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Increased survivin expression and its association with clinical parameters of congenital choledochal cysts

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ABSTRACT. The aim of the current study was to investigate survivin expression in congenital choledochal cysts (CCCs), and its associations with clinical parameters of CCCs. In total, 121 children with CCCs were included in this study as the case group, and their cysts were staged according to the Todani classification system. Additionally, 49 normal gallbladder specimens from healthy children were included as the control group. Survivin detection was conducted using immunohistochemical staining. Associations between positive survivin expression and clinical parameters of CCCs were then analyzed. Positive survivin expression was observed in the cytoplasm, and was seen as granular with yellow or dark brown staining. In the case group, positive survivin expression was detected in most tissues. Specifically, compared to that of normal tissues, the cystic-shaped and fusiform-shaped CCC tissues had significantly higher positive survivin expression rates (all $P < 0.05$). Importantly, positive survivin expression was also shown to be significantly associated with gender and histological type (both $P <$

0.05). In conclusion, increased survivin expression was observed in CCC tissues, and was correlated with certain clinical parameters of CCCs, suggesting a possible prognostic value of survivin for CCC progression.

Key words: Congenital choledochal cysts; Protein expression; Survivin

INTRODUCTION

Congenital choledochal cysts (CCCs) are rare anomalies of the biliary tree found in the pediatric population that are characterized by either congenital cystic dilation of the extrahepatic or intrahepatic biliary ducts, and which are often associated with an anomalous junction of the pancreaticobiliary tracts (Liu et al., 2013). The prevalence of CCCs is generally higher among Asian populations than in the European and United States populations. Moreover, the incidence of CCCs among females is significantly higher than that among males, and accounts for 60 to 80% of the total incidence (Hung et al., 2011). Based on the Todani classification system first described in 1997, type I is the most common type of CCC, accounting for approximately 90% of all CCC cases (Todani et al., 2003). Although many theories have been put forth, the etiology of CCCs remains disputed and unclear, although most researchers insist that this disease is related to congenital pancreaticobiliary malformation and obstruction of the common bile duct (Liu et al., 2014). Additionally, bile duct dysplasia and viral infection may increase the risk of developing CCCs (Rauschenfels et al., 2009; Ohashi et al., 2013). Early diagnosis and appropriate treatment of CCCs are important, as these cysts are associated with a risk of carcinogenesis, and this risk increases with age. Although surgical treatments have significantly improved the survival outcomes of CCC patients, the long-term postoperative rate of complications such as cystolithiasis, cholangitis, acute pancreatitis, hepatolithiasis, and malignancies remains high (Gadelhak et al., 2014). Therefore, early recognition and proper management of CCCs are a focus of the current research.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, and is involved in the inhibition of apoptosis and regulation of cell division (Mita et al., 2008). Various clinical and experimental studies have shown that increased expression of survivin plays an important role in the development and progression of malignant neoplasms by reducing tumor cell apoptosis (Kim and McNiff, 2008; Mahalingam et al., 2010). This makes survivin a primary chemotherapeutic target, and a biomarker for the development and prognosis of various malignancies (Church and Talbot, 2012), including gastric (Bertazza et al., 2009), colorectal (Rödel et al., 2008), and bladder cancers (Margulis et al., 2008). As a multi-functional protein involved in inhibiting apoptosis, regulating cell division, and promoting angiogenesis, survivin can be expressed at significantly different levels in normal and malignant cells, where extremely low or absent expression levels have been observed in normal tissues, whereas elevated levels have been observed in various solid tumors (Li and Ling, 2006; Mishra et al., 2015). To date, few studies have reported on the relationship between survivin and the clinicopathological features of CCCs. Therefore, the aim of the current study was to investigate the associations between survivin expression and the clinical parameters of CCCs.

MATERIAL AND METHODS

Ethics statement

This case-control study was approved by Shenzhen Children's Hospital. Written

informed consent was obtained from the legal guardians of all enrolled pediatric subjects, and the study conformed to the guidelines and principles of the Declaration of Helsinki (General Assembly of the World Medical Association, 2014).

Subjects

One hundred and twenty-one children with CCCs who were treated at Shenzhen Children's Hospital from January 2010 to January 2014 were enrolled in this study as the case group. All patients were pathologically diagnosed with CCCs and the diagnoses were confirmed by surgery, with complete pathological and clinical data obtained for all patients. Additionally, all included patients had no other systemic diseases nor were their cases complicated by tumors. The study population consisted of 50 males and 71 females with a mean age of 5.44 ± 4.10 years. The cysts of all included CCC patients were staged according to the Todani classification system as follows: stage I, N = 111; stage III, N = 2, and stage IV, N = 8 (Todani et al., 2003). Additionally, 49 normal gallbladder specimens from healthy children were obtained as the control group. This normal control group was examined by abdominal B ultrasound or CT for the exclusion of liver and gallbladder diseases, and all included normal tissues were confirmed by histopathologic examination.

Immunohistochemistry (IHC)

All collected specimens were fixed in 10% neutral buffered formalin, dehydrated through an ethanol gradient (30, 50, 70, 80, 95, and 100% ethanol), hyalinized in xylene, and embedded in conventional liquid paraffin for immunohistochemical staining. The paraffin-embedded specimens were sectioned into four consecutive 5 μm-thick slices. All standard IHC reagents were provided by the Immunohistochemistry Laboratory of the Department of Pathology, Tongji Medical College, China. The primary mouse anti-human survivin monoclonal antibody was purchased from Wuhan Boster Biological Engineering Co., Ltd. (China). Known positive sections were used as a positive control, and phosphate-buffered saline (PBS) was substituted for the primary antibody as a negative control. After dewaxing and hydration, the paraffin sections were washed three times for 3 min each in PBS, pH 7.4. Subsequently, 50 μL (1 drop) peroxidase blocking solution was added onto each section to block the activity of endogenous peroxidases, followed by a 10-min incubation at room temperature. Following another three washes in PBS (3 min each), 50 μL (1 drop) non-immune animal serum was added onto each section, and then incubated for 10 min at room temperature. After removing the serum, 50 μL (1 drop) primary anti-survivin antibody was added onto each section, followed by a 60-min incubation at room temperature or overnight at 4°C. Then, the sections were washed three times in PBS (5 min each). After removing the PBS, 50 μL (1 drop) biotin-labeled secondary antibody (goat anti-mouse IgG, purchased from Southern Biotechnology Associates, Birmingham, AL, USA) was added onto each section, and then incubated at room temperature for 30 min. Following another three washes in PBS (3 min each), the PBS was removed, and 50 μL (1 drop) streptavidin-biotin-peroxidase solution was added onto each section, and the specimens were incubated at room temperature for 10 min. The sections were then washed three times with PBS (3 min each), then washed under tap water, slightly counterstained with hematoxylin, decomposed by 0.1% HCl, rinsed with 0.1% ammonia or PBS, and then converted to blue. The sections were then colored with the

StreptABComplex/DAB Kit (Lab Vision Corporation, Fremont, CA, USA) in accordance with manufacturer protocols. Finally, the sections were dehydrated and dried through an ethanol gradient (as mentioned above), hyalinized in xylene, and sealed with neutral gum.

Immunohistochemical analysis

Positive expression of survivin was mainly observed in the nucleus/cytoplasm, and was visualized as yellowish-brown or yellow granular staining. In contrast, ≤5% cells exhibited brown particles in the cytoplasm, which were interpreted as survivin-negative under normal light microscopy. The slides were observed using a Nikon Eclipse E600 light microscope (Tokyo, Japan), and the positively labeled cells were counted in five randomly selected fields and expressed as the percentage of total cells. Each specimen was scored based on the percentage of positive cells (<1%: 0 points; 1-25%: 1 point; 25-50%: 2 points; 51-75%: 3 points; and >75%: 4 points) and staining intensity (colorless: 0 points; light yellow: 1 point; brownish-yellow: 2 points; and dark yellow: 3 points). The final scoring was determined according to the product of the two scores, with 0 points defined as negative (-), 1-2 points as positive (++), and >2 points as strongly positive (+++). Determination was conducted under double-blind conditions.

Statistical analysis

The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all data analyses. χ^2 and Spearman rank correlation tests were performed for correlation analyses. P values <0.05 indicated significant differences.

RESULTS

Clinicopathological characteristics

Of the 121 CCC patients aged 1 month to 15 years old (mean age of 5.44 ± 4.10 years), 50 were males and 71 were females. Of these CCC cases, 67 were cystic type and 54 were fusiform type. The CCC patients were further categorized into stage I (N = 111), stage III (N = 2), and stage IV (N = 8) based on the Todani classification system. Furthermore, there were 81 cases with combined bile duct, 15 cases with stenosis of the left hepatic duct, 11 cases with stenosis of the right hepatic duct, 8 cases of stenoses of both the right and left hepatic ducts, as well as 6 cases of stenosis of the common hepatic duct. The current study also included 49 healthy children aged 4 months to 11 years old (mean age of 4.84 ± 3.87 years) as the control group, which consisted of 16 males and 33 females. There were no significantly differences regarding age or gender between the case and control groups (both P > 0.05).

Evaluation of survivin protein expression by IHC

As shown in Figure 1, the cytoplasm showed a high expression of survivin as seen as positive staining, which presented in granular form, with yellow or dark brown color, whereas the nuclei were hardly stained. Sixteen of 49 normal tissues (32.65%) in the control group stained positive for survivin (Table 1). In contrast, most of the tissues in the case group stained

positive for survivin conferring survivin expression, including 56 of the 67 cystic-shaped tissues (83.58%) and 32 of the 54 fusiform-shaped tissues (59.26%), and these surviving-positive rates were found to be significantly higher than that of normal tissues (both $P < 0.05$).

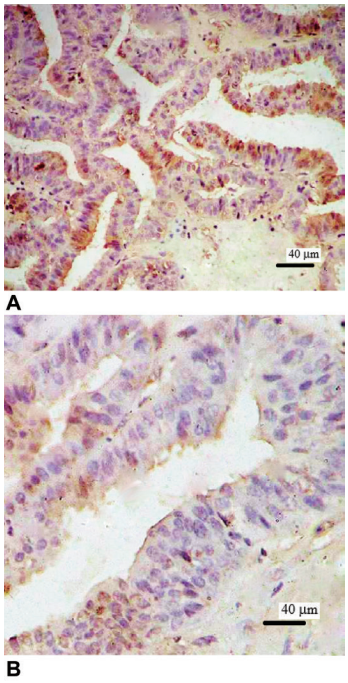

Figure 1. Immunohistochemical staining results for the protein expression of survivin. The cytoplasm showed high survivin expression, which presented as granular form, with yellow or dark brown staining, while the nuclei were hardly stained (**A.** cytoplasm, 200X; **B.** nuclei, 400X).

Table 1. Expression of survivin in cystic-shaped and fusiform-shaped tissues of congenital choledochal cyst patients, and in normal tissues from healthy children.

	N	Survivin expression			Positive rate (%)
		-	+	++	
Normal tissues	49	33	16	0	32.65
Cystic-shaped tissues	67	11	25	31	83.58
Fusiform-shaped tissues	54	22	19	13	59.26

In the comparison of the cytoplasm of normal tissues with the cystic-shaped and fusiform-shaped tissues, $P < 0.05$.

Associations between survivin expression and clinicopathologic parameters of CCCs

As shown in Table 2, survivin expression was not markedly correlated to age, Todani stage, or diseases sub-type (all $P > 0.05$). On the other hand, survivin expression was found to be statistically correlated with sex and histological type. Specifically, there were significantly higher positive survivin expression rates in female patients and patients with cystic-shaped CCCs than those of male patients and patients with fusiform-shaped CCCs, respectively (both $P < 0.05$).

Table 2. Associations between survivin expression and clinical pathologic parameters of congenital choledochal cysts.

Parameters	Cases	Positive expression of survivin		χ^2	P value
		Positive (%)	Negative (%)		
Gender					
Male	50	30 (60.00)	20 (40.00)	6.959	0.008
Female	71	58 (81.69)	13 (18.31)		
Age					
≤5 years	78	55 (70.51)	23 (29.49)	0.543	0.461
>5 years	43	33 (76.74)	10 (23.26)		
Histological type					
Cystic-shaped	67	56 (83.58)	11 (16.42)	5.755	0.016
Fusiform-shaped	54	32 (59.26)	22 (40.74)		
Todani stage					
Stage I	111	78 (70.27)	33 (29.73)	2.726	0.099
Stages III + IV	10	10 (100.00)	0 (0.00)		
Diseases sub-types					
Combined bile duct	81	63 (77.78)	18 (22.22)	5.055	0.271
Stenosis of the left hepatic duct	15	9 (60.00)	6 (40.00)		
Stenosis of the right hepatic duct	11	6 (54.55)	5 (45.45)		
Stenoses of the right and left hepatic ducts	8	5 (62.50)	3 (37.50)		
Stenosis of the common hepatic duct	6	5 (83.33)	1 (16.67)		

DISCUSSION

The aims of the current study were to investigate associations between survivin expression and clinical parameters of CCCs, and to explore the relationship of survivin expression and CCC progression. According to our observations, survivin was positively expressed in nearly twice as many cystic-shaped and fusiform-shaped tissues compared with that in normal tissues, indicating that the upregulation of survivin may occur in the early stages of CCC development. The deregulation of apoptosis has emerged as a hallmark of human malignancies and carcinogenesis (Fuhrman et al., 1982; Wang et al., 2013; Uchida et al., 2013). In this regard, the IHC results of this study showed that there was a significantly higher positive expression rate of survivin in CCC tissues than that in normal tissues, which in turn provides evidence of the occurrence of apoptosis deregulation in CCC tissues, and strengthens the case for survivin to predict the existence of deregulated apoptosis. Survivin exhibits many biological effects, including multiple anti-apoptotic functions, which are mainly attributable to its differential subcellular localization, phosphorylation, and acetylation (Tyner et al., 2012; Chang et al., 2013). Although belonging to the IAP family, survivin is prominently expressed in the vast majority of neoplasms and not in normal differentiated tissue, unlike other IAP members (Brustmann et al., 2011). Through survivin detection, several previous studies have shown that the selective expression of this protein in malignant tissues makes it an attractive therapeutic target for different malignancies (Fukuda and Pelus, 2006; Vandghanooni et al., 2011). From a biological standpoint, survivin suppresses apoptosis, regulates cell division, and promotes angiogenesis (Mita et al., 2008). For example, Margulis et al. (2008) demonstrated that inhibition of survivin expression impedes tumor cell proliferation and promotes spontaneous or chemotherapy-induced apoptosis in a pre-clinical bladder tumor model. Additionally, Liu et al. (2011) found that gallbladder cancer, which is characterized by rapid progress, poor prognosis, and aberrantly high survivin expression, was inhibited by survivin promoter-regulated oncolytic adenovirus carrying the *P53* gene. These

aforementioned studies, in addition to the results herein, suggest that detection of survivin may have a diagnostic/prognostic value for CCCs. Importantly, there was an evident difference in survivin expression between the cystic-shaped and fusiform-shaped tissues, where higher survivin expression was found in the former. A possible explanation for these observations may be that the apoptotic rate of epithelial cells has been shown to be positively correlated with the degree of duct damage, highlighting the essential role of cell apoptosis and relevant markers thereof in the progression of this disease. Cystic-shaped CCCs are characterized by relatively large sizes and thick cyst walls, with moderate to large numbers of elastic and reticular fibers (Ahanatha Pillai et al., 2012). In contrast, fusiform-shaped CCCs are relatively smaller and have thinner walls without elastic or reticular fibers (De and Basu, 2007). Survivin expression may therefore be different between cystic-shaped and fusiform-shaped CCCs due to their structural differences. Additionally, the results of this study demonstrated that positive survivin expression was significantly higher in female CCC patients than that in males. Since CCCs have a high rate of carcinogenesis, the identification of useful early detection biomarkers would be important for the diagnosis and treatment of this disease. Epidemiological investigation has found that CCCs have a relatively higher incidence in females than in males (Shanmugam et al., 2005), which in turn may explain why survivin expression levels were observed to be higher in females herein.

In the current study, we did not investigate the signaling pathways of survivin in CCCs, and thus our data do not provide any mechanistic explanations for our observations. Another limitation of our study lies in the relatively small sample size, which may have affected our results. Additionally, our study measured survivin *in vitro*, which may also have biased our results. Therefore, future efforts should focus on the mechanistic role of survivin and its complex interactions with other clinical biomarkers in CCCs, include lager sample sizes, and measure survivin both *in vivo* and *in vitro*.

In conclusion, we provide strong evidence that survivin is positively expressed in CCC tissues, and may be correlated with clinicopathological features of CCCs. The results of the current study could be enhanced by further studies and effective meta-analyses of the cumulative results, which may lead to better diagnostics, prognostics, and treatments for CCCs in the future. Therefore, additional efforts are needed to provide more powerful insights on this topic.

Conflicts of interest

The authors declare no conflict of interest.

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