Original Article

Digestive Diseases

Dig Dis 2016;34:702–707
DOI: 10.1159/000448860

Impact of Tight Junction Protein ZO-1 and TWIST Expression on Postoperative Survival of Patients with Hepatocellular Carcinoma

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Key Words
Epithelial-mesenchymal transition · Tight junction protein ZO-1 · TWIST · Survival · Hepatocellular carcinoma

Abstract
Background: Epithelial-mesenchymal transition (EMT) is considered to play a critical role in cancer progression and metastasis. However, the impact of EMT on the prognosis of hepatocellular carcinoma (HCC) is still elusive. In this study, we examined the relationship between the expression of EMT markers and recurrence-free survival (RFS) and overall survival (OS) in HCC patients after hepatic resection. ***Summary:*** The mRNA expression of 15 genes related to EMT was assessed by quantitative real-time polymerase chain reaction in cancerous tissues from 72 patients who underwent hepatic resection of HCC between January 2005 and December 2010 at our hospital. The upregulation of TWIST and the downregulation of tight junction protein ZO-1 (TJP1) were significantly associated with shorter RFS as well as OS. Increased levels of TWIST and decreased levels of TJP1 should be predictive markers for poor prognosis in patients with HCC after hepatectomy; those could serve as potential biomarkers for the treatment of HCC. ***Key Messages:*** A low level of TJP1 and high level of TWIST expression were prognostic factors predicting HCC after hepatic resection.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cause of cancer-related death in the world annually, and the development of new primary tumors, recurrences, and metastasis are the most common causes of mortality among patients with HCC [1–3]. HCC develops predominantly in an established background of chronic liver diseases mainly caused by persistent infection of hepatitis B [4] and/or hepatitis C virus, alcoholic liver disease, or non-alcoholic steatohepatitis [5, 6]. Meanwhile, growing evidence indicates that the epithelial-mesenchymal transition (EMT), a developmental process by which epithelial cells reduce intercellular adhesions and acquire fibroblastoid properties, has important roles in the acquisition of the invasive and metastatic potentials of cancer progression [7–10]. We have recently

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reported that sorafenib [11, 12] exerts a potent inhibitory activity against the EMT by inhibiting MAPK signaling and SNAI1 expression in HCC [13]. Chen et al. [14] reported that sorafenib inhibits transforming growth factor β1-mediated EMT and apoptosis in mouse hepatocytes. These papers reported that the anti-EMT effect of sorafenib may prolong overall survival (OS) in patients with unresectable HCC. Based on the previous analyses, it is conceivable that analyzing the relationship of the expressions of EMT markers with survival of HCC patients will provide critical information for the management of this disease. As EMT is critical for the invasiveness and metastasis of cancers, we specifically focused on the expression profiles of the 15 genes related to EMT in the context of survival of the HCC patients who had undergone hepatic resection. Here, we show the unique expression profile of the EMT-related molecules that affect the prognosis of the HCC patients who underwent hepatic resection.

Table 1. Patient characteristics

Age, years, median (range)	71 (40–80)
Gender (male/female)	54/18
TNM (I/II/III/IV)	4/20/28/20
Cause of disease (HBV, HCV/non B, non C)	13/39/20
Child–Pugh (5/6/7)	60/10/2
Histology (well/mod/poor)	16/46/10
Tumor size (<5/≥5 cm)	43/29
Previous therapy (yes/none)	23/49
Vascular invasion (yes/no)	34/38
Tumor number (1/2/≥3)	34/18/20
AFP, median (range)	15 (1–23,811)
PIVKA-II, average (SD)	101 (10–46,416)

Total 72 patients who underwent hepatic resection for HCC at Kinki University from 2005 to 2010.

Materials and Methods

Patients

Clinical research included 72 patients who underwent hepatic resection for primary HCC between January 2005 and December 2010 in the Department of Surgery, Kinki University Hospital. The mean age (range) of patients is 71 (40–80), 54 are male and 18 are female. The numbers of the patients carrying each stage of HCC are as follows: 4 for stage I, 20 for stage II, 28 for stage III and 20 for stage IV. Details of the patient characteristics and the clinical backgrounds are summarized in table 1.

RNA Extraction, cDNA Synthesis, and Real-Time Reverse-Transcription Polymerase Chain Reaction

We examined the mRNA expression of the following EMT-related molecules: Snail, Slug, zinc finger E-box binding homeobox 1, zinc finger E-box binding homeobox 2, TWIST, E-cadherin, N-cadherin, vimentin, fibronectin (FIN), discoidin domain receptor 2 (DDR2), S100 calcium-binding protein A4 (S100A4), tight junction protein ZO-1 (TJP1), forkhead box protein C2 (FOXC2), SIX homeobox 1 (SIX1), and Goosecoid (GSC). The mRNA was extracted from frozen surgical HCC samples. Real-time reverse-transcription (RT)-polymerase chain reaction (PCR) amplification was performed for quantification of target mRNA using glyceraldehyde 3-phosphate dehydrogenase (GAPD) as an internal control for normalization.

The condition of real-time RT-PCR has been described previously [15]. Briefly, 1 μg of total RNA from the cultured cells was converted to cDNA using a GeneAmp RNA-PCR kit (Applied Biosystems, Calif., USA). Amplification was performed using a Thermal Cycler Dice (Takara, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95°C for 6 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. GAPD was used to normalize the expression levels in the subsequent quantitative analyses. To amplify the target genes, the following primers were purchased from TaKaRa (Yotsukaichi, Japan): *CDH1* (E-cadherin), forward: 5′-TTA AAC TCC TGG CCT CAA GCA ATC-3′ and reverse: 5′-TCC TAT CTT GGG CAA AGC AAC TG-3′; *CDH2*, forward: 5′-CGA ATG GAT GAA AGA CCC ATC C-3′ and reverse: 5′-GGA GCC ACT GCC TTC ATA GTC AA-3′; SNAI1 (Snail1), forward: 5′-TCT AGG CCC TGG CTG CTA CAA-3′ and reverse: 5′-ACA TCT GAG TGG GTC TGG AGG TG-3′; SNAI2 (Snail12), forward: 5′-ATG CAT ATT CGG ACC CAC ACA TTA C-3′ and reverse: 5′-AGA TTT GAC CTG TCT GCA AAT GCT C-3′; VIM (vimentin), forward: 5′-TGA GTA CCG GAG ACA GGT GCA G-3′ and reverse: 5′-TAG CAG CTT CAA CGG CAA AGT TC-3′; FIN, forward: 5′-GGA GCA AAT GGC ACC GAG ATA-3′ and reverse: 5′-GAG CTG CAC ATG TCT TGG GAA C-3′; DDR2, forward: 5′-CCC AGC TGT CAG ATC AAC AGG TTA-3′ and reverse: 5′-TCA GGA CAA ATG GCT GGT TGA G-3′; S100A4, forward: 5′-GGG TGA CAA GTT CAA GCT CAA CAA-3′ and reverse: 5′-ATC ATG GCG ATG CAG GAC AG-3′; TJP1, forward: 5′-CGG GAC TGT TGG TAT TGG CTA GA-3′ and reverse: 5′-GGC CAG GGC CAT CAT AGT AAA GTT TG-3′; FOXC2, forward: 5′-CTA CAG CTA CAT CGC GCT CAT CA-3′ and reverse: 5′-ACT GGT AGA TGC CGT TCA AGG TG-3′; SIX1, forward: 5′-AAT GCC ATT ACT CAT GCC CTC A-3′ and reverse: 5′-CCA GGT TGC CAG ATT CGT TG-3′; GSC, forward: 5′-CAC CTC CGC GAG GAG AAA GT-3′ and reverse: 5′-GAC GAC GAC GTC TTG TTC CAC-3′; GAPD, forward: 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse: 5′-ATG GTG GTG AAG ACG CCA GT-3′.

Cell Culture and Migration Assay

The human HCC cell lines Huh7 were maintained in DMEM medium (Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37°C. The migration assays were performed using the Boyden-chamber methods and polycarbonate membranes with an 8-μm pore size (Chemotaxicell; Kurabo, Tokyo, Japan), as previously described [13]. The membranes were coated with FIN on the outer side and dried for 2 h at room temperature. The cells to be analyzed (2×10^4 cells/well) were then seeded onto the upper chambers with 200 μl of migrat-

Table 2. Univariate Cox regression analyses of RFS and OS of the patient characteristics and the expression of 15 genes related to EMT. Multivariate Cox regression analyses of RFS and OS of the patient characteristics of the patient characteristics and the expression of 15 genes related to EMT

	RFS			OS		
	univariate, p value	multivariate		univariate, p value	multivariate	
		HR (95% CI)	p value		HR (95% CI)	p value
Age, years	0.22		NA	0.88		NA
Gender (male/female)	0.86		NA	0.46		NA
TNM stage (I + II/III + IV)	0.17		NA	0.03*	9.22 (1.121–75.84)	0.039*
HBV history (no/yes)	0.71		NA	0.58		NA
HCV history (no/yes)	0.48		NA	0.59		NA
Child–Pugh (5/>5)	0.233		NA	0.168		NA
Histology (well + mod/poor)	0.003*	1.657 (0.721–3.81)	0.234	0.008*	5.526 (1.385–22.05)	0.015*
Tumor size (<5/≥5 cm)	0.28		NA	0.03*		NA
Previous therapy (no/yes)	0.752		NA	0.604		NA
Vascular invasion (no/yes)	0.02*	1.844 (0.947–3.59)	0.072	0.003*		NA
Tumor number (1/≥2)	0.06	1.891 (0.961–3.72)	0.065	0.48		NA
AFP (≤20/>20)	0.00017*	3.099 (1.514–6.344)	0.002*	0.07	1.427 (0.46–4.432)	0.538
PIVKA-II (≤120/>120)	0.21		NA	0.046*	3.331 (1.054–11.823)	0.041*
CDH1	0.68		NA	0.66		NA
CDH2	0.28		NA	0.12		NA
Snail	0.79		NA	0.79		NA
Slug	0.88		NA	0.83		NA
ZEB1	0.65		NA	0.37		NA
ZEB2	0.90		NA	0.65		NA
TWIST	0.004*	2.027 (1.143–3.595)	0.016*	0.019*	2.553 (1.309–4.979)	0.006*
Vimentin	0.51		NA	0.08		NA
FIN	0.61		NA	0.35		NA
DDR2	0.66		NA	0.33		NA
S100A4	0.48		NA	0.73		NA
TJP1	0.045*	0.45 (0.14–1.451)	0.181	0.002*	0.148 (0.027–0.805)	0.027*
FOXC2	0.83		NA	0.46		NA
SIX1	0.77		NA	0.87		NA
GSC	0.41		NA	0.98		NA

Variables with $p < 0.1$ in univariate analysis were adopted for multivariate analysis. TNM stage was included; other covariates including tumor size and vascular inavasion were excluded.

NA = Not applicable. * $p < 0.05$.

ing medium (DMEM containing 0.5% FBS), and the upper chambers were placed into the lower chambers of 24-well culture dishes containing 600 μl of DMEM containing 10% FBS. After incubation for 24 h, the media in the upper chambers were aspirated and the non-migrated cells on the inner sides of the membranes were removed using a cotton swab. The cells that had migrated to the outer side of the membranes were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% Giemsa stain solution for 15 min; they were then counted using a light microscope. The experiment was performed in triplicate.

Western Blot Analysis

The following antibodies were used in this study: TJP1 and β-actin antibody and HRP-conjugated secondary antibody (Cell Signaling Technology, Beverly, Mass., USA). All the experiments were performed in duplicate. The western blot analysis was performed as described previously [10].

Transfection of Small Interfering RNA

Three different sequences of small interfering RNA (siRNA) targeting human TJP1 (Hs-TJP1-5302, Hs-TJP1-7406) and those of 2 scramble control siRNA were purchased from Sigma Aldrich Japan (Tokyo). The details of the conditions of transfection have been described previously [13].

Statistical Analysis

Statistical analyses were performed to calculate the SD and to test for statistical significances between the samples using a Student t test and Pearson chi-square test. The gene expression data were normalized by means of the log10 transform. Univariate Cox regression analyses were run for each gene, as summarized in table 2. Subsequently, a multivariate Cox proportional hazard model was applied to identify independent prognostic factors for recurrence-free survival (RFS) and OS. The analysis of OS after hepatic resection was calculated using the Kaplan–Meier method, and the

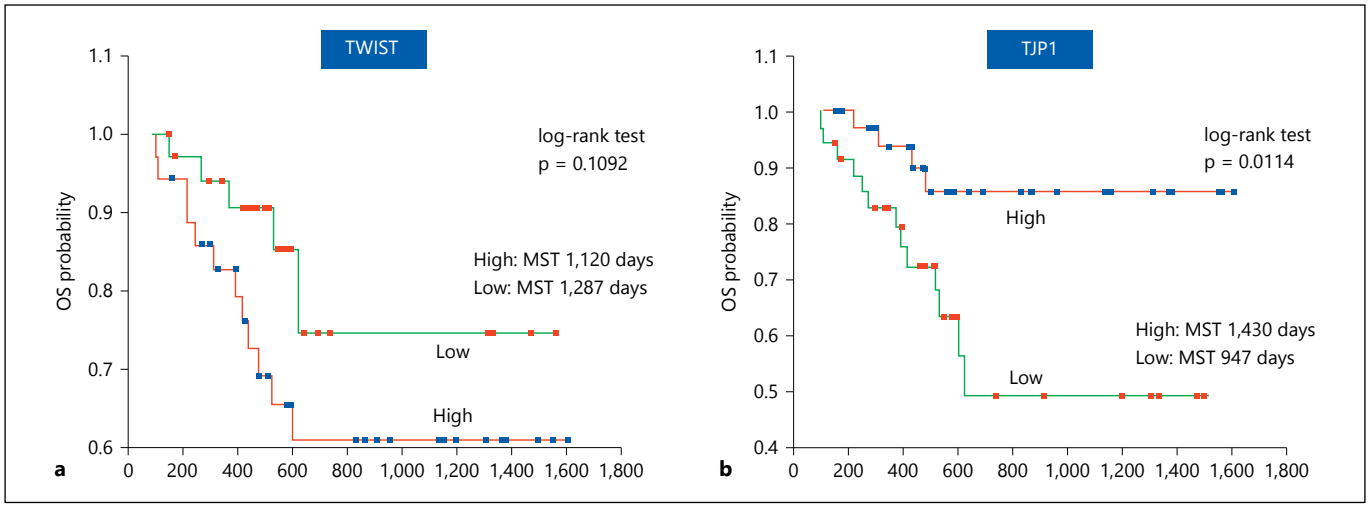

Fig. 1. Low-TJP-1 expression is associated with worse clinical outcomes in HCC after operation. Kaplan–Meier survival curves for high-TWIST expression versus low-TWIST expression (**a**) and high-TJP-1 expression versus low-TJP-1 expression in 72 HCC patients and p values = 0.1092 (**a**) and p values = 0.0114 (**b**).

differences in survivals between the groups were examined by the log-rank test. A p value of <0.05 is considered statistically significant. All statistical analyses were done with SPSS 19.0 (SPSS Inc., Chicago, Ill., USA).

Results

The TJP1 as a Protective Gene and the TWIST as a Risk Gene for the Survival of HCC Patients

For identification of prognostic factors associated with RFS and OS, we examined the expression of 15 kinds of EMT-related genes in HCC tissues and analyzed the RFS and OS of the patients. The follow-up was completed on December 31, 2010, with median follow-up time of 17 months (range 3.3–53.5 months). Patients' characteristics and factors revealed to be significant in univariate analysis were subsequently applied into the multivariate Cox proportional hazards regression model. The associations between patients' backgrounds and RFS and OS by univariate Cox regression analysis are shown in table 2. Poorly differentiated phenotype, the presence of vascular invasion, and high serum level of alpha-fetoprotein (AFP) are significantly associated with RFS. Similarly, larger tumor size, TNM stage, vascular invasion, and high levels of AFP and prothrombin induced by vitamin K absence-II (PIVKA-II) are significantly associated with OS. In addition, the expression of TWIST and TJP1 are correlated with RFS and OS by univariate Cox regression analysis (table 2).

Figure 1 showed survival curve of the patients with high expression and low expression of TWIST and TJP1. Low expression of TWIST shows a trend with longer OS in HCC patients after hepatic resection (fig. 1a). And the high expression of TJP1 is significantly associated with longer OS in those patients (fig. 1a). The high expression of TJP1 is related to longer OS. Multivariate analysis revealed that AFP levels (>20) and high expression of TWIST were independent predictors for shorter RFS (table 2). Regarding the OS, high expression of TWIST, low expression of TJP1, poorly differentiated phenotype, high serum level of PIVKA-II, and stage were the variables related to shorter OS (table 2). Therefore, TJP1 is considered a protective gene (a hazard ratio of less than 1) and TWIST a risk gene (a hazard ratio of more than 1) for patients' survival.

Knocking Down of TJP1-Induced Migration of HCC Cell line

Previously, we reported that sorafenib suppressed the EMT and cellular migration in an HCC cell line, Huh7. Therefore, we performed a migration assay using the Boyden-chamber method. The siRNA knockdown of TJP1 strongly induced migration of an HCC cell line Huh7 (fig. 2).

Discussion

In this study, we demonstrated that the low expression of TJP1 was significantly associated with poor survival in HCC patients after hepatic resection. Yang et al. [16] re-

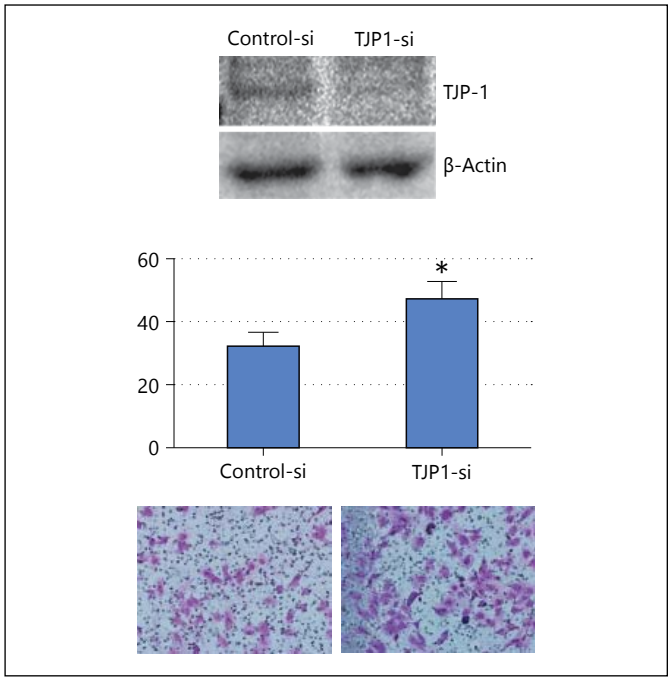

Fig. 2. Cells transfected with si-TJP-1 showed higher potential for invasion. By transwell assay, si-TJP-1-transfected cells penetrated into the lower chamber when compared to the action of si-control cells.

ported that the coexpression of Snail and TWIST was associated with the worst prognosis for HCC patients. On the other hand, our data demonstrated that the low level of TJP1 is also an independent factor for poor prognosis in HCC patents, which have not been reported so far. TJP1 is a 210–225 kDa protein that is found at the submembranous domain of tight junction in epithelia and endothelia; it represents a scaffolding function and reportedly plays an important role in signal transduction by clustering critical membrane proteins [17, 18]. TJP1 also known to establish a link between occluding and the actin cytoskeleton, and play a role on the regulation of gene expression mediated by the signal from the tight junction [19–21]. Orban et al. [22] reported that the expression of occludin and TJP1 mRNA in HCC was lower than those in normal liver tissue, while the protein expression of occludin and TJP1 was higher in metastatic lesion compared with those in normal liver. Ohtani et al. [23] reported the expression of tight-junction–associated proteins in human gastric cancer. Kaihara et al. [24] also reported the association between the dedifferentiation of tumors and decreased expression of adhesion molecules; E-cadherin and TJP1 are closely related to liver metastasis of colorectal cancer. Ni et al. [25] reported that increased TJP1 expression predicted valuable prognosis in non-small cell lung cancer. Decreased TJP1 expression was also shown to be associated with increased invasiveness in breast cancer [26]. In addition, the altered expression of the tight junction protein, such as TJP1, should play an important role in the process of cell dissociation. TWIST is also considered one of the major EMT regulators [22]. Taking these facts into consideration, understanding the role of TJP1 and TWIST expression in human cancer is quite important from both basic and clinical aspects.

The results of our analysis revealed that RFS and OS were independently influenced by different prognostic factors. For example, the multivariate Cox proportional hazards regression model revealed that TWIST was an independent factor of RFS and OS. And there was a trend between with the high expression of TWIST and shorter OS ($p = 0.1092$). The analysis of survival curves with the Kaplan–Meier method for high-TWIST expression versus low-TWIST expression is not significantly different. The one of reason is that the groups of high- and low-TWIST expression are divided in the median.

On the other hand, TJP1 was not found to be an independent prognostic factor affecting the RFS. It is probably due to the different follow-up schedule of the patients among the physicians; the date of recurrence was not defined precisely. Therefore, RFS should be affected by the variation of follow-up schedule among the physicians greater than the way in which OS is affected. On the other hand, the high expression of TJP1 and low expression of TWIST are significantly associated with longer OS, suggesting the critical role of TJP1 as a tumor suppressor and TWIST as an oncogenic protein on survival of the HCC patients.

Although TJP1 is known as an epithelial marker, its function is still largely unclear. To confirm the biological function of TJP1, we knocked down TJP1 using siRNA in a Huh7 HCC cell line, which accelerated a migration of Huh7. Therefore, it is possible that TJP1 may act as a metastatic suppressor because knockdown of TJP1 led to an increased migration of Huh7 cells. In conclusion, we found that the low expression of TJP1 is associated with poor prognosis; it could be a predictive factor for poor prognosis in patients with HCC after hepatic resection and may serve as a potential biomarker.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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