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Original Article

Expression of Wnt-5a and β-catenin in primary hepatocellular carcinoma

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Abstract: It has been reported that changes in Wnt5a expression are closely related to hepatocellular carcinoma (HCC) development, while decreased or abnormal β-catenin expression may promote the invasion and metastasis of tumor cells. In this study, the roles and clinical significance of Wnt-5a and β-catenin expression were analyzed in primary HCC. Real-time PCR (RT-PCR) analysis of Wnt-5a mRNA expression was performed in 26 fresh HCC samples and the corresponding para-carcinoma tissues. Wnt-5a and β-catenin protein expression was detected by immunohistochemical staining of paraffin-embedded tissues of 85 cases of HCC and corresponding para-carcinoma tissues and 15 cases of hepatic cirrhosis. Results showed that Wnt-5a mRNA levels were significantly higher in HCC tissue than in the para-carcinoma tissue (0.102 ± 0.159 and 0.020 ± 0.022, respectively; $P < 0.05$), while Wnt-5a protein was absent or low in HCC. Wnt-5a expression was detected in significantly fewer HCC tissue samples than in the para-carcinoma and hepatic cirrhosis tissue samples (21.2% (18/85), 81.26% (69/85) and 86.7% (13/15), respectively; $P < 0.01$). Abnormal localization of β-catenin protein shown by intracytoplasmic or intranuclear staining was observed in 72.94% (62/85) of HCC samples. These observations indicate that the role of Wnt-5a in HCC is mediated at the protein level rather than the transcriptional level. Furthermore, the abnormal localization of β-catenin observed in HCC tissues may be associated with gene mutation leading to the generation of truncated β-catenin proteins, which in turn, may represent an initiating or contributing factor in the development of HCC.

Keywords: Hepatocellular carcinoma, Wnt-5a, β-catenin, RT-PCR, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide; however, the mechanism of hepatocarcinogenesis remains poorly understood. It has been confirmed that genetic mutation and abnormal activation of signal transduction pathways are involved into the pathogenesis of HCC [1-3]. The genes of the Wnt family encode a large and diverse group of signaling molecules involved in embryogenesis, proliferation, and differentiation [4]. As one of the most important members, Wnt-5a is involved in intercellular signal transduction via the non-canonical Wnt signaling pathway [5]. β-catenin is the key mediator in the canonical Wnt signal pathway. Furthermore, as a cell-cell adhesion molecule, decreased β-catenin expression in the cell membrane and abnormal localization in the nucleus or cytoplasm can lead to decreased intercellular adhesion and promote the invasion and metastasis of tumor cells [6].

It has been reported that changes in the transcription and translation levels of Wnt-5a are closely related to HCC development [7, 8]. In this study, we analyzed Wnt-5a mRNA levels in HCC samples and the corresponding para-carcinoma tissues through RT-PCR. The Wnt-5a and β-catenin protein levels were also analyzed in normal liver, cirrhotic liver as well as HCC samples and the corresponding para-carcinoma tissues. The elucidation of Wnt-5a and β-catenin expression and function provide new insights into the mechanism of hepatocarcinogenesis.

Materials and methods

Specimens

HCC samples and the corresponding para-carcinoma tissues were collected from 26 patients who had undergone partial liver resection for HCC at the Jinan Military General Hospital. The

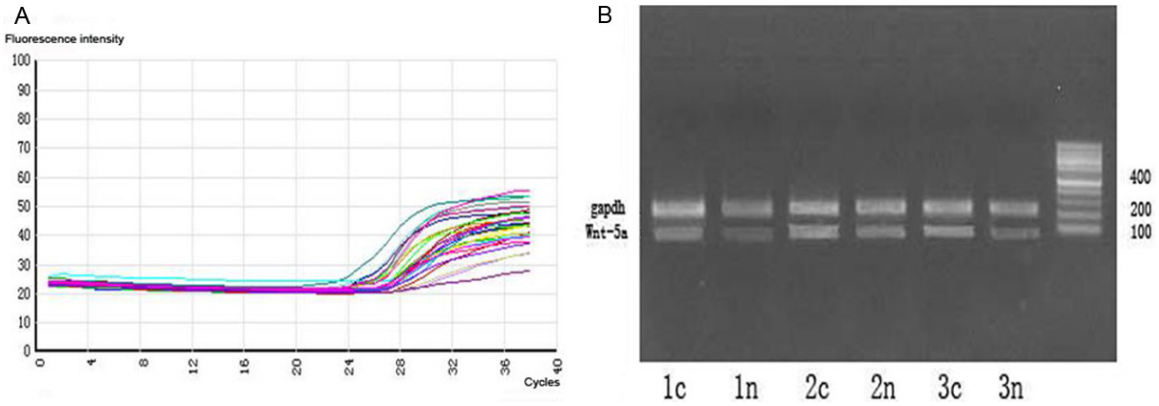

Figure 1. Wnt5a mRNA expression. A: RT-PCR results of Wnt5a mRNA expression in HCC; B: Agarose gel electrophoresis of PCR-amplified Wnt5a and GAPDH gene products. (n: para-carcinoma, c: HCC).

samples were stored at -80°C immediately for later real-time PCR (RT-PCR) analysis.

HCC samples and the corresponding para-carcinoma tissues from 85 HCC patients (67 male and 18 female; average age, 52.6 y). The maximum tumor diameters ranged from 1.5 cm to 17 cm. Normal liver tissues without HBV-infection (n = 6) and liver cirrhosis tissues (n = 15) were studied for comparison.

RT-PCR

Total RNA was extracted from the frozen tissues using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. The extracted RNA was digested with DNase I (Invitrogen) for use in the synthesis of single-stranded cDNA using the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

RT-PCR was carried out using SYBR green dye (TaKaRa Biotechnology Co. Ltd., Dalian, China). Each SYBR 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 (Wnt5a: Forward: 5'-accacatgcagtacatcggag-3, Reverse: 5'-gaggtgttatccacagtgctg-3; GAPDH: Forward: 5'-ggacctgacctgccgtctag-3', Reverse: 5'-tagcccaggatgcccttgag-3' [Shenergy Biocolor Bioscience & Technology Company, Shanghai, China]). 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 38 cycles. The amplified fragment of the Wnt-5a gene was 106 bp. The GAPDH gene (203 bp) was amplified as an internal control. The relative content of the gene amplification product was calculated using the $2^{-\Delta Ct}$ method.

Immunohistochemistry

Immunohistochemical staining of Wnt5a (Santa Cruz, Texas, USA) and β-catenin (Zhongshan, Peking, China) proteins was performed using the streptavidin-peroxidase method on formalin-fixed paraffin-embedded tissue. The Dako Envision Plus System (K5007, Dako, Carpinteria, CA, USA) was used following the manufacturer's recommendations. Blank controls were prepared by replacing the primary antibodies with PBS. Wnt-5a protein appeared as cytoplasmic brown-yellow staining. β-catenin protein expression was localized to the cell membrane with linear brown staining; cytoplasmic or nuclear staining was regarded as abnormal expression.

Statistical analysis

Statistical analysis was carried out using SPSS 17.0 for Windows; P-values were two sided, and $P < 0.05$ was considered significant. The relative mRNA contents were expressed as the mean ± SD, and expression differences were compared using t-tests. Protein expression was analyzed using Chi-square tests.

Results

Wnt5a mRNA expression in hepatocellular carcinoma

The OD260/OD280 ratio of each total RNA sample ranged from 1.8 to 2.1, demonstrating

Table 1. Expression of Wnt5a protein in liver disease

Groups	Cases (*n*)	Wnt-5a protein expression		χ^2	*P*-value
		Positive	Negative		
HCC	85	18	67		
Para-carcinoma	85	69	16	61.234*	< 0.001
Hepatic cirrhosis	15	13	2	25.565*	< 0.001
Normal liver	6	1	5	0.069*	0.793

*Compared with HCC.

that the purity of RNA was suitable for RT-PCR analysis. Agarose gel (0.5%) electrophoresis of the samples showed distinct specific amplification bands for the PCR amplification products of the Wnt-5a and GAPDH genes.

Expressed as fold changes compared with GAPDH mRNA expression levels, Wnt5a mRNA expression was 0.102 ± 0.159 and 0.020 ± 0.022 in HCC and para-carcinoma tissues, respectively. A marked increased in Wnt-5a mRNA expression was detected in 73.1% (19/26) cases of HCC samples (**Figure 1**). There was a statistically significant difference between the Wnt5a mRNA expression of HCC and para-carcinoma tissues (t = 2.22, P = 0.039).

Wnt-5a protein expression

Wnt5a protein expression was detected in HCC tissue, para-carcinoma tissues and hepatic cirrhosis tissues at 21.2% (18/85), 81.26% (69/85) and 86.7% (13/15) of the samples, respectively (**Table 1**). Immunohistochemical staining showed weak Wnt-5a protein expression with yellowish staining in HCC, while moderately or strongly positive expression with diffuse granular staining was observed in hepatic cirrhosis and para-carcinoma tissues (**Figure 2**). Compared with hepatic cirrhosis and para-carcinoma tissues, Wnt-5a protein expression in HCC was significantly reduced or absent (P < 0.001). In contrast, 16.7% (1/6) of normal liver tissue samples showed weakly positive Wnt-5a expression, with no statistical differences compared with the HCC group (P = 0.793).

β-catenin protein expression

In normal liver tissue, β-catenin protein expression was located in the hepatocyte membrane by clear linear brown staining. However, the incidence of abnormal β-catenin protein expression (decreased membrane expression and increased cytoplasm and nuclear expression) was significantly greater in HCC tissue compared with that in para-carcinoma tissues and hepatic cirrhosis tissues (72.94% (62/85), 22.35% (19/85) and 26.67% (4/15), respectively; P < 0.001) (**Figure 3** and **Table 2**).

Discussion

The Wnt signal transduction pathway, which is involved in almost all common human tumors [9-11], comprises two major pathways; the canonical pathway and the non-canonical pathway. The canonical Wnt-β-catenin signaling pathway regulates cell adhesion, cell morphology and gene expression controlling cell proliferation and tissue remodeling. The non-canonical Wnt pathway includes Wnt-5a and Wnt11 which regulate cell migration, invasiveness and metastasis. It has been reported that the non-canonical pathway can antagonize the role of the canonical pathway in tumors [12]. Recently, the relationship between Wnt signaling and HCC has become an important focus of research [13, 14]. Yam *et al.* suggested that the occurrence of HCC is closely related to allelic loss, chromosomal changes and mutations in the Wnt/β-catenin signaling pathway genes [15]. However, the precise molecular mechanism remains uncertain.

Our current study showed that Wnt5a gene transcription and translation are closely related to the occurrence and development of HCC. RT-PCR analysis showed significantly increased Wnt-5a mRNA expression in HCC tissues compared with that in corresponding para-carcinoma liver tissues (P = 0.039), thus revealing the oncogene-like effects of Wnt-5a. In contrast, immunohistochemical analysis showed that there was little or no Wnt5a protein expression in HCC tissue (P < 0.01), while, all hepatic cirrhosis and para-carcinoma liver tissues exhibited moderately or strongly positive immunostaining for Wnt5a. This contradictory expression of Wnt-5a mRNA and protein expression in HCC tissues is similar to expression patterns observed in thyroid carcinoma and malignant melanoma. Bachmann *et al*. found that Wnt5a mRNA was increased obviously in malignant

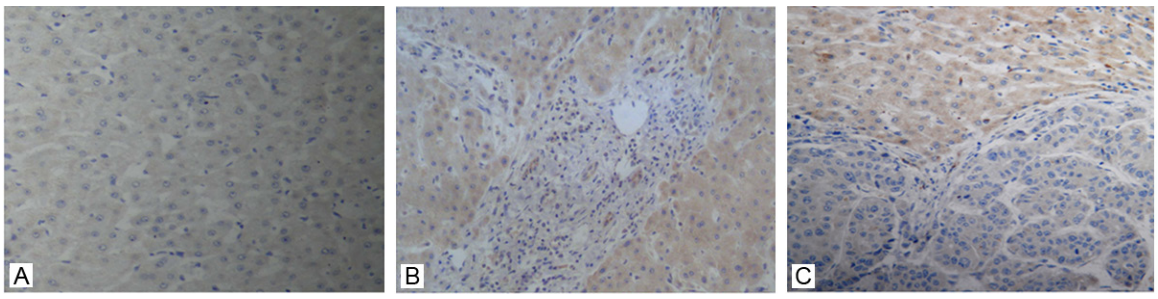

Figure 2. Immunohistochemical analysis of Wnt-5a protein expression in normal, hepatic cirrhosis, para-carcinoma and HCC tissues. A: Negative Wnt-5a expression in normal hepatic tissue; B: Strong Wnt-5a expression in hepatic cirrhosis; C: Strong Wnt-5a expression in para-carcinoma tissues and reduced Wnt-5a expression in HCC tissues.

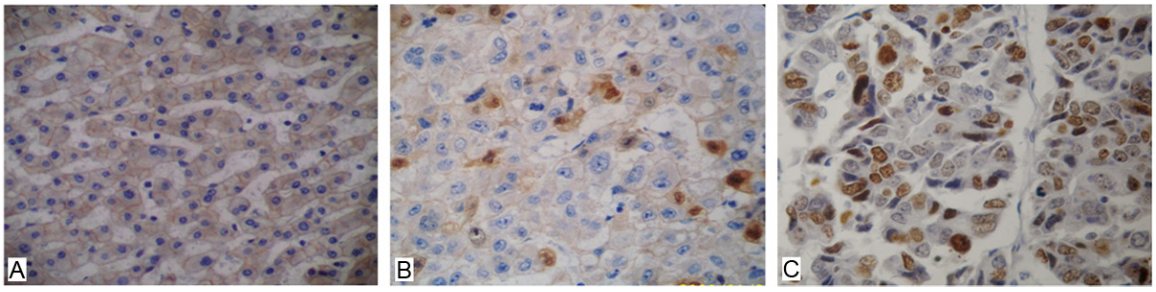

Figure 3. Immunohistochemical analysis of β-catenin protein expression in normal (A), hepatic cirrhosis (B) and HCC tissues (C).

melanoma [16]. However, compared with the benign nevus, the Wnt5a protein and its receptor Frizzled protein in malignant melanoma showed reduced expression or translocation from the nucleus to the cytoplasm. Kremenevskaja *et al*. found a similar pattern of Wnt5a expression in thyroid anaplastic carcinoma, while Wnt5a RNA and protein were both increased in thyroid follicular carcinoma and papillary carcinoma [17].

Wnt5a protein expression patterns vary in different tumors. For example, Wnt5a protein expression is reduced or absent in breast cancer and colorectal cancer compared with noncancerous tissue [18, 19], indicating that Wnt5a plays a suppressor role in these tumors. However, high levels of Wnt5a expression in non-small cell lung cancer, gastric cancer and pancreatic cancer indicate tumor-inducing roles in these malignancies [20-22]. In our study, Wnt5a mRNA expression was higher in HCC than that in para-carcinoma liver tissues, indicating that Wnt5a plays an oncogenic role in HCC. Although Wnt5a gene transcription was upregulated in HCC, at the translational level, expression was obviously reduced or absent. This suggested that the function of the Wnt5a gene is disturbed at the translational level rather than the transcriptional level. The loss of Wnt5a protein expression is related to the loss of tumor suppressor function, and occurs in the advanced stage of HCC tumorigenesis. Mikels *et al*. reported that purified Wnt5a protein activated or inhibited β-catenin-T-cell factor signaling depending on the receptor context in the HEK293 cells [23]. Wnt5a protein inhibits canonical Wnt signaling in a dose-dependent manner, not by influencing β-catenin levels but by downregulating β-catenin-induced reporter gene expression. In addition, Wnt5a can also activate β-catenin signaling in the presence of the appropriate Frizzled 4 [23].

The Wnt/β-catenin signal transduction cascade is a major regulator of liver development and hepatocyte function. Moreover, β-catenin is an established oncogene and defines a genetically distinct subset of HCC [24, 25]. Its activity therefore needs to be tightly controlled. β-catenin binds to E-cadherins on the intracellular side of the membrane. This helps to enable cadherin-based adhesion, establish cell polarity, and establish tight junctions in normal cells. In our study, linear brown β-catenin staining was observed in normal hepatocyte mem-

Table 2. Expression of β-catenin protein in liver diseases

Groups	Cases (*n*)	β-catenin		χ^2	*P*-value
		Membrane positive	Cytoplasm/nucleus positive		
Normal liver	6	6	0	13.733*	< 0.001
Hepatic cirrhosis	15	11	4	12.167*	< 0.001
Para-carcinoma	85	66	19	43.602*	< 0.001
HCC	85	23	62		

*Compared with HCC.

branes, while aberrant cytoplasmic and nuclear expression was observed with increased incidence in liver cirrhosis tissue, para-carcinoma tissue and HCC tissues. It can be speculated that this abnormal pattern of localization is related to tumorigenesis and the development of HCC, with β-catenin exerting oncogene-like effects. Suzuki *et al*. [26] found β-catenin protein expression was located mainly in the cell membrane of highly differentiated HCC tissue with a low Ki-67 proliferation index. In contrast, cytoplasmic or nuclear β-catenin protein expression was observed in less differentiated HCC tissue with a high proliferation index. It can be hypothesized that, to some extent, the change in localization of β-catenin expression reflects the dedifferentiation of HCC tissues [26].

The abnormal localization of β-catenin correlates with gene mutation, which leads to the early appearance of the transcription terminator signal and the generation of truncated proteins [27, 28]. These proteins have no transmembrane domain or β-catenin binding region, resulting in an increase in intracytoplasmic free β-catenin. Miyoshi *et al*. reported that β-catenin mutations, mainly in exon 3 and missing of base fragment, are present in 18.7% of primary HCC [27]. Chan *et al*. reported that CTNNB1 (β-catenin encoding gene) exon 3 deletion or missense mutations are the most frequent event leading to the occurrence of HCC [28]. There is a significant correlation between CTNNB1 missense mutations and the increased nuclear and cytoplasmic expression of β-catenin. Therefore, abnormal activation of the Wnt signaling pathway caused by CTNNB1 missense mutations is an important mechanism underlying the occurrence and development of HCC. Furthermore, it was reported that the incidence of HCC was 100% in mice with β-catenin and H-Ras gene mutations [29].

Based on the results of the present study, it can be hypothesized that β-catenin gene mutations lead to abnormal transposition of β-catenin protein from the membrane to the cytoplasm and nucleus during the process of transformation from cirrhosis to liver cancer. Transcription complexes, formed by a combination of intranuclear β-catenin and transcription factors, activate downstream target genes, and regulate the expression of corresponding genes, leading to HCC tumorigenesis. Therefore, β-catenin mutations may be an initiating or contributing factor in the development of HCC.

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Disclosure of conflict of interest

None.

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