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Overexpression of Thy1/CD90 in human hepatocellular carcinoma is associated with HBV infection and poor prognosis

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ABSTRACT

Thy1/CD90 is an important marker of many types of stem cells. It functions as a tumor suppressor in ovarian cancer and in nasopharyngeal carcinoma. In this study, the expression status of Thy1 in clinical hepatocellular carcinoma (HCC) tissue samples was investigated. Relationships of Thy1 expression with clinical parameters and patient survival rate were analyzed. The quantities of Thy1 mRNA were statistically higher in tumor tissues than those in the adjacent non-tumor tissues ($p < 0.001$). Immunohistochemical data confirmed that Thy1 protein was increased in 73% of HCC samples. Thy1 expression was not influenced by chronic alcohol exposure or cirrhosis. Overexpression in Thy1 was correlated with age ($p = 0.006$), hepatitis B virus (HBV) infection ($p = 0.044$), and histological grade ($p = 0.014$). Patients with the highest level of Thy1 expression showed the poorest prognosis ($p = 0.040$). In conclusion, overexpression of Thy1 may not suppress the development of HCC. Thy1 could provide a clinical prognostic marker for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality in Taiwan (Chen et al., 2002) and many other countries in Asia and Africa (El-Serag, 2002). Despite considerable progress in diagnosis and treatment, the prognosis for patients with HCC is still unsatisfactory and unpredictable because of high rates of recurrence and metastasis (Ding et al., 2005; Tung-Ping Poon et al., 2000; Yeh et al., 2003). The development of HCC is closely related to chronic hepatitis B or C, cirrhosis of any etiology, and aflatoxin B1 exposure (El-Serag, 2002). However, genetic events in hepatic carcinogenesis are still poorly understood. Identification of the key genes and biological pathways involved in the carcinogenesis, and the characterization of better prognostic markers to predict individual risk of recurrence and subsequent prognosis will be useful to develop anti-cancer drugs and guide surgical and chemotherapeutic treatment of HCC patients.

Thy1 is a 25–37 kDa cell surface glycoprotein. Human Thy1 maps to chromosome 11q22.3. It is expressed on several types of cells including: fibroblasts, blood stem cells, endothelial cells and adult neurons (Rege and Hagood, 2006). Therefore, it is often used as a marker protein in cell-typing and isolation. The exact function and physiologic role of Thy1 in the cell remains unclear. However, recent research results demonstrate that Thy1 is an important regulator of cell–cell and cell–matrix interactions (Rege and Hagood, 2006). It is involved in lymphocyte activation (Haeryfar and Hoskin, 2004), cell reorganization and signaling (Saalbach et al., 2002, 2005), apoptosis (Fujita et al., 1996, 1997), cell adhesion and migration (He et al., 1991; Saalbach et al., 2005), tumor suppression (Abeysinghe et al., 2003, 2004, 2005; Lung et al., 2005), and neurite outgrowth modulation (Tiveron et al., 1992).

Thy1 also plays important roles in oncogenesis. It may function as a tumor suppressor in ovarian cancer (Abeysinghe et al., 2004) and nasopharyngeal carcinoma (NPC) (Lung et al., 2005, 2010). In an ovarian cancer cell line, Thy1 upregulates thrombospondin-1 and fibronectin, which prevent tumor angiogenesis and metastasis (Akiyama et al., 1995). Thy1 is down-expressed in NPC. Transfection of Thy1 reduces the colony forming and invasive activities in NPC cells (Lung et al., 2005, 2010). Loss of Thy1 expression is correlated with poor survival rate in neuroblastoma patients (Fiegel et al., 2008).

Thy1 is expressed on hematopoietic progenitor cells in human fetal liver, cord blood, and bone marrow (Fiegel et al., 2008). It has

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been used as a cell marker for isolation of stem cells for potential transplantation (Dennis et al., 2007; Fiegel et al., 2008). It is also an important marker on hepatic stem/progenitor cells from fetal and adult human liver (Dan et al., 2006; Herrera et al., 2006). However, Thy1$^+$ cells isolated from HCC cell lines, tumor specimens, and blood samples display tumorigenic capacity, indicating that Thy1 is also a marker of cancer stem cells (Fiegel et al., 2008). The role of Thy1 in hepatocarcinogenesis is not clear. In this study we evaluated the expression of Thy1 in HCC patients by real-time quantitative PCR and immunohistochemical analysis, and correlated Thy1 expression with the clinical outcome and survival in 59 patients.

Materials and methods

Patients and tissue specimens

Fifty-nine histologically confirmed resected HCC and paired non-tumor tissue samples were obtained under protocols approved by the Institutional Review Board (IRB) from Changhua Christian Hospital, Taiwan. The age of the patients ranged from 17 to 78 years (mean, 55 years). All samples were confirmed pathologically. Histological grade of HCC was based on the WHO grading system (Hirohashi et al., 2000). Tumor stage was based on the pTNM staging system of the American Joint Committee on Cancer (Lippincott-Raven, 2009). Slides from tumors were reviewed by two pathologists to define the histological grading. The tissues were frozen immediately after surgical resection and stored in liquid nitrogen until use for RNA extraction.

Real-time quantitative RT-PCR

The total RNA samples were isolated from tissues using Trizol total RNA isolation Kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. Five micrograms of the extracted RNA was reversely transcribed into cDNA in a final volume of 100 μl. Random primers, oligo-dT, and MMLV-RT (Promega, Madison, WI, USA) were used according to the manufacture's protocol. PCR amplification for Thy1 mRNA was carried out in a LightCycler system (Roche Applied Science, Indianapolis, IN, USA) using Fast-Start Universal Probe Master kit (Roche Diagnostics, Mannheim, Germany) and primers Thy1-F/-R (5′-CACCACTCTGGCCATTCC-3′/5′-CTCACACTTGACCAGTTTGTCTCT-3′), with a cycling profile of one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 9 s (Lu et al., 2009). HMBS, an internal control (HMBS gene), was amplified with primers HMBS-F/-R (5′-AGCTATGAAGGATGGGCAAC-3′/5′-TTGTATGCTATCTGAGCCGTCTA-3′). The relative quantity for a target gene was given by the Ct value. The differential expression of Thy1 between cancer and paired non-cancer tissues was determined as $2^{-\Delta\Delta Ct}$, where $^{\Delta\Delta}Ct = tumor^{\Delta}Ct - non\text{-}tumor^{\Delta}Ct$. All experiments were performed in triplicate, and the means of three values were presented.

Immunohistochemistry

Formalin-fixed, paraffin-embedded HCC and paired non-tumor tissue sections (5-7 μm) on poly-L-lysine coated slides were deparaffinized. The slides were treated with 3% H_2O_2 to block endogenous peroxidase activity, and heated in 10 mM citrate buffer at 100 °C for 20 min to retrieve antigen. After being incubated with primary antibody (goat anti-Thy1, Santa Cruz, CA, USA, at a 1:120 dilution) for 20 min at room temperature, samples were incubated with a secondary antibody conjugated with horseradish peroxidase polymer conjugate (Rabbit anti-Goat, Zymed, S. San Francisco, CA, USA, at a 1:500 dilution) for 10 min and then developed with H_2O_2 and diaminobenzadine (Dako Cytomation, Glostrup, Denmark). Hematoxylin was used as a counterstain (Lu et al., 2010; Tseng et al., 2009). The staining intensity of Thy1 was scored as 0 (<5%), 1 (5–50%), or 2 (>50%), respectively, according to the percentage of positively stained cells (Lu et al., 2010; Tseng et al., 2009). Since Thy-1 was expressed at a basal level in most of the liver cells, only cells that express a significant high level of Thy1 were considered as "positive".

Statistics

Paired Student's *t*-test was used to compare the mRNA levels between tumor and non-tumor samples. Wilcoxon signed rank test was used to compare Thy1 protein scores between tumor and non-tumor samples. Correlation of the Thy1 expression levels and clinicopathological variables was analyzed by Chi-square test. The survival curves were obtained by the Kaplan–Meier method (Mantel, 1966) and the comparison between the curves was made by the log-rank test. In all statistical analyses, p values lower than 0.05 were considered statistically significant.

Results

Thy1 mRNA level in clinical tissue samples

Real-time quantitative PCR analyses of 59 HCC patients showed that the quantity of Thy1 mRNA was higher in tumor tissues than in corresponding non-tumor tissues (Table 1). The results demonstrated that Thy1 was overexpressed in 76% of the HCC cases ($p < 0.001$).

Evaluation of Thy1 by immunohistochemical staining

Immunohistochemical results showed that Thy1 was expressed at different levels in different samples which can be scored into three classes according to the percentage of positively stained cells in the tissue (Fig. 1A–C). Most of the non-tumor samples had a score of 0 or 1, while most of the tumor samples had a score of 1 or 2 (Fig. 1D). The difference between tumor and the paired non-tumor samples ($T - N$), ranging from −2 to 2, revealed that Thy1 expression was increased in 73% of the tumor tissues ($T - N > 0$) (Fig. 1E). Wilcoxon signed rank test further confirmed that tumor samples had a significantly higher Thy1 expression than non-tumor samples ($p < 0.001$).

Table 1
Differential expression of Thy1 in 59 HCC and paired non-tumor tissues.

Tumor $^{\Delta}$Ct (Thy1 – HMBS)	Non-tumor $^{\Delta}$Ct (Thy1 – HMBS)	$^{\Delta\Delta}$Ct (Tumor$^{\Delta}$Ct – non-tumor$^{\Delta}$Ct)	$2^{-\Delta\Delta}$Ct	p value [a]
1.201	3.215	−2.013	3.797	5.470E−08*

[a] p-value by t-test.
* $p < 0.05$.