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• ORIGINAL RESEARCH •

Abnormal β-catenin gene expression with invasiveness of primary hepatocellular carcinoma in China

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Abstract

AIM **To study the abnormal expression of β-catenin gene and its relationship with invasiveness of primary hepatocellular carcinoma among Chinese people.**

METHODS **Thirty-four hepatocellular carcinoma (HCC) specimens and adjacent paracancerous tissues,** 4 **normal liver tissues were immunohistochemically stained to study subcellular distribution of β-catenin. Semiquantitive analysis of expression of β-catenin gene exon** 3 **mRNA was examined by RT-PCR and *in situ* hybridization. The relationship between expressions of both β-catenin protein, mRNA and clinicopathological characteristics of HCC was also analyzed.**

RESULTS **Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined displayed membranous type staining for β-catenin protein, occasionally with weak expression in the cytoplasm. While** 21 **cases** (61.8**%**) **of HCC examined showed accumulated type in cytoplasms or nuclei. The accumuled type Labling Index** (**LI**) **of cancer tissue and para-cancarous tissue was** (59.9±26.3) **and** (18.3±9.7) **respectively** (***P***<0.01)**. Higher accumulated type LI was closely related with invasiveness of HCC. Results of RT-PCR showed the β-catenin gene exon** 3 **mRNA Expression Index** (**EI**) **of** 34 **HCCs was higher than that of para-cancerous tissue and normal liver tissue. Using *in situ* hybridization, the signal corresponding to β catenin gene exon** 3 **mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous and normal liver tissues. Over expression of β-catenin exon** 3 **was also found to be correlated with high metastatic potential of HCC.**

CONCLUSION **Abnormal expression of β-catenin gene may contribute importantly to the invasiveness of HCC among Chinese people.**

Subject headings hepatocellular carcinoma; wnt pathway; β-catenin gene; metastasis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is quite common in China. In recent years, great progresses have been made in the treatment of HCC, but the major problem is the high malignancy of HCC, that is, more than 50% of the patients receiving grossly radical treatment will suffer from recurrence within two years. So much effort has been put to investigate the molecular biological characteristics of HCC in order to lower the recurrence rate[1-20]. β-catenin is a ubiquitous intracellular protein which is important in both intercellular adhesion and Wingless/Wnt developmental signaling transduction pathway[21]. β-catenin plays an important role in the interactions between cadherins and other transmembrane receptor proteins, such as the epidermal growth factor receptor. In addition, it is also a signaling molecule and can activate gene transcription by forming a heterodimer with the T-cell factor/lymphoid enhancer-binding factor family of DNA binging proteins[22]. Previous studies have shown that β-catenin is involved in pathways that regulate cellular differentiation and proliferation. In the absence of growth or differentiation signals, cytoplasm β-catenin is rapidly turned over under the control of the APC protein and the GSK-3β, resulting in low level of cytoplasm β-catenin level in normal cells[23,24]. The presence of a wingless-Wnt signal in normal embryonic cells stabilizes β-catenin, which accumulates in the cytoplasm, where it binds to Tcf-lymphoid enhancer factor and triggers gene transcription. Abnormal expression and/or structural abnormalities of catenins are closely associated with tumor development for human esophageal, gastric and colon cancers[25,26]. Previous study has shown that E-cadherin expression was significantly lowed and is closely related with the metastatic potential of HCC[27], and abnormal β-catenin expression has been observed by immunohistochemistry in many malignant human tumors including HCC[28], so it is our logical thoughts whether abnormality of β-catenin gene existed and what its relationship with malignancy in HCC among Chinese people is because of the close relationship between E-cadherin and β-catenin.

MATERIALS AND METHODS

Tissue

Thirty-four HCC specimens and adjacent para-cancerous tissues, four normal liver tissues obtained from patients who underwent surgery in Liver Cancer Institute, Zhongshan Hospital, Shanghai Medical University were analyzed. The tissues were each cut into three parts: one was fixed in formalin, and then embedded in paraffin. Paraffin sections were stained with HE for histological examination of HCC and were also used for immunohistochemistry. One was immediately frozen by liquid nitrogen and stored at -80℃, which was to be used for DNA and RNA extraction. Genomic DNA was purified from all samples using standard proteinase K digestion and phenol/chloroform extraction. Total RNA was extracted using a Trizol reagent (Promega) according to

the protocol recommended by the manufacturer. And the last was rinsed in cold PBS, placed in OCT compound, and immediately frozen in liquid nitrogen, which was to be used for *in situ* hybridization.

Immunohistochemical staining

Immunohistochemical analysis was carried out with the avidin-biotin complex immunoperoxidase technique as described previously[29]. As the primary antibody, polyclonal human anti-β-catenin antibody (Sigma) was used at 500×dilution. As the secondary antibody, biotinylated anti-rabbit IgG (Dako) was used at 100× dilution. Staining was performed using avidin-biotin reagents, 3, 3'-diaminobenzidine, and hydrogen peroxide. As a negative control, duplicate sections were immunostained without exposure to the primary antibodies. All cases were divided into two groups according to immunostaining pattern. Cases with a membrancous staining pattern similar to that in normal hepatic cell were classified as membraneous or normal and cases with marked cytoplasmic and nuclear staining in addition to the membranous staining were defined as accumulated or abnormal. Cells from five randomized views were counted and the cell labeling index (LI) was arbitrarily defined as: (positive cells counted/all cells counted)×100.

RT-PCR

Primers for PCR were designed to amplify the consensus sequence for GSK-3 β phosphorylation in exon 3 of β-catenin gene, based on the published cDNA sequence of human β-catenin gene. To verify the validity of amplification, the primers were designed within the region of exon 3 of β-catenin gene, and the amplification was performed by direct PCR and RT-PCR respectively. Primers, F: AAAGCGGCTGTTA-GTCACTGG R: GACTTGGGAGGTATCCACATCC. PCR: PCR mixture, containing 100pM of primer A and B each, deoxyribonucleotide triphosphates at 200μmol·L^{-1} each, 1.5mmol·L^{-1} $MgCl_2$, 2U *Taq* polymerase (Promega) and 2μL DNA template was adjusted to 50μL by adding double distilled water. Then the mixture was overlaid with 50μL mineral oil and subjected to amplification for 40 cycles. Each cycle consisted of 95℃ for 60s, 55℃ for 45s, 72℃ for 45s. RT-PCR: Total RNAs were reverse-transcribed to obtain the cDNA that was going to be amplified. PCR was also performed under the above same condition except for adding 1μL cDNA to the PCR mixture. A 450bp fragment of β-actin mRNA was also amplified by RT-PCR as the internal control. The PCR products were identified first onto 20g·L^{-1} agrose gel and photographed. The photos of RT-PCR were scanned by optical density scanner (Shimadzu C-9000) and the gene expression index (EI) was arbitrarily defined as density Lum of β-catenin/density Lum of β-actin.

In situ hybridization

Cryostat sections (6μm) were obtained, dried for 2h at RT, and delipidated in chloroform for 5min. Sections were fixed in 40g·L^{-1} paraformaldehyde/PBS for 7min, rinsed in PBS for 3min, rinsed twice in 2× SSC for 5min, and prehybridized at 42℃ for 60min in 4× SSC/100g·L^{-1} dextran sulfate/1× Denhardt's solution/2mM EDTA/500g·L^{-1} deionized formamide/ 500mg·L^{-1} salmon sperm DNA. Hybridization was for 16h in 100μL of prehybridization solution and 20μg·L^{-1} digoxin labeled oligonucleotides (TGTTCC-CACTCATACAGGACTTGGGAGGTATCCACATCCTCTT CCTCAGGA). After hybridization, sections were rinsed twice in 2×SSC for 5min at 37℃, 3 times for 5min each in 60g·L^{-1} formamide and 0.2×SSC at 37℃ and twice for 5min each in 2×SSC at RT. Sections were then rinsed in 100 mol·L^{-1} Tris · HCl, pH 7.5/150 mol·L^{-1} NaCl for 5min, and treated with the same solution saturated with blocking mix for 30min, and then reacted with a 1:2000 dilution of alkaline phosphatase-conjugated sheep antidigoxigenin Fab fragments (750×10^3·L^{-1}) in the same solution. They were rinsed twice in 100mol·L^{-1} Tris · HCl, pH 7.5 and 150mol·L^{-1} NaCl for 5min each, then in 100 mol·L^{-1} Tris · HCl, pH 9.5/100 mol·L^{-1} NaCl/ 50mol·L^{-1} $MgCl_2$ for 10 min, and then reacted with 0.18g·L^{-1} 5-bromo-4-chloro-3-indolyl phosphate, 0.34g·L^{-1} nitroblue tetrazolium, and 240mg·L^{-1} levamisole (Sigma) in the same solution for 6h in the dark at RT. The reaction was stopped with 10mol·L^{-1} Tris · HCl (pH 8.0) and 1mol·L^{-1} EDTA. Sections were counterstained in nuclear methyl green, mounted with aqueous solution, and the final results of average density area and density lum of 500 signal positive cells were analyzed by a multifunctional true digital system (MTDS) using a computer. Albumin oligonucleotide probe and hybridization solution without probe were used as positive and negative control respectively.

RESULT

Immunohistochemical analysis

Immunostaining with polyclonal antibody was performed to evaluate the significance of β-catenin accumulation in HCC. Immunohistochemistry showed that all normal liver tissues and para-cancerous tissues examined showed membranous type, occasionally with weak expression of β-catenin in the cytoplasm, but no β-catenin accumulation in nuclei was found. While for HCC, 21 cases (61.8%) showed accumulated type (Figure 1). The LI of accumulated type for tumor tissue and paracancerous tissue were 59.9±26.3 and 18.3±9.7 respectively (P<0.01), while the LI of membraneous type for tumor tissue and paracancerous tissue were 24.6±8.5 and 91.8±10.6 respectively (P<0.01, Table 1). When LI of accumulated type was analyzed according to the clinicopathological characteristics of HCC, close relationship could be seen with capsule, portal vein tumor thrombus, pathological grade, intrahepatic metastasis (Table 2) and postoperative recurrence (Figure 2).

β-catenin exon 3 mRNA expression

Since the primers were designed in such a way that the product was within β-catenin gene exon 3, direct PCR and RT-PCR were used separately to verify the amplification. Agrose gel electrophoresis showed that PCR and RT-PCR amplification products were both 132bp, which were the same as those of normal liver tissues, para-cancerous tissues and HCC tissues. None of amplification products showed fragment that was shorter. RT-PCR results showed the β-catenin exon 3 mRNA EI were (0.77±0.16) and (0.50±0.05) for HCC tissues and para-cancerous tissues respectively (P<0.05, Figure 3). In HCC, higher EI of β-catenin mRNA attempted to be seen in cancer with incomplete capsule, intrahepatic metastasis and portal vein thrombus (Table 2). Using *in situ* hybridization, we also found the signal corresponding to β-catenin exon 3 mRNA was particularly strong in cytoplasm of HCC when compared with those of para-cancerous tissues and normal liver tissues (Figure 4) and stronger signal of β-catenin mRNA was also closely related to incomplete capsule, intrahepatic metastasis and portal vein thrombus.

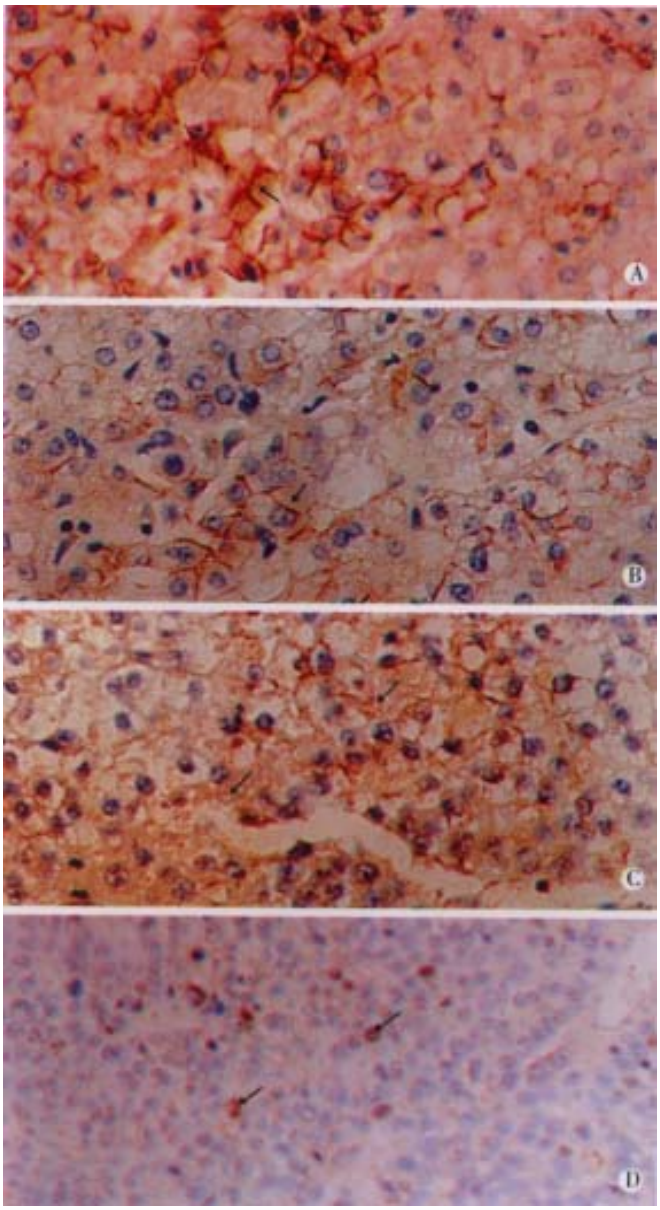

Figure 1 Immunohistochemistry of β-catenin.
A: In normal liver tissue, the staining was mainly positive on the cellular membrane (arrowpoint), with very weak cytoplasmic staining. ×200 B: Para-cancerous cirrhotic liver tissue showed membrane staining (arrowpoint) like normal liver tissue. C,D: For HCC, cytoplasmic and nuclear staining was dominant (arrowpoints), whereas membrane staining was rare. ×200

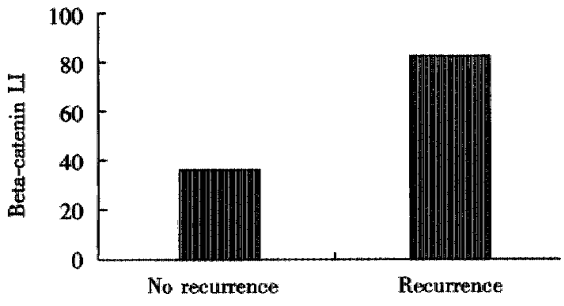

Figure 2 Labeling index (LI) of β-catenin.
Recurrent patient (n = 15) was much higher than that of non-recurrent patient (n=19) (84.9±17.4) *vs* (39.1±14.3).

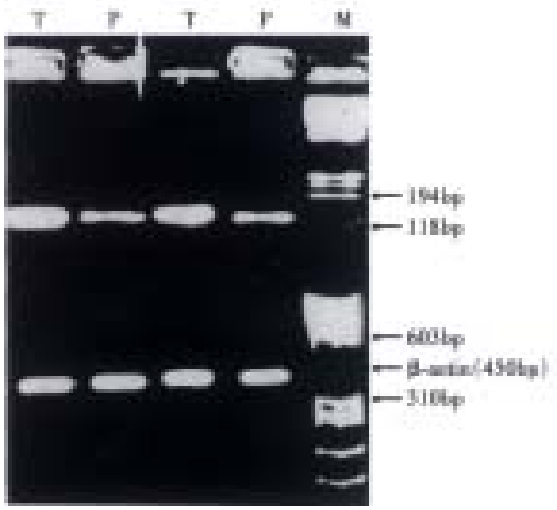

Figure 3 β-catenin mRNA expression index (EI).
HCC was higher *vs* para-cancerous tissue (P<0.05). P: para-cancerous tissue; T: HCC; M: nucleic acid molecular mass marker.

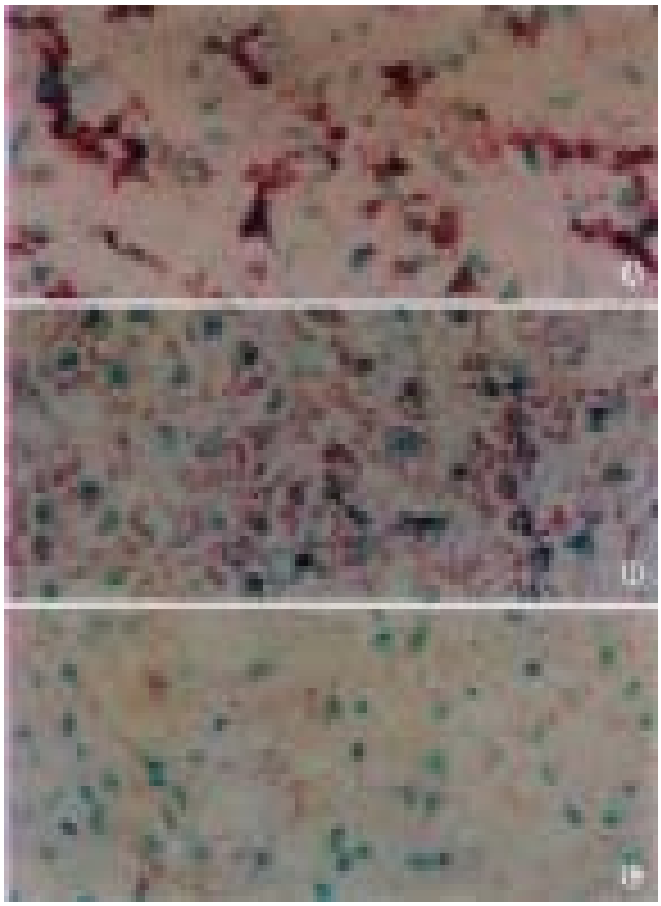

Figure 4 *In situ* hybridization of β-catenin gene mRNA. Stronger in HCC (A) *vs* para-cancerous cirrhotic liver tissue and (B) normal liver tissue (C).

Table 1 Labeling index for β-catenin accumulated type and membraneous type in HCC and para-cancerous tissues (n=34, $\bar{x}$±s)

Tissue	Membraneous	Accumulated
HCC	59.9±26.3	24.6±8.5
Para-cancerous tissue	18.3±9.7[b]	91.8±10.6[b]

[b]P<0.01 *vs* HCC.

Table 2 Relationship between labeling index of β-catenin accumulated type, expression index of β-catenin mRNA and clinicopathological characteristics of HCC

	n	LI of β-catenin accumulated type	EI of β-catenin mRNA
Male	31	58.4±14.2	0.8±0.2
Female	3	54.1±15.3	0.9±0.1
AFP ≤20ng/mL	9	49.3±17.2	0.8±0.1
AFP>20ng/mL	25	54.3±13.7	0.8±0.1
Tumor size			
≤5cm	15	58.7±20.4	0.8±0.2
5cm~10cm	7	54.4±21.3	0.8±0.2
>10cm	12	55.9±17.9	0.8±0.1
Capsule			
Complete	15	72.2±23.4	0.7±0.1
Incomplete	19	44.4±21.1[b]	0.9±0.1[a]
Intrahepatic Metastasis Yes	14	77.2±25.5	0.9±0.2
Intrahepatic Metastasis No	20	41.3±19.6[b]	0.7±0.1[a]
Portal vein thrombus Yes	19	79.8±14.9	0.9±0.2
Portal vein thrombus No	15	52.8±25.9[a]	0.6±0.2[a]
Edmondson's Grade II	19	39.7±20.0	0.7±0.4
Edmondson's Grade III	15	75.9±18.7[b]	0.8±0.2
Cirrhotic nodule ≤ 0.5cm	23	54.3±12.5	0.8±0.2
Cirrhotic nodule >0.5cm	11	62.2±16.6	0.8±0.1

[a]$P<0.05$, [b]$P<0.01$.

DISCUSSION

Previous studies have shown that activation of the wnt pathway results in up-regulation of cytoplasmic β-catenin and its translocation to the nucleus, presumably via the binding of β-catenin to T-cell factor/lymphoid-enhancing factor family members[25,26,30]. Thus, as a first assessment, we examined the subcellular localization of β-catenin in 34 HCC specimens with the result that 61.8% of HCC specimens showed to be accumulated type, suggesting cytoplasmic stabilization of the protein. This showed that activation of Wnt pathway maybe of importance in the carcinogenesis of HCC among Chinese people. Although either β-catenin mutations involving the GSK-3 β phophorylation sites or inactivation of APC and some other factors are related to activation of the Wnt pathway in colon cancer and melanomas[31,32], loss of heterozygosity at the APC locus on chromosome 5 has been detected only at low frequency in human HCC, indicating that inactivation of APC may be infrequent[33]. So mutation of exon 3 of β-catenin gene is probably one of the most important factors activating Wnt pathway and thus causing β-catenin protein accumulated in the cytoplasms in HCC.

Although some studies have been made to investigate β-catenin mutation and abnormal Wnt pathway in HCC[34-41], no previous results have been reported concerning about the relationship between expression abnormality of β-catenin and clinocopathological features of HCC. Furthermore, research reports about the relationship between β-catenin abnormal expression and clinicopathological features of tumors such as colon cancer[42,43], melanoma[44,45], breast carcinoma[46,47], gastric carcinoma[48,49], and lung carcinoma[50,51] are rather various and some of the results were even totally contradictory. That is partly due to most of the previous immunohistochemical studies on β-catenin did not differentiate between membrane-associated type and intracellular accumulated type. Most tumors showed reduced β-catenin in the cytoskeletal fraction but increased β-catenin in the cytosolic fraction and truncated β-catenin protein which was encoded by mutational β-catenin gene was found bound weakly to β-catenin monoclonal antibody when compared with non-truncated β-catenin[52]. This is the reason why we chose polyclonal antibody instead of monoclonal antibody in our study. In this study we aimed to determine which type of expression abnormalities for β-catenin correlate with clinicopathological features and postoperative recurrence in HCC. Our results demonstrated that although great difference existed between cancer tissue and non-cancer tissue, we failed to show the LI of membraneous type to be correlated with the invasiveness of HCC (data not shown here). But, the LI of accumulated type was discovered closely related with the invasive characteristics of HCC, higher EI would predict high ability of invasiveness of HCC and thus a worse prognosis. This was different from another article about gastric carcinoma, which showed that membraneous type, instead of accumulated type, was related to the invasiveness and prognosis of the tumor[47].

Since abnormal expression of β-catenin protein can be caused by both β-catenin gene mutation and over expression, and in some HCCs, both strong membraneous type and accumulated type of staining could be observed, it is our logical thoughts to figure out whether over expression of β-catenin gene existed and what its relationship with the invasiveness of HCC was. This article is the first one to study the β-catenin gene expression in HCC at mRNA level. First we used RT-PCR to examine the expression of β-catenin gene exon 3 mRNA. Since RT-PCR was not very accurate in semi-quantitive analysis of gene expression, we chose *in situ* hybridization to reconfirm the results of RT-PCR. The results of them are the same, that is over expression did exist in HCC and it showed relationship with the invasiveness of HCC (data of relationship between *in situ* hybridization and HCC clinicopathological characteristics not shown). This could give some explanation why strong membranous and cytoplasmic distribution of β-catenin was observed on immunohistochemistry in some HCC while β-catenin gene exon 3 mutation was not observed. It was the accumulation of β-catenin, though apparently normal, that exceeded the capacity of E-cadherin combination and GSK-3 βdegradation, resulting in increase and stabilization of this protein in the cytoplasm.

Although we found that LI of β-catenin accumulated type was related with HCC recurrence, we were unable to find there was such relationship between β-catenin gene EI and HCC recurrence, either by RT-PCR or *in situ* hybridization. This implies that the LI of β-catenin accumulated type would be of greater value in predicting recurrence of HCC. From above we can see that abnormal expression of β-catenin protein, especially the accumulated type, which is closely related to the invasiveness of HCC among Chinese people. Further study should be carried out to confirm this and investigate what the other mechanism causing abnormal expression of β-catenin gene is.

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