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Expression of Wnt-5a and its clinicopathological significance in hepatocellular carcinoma

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Abstract

Background. The functions of Wnt-5a in human cancers are controversial and unclear.

Aim. To investigate the clinical significance of Wnt-5a expression in hepatocellular carcinoma.

Patients and methods Real-time quantitative Reverse transcriptase Polymerase Chain Reaction was done to evaluate Wnt-5a gene expression. Wnt-5a, β-catenin, E-cadherin and Ki-67 were examined immunohistochemically in 114 hepatocellular carcinoma cases.

Results. Compared to normal tissue, Wnt-5a mRNA expression was clearly increased in hepatocellular carcinoma, chronic hepatitis and cirrhosis. On immunohistochemistry, immunostaining of Wnt-5a showed a bell-shaped pattern: low to undetectable levels were present in normal tissue and in tumour samples, whereas strong immunostaining was seen in chronic hepatitis, cirrhosis and dysplastic liver cells. Reduction or loss of Wnt-5a protein expression was found in 80.7% of hepatocellular carcinoma cases ($n = 92$) and was significantly associated with higher tumour stage ($p < 0.001$), serum AFP level ($p = 0.025$), low membranous expression of E-cadherin ($p < 0.0001$) and β-catenin ($p = 0.036$) and high Ki-67 labelling indices (LIs, $p = 0.001$).

Conclusion. Wnt-5a mRNA and protein levels are higher than normal in hepatitis and cirrhosis and appear to be related to the presence of hepatitis B virus infection. However, Wnt-5a protein expression is frequently lost in hepatocellular carcinoma; this supports the notion that this protein has a tumour suppressor function in hepatocellular carcinoma.

Keywords: β-Catenin; E-cadherin; Hepatocellular carcinoma; Wnt-5a

1. Introduction

Hepatocellular carcinoma (HCC) is endemic primarily in China, Taiwan, Korea and sub-Saharan Africa, and is a leading cause of cancer-related deaths in these countries. The major HCC risk factors include various chemicals and viruses. Among these, chronic HBV and HCV infections account for the development of more than 80% of HCC cases worldwide. Other known risk factors, including AFB1 uptake, cigarette smoking, and heavy alcohol consumption, are capable of inducing HCC alone, but they also have synergetic effects [1,2]. Although the major risk factors for the development of HCC are well recognized, the molecular basis of liver cell malignant transformation is still not completely understood. Recent advances have shown that apart from autocrine stimulation by growth factors such as insulin-like growth factor-II [3] and transforming growth factor-α [4], the dysregulation of at least four different growth regulatory pathways is frequently involved in hepatocarcinogenesis. These include the RB-1, the transforming growth factor-β, the p53 and the Wnt/β-catenin signalling pathways; these pathways also interfere with each other at different levels [1,5,6].

Wnt proteins are secreted and exert their effects through activation of distinct intracellular signalling pathways. Based on their biological activities in specific assays, vertebrate Wnt proteins have been divided into those involved in canonical signalling with transforming activities in mammary epithelial cells and those involved in non-canonical pathways [7–9]. Members of the canonical Wnt signalling pathway act via frizzled (Fz) receptors to induce β-catenin stabilization by preventing its phosphorylation, which targets β-catenin for

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proteosomal degradation. In this canonical Wnt pathway, stabilized β-catenin enters the cell nucleus, associates with members of the T-cell factor and lymphoid enhancer factor (TCF/LEF-1) family of transcription factors, and stimulates the expression of Wnt/β-catenin target genes, including regulators of cell growth and proliferation, as well as modulators of cell death pathways and cell–cell communication [10–13]. The inappropriate activation of canonical signalling, which induces intracellular β-catenin accumulation, has recently been implicated in several different malignancies [14,15]. β-catenin is a multifunctional protein that integrates the E-cadherin–catenin intercellular adhesion system with Wnt signalling during embryonic development [16]. Furthermore, reduced expression of E-cadherin is a frequent finding in HCC; it is most often due to CpG-island methylation within the promoter region of the gene [17]. The loss of E-cadherin not only affects cell adhesion, but may also lead to the redistribution of submembranously sequestered β-catenin into the cytoplasm [18,19].

Currently, the signalling and physiological functions of the non-canonical group of Wnt-proteins are not completely understood. Some reports indicate that Wnt-5a has oncogenic properties, based on the findings that the Wnt-5a mRNA level is upregulated in lung, prostate, gastric, and breast cancers [20,21]. Wnt-5a expression is correlated with increased cell motility and invasiveness of melanoma and breast cancer cell tumour-associated macrophages [22,23]. On the other hand, it has also been suggested that Wnt-5a acts as a tumour suppressor, since Wnt-5a can inhibit the β-catenin pathway. For instance, Wnt-5a has been shown to induce the down-regulation of β-catenin through Siah2 [24] and to inhibit the transcriptional activity of TCF/LEF [25,26]. Antisense Wnt-5a mimics Wnt-1-mediated C57MG cell transformation [27]. Wnt-5a negatively regulates B-cell proliferation, and Wnt-5a heterozygous mice develop B-cell lymphoma [28]. Furthermore, Wnt-5a inhibits the proliferation, migration, and invasiveness of thyroid tumour and colorectal cancer cell lines [29,30]. Thus, the functions of Wnt-5a in human cancers are controversial and still unclear.

The present study investigated the clinical significance of Wnt-5a expression in hepatocellular carcinoma. Since Wnt-5a is considered to be multifunctional, the expressions of β-catenin and E-cadherin, as well as the tumour proliferation rate using the Ki-67 index, were also evaluated.

2. Materials and methods

2.1. Patients and specimens

We consecutively collected tumours from 114 patients (mean age, 52; range, 12–78 years; male:female, 106:8) who had undergone surgery for HCC at the Jinling Hospital or the Nanjing 81 hospital from January 2002 to September 2006. Among the 114 patients, 75 had serum alpha-fetoprotein (AFP) ≥30 μg/l, 98 were serum positive for hepatitis B surface antigen (HBsAg), which HCV infection is negative by serum assay, and the remainder were negative for HBV or HCV infection by serum and immunohistochemistry assay. On gross examination, 11 cases had tumour sizes that were ≤2 cm and 103 had tumour sizes >2 cm (median tumour size, 5.6 cm; range, 1.0–17 cm). The histopathological diagnosis was made according to the WHO classification (2000) [31] 16 cases were well differentiated, 63 cases were moderately differentiated, and 35 cases were poorly differentiated. The tumour T classification was according to the tumour-node-metastasis classification of the International Union against Cancer. In total, 69 HCC cases had liver cirrhosis, 37 cases had chronic hepatitis, and 8 cases had basically normal liver tissue. Furthermore, normal liver tissues obtained during surgery for liver cholelithiasis ($n = 3$) and HBV-infected liver biopsy tissues ($n = 10$) were studied.

Of the 114 cases, fresh tissue, including tumour and adjacent non-tumourous liver tissues of HCC, which included chronic hepatitis ($n = 5$) and cirrhosis ($n = 17$), were obtained immediately after resection of the tumours in 22 cases. Normal liver tissues ($n = 3$) with no HBV or HCV infection were obtained during surgery for liver cholelithiasis. In these 25 cases, one part of the fresh tissue was snap-frozen in liquid nitrogen immediately and stored at −80 °C. The other part was fixed in 10% buffered formalin and embedded in paraffin.

2.2. Extraction of RNA and real-time RT-PCR

Total RNA was extracted from the tumour, adjacent non-tumourous liver tissues ($n = 22$) and normal liver tissues ($n = 3$) using 10-mm frozen sections. To isolate the RNA from defined areas containing at least 80% tumour cells, all tumours were manually microdissected under direct visual control through a dissecting microscope. Total RNA in the frozen tissues was extracted using TRIZOL (Invitrogen), following the manufacturer's recommendations. Total RNA was digested with DNase I (Invitrogen) and was used for the first-strand cDNA reaction. The reaction mixture consisted of 5 μg of DNase I-treated RNA, 1×reverse transcriptase buffer, 2.5 mM dNTP mix, 3.5 μM oligo primer, and 2.5 U/ml multiScribe reverse transcriptase (PE Applied Biosystems). Each sample was handled using the same protocol, with the exception that reverse transcriptase was added to exclude the presence of interference from genomic DNA.

Real-time RT PCR was carried out using SYBER green dye in a Rotor Gene 3000 Detection System (Corbett Research, Sydney, Australia). Each SYBER green reaction (25 μl) contained 1 μl diluted cDNA and 10.5 μl SYBR Green PCR Master Mix, as well as 5 pmol forward and reverse primer (Wnt-5a: forward 5′ ATTCTTGGTGGTCGCTAGG 3′, reverse 5′ CTGTCCTTGAGAAAGTCCTG 3′). Samples were activated by incubation at 94 °C for 5 min and denatured at 94 °C for 20 s. This was followed by annealing at 60 °C for 20 s and extension at 72 °C for 20 s for 40 cycles. In all of the cDNA samples, gene expressions of Wnt-5a and beta-actin (forward 5′ CCTGTACGCCAACACAGTGC 3′; reverse 5′