

RESEARCH COMMUNICATION

Up-regulation of Thy-1 Promotes Invasion and Metastasis of Hepatocarcinomas

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Abstract

Increasing evidence has revealed that thy-1 was a potential stem cell marker of liver cancer, but no data have been shown on how thy-1 regulates the pathophysiology of liver cancer, such as proliferation, apoptosis, invasion and migration. We previously demonstrated that thy-1 was expressed in about 1% of hepg2 cells, thy-1+ hepg2 cells, but not thy-1-, demonstrating high tumorigenesis on inoculation 0.5×10^5 cells per BACA/LA mouse after 2 months. In the present study, our results showed that higher expression of thy-1 occurs in 72% (36/50 cases) of neoplastic hepatic tissues as compared to 40% (20/50 cases) of control tissues, and the expression of thy-1 is higher in poorly differentiated liver tumors than in the well-differentiated ones. In addition, thy-1 expression was detected in 85% of blood samples from liver cancer patients, but none in normal subjects or patients with cirrhosis or hepatitis. There was a significant negative correlation between thy-1 expression and E-cadherin expression (a marker of invasion and migration), but not between thy-1 expression and AFP expression in all the liver cancer and blood samples. We further investigated the relationship between thy-1 and E-cadherin in liver cancer hepg2 cell line which was transfected with pReceiver-M29/thy-1 eukaryotic expression vector followed by aspirin treatment. Lower expression of E-cadherin but higher expressions of thy-1 were detected in hepg2 cells transfected with pReceiver-M29/thy-1. Taken together, our study suggested that thy-1 probably regulates liver cancer invasion and migration.

Keywords: Invasion - migration - thy-1 - hepatocarcinomas

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Introduction

Hepatocarcinoma is the fifth most common cancer and one of the leading causes of cancer death worldwide. Especially with quick development of liver disease due to hepatitis B virus infection, incidence of hepatocarcinoma has been on the rise and earlier recurrence after operation or liver transplant is common. The underlying root cause is the existence of cancer stem cells (CSCs) (Shupe et al., 2005; Sell et al., 2008; Alison et al., 2009; Chiba et al., 2009; Lee et al., 2009), which, despite their small number, play a major role in tumor origination, development, invasion and earlier metastasis (Reya et al., 2001; Couzin et al., 2003). Oval cells are the stem cells of liver (Bird et al., 2008; Strick-Marchand et al., 2008; Okabe et al., 2009; Shupe et al., 2009). Recently a great deal of research suggested a positive relationship between its proliferation and the incidence of hepatocarcinoma (Alison et al., 2005; Alison et al., 2006; Wu et al., 2006). Thy-1, as a marker protein of oval cells, has recently been suggested to play a role in potential liver cancer stem cells (Dahlke et al., 2003; Ceafalan et al., 2005; Masson et al., 2006; Yang et al., 2008; Yang et al., 2008; Xu et al., 2009). As a gene expressed during embryonic period or under pathologic state, AFP (alpha fetoprotein) participates in

embryonic development and cell division process (Kang et al., 2006). Recent research suggested (Kuhlmann et al., 2006) AFP possessed stem cell characteristics and could be regarded as an ideal marker predicting earlier liver cancer recurrence after operation. However, the shortcoming of AFP loss expression in some samples required a more accurate and specific marker to predict liver cancer occurrence and recurrence independent of or in combination with AFP marker. E-cadherin, a member of cadherin family, mediates cell-cell adhesions and its loss of function could cause breakdown of cell-cell junctions. It is therefore commonly used to predict invasion and metastasis of various kinds of malignant tumor. Based on findings above, this article was aimed to investigate the relationship between thy-1, AFP and E-cadherin and to unravel the function of thy-1 in hepatocarcinoma.

Materials and Methods

Cell lines

The human cell line hepg2 (ATCC, American) was maintained in RPMI1640 medium (Hyclone, American) containing 10% fetal bovine serum (Hyclone, American) and 1% penicillin at 37 °C in a humidified incubator with 5% CO₂.

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Patients and sample collection

Fifty paraffin-embedded tissues with liver cancer who had undergone laparotomy (46/50 had curative hepatic resection and 4/50 had liver transplantation; 18/50 well-differentiated and 32/50 poorly differentiated) were included in the study. Pathology diagnosis was made according to the histology of tumor specimens and examinations were performed by experienced pathologists. Fifty corresponding peri-tumorous healthy tissues were used as controls which were confirmed according to pathological diagnosis. The majority of HCC (53 cases) and cirrhosis or hepatitis patients (36 cases) were positive for serum hepatitis B surface antigen. All the blood sample studies were approved by the Institutional Review Board of the fuzhou general hospital.

Immunohistochemistry and Immunocytochemistry

Immunohistochemical evaluation on 4mm-thin tissue sections from formalin-fixed and paraffin-embedded samples was performed for thy-1 (sc-53456, dilution 1:200; Santa Cruz Biotechnology Inc., Santa Cruz CA), AFP (sc-51506, dilution 1:200; Santa Cruz Biotechnology Inc., Santa Cruz CA), E-cadherin (sc-8426, dilution 1:200; Santa Cruz Biotechnology Inc., Santa Cruz CA). The staining reactions were interpreted in the presence of tissue mast cells in portal fields of the liver as internal controls, while AFP and E-cadherin as external controls for embryonic tissues and breast duct carcinoma respectively.

Staining was performed according as per manufacturer's instructions of ultrasensitive TMS-P (mouse/rabbit) kit (Maixin, Fujian China). Briefly, paraffin-embedded sections were deparaffinized and rehydrated through graded alcohols to phosphate buffered saline (PBS). 0.01M citrate buffer solution (pH 6.0) was used in antigen recovery. After sequential incubation with 3% endogenous peroxidase blocking solution and 1% normal nonimmune serum, 10 min for each at room temperature, primary antibodies were incubated over night at 4 °C. Anti-mouse IgG was used instead of primary antibody as additional control. Other negative controls included the omission of either primary or secondary antibodies. Nonspecific reactions were detected in the negative controls. According to Fromowitz score standard, when there were different differentiation degree in the same sample, predominate area was selected (Fromowitz et al., 1987) according to positive staining percent, score was classified as follows: 0: ≤ 25%; 1: 26%~50%; 2: 51%~75%; 3: > 75%. In addition, staining intensity was rated as 0 (negative), 1 (weakly positive), 2 (intermediate) and 3 (strongly positive). Finally, a summation of the two scores above was further classified into: 0: negative; 1~2: "+"; 3~4: "++"; 5~6: "+++" (Jawhari et al., 1997).

Immunocytochemistry

HepG2 cells were plated at 100,000 cells per well one night before the experiment. Breast carcinoma cell line DBA-DA231 and un-transfected hepg2 cells were used as positive and negative controls respectively. Hepg2 cells were transfected with pReceiver-M29/ thy-1 expression vector and treated with Aspirin in the test

group according to the manufacturer's instructions of ultrasensitive TMS-P (mouse/rabbit) kit (Maixin, Fujian China). Briefly, cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton-100 for 10 min. The following steps were performed in the same way as immunohistochemistry.

Immunosorbent assay

Thy-1, AFP and E-cadherin ELISA were performed using ELISA kit (R&D) as per manufacturer's instructions. Briefly, 50 μl standard or 50 μl of 1:5 diluted samples was dispensed to standard or sample wells, respectively. Thirty minutes after the addition of 50 μl enzyme label reagent at 37 °C, chromogenic agent A and B were added to react for 10 min at 37 °C with protection from light, following which stop buffer was added. Thereafter, the absorbance at 450 nm was measured with an enzyme-labeling instrument (ELX-800 type).

RT-PCR

Experiments were performed using RT-PCR kit (TakaRa, code: DRRO14A). Total RNA was isolated with trizol reagent (Invitrogen, Cat: 15596-026) according to the manufacturer's instructions using 1ml trizol per 1*10⁷ cells. Total RNA concentration was quantified with eppendorf Bio photometer. The cDNAs were generated from 5 μg of total RNA per sample using 20 μl reaction containing 1 μl random 6 primers, 1 μl dNTP mixture, 0.5 μl prime Script™ Rtase and 0.5 μl RNase inhibitor. Gene-specific primers for thy-1 (primer A: 5'-AAGGTGACCAGCCTAACGG-3', primer B: 5'-CCCTCGTCCTTGCTAGTGAA-3'), for β-actin (primer A: 5'-GGAAATCGTGCGTGACATT-3', primer B: 5'-CGTCATACTCCTGCTTGCTG-3'), and for E-cadherin (primer A: 5'-CGCCGACGAGAGCTACACGTTCA-3', primer B: 5'-CCC AGGCGTAGACCAAGAAA-3') were designed using the primer 3 software and synthesized (Shanghai Biologic company, China).

Statistical analysis

Descriptive statistics, t-test, χ² test and One-way ANOVA with LSD multiple comparison were performed. Fisher precise probabilistic method was used to analyze expression intensity, Spearman for correlation analysis. SPSS software (version 11.5 for Windows; spss Inc., Chicago, IL) was used for all statistical analyses. P value less than 0.05 was considered significant.

Results

Altered expression pattern of thy-1, AFP and E-cadherin in liver cancers. Immunohistochemical staining showed reactivities of thy-1, AFP and E-cadherin in 40%, 20%, and 30% of peritumoral normal tissues, whereas 72%, 76% and 64% of neoplastic liver tissues respectively. Staining intensities for thy-1 and AFP in normal peritumoral samples were significantly weaker than in tumor samples, but E-cadherin staining showed the opposite results (Figure 1). In addition, we detected a significant correlation between the degree of differentiation of hepatocarcinomas and the expression of

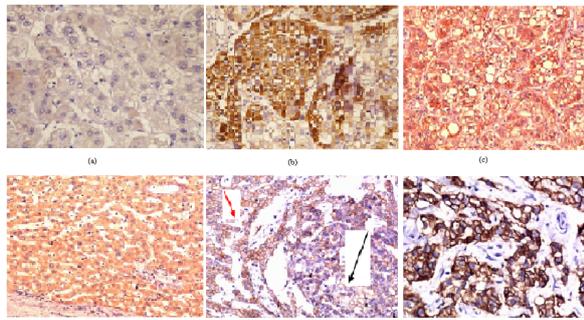


Figure 1. Immunohistochemical Staining of Thy-1, AFP and E-cadherin in Hepatocarcinoma and Peritumoral Normal Liver Samples. (a) Peritumoral normal tissues demonstrating weak AFP reactivity (magnification 200x). (b) Hepatocarcinoma exhibiting strong expression of AFP (magnification 200x). (c) Hepatocarcinoma exhibiting strong expression of thy-1 (magnification 200x). (d) Peritumoral normal liver tissues revealing intermediate thy-1 expression (magnification 100x). (e) Liver cancer cells demonstrating weaker expression of E-cadherin (black arrow) compared to higher expression in normal tissues (red arrow) (magnification 200x). (f) Breast cancer as control exhibiting strong expression of E-cadherin (magnification 200x)

Table 1 Immunohistochemical Reactivity in Liver Cancers and Peritumoral Normal Tissues

protein of interest	tumor (n=50)	peritumous (n=50)	Well-differentiated (n=18)	Poorly differentiated (n=32)
Thy-1	36	20	7	26
AFP	38	10	7	22
E-cadherin	32	15	15	17

Table 2 Correction Between thy-1 and AFP, E-cadherin by Immunohistochemistry

Protein of interest	Thy-1		rs	p
	+	-		
AFP	+	27	0.116	0.421
	-	11		
E-cadherin	+	18	-0.336	0.117
	-	14		

Table 3 Correction Between thy-1 and AFP, E-cadherin by ELISA

groups	Serum (U/ml)	rs	p
Thy-1	3.694		
E-cadherin	2.961	-0.334	0.027
	1.591		
AFP	0.696	0.046	0.746
	3.121		
	1.965		

thy-1, AFP and E-cadherin, whereby poorly differentiated hepatocarcinomas were more likely to express thy-1 and AFP but less likely to express E-cadherin than well-differentiated ones (poorly differentiated: thy-1, AFP and E-cadherin expressed in 81.2%, 68.8% and 53.1% of samples respectively; well-differentiated: thy-1, AFP and E-cadherin expressed in 38.8%, 38% and 83.3% of samples respectively) (Table1).

Lack of correlation between thy-1 and AFP, but negative correlation between thy-1 and E-cadherin.

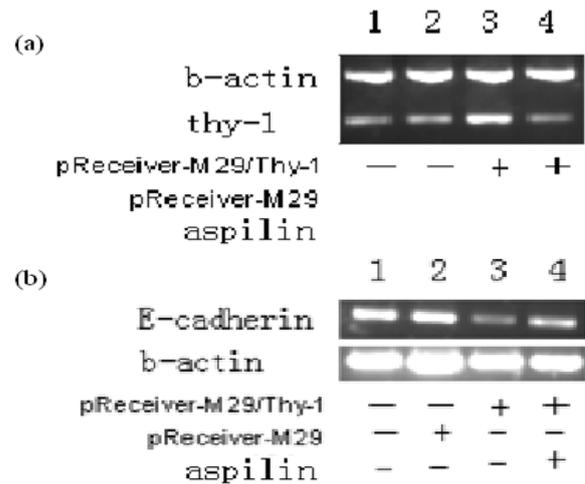


Figure 2. RT-PCR Analysis of Thy-1 mRNA and E-cadherin mRNA Expression. (a) Expression of thy-1 was increased in pReceiver-M29/Thy-1 transfected hepg2 cells but dramatically reduced after treatment with Aspirin. (b) E-cadherin expression was lowest in in pReceiver-M29/Thy-1 transfected hepg2 cells but slight increased with Aspirin treatment

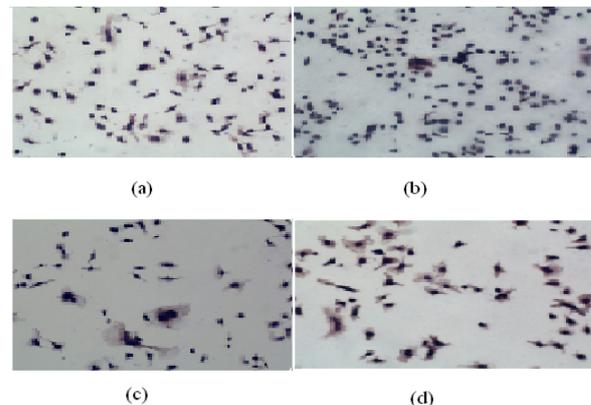


Figure 3. Immunocytochemical Staining for E-cadherin in Hepg2 Cells after Different Treatments. (a) E-cadherin staining in non-transfected hepg2 cells (magnification 200x). (b) Obviously weaker staining for E-cadherin in hepg2 cells transfected with pReceiver-M29/thy-1 (magnification 200x). (c) Slightly weaker staining in pReceiver-M29/thy-1 hepg2 transfected cells treated with Aspirin (magnification 200x). (d) E-cadherin staining in breast cancer DAB-DA231 cells (magnification 200x)

Both positive expression samples or negative for thy-1 and AFP was 27 or 5, Positive expression samples for thy-1 of 7 cases but negative 1 for AFP and negative 11 for thy-1 but positive for AFP was also observed, there was no significant difference between them (rs=0.116, p=0.421). Both positive expression samples for thy-1 and E-cadherin was 18, both negative for them was 2, positive for thy-1 was 16 but negative for E-cadherin was 14, negative correlation was detected between them (rs=-0.336, p=0.017) (Table 2). In addition, from 53 liver cancer serum samples, levels of thy-1, AFP and E-cadherin detected were 3.694 2.961, 3.121 1.965, and 1.591 0.696 respectively. No correlation was found between AFP and thy-1 (rs=0.046, p=0.746), but there was a negative correlation between thy-1 and E-cadherin (rs=-0.334, p=0.027) (Table 3).

Enhanced Thy-1 expression reduces E-cadherin level. In order to further understand the relationship

between Thy-1 and E-cadherin, we transfected hepg2 cells with pReceiver-M29/thy-1 expression vector and looked for changes in expression of E-cadherin and thy-1 using RT-PCR. Immunocytochemistry revealed higher expression of thy-1 but lower expression of E-cadherin in pReceiver-M29/thy-1 transfected hepg2 cells as compared to non-transfected hepg2 cells and differences in expression levels were statistically significant ($p < 0.05$). Moreover, when $0.5 \mu\text{mol/ml}$ Aspirin was used to inhibit the wnt/b-catenin signaling pathway (Dihlmann et al., 2003); E-cadherin level was slightly elevated but thy-1 expression was dramatically reduced in hepg2 cells transfected with pReceiver-M29/thy-1 (Figure 2). In addition, E-cadherin staining was obviously weaker in transfected cells, but slightly higher than in non-transfected hepg2 cells treated with Aspirin (Figure 3). Transfected hepg2 cells also frequently showed double or multiple nuclei, but this phenotype did not change after Aspirin treatment.

Discussion

Thy-1 is a 25-37KD glycosylphosphatidylinositol-anchored glycoprotein expressed mainly in leukocytes and involved in cell-cell and cell-matrix interactions (Rege et al., 2006; Rege et al., 2006). Recently research showed its expression in thymocytes, mesenchyme stem cells, embryo haemopoietic stem cells, smooth muscle cells, and cd34+myeloma cells. Moreover, thy-1 is regarded as a tumor stem cell marker in some cancers (Chen et al., 2005; Yamazaki et al., 2009; Mercati et al., 2009; True et al., 2010). Yang et al findings suggested thy-1+/CD45+ (Donnenberg et al., 2010, Yang et al., 2008) hepatocarcinoma compared to thy-1-/CD45- in liver cancer possessed the ability of conspicuous neoplasia, and thy-1+/CD44+ hepatocarcinoma cells showed enhanced tendency of invasion and metastasis. Subsequent research also supported thy-1 as liver cancer stem cells. In this study we found positive expression of thy-1 in 72% (36/50 cases) of liver cancer samples with hepatitis B surface antigen positive compared with 40% (20/50 cases) of peritumoral liver tissues including cirrhosis or hepatitis, and the difference was significant ($p = 0.000?$). We further investigated the relationship between thy-1 expression and differentiation degree of liver cancers. Higher expression of thy-1 was found in poorly differentiated hepatocarcinoma than in well-differentiated ones with staining intensity correlating to degree of differentiation and these findings are similar to a former smaller-scale study (Rege et al., 2006). They found decreasing number of CD117+/CD90+ cells from the neoplastic (2.48 +/- 0.67) and peritumoral region (0.88 +/- 0.12) to the area of para-tumoral (normal) parenchyma (0.13 +/- 0.04). This suggested thy-1 could be a tumor stem cell marker of liver cancer.

Tumorigenesis is a multi-step genetic event with activation of oncogenes and suppression of anti-oncogene. We aimed to determine whether thy-1 could interact with other proteins or genes to influence hepatocarcinoma development. Our study detected a correlation between AFP and thy-1, as well as between E-cadherin and thy-1. A protein expressed during embryo period or pathologic

state, AFP participates in embryonic development and cell division. Some recent studies consider (Kuhlmann et al., 2006) AFP as a super marker of oval cells, showing conspicuous clonal expansion in cells of liver cancers. Form tumor and self-renewal capability, AFP possess stem cell characteristic, play an important role in carcinogenesis and recurrence and metastasis of liver cancer. E-cadherin is a calcium-dependent cellular adhesion molecule existing in normal endothelial cells which maintains the structural integrity of tissues. Down-regulation of E-cadherin caused invasion and metastasis of tumor. Here we investigated the expression of AFP and E-cadherin in all liver cancer samples and interestingly their expression levels correlated to thy-1. AFP expression is higher in poorly differentiated hepatocarcinomas than in well-differentiated ones, but the reverse is true for E-cadherin. In addition, a negative correlation was detected between thy-1 and E-cadherin but there is no relationship between AFP and thy-1. The same expression patterns were observed in 53 blood samples collected from liver cancer patients with blood analytes quantified using ELISA. From findings above we speculate that high expression of thy-1 but low expression of E-cadherin in hepatocarcinoma could be regarded as markers to predict invasion and early metastasis.

In order to further prove that thy-1 possibly participates in invasion and metastasis, we transfected pReceiver-M29/Thy-1 expression vector (this part was not presented in this article) into hepg2 cells. Immunocytochemistry and RT-PCR results indicated obvious down-regulated expression of E-cadherin but up-regulated expression of thy-1 in transfected hepg2 cells. Under normal condition, E-cadherin is connected to actin cytoskeleton thereby regulates intercellular adhesion by forming a E-cadherin/catenins Complex with β -catenin and α -catenin (Nieset et al., 1997). Previous study showed that down-regulation or mutation of any member of the complex would result in loss of adhesion ability that is related to tumor invasion and metastasis and therefore the prognosis of the disease (Czyzewska et al., 2010). β -catenin/TCF dimer could also down-regulate E-cadherin transcription and attenuate cell adhesion. On the other hand, Aspirin was found to attenuate β -Catenin expression, suppress cell proliferation, while induce apoptosis (DiMmann et al., 2001). Cording to findings of a previous study (Fromowitz et al., 1987), $0.5 \mu\text{mol/ml}$ Aspirin was used to treat hepg2 cells transfected with thy-1. After 48h treatment, significantly lower expression of β -catenin was detected (this part result was not presented in this article data no shown). In addition, E-cadherin expression slightly increased but thy-1 level decreased compared to hepg2 cells which were transfected but not treated with Aspirin, suggesting that thy-1 and E-cadherin participate in invasion and metastasis of liver cancer.

Taken together, our study demonstrated for the first time that there is no correlation between thy-1 and AFP in liver cancer, but up-regulated expression of thy-1 plays critical roles in the invasion and metastasis of hepatocarcinoma. Furthermore, we also presented the mechanism of thy-1-induced invasion and metastasis of liver cancer with down-regulation of E-cadherin and up-regulation of β -catenin. However, high recurrence and

early invasion/metastasis of liver cancer are complex processes. Therefore, understanding detailed actions of thy-1 in hepatocarcinoma and designing therapeutic strategies such as RNAi or gene knock-out directed towards thy-1 would hold promise for future liver cancer therapy.

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