statistical software package (SPSS, Version 17, Inc., Chicago, IL).

Results

The age of patients with BA (M = 13, F = 15) and INH (M = 6, F = 4) were 2.78±1.33 months and 2.40±1.26 months, respectively, which were not significantly different. Villin mRNA expression in patients with BA and INH were 15.98 (2.85, 45.25) and 0.09 (0, 21.25), respectively. Villin mRNA expression in BA patients was significantly higher than in patients with INH (p = 0.01) as shown in **Figure 1**.

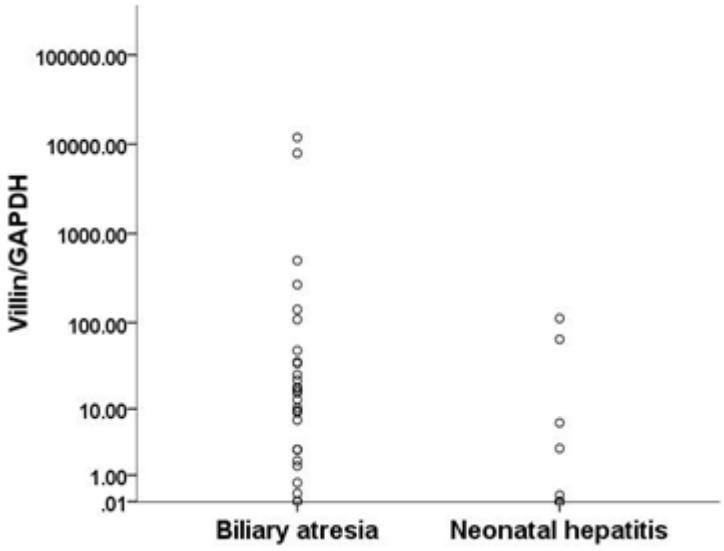

Figure 1. Villin mRNA expression using real-time PCR

In addition, villin mRNA expression in BA patients with unfavorable outcome (n = 13) and favorable outcome (n = 15) was 16.12 (2.19, 384.16) and 15.83 (2.85, 35.72), which was not significantly different (p = 0.63). Villin mRNA expression and villin immunostaining were undetectable in two cases of INH. Positive villin staining of biliary epithelial cells was demonstrated in the rest of the specimens (**Figure 2**).

Discussion

Bile secretion depends on a series of transport systems embedded in the membranes of hepatocytes and cholangiocytes. The bile canaliculus is the site at which the active transport of bile salts and other organic solutes initiate the bile formation [6]. Canalicular secretion of the bile salts is a rate-limiting step in the bile formation. The complex cytoskeleton includes actin filaments that supports the canaliculus and exhibits contractions that facilitate the bile flow. This contractile mechanism is altered in cholestasis.

Villin is situated in the core of actin filaments, supporting the microvillus membrane, forming the brush border [7]. In the assembly of the brush border, the function of villin in severing or bundling actin filaments is calcium-dependent [8, 9]. It has been found that the distribution of villin expression protein in normal adult tissues is restricted to some cell types of the digestive and urogenital tracts, which perform an absorptive role, and/or exhibit well-developed microvilli at their surfaces [10, 11].

Biliary atresia, an idiopathic obliterative cholangiopathy, rapidly leads to liver cirrhosis and liver failure if untreated with Kasai portoenterostomy to establish the bile flow. A recent genome-wide association study (GWAS) demonstrates that one of the BA-associated SNPs resides in contiguous genomic region of *ADD3* genes, which encodes adducin 3 [12]. Both adducin 3 and villin belong to the skeletal proteins playing a role in the gathering of spectrin-actin network in the epithelial tissues, including that of the biliary tracts [5, 13, 14]. Although the present study reveals increased villin expression in BA, it is not exactly known whether this finding is a cause or a consequence of biliary tract abnormality in BA.

Liver biopsy is the common diagnostic method in infants with cholestasis to exclude other neonatal liver diseases with similar clinical presentations as BA. Liver biopsy samples taken early in development of BA (before 6 weeks of age) might not have typical histological features, and serial biopsy samples might be required for diagnosis. This limitation put the infants through the risk of repeated invasive procedures and the possibility of delayed operation. The present study shows that patients with BA had significantly higher villin mRNA expression than patients with INH. Therefore, hepatic villin expression might increase the accuracy of liver biopsy in diagnosis of BA. The higher villin expression in BA might have been due to more pronounced bile duct proliferation in comparison with INH. Increased actin and myosin deposition around the bile canaliculi has been observed in BA patients who did not exhibit bile flow after surgery [15]. However, we found that the expression of villin in BA patients was not correlated with the outcome in term of the bile flow establishment after Kasai portoenterostomy.

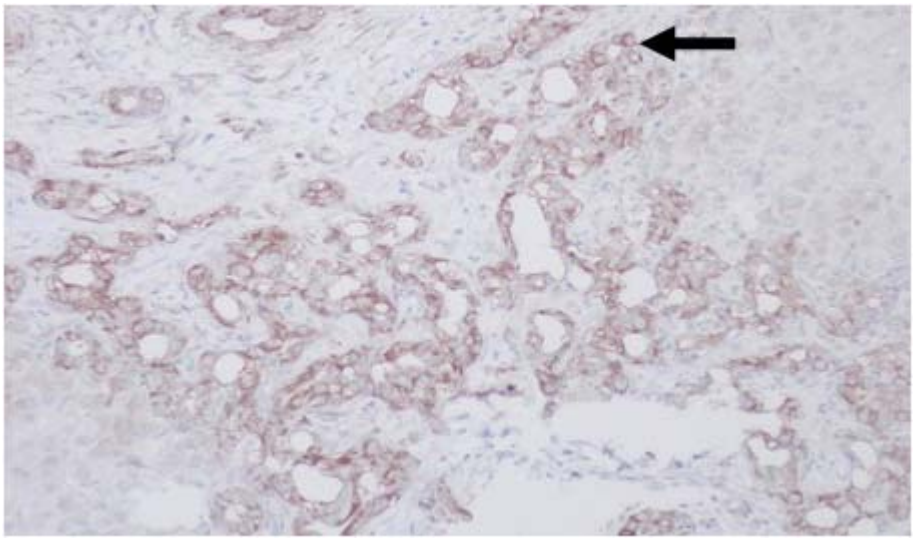

Figure 2. Positive villin staining of biliary epithelial cells in a patient with biliary atresia (arrow)

Phillips MJ et al. reported a complete absence of villin protein and mRNA in purified canalicular membranes in conjunction with highly abnormal canalicular microvillus structure in children with cholestatic liver disease resembling BA [16]. They proposed that the functional absence of villin protein results in microvillus structural and functional changes, leading to progressive canalicular cholestasis and liver failure. Nevertheless, our study discloses the absence of villin immunohistological staining and villin mRNA expression in the liver of two infants with INH. Based on the long-term follow-up, all INH patients in the present study are free from jaundice. This finding implies that lack of villin does not cause biliary tract abnormality and chronic liver disease. Our finding is consistent with several studies showing that homozygous villin-deficient mice have intact microvilli in the small intestine, colon, kidney proximal tubules, and liver bile canaliculi [4, 17]. An *in vitro* study also demonstrated that villin mRNA was undetectable in normal rat liver [18].

Although villin is an important actin-binding protein, it plays a minor or redundant role in the generation of microvilli in multiple absorptive tissues. Villin may not be required for biliary microvillar morphogenesis or maintenance in human. The exact functional significance of villin on hepatic bile canaliculi remains to be elucidated.

Conclusion

The significant higher hepatic villin expression in BA might improve the value of standard liver biopsy in discrimination BA from INH. Consequently, BA patients can be timely diagnosed and surgery can be pursued promptly.

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