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Wntless Is Highly Expressed in Advanced-Stage Intrahepatic Cholangiocarcinoma

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Intrahepatic cholangiocarcinoma (ICC) is an aggressive malignancy of the bile duct, representing the second most common primary liver cancer. Wntless (Wls) is a highly conserved transmembrane protein that shuttles palmitoylated Wnt proteins from the endoplasmic reticulum to the plasma membrane. Wls is highly expressed in various types of cancers and is essential for cell proliferation, anti-apoptotic activity, and survival. The profile of Wls expression and its clinical significance has not been well clarified in ICC. In the present study, we analyzed Wls expression in a set of ICC tissues (n = 44) by immunohistochemistry and the relationship between Wls expression and clinicopathological parameters. Immunoreactive Wls was detected in normal cholangiocytes, but was undetectable in normal hepatocytes. The intensity for immunoreactive Wls was varied, depending on ICC specimens. The degree of Wls expression was scored as 0 or 1+ in 8 specimens (18.2%), 2+ in 24 (54.5%), and 3+ in 12 (27.3%) out of the 44 ICC specimens, based on the staining intensity and percentage of Wls-positive cells. In normal cholangiocytes, the scores were varied from 0 to 2+. The intensity of Wls expression was positively associated with tumor stage (T stage, $P = 0.005$, $r = 0.413$), tumor-node-metastasis stage (TNM stage, $P = 0.000$, $r = 0.548$), and lymphatic invasion ($P = 0.000$, $r = 0.548$). Our results show that Wls is differentially expressed in ICC tissues and positively related to tumor stage and lymphatic invasion. Wls is a potential marker for advanced tumor stage and metastasis in ICC.

Keywords: immunohistochemistry; intrahepatic cholangiocarcinoma; tumor stage; Wnt; Wntless
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Introduction

Cholangiocarcinoma (CCA) is anatomically classified as intrahepatic, perihilar, or distal cholangiocarcinoma. Intrahepatic cholangiocarcinoma (ICC) is an aggressive epithelial malignancy of the bile ducts within the liver that is often locally advanced to metastatic disease and has an extremely poor prognosis. The incidence of CCA varies among different countries. The highest rate is in Northeast Thailand (> 80 per 100,000 population), whereas the rate is much lower in Western countries such as Canada (0.3 per 100,000 population) (Bridgewater et al. 2014). ICC is the second most common primary liver cancer in adults, next to hepatocellular carcinoma. The incidence and mortality of ICC has increased recently worldwide, including the United States (Khan et al. 2012; Saha et al. 2016). Despite improved diagnostic and operative techniques, the prognosis of ICC remains very poor (Yao et al. 2014). ICC is a highly fatal disease and is due to its widespread metastasis, early invasion, and no effective therapy. Complete surgical resection with histologically negative resection margins is currently the only curative treatment, therefore there is an urgent need to find the new molecular mechanisms of ICC and find new molecular targets to improve the treatment outcomes.

Wntless (Wls), also known as Evi, Sprinter, or GPR177, is a highly conserved transmembrane protein located in compartments of the secretory pathway including the Golgi apparatus, endosomes, and plasma membrane (Banziger et al. 2006). Wls shuttles palmitoylated Wnt proteins from the endoplasmic reticulum to the plasma membrane as a Wnt cargo receptor. It is also required for exocytosis of Wnt protein from Wnt-producing cells (Belenkaya et al. 2008; Franch-Marro et al. 2008; Yang et al. 2008). Activation of Wnt signaling has been implicated in the development of many cancers, and there are a few studies

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on the role of Wnt proteins in ICC. Wang et al. (2015) reported the underexpression of liver kinase B1 (LKB1), a tumor suppressor, in ICC as a result of gene deletion and missense mutation. They knocked down the *LKB1* gene in ICC cells and found dramatically enhanced Wnt/*β*-catenin signaling (Wang et al. 2015). Zhao et al. (2014) investigated the clinical significance of a chemokine receptor gene, *CXCR4*, in ICC. They showed that CXCR4 expression was correlated with ICC progression and metastasis-related characteristics. *CXCR4* knockdown resulted in downregulation of Wnt target genes. Their data indicated that high *CXCR4* expression is associated with ICC progression and metastasis via the canonical Wnt pathway. In previous studies, we investigated Wls expression and its clinical significance in gastric cancer and colorectal cancer and showed that Wls protein was differentially expressed in gastric cancer cell lines and tissue samples (Zhang et al. 2017). High expression of Wls in gastric cancer was positively associated with well-differentiated tumor, lymph node metastasis, and advanced TNM stage. High expression of Wls was also observed in most colorectal cancers, and was related to sex, depth of invasion, lymph node metastasis, and TNM stage (Xu et al. 2016). More recently, we investigated Wls expression in hepatocellular carcinoma (HCC) and its clinicapathological significances (Zhou et al. 2017). Wls expression was hardly detected in normal liver tissues, weakly detected in liver cirrhosis, and differentially detected in HCC. Wls expression was related to tumor size, TNM stage, and HBV infection.

In the present study, we detected Wls protein in a set of ICC tissues and statistically analyzed the relationship between Wls expression and clinicopathologic parameters. To the best of our knowledge, this is the first report that shows the Wls expression in ICC.

Materials and Methods

Patients and specimens

Formalin-fixed, paraffin-embedded tissues from 44 patients with ICC between 2014 and 2016 were collected from the Department of Pathology of the First Affiliated Hospital of Nanjing Medical University. All patients who enrolled in this study underwent hepatectomy without neoadjuvant chemotherapy and other preoperative treatments. The histology of each specimen was confirmed by review of Hematoxylin and Eosin staining slides. Clinicopathological parameters are listed in Table 1. Twenty out of 44 ICC specimens included normal liver tissues and 7 included normal bile duct tissues. This project was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University.

Immunohistochemistry

Immunohistochemistry was routinely carried out using an Envision Plus system and DAB kit (Kako, Glostrup, Denmark), the detailed protocol was reported previously (Sun et al. 2017). Briefly, the slides were deparaffinized using xylene, and then dehydrated and rehydrated. The antigen was retrieved by autoclave (120°C for 2 min) with 1 mM EDTA (pH 8.0). Hydrogen peroxide (3%) in methanol was used to block endogenous peroxidase activity and 10% goat serum was used to block nonspecific binding. A monoclonal antibody specific for human Wls (dilution 1:200, EMB Millipore, Temecula, CA, USA) was incubated overnight at 4°C. A secondary antibody (Dako, Cambridge, UK) was added. Slides were colored with 3, 3′-diaminobenzidine (DAB) and then counterstained. A colorectal cancer tissue specimen expressing Wls was used as a positive control and PBS replaced the relevant primary antibody as a negative control (data not shown).

Immunohistochemistry score

Wls expression level in ICC was semi-quantitatively analyzed, as described previously (Zhou et al. 2017). The specimens were scored as 1-3 based on the intensity of staining. The staining was scored as 0: no color; 1: weak light yellow; 2: medium brown-yellow; and 3: dark brown. Wls expression level was further graded on the percentage of stained ICC cells as 0 for complete absence of staining; 1 for < 25%; 2 for 25-50%, and 3 for > 50%. The final Wls expression level was summed by staining intensity and staining extent scores and was as follows: 0-2, score 0 or 1+ (0/1+); 3-4, score 2+; and 5-6, score 3+.

Statistical analysis

SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Spearman test was used to analyze the association of different levels of Wls expression (0/1+, 2+, and 3+) with clinicopathological features. *P* values less than 0.05 were considered as statistical significance.

Results

Wls expression in ICC specimens

Wls expression level and its cellular distribution in ICC cells and normal hepatocytes were checked using an immunohistochemical staining. Wls protein staining was observed as brown distributed in the cytosol. The intensity of Wls immunoreactivity was low in normal hepatocytes, scored 0 or 1+ in all specimens (Fig. 1A). The intensity of Wls immunoreactivity was variable in normal cholangiocytes (Fig. 1B).

The expression of Wls in ICC specimens was heterogeneous inter-specimens. As listed in Table 1, Wls expression was categorized as score 0/1+ in 8 (18.2%), score 2+ in 24 (54.5%), and score 3+ in 12 (27.3%) out of 44 samples (Fig. 2).

Association of clinicopathologic characteristics with Wls in ICC

The relationship between clinicopathologic features and Wls expression in 44 ICC patients are listed in Table 1. The association of Wls expression level with clinicopathologic characteristics was statistically analyzed. Our data show that Wls expression was positively associated with tumor stage (T stage, $P = 0.005$, $r = 0.413$), tumor-node-metastasis stage (TNM stage, $P = 0.000$, $r = 0.548$) and lymphatic invasion ($P = 0.000$, $r = 0.548$). We did not found any significant relationship between Wls expression and age, sex, tumor differentiation, tumor diameter, or vascular invasion.

Table 1. Relationship between Wls expression and clinicopathological features (n = 44).

Variables		Wls expression levels			*P**	r_s
		0/1+	2+	3+		
		8	24	12		
Age (years)						
	< 55	2	10	4	0.831	–0.033
	≥ 55	6	14	8		
Sex						
	Male	6	14	7	0.527	0.098
	Female	2	10	5		
Differentiation						
	poorly	0	0	0		
	moderately	8	14	10	0.730	0.054
	well	0	10	2		
Diameter						
(cm)	< 2	2	2	2	0.765	0.046
	≥ 2	6	22	10		
T stage						
	T1/T2	6	14	2	0.005	0.413
	T3/T4	2	10	10		
TNM stage						
	I+II	7	14	1	0.000	0.548
	III+IV	1	10	11		
Lymphatic						
invasion	Absent	7	14	1	0.000	0.548
	Present	1	10	11		
Vascular						
invasion	Absent	2	4	5	0.284	–0.165
	Present	6	20	7		

*The Spearman test was used to analyze the association of Wls expression with clinicopathological features among three groups.
$P < 0.05$, considered statistically significant.

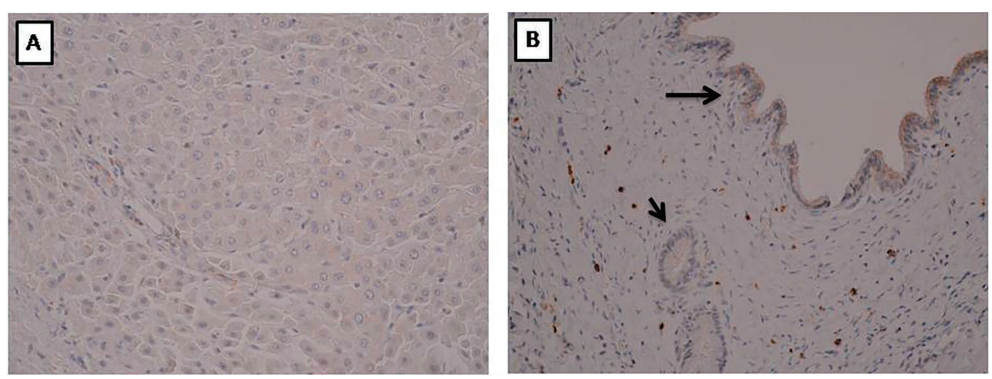

Fig. 1. Wls expressed in normal hepatocytes and cholangiocytes.
A: Immunoreactive Wls was undetectable in normal hepatocytes (score 0, magnification × 400). B: Wls was expressed in normal cholangiocytes. Wls was expressed as score 2+ in big bile duct (big arrow) and as score 0 in normal small bile duct as small arrow showed (magnification × 200).

Discussion

Despite recent developments in imaging techniques and laboratory tests, the diagnosis of ICC remains challenging. Identifying ICC-specific diagnostic and prognostic biomarkers has been a focus of many researchers. Some biomarkers underlying ICC carcinogenesis with potential diagnostic and prognostic application were previously reported (Terashita et al. 2016; Yang and Zong 2016; Yang et al. 2016; Huang et al. 2017; Rahnemai-Azar et al. 2017).

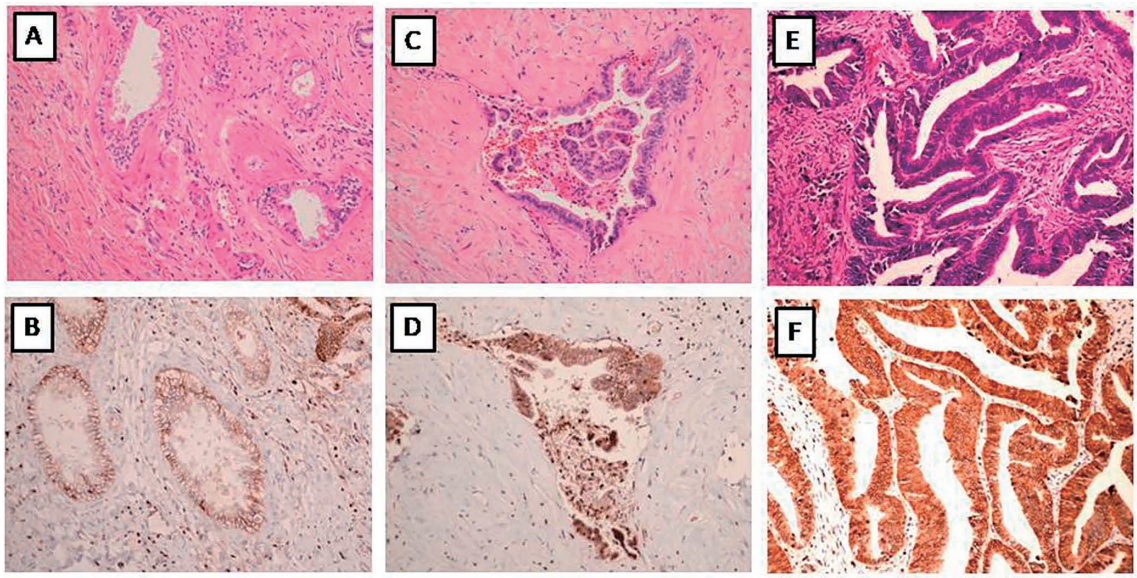

Fig. 2. Representative examples of Wls expression in ICC.
A: H&E staining to confirm the pathological diagnosis of ICC. B: Immunohistochemistry of the consecutive tissue section of A. Wls expressed in ICC as score 1+ (weak light yellow staining). C: H&E staining of ICC. D: Immunohistochemistry of the consecutive tissue section of C. Wls expressed in ICC as score 2+ (medium brown-yellow staining). E: H&E staining of ICC. F: Immunohistochemistry of the consecutive tissue section of E. Wls expressed in ICC as score 3+ (dark brown staining). (All magnification × 400).

This study is the first report of the Wls expression in ICC. Wls protein was expressed in most of the ICC tissues tested (81.8%, score 2+ and 3+). Wls has been reported to be involved in the development of certain human cancers (Maruyama et al. 2013; Lu et al. 2015; Stewart et al. 2015; Zhang et al. 2017). Wls is overexpressed in malignant astrocytomas and promotes downstream Wnt signaling (Augustin et al. 2012). Augustin et al. (2012) examined Wls protein level in normal and astrocytic tumor tissues and found that Wls was highly expressed in tumor cells of both low-grade and high-grade gliomas. Lu et al. (2015) reported high expression levels of Wls mRNA and protein in breast cancer samples. The Wnt signaling pathway was previously shown to be involved in progression and metastasis of ICC (Zhao et al. 2014; Wang et al. 2015). Thus, we hypothesized that the tumor promotive effect of Wls in ICC might be mediated through regulation of Wnt secretion and its downstream signaling. Further studies to explore the roles of Wls in Wnt signaling and in carcinogenesis of ICC are needed.

ICC is a rare and highly aggressive malignancy. The incidence of ICC in the United States continues to increase, whereas the incidence of extrahepatic cholangiocarcinoma is stable (Saha et al. 2016). ICC is considered as primary intrahepatic liver cancers along with hepatocellular carcinomas, while extrahepatic tumors along with malignancies of the gall bladder are derived from bile duct(Farges and Fuks 2010). Patients with ICC typically present at advanced stages, and the vast majority of patients with unresectable disease die between 6 to 12 months following diagnosis (Bridgewater et al. 2014).The lack of effective therapeutic strategies is partially attributed to poor understanding of the underlying molecular mechanisms. In this study, we demonstrated that Wls is highly expressed in ICC and that Wls expression is positively associated with tumor stage, tumor-node-metastasis stage, and lymphatic invasion. Wls may be used as a molecular marker for advanced tumor stage and metastasis in ICC.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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