RESEARCH ARTICLE

Expression of Smad7 in Cholangiocarcinoma: Prognostic Significance and Implications for Tumor Metastasis

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Abstract

Background: There are few molecular markers known to predict cholangiocarcinoma (CCA) prognosis. Smad7 has a certain relationship with epithelial-mesenchymal transition (EMT), but its relevance to CCA in unclear. Therefore expression and clinical significance of Smad7 in CCA was the focus of this study. Methods: Expression of Smad7, E-cadherin and vimentin was assessed in 41 patients with CCA by immunohistochemistry and analyzed for associations with clinical parameters. Results: Smad7 and vimentin expression in the CCA tissue was dramatically higher than that in adjacent tissues. In addition, Smad7, vimentin and E-cadherin expression was significantly associated with CCA lymph node metastasis and perineural invasion(P≤0.05), but not other factors, such as gender, age, tumor location, tumor type and tumor differentiation degree (P>0.05). The overall survival and relapse-free survival rate was significantly higher in patients with negative Smad7 expression than those with positive Smad7 expression. Conclusion: EMT phenomena may occur in the process of CCA invasion and metastasis. Smad7, which was highly expressed in CCA, may be considered to be one feedback regulator in late stages and could have potential as a prognostic indicator for clinical assessment.

Keywords: Smad7 - epithelial mesenchymal transition - cholangiocarcinoma - metastasis - prognostic indicator

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Introduction

Cholangiocarcinoma(CCA) is aggressive tumor originated from bile duct epithelial cells. It is the second most common subtype of primary hepatobilliary cancer, accounting for about 3% of gastrointestinal tumors and its incidence is rising (Aljiffry et al., 2010). Only radical resection can make patients get cured.But postoperative tumor recurrence rate is very high, which also often associated with tumor invasion and metastasis (Li et al., 2011).

Within the primary tumor microenvironment, there were many factors can induced tumor metastasis. A large number of studies showed that these metastasis-promoting effects of factors are through the stimulation of tumor cell migration, invasion, intravasation, and enhancement of angiogenesis(Iiizumi et al., 2008; Li et al., 2010; Berge et al., 2011).Recently, increasing evidence has suggested that epithelial mesenchymal transition (EMT) is one of the key mechanisms which is often activated during cancer invasion and metastasis (Peinado et al., 2007; Iwatsuki et al., 2010). EMT is a biological process that allows a epithelial cell to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. During this process, there is an induction of mesenchymal markers, such as vimentin, and epithelial markers disappear, like E-cadherin that is essential for the structural integrity of epithelium (Neilson et al., 2006). Because E-cadherin loss can causes the disassembly of inter-cellular adhesion complexes, thus loosening contacts between neighbouring epithelial cells and disrupting the overall tissue architecture. However, Vimentin is a component of type III intermediate filaments and the archetypal mesenchymal marker most commonly used to categorize EMT. That vimentin expression is a late event in EMT points to a temporal sequence of genetic events in which loss of epithelial features directly precedes and leads to up-regulation of mesenchymal genes (Kokkinos et al., 2007; Polette et al., 2007).

Smads signal pathways is one of the important pathways involved in EMT process (Sabe et al., 2007; Liu et al., 2007). As we all know Smad7 is an inhibitory Smad, which can inhibit TGF-β. Recent research has documented the role of Smad7 in tumorigenesis and progression in cancer.It is regulate TGF-β1 signal pathway by inhibiting the phosphorylation of Smad2,3 (Hayashi et al., 1997; Lan et al., 2011). Despite the importance of Smad7 in EMT and cancer progression, its expression and underlying molecular mechanisms of action have not been well characterized in CCA. In this study, we detected the expressions of Smad7, E-cadherin, Vimentin in CCA and adjacent tissues,exploring the relationship between Smad7

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and EMT process and possible molecular mechanism in CCA. Further,we explore its prognostic significance in patients with CCA. To our knowledge, this also is the first study that establishes the link among Smad7 and EMT in human CCA .

Materials and Methods

Patients and tissues

Surgical specimens from 41 patients who had been surgically treated for CCA at Anhui Provincial Hospital, Hefei, form 1 January 2005 to 1 January 2010 were employed.All without radiotherapy or chemotherapy before operation. There were 25 cases of men,16 women, from 32 to 80 years old (mean 60±2.6 years). All tumors were clinically and histologically diagnosed as cholangiocarcinoma. Inclusion criteria for all cases included: (i) unambiguous histology and absence of mixed tumour types; (ii) sufficient viable tissue available for RNA extraction; (iii) absence of any treatment prior to surgery; and (iv) age of tissue block less than 7 years. The clinicopathological characteristics of the patients are given in Table 1. There were 10 cases of adjacent tissues form 2cm outside of the CCA were used as control.

Immunohistochemical staining

Tissues were fixed in 4% neutral buffered formalin, paraffin embedded, and sectioned (2μm). Smad7,E-cadherin,Vimentin were stained with SABC method used as instructions. The sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water,and antigen retrieval with citric acid (pH 6.0), heating in a microwave oven at high power for 3 min, and low power for 7 min.The sections were blocked with normal goat serum for 15 min then incubated at 4°C overnight with primary antibody (Smad7, mouse monoclonal IgG antibody, Stancruz, USA, E-cadherin, Vimentin, mouse monoclonal IgG antibody, Zhong san, China) and for 15 min each with biotinylated secondary antibody and S-A/HRP reagent.

Evaluation of immunostaining

There have been few studies dealing with the definite level of Smad7 positivity in immunohistochemical staining, so we applied the previously reported methodology on Smad7 positivity in immunohistochemical staining (Sandusky et al., 2002). For E-cadherin, ≥90% tumor cells with cytomembrane staining was taken to indicate normal expression, and <90% cytomembrane staining was taken to indicate decreased expression.For Vimentin, ≥20% tumor cells with cytoplasm staining was taken to indicate positive expression, with negative expression recorded otherwise (Nakajima et al., 2004). All slides were re-reviewed by two pathologists.

Statistical analysis

The associations between Smad7, E-cadherin,Vimentin and clinicopathologic features were assessed using Chi-square tests, the correlations of expression between different proteins were done by employing Spearman's rank Correlation tests.The overall Survival and relapse-free Survival curves were calculated using the Kaplan-Meier method and analyzed using the Log-rank test. The level of statistical significance was set at $P \leq 0.05$. These analyses were performed with SPSS Version 17.0 statistical software package (SPSS Corporation).

Results

Smad7, E-cadherin and Vimentin expression in CCA

Immunohistochemical shown the positive rate for Smad7, Vimentin respectively were 68.3% (28/41), 70.7% (29/41), expression of E-cadherin was decreased 51.2 (21/41)% in CCA.However, Smad7, E-cadherin and Vimentin protein positive expression rate respectively were 30% (3/10), 90% (9/10), 30% (3/10) in 10 cases of adjacent tissues. Smad7,E-cadherin and Vimentin expression in CCA (Figure 1). According to whether have nerve vascular invasion or lymph node metastasis is divided into the metastasis group and non-metastasisl

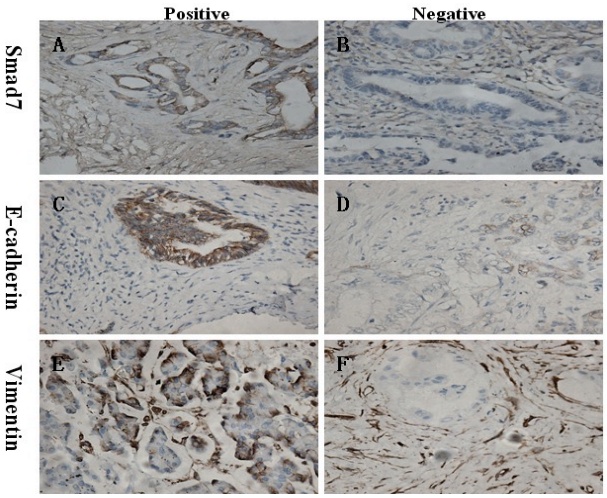

Figure 1. Smad7, E-cadherin and Vimentin Expression in CCA tissue. Smad7 and Vimentin in CCA Cytoplasm; E-cadherin in CCA Cytomembrane (A-F. 40×)

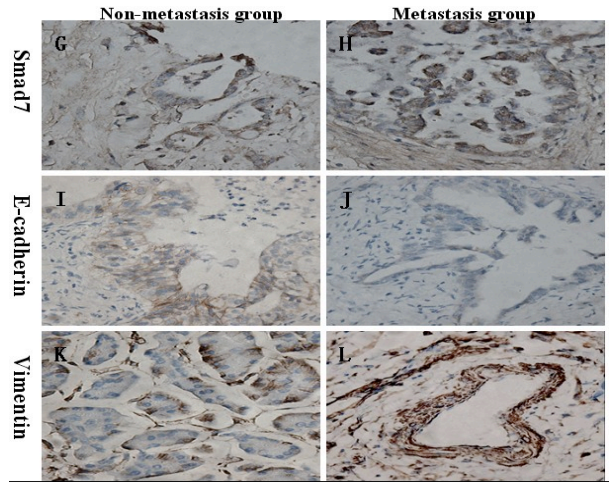

Figure 2. Smad7, E-cadherin and Vimentin in the Metastasis Group and Non-metastasisl Group Expression. The potisive rate for Smad7 expression was significantly higher in the metastasis group (H)than non-metastasisl group (G). However, E-cadherin expression result was contrary (I>J). The potisive rate for Vimentin was significantly higher in the metastasis group (L) than non-metastasisl group (K) (40×)

Table 1. Correlation of Clinicopathologic Parameters and Smad7 (E-cadherin) Vimentin Expression

Characteristic	n	Smad7		P	E-cadherin		P	Vimentin		P
		Positive	Negative		Decrease	Normal		Positive	Negative	
Gender				0.96			0.606			0.823
Male	25	17	8		12	13		18	7	
Female	16	11	5		9	7		11	5	
Age				0.691			0.91			0.514
<60 years	14	9	5		7	7		9	5	
≥60 years	27	19	8		14	13		20	7	
Tumor location				0.698			0.796			0.545
Hepatic portal	11	7	4		6	5		7	4	
Middle or inferior	30	21	9		15	15		22	8	
Differentiation				0.894			0.414			0.098
Well	10	7	3		4	6		5	5	
Moderate or Poor	31	21	10		17	14		24	7	
Histological type				0.408			0.959			0.337
Adenocarcinoma	37	26	11		19	18		27	10	
Others	4	2	2		2	2		2	2	
Lymph metastasis				0.009			0.031			0.016
Positive	15	14	1		11	4		14	1	
Negative	26	14	12		10	16		15	11	
Perineural invasion				0.025			0.019			0.008
Positive	20	17	3		14	6		18	2	
Negative	21	11	10		7	14		11	10	

Table 2. Association of Smad7 Expression and E-cadherin, Vimentin Expression in CCA (Spearman's Rank)

Characteristic	Smad7		r	P
	Positive	Negative		
E-cadherin			0.384	0.013
Decrease	10	10		
Normal	18	3		
Vimentin			0.368	0.018
Positive	23	6		
Negative	5	7		

group. The Smad7 and Vimentin expression was significantly higher in the metastasis group than that in the non-metastasisl group. However, E-cadherin expression results contrary (Figure 2).

Associations between Smad7, E-cadherin and Vimentin expression and clinicopathologic features

Smad7,E-cadherin and Vimentin expression was significantly higher in tumors showing in lymph node metastasis, perineural invasion (P≤0.05). Its has nothing to do with gender,age,tumor location,tumor type,tumor differentiation degree (P>0.05) (Table 1).

Relationship between Smad7, E-cadherin and Vimentin expression in CCA patients

We also investigated the relationship between Smad7 and E-cadherin,Vimentin expression. We found positive Smad7 expression was associated with decreased expression of E-cadherin and with increased expression of Vimentin (P≤0.05) (Tables 2). The results showed that a positive correlation between the degree of EMT and the protein expression level of Smad7.

Association of Smad7 expression with overall Survival and relapse-free Survival

We then performed Kaplan-Meier analyses to

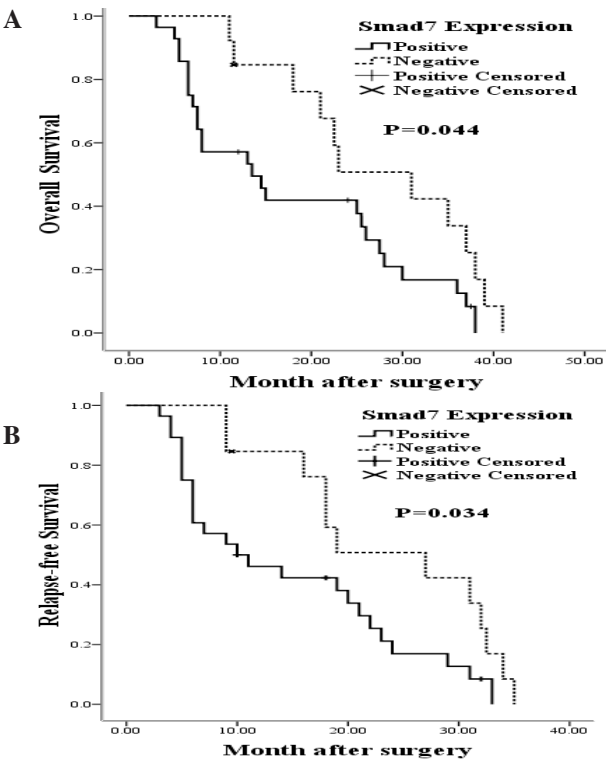

Figure 3. Association of Smad7 Expression with Overall Survival and Relapse-free Survival of the Patients with CCA. A, Overall survival (OS). B, Relapse-free survival (RFS)

determine whether Smad7 expression was associated with overall survival and relapse-free survival of the CCA patients. The 3-year survival rate was 14.3% in patients with Smad7-positive tumors and 30.8% in patients with Smad7-negative tumors. The overall survival rate was significantly higher in patients with negative Smad7 expression than in patients with positive Smad7 expression. The difference was statistically significant (P = 0.044, Figure 3A). Similarly,a statistically significant

association of Smad7 with relapse-free survival was also found (P = 0.034, Figure 3B).

Discussion

Smad7 play a feedback regulatory role in maintaining the balance of the TGF-β/Smads pathway and Its abnormal expression can promoting cell malignant progression ,So Smad7 has been considered as an oncogene (Pittman et al., 2009; Drabsch et al., 2012). This prediction has been supported in some cancer cases. Some studies found that with the degree of malignancy increasing in Oral tumors,the expression of Smad7 is becoming more and more obvious,indicating it extensive involvement with the tumor malignant process (Chen et al., 2012). This experiment found that the positive rates for Smad7 was 68.3% in CCA, however, Smad7 positive expression rate only was 30% (3/10) in 10 cases of adjacent tissues.The difference was statistically significant.It suggests that the abnormal expression of Smad7 may be involved in the malignant process of CCA, especially in tumor invasion and metastasis recurrence. Furthermore, some studies have shown that Smad7 also blocks TGF-β1-mediated growth inhibition in a variety of cell lines such as Mv1-Lu,keratinocytes, mammary epithelial cells, and COLO-357 pancreatic cancer cells (Kleeff et al., 1999; Zhu et al., 1999; Dahler et al., 2001). Some Researcher think increase in the incidence rate of CCA by Smad7 may involve (1) blockade of TGF-β-mediated tumour suppressor function by Smad7, (2) cooperation between mutated K-Ras and activated Smad2 generated as a result of metastasis and (3) cooperation between activated K-Ras and higher levels of Smad7 (Halder et al., 2012).

EMT in tumor leads to the loss of cell-cell adhesion and cell polarity as well as altered cell-extracellular matrix interactions,resulting in invasion and metastasis (Kalluri et al., 2012). Decreased expression of E-cadherin or Vimentin abnormal expression has gradually become an indicator of EMT changes (Sethi et al., 2012). With the development of tumor progression, tumor EMT phenomenon will become more apparent. In our experiment found that Vimentin expression was significantly higher in the metastasis group than non-metastasisl group (P≤0.05). However, E-cadherin contrary. We therefore deduce that EMT involved in the malignant process of CCA and it can promote CCA invasion and metastasis.

Some studies (Aimin et al., 2009) reported that Smad7 almost 100% expression in poorly differentiated gastric cancer tissues. The positive rates for Smad7 respectively were 70.0%, 87.50%, and 97.4% in highly, moderate and poorly differentiated cancer tissue. However, the expression was only 17.95% in adjacent tissues, these means the smad7 expression are widely involved in gastric cancer malignant process. Although there are some other experiments shown that Smad7 can hinder the EMT process,we found that the positive Smad7 expression was associated with decreased expression of E-cadherin and increased expression of vimentin in CCA .With the development of EMT phenomenon,the positive rate of Smad7 expression was becoming more obvious. We think it may be partly due to tumors of different or insufficient number of specimens.

Some researchers found that there have some significant correlations between Smad7 expression and postoperative disease-free survival. Smad7 expression,tumor invasion depth and lymph node metastasis as independent risk factors for the prognosis of gastric cancer (Kim et al., 2004). In this study, Kaplan-Meier analyses indicate that the overall survival rate was significantly higher in patients with negative Smad7 expression than in patients with positive Smad7 expression (P=0.044) in CCA. Similarly,a statistically significant association of Smad7 with relapse-free survival was also found (P=0.034). These results indicate that Smad7 protein expression may be as a postoperative prognostic evaluation indicator for clinical application, In addition, it should also be noted that in this study, the total sample size was small and the follow-up duration was relatively short, which might influence the overall validity of our analysis to some extent.

In summary, this study found that Smad7 expression is related closely with the malignant process of CCA, also confirmed that the EMT phenomenon occurred in CCA.However, in Yu H et al.'s study, found that with increased of Smad7 expression, EMT induced by TGF-β could was inhibited (Zhu et al., 2009; Yu et al., 2012), with the results of this paper is different, on the one hand, the authors take into account the activation of several signaling pathways involved in the process of tumor EMT, such as TGF-beta and Wnt, Notch, etc (Moustakas et al., 2007). Smad7 regulation the TGF-β/Smads pathway may not the major molecular mechanisms of participate in regulating cholangiocarcinoma EMT.On the other hand,with the development of the EMT process. Smad7 positive expressing rate was higher, which may be a negative feedback regulation of the body response to TGF-β1-induced tumor invasion and metastasis (Park et al., 2004; Gratchev et al., 2008).

To our knowledge, this is the first study that establishes the link among Smad7 and EMT in human CCA. In conclusion, our study demonstrated that Smad7, which proved to be a significant prognostic factor, might be an attractive target for the treatment of patients with CCA. Furthermore, further studies are needed to evaluate Smad7 functional role and signaling pathway in CCA cells EMT in vitro,and make we can effective inhibit tumor EMT process, thereby slow the process of tumor recurrence and metastasis.

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