Association of hTcf-4 gene expression and mutation with clinicopathological characteristics of hepatocellular carcinoma

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METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) method was used to detect the expression of hTcf-4 mRNA in 32 HCC and para-cancerous tissues and 5 normal liver tissues. PCR-single strand conformation polymorphism (PCR-SSCP) method was used to detect the mutation of hTcf-4 exons 1, 4, 9 and 15 in HCC. The correlation of expression and mutation of the hTcf-4 gene with clinicopathological characteristics of HCC was also analyzed.

RESULTS: RT-PCR showed that the expression rate of hTcf-4 mRNA in HCC, para-cancerous tissues and normal liver tissues was 90.6 %, 71.9 % and 80 %, respectively. The gene expression level in tumor was 0.71±0.13, much higher than that in para-cancerous liver 0.29±0.05 and normal liver 0.26±0.05 (P<0.001), although there was no significant difference in gene expression level between para-cancerous tissues and normal liver (P>0.05). Furthermore, hTcf-4 gene expression was closely associated with tumor capsule status and intrahepatic metastasis of HCC. On SSCP, 2 of 32 cases of HCC (6.25 %) displayed characteristic mutational mobility in para-cancerous tissues. Of the 32 patients, 16 showed abnormal serum concentration of alpha-fetoprotein (AFP) (>20 µg/L), 18 had macronodular cirrhosis (cirrhotic nodules measured <0.3 cm in greatest dimension) and 14 had micronodular cirrhosis (cirrhotic nodules measured <0.3 cm). HCC was large in 17 patients (>5 cm in greatest dimension) and 14 had micronodular cirrhosis (cirrhotic nodules measured <0.3 cm). HCC was large in 17 patients (>5 cm in greatest dimension) and 14 had micronodular cirrhosis (cirrhotic nodules measured <0.3 cm). The association of hTcf-4 gene with clinicopathological characteristics of hepatocellular carcinoma was analyzed.

CONCLUSION: The highexpression level of hTcf-4 in HCC, especially in tumors with metastasis, suggests that the overexpression of hTcf-4 gene may be closely associated with development and progression of HCC, but the mutation of this gene seemed to play less important role in this respect.

INTRODUCTION
Recent work has shown that APC/β-Catenin/Tcf pathway, also known as Wnt signaling pathway, plays a key role in regulation of development and growth of the cells[1-3]. The alteration of APC/β-Catenin/Tcf pathway leading to cancer has been described. In differentiated cells, the cytoplasmic level of β-catenin is maintained very low through degradation by the ubiquitin-proteasome pathway, whereby serine and threonine residues in exon 3 are phosphorylated by GSK3β[4-6] and ubiquitinylated by binding to proteins such as APC, axin and conductin[7-11]. In experiments with colorectal cancer and melanoma cell lines, dysfunction of APC resulted in stabilization of β-catenin and binding of excess β-catenin to Tcf/Lef to activate transcription in the nucleus[12-16]. Furthermore, in cell lines having no APC mutations, mutations of the β-catenin gene that altered amino acid residues representing potential GSK3β phosphorylation sites could confer resistance to degradation and lead to intracellular accumulation of β-catenin[12,17,18]. Activated cytoplasmic β-catenin, probably bound to Tcf/Lef, is thought to migrate into the nucleus and stimulate transcription of downstream genes in a constitutive manner[12,13]. However, recent studies focused mainly on the relationship between cancers and mutations of APC and β-catenin gene, and little is known about the change of human T-cell transcription factor-4 (hTcf-4) gene in tumors, especially about its expression and mutation in hepatocellular carcinoma (HCC). To further understanding the role of the APC/β-Catenin/Tcf pathway in HCC, the present study examined expression and mutation of the hTcf-4 gene in HCC by reverse transcription-polymerase chain reaction (RT-PCR) and PCR-single strand conformation polymorphism (PCR-SSCP).

MATERIALS AND METHODS

Patients
Thirty-two fresh HCC specimens and the para-cancerous tissues and 5 normal liver tissues were analyzed. All specimens were obtained from patients who underwent surgery for HCC or hemangiomia between 1999 and 2000 at Liver Cancer Institute, Fudan University, and stored frozen at -70°C until use. The diagnosis was confirmed by pathological examination. The patients with HCC consisted of 12 women and 20 men with the mean age of 56 years and range from 16 to 75 years.

RNA Extraction and RT-PCR
Total RNA was isolated from tissues using Trizol Reagent (Life Technologies, United States).
Technologies, Inc.) according to the manufacturer’s protocol. A 3-µg aliquot of total RNA from each specimen was reverse-transcribed into single-strand cDNA using oligo (dT)$_5$ primer and SuperscriptII (Life Technologies, Inc.). Each single-strand cDNA was diluted for subsequent PCR amplification of hTcf-4 and β-actin with the latter used as an internal quantitative control. The PCR was carried out in a reaction volume of 25 µl for 5 min at 95 °C for initial denaturing, followed by 25 (for β-actin) or 30 (for hTcf-4) cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s on the Gene Amp PCR system 9 600 (Perkin-Elmer Corp.). The primer sequences used for amplification were 5'-CTTCCTCTTGGCAGTGGAG-3' and 5'-TGGAGGGGCGCGA CTCGTTCA-3' for β-actin and 5'-GTACCCCAATACGACAGGA-3' and 5'-GCCAGCT CGTAGTATCAGC-3' for hTcf-4. PCR products were resolved in 2 % agarose gels and visualized by staining with ethidium bromide. To quantify PCR products, the bands representing amplified products were analyzed by Pharmacia Biotec Image MASTER VDS.

**PCR-SSCP-silver staining**

DNA was isolated from tissues using a DNA extraction kit (BBST Corporation) according to the manufacturer’s protocol. The primers of PCR amplification of exons 1, 4, 9 and 15 of hTcf-4 were as follows: 5'-AATTGCTCCTGGTGGGTA-3' and 5'-CCCAGGGCTTTT CTA-3' for exon 1 (234bp); 5'-GAGCGTTTGAATGGTTTCC-3' and 5'-GCCCTCGAATCTCTTGGTG-3' for exon 4 (124bp); 5'-GATTCTGACGATTACAGG-3' and 5'-GCTACGAGAAAGTGAGAA-3' for exon 9 (196bp) and 5'-GCAGAACACCATTTTGTTGA-3' and 5'-AAACGCTCTGACTGATAT-3' for exon 15 (144bp). The PCR reaction was performed by denaturation at 94 °C for 40 s, annealing at 60 °C for 40 s and extension at 72 °C for 40 s for 30 cycles using 2.5 units of Taq DNA polymerase (BBST Corporation) per 25 µl reaction volume. The PCR products were detected on 2.5 % agarose gels. SSCP analysis was performed as follows: 15 µl of PCR sample plus 20 µl of formamide loading dye (95 % formamide, 0.05 % bromphenol blue, 10 mmol/L EDTA) were boiled for 10 min, frozen- on-ice and electrophoresed on a 12 % non-denaturing polyacrylamide gel at 300 V for 5 min, then 120 V for 3-4 h, depending on the size of PCR fragment. Silver Staining for SSCP consisted of fixation in 10 % alcohol for 5 min, sensitizing in 1 % HNO$_3$, for 5 min, washing twice with distilled water for 2 min, silver reaction (silver nitrate 0.25 g, formaldehyde 50 µl, topped up with distilled water to 100 ml) for 10 min, washing with distilled water for 10s, developing (anhydrous sodium carbonate 6.0 g, formaldehyde 200 µl, 10 % sodium thiosulfate 20 µl, topped up with distilled water to 200 ml) for 10 min, stopping in 10 % glacial acetic acid for 10 min and anhydration in absolute alcohol for 2 min.

**Statistical analysis**

The statistical differences between different groups were analyzed by Student t-test or One-Way ANOVA. A value of $P<$0.05 was considered significant. All data were disposed by SPSS 9.0 statistical software.

**RESULTS**

**Expression of hTcf-4 mRNA in HCC specimens, para-cancerous tissues and normal liver tissues**

2 % Agarose gel electrophoresis showed a 406bp hTcf-4 fragment by RT-PCR amplification from normal liver tissues, para-cancerous tissues and HCC tissues. The hTcf-4 mRNA amplification was successful in 29 of 32 HCC tissues (90.6 %), 23 of 32 para-cancerous liver tissues (71.9 %) and 4 of 5 normal liver tissues (80 %). The expression level was 0.71±0.13 in tumor, much higher than that in para-cancerous liver (0.29±0.05, $P<0.001$) and normal liver (0.26±0.05, $P<0.001$) (Figure 1). However, there was no significant difference in hTcf-4 expression level between para-cancerous tissues and normal liver tissues ($P>0.05$).

**Figure 1** Expression of hTcf-4 and β-actin mRNA in HCC (Ca), para-cancerous tissue (P) and normal liver (N). Semiquantitative RT-PCR analysis revealed that the expression level of hTcf-4 gene in HCC was much higher than that in para-cancerous tissues and normal livers. M: 100bp DNA Ladder

**The relationship between hTcf-4 mRNA expression and clinicopathological features of the patients**

Statistical analysis showed that the expression level of hTcf-4 mRNA in poorly encapsulated tumors and in tumors with intrahepatic metastasis was much higher than that in well encapsulated tumors and in tumors without metastasis ($P<0.05$). However, no significant difference in hTcf-4 mRNA level was observed with variants of serum AFP levels, liver cirrhosis degree and tumor size (Table 1).

**Table 1** The relationship between hTcf-4 mRNA expression and clinicopathological features of the patients

| n | hTcf-4/β-actin | P    |
|---|----------------|------|
| ≤20µg/L | 0.71±0.14 | >0.05 |
| >20µg/L | 0.67±0.12 |      |
| Cirrhotic nodules ≤0.3cm | 0.75±0.11 | >0.05 |
| >0.3cm | 0.67±0.14 |      |
| Tumor size ≤5cm | 0.66±0.13 | >0.05 |
| >5cm | 0.76±0.12 |      |
| Capsule well capsule | 0.61±0.10 | <0.05 |
| poor capsule | 0.79±0.10 |      |
| Metastasis Yes | 0.78±0.12 | <0.05 |
| No | 0.66±0.12 |      |

N: number; Yes: tumors with cancerous thrombi in portal vein or intrahepatic metastasis; No: tumors without cancerous thrombi in portal vein and intrahepatic metastasis.
Detection of mutation by PCR-SSCP-silver staining
Abnormal SSCP migration bands were detected in exon 15 of the hTcf-4 gene from 2 of 32 tumor tissues (6.25 %), compared with the mobility pattern of the para-cancerous liver, demonstrating that there existed mutation in the exon 15. Among these two cases, one was complicated by portal vein thrombosis and the other had tumor without metastasis. The mobility pattern of PCR products from exons 1, 4, and 9 of the hTcf-4 gene was not altered (Figure 2).

Figure 2 SSCP analysis of hTcf-4 exon 15 in HCC (Ca) and para-cancerous liver (P). Lane 1, abnormal SSCP pattern detected in exon 15 of patient 1, compared with the pattern of exon 15 from the corresponding para-cancerous liver in lane 2. Lane 3, normal SSCP pattern of exon 15 from patient 2, compared with the pattern of exon 15 from the corresponding para-cancerous liver in lane 4.

DISCUSSION
The Tcf-4 gene is a member of the APC/-catenin/Tcf pathway that is well known to play a crucial role in many developmental processes and human carcinogenesis[19-27]. Mapping to chromosome band 10q25.3[28], Tcf-4 encodes a transcription factor that interacts functionally with -catenin to transactivate target genes[29-31]. Morin has recently shown that the nuclei of colon carcinoma cell lines contain constitutively active Tcf-4/ -catenin complexes as a direct consequence of either loss of function of the tumor suppressor protein APC or gain of function by mutations in -catenin itself[32]. This is believed to result in the uncontrolled transcription of Tcf target genes, leading to transformation of colon epithelial cells and initiation of polyp formation. High level of hTcf-4 expression has been identified in colon cancer, mammary carcinoma and a variety of colorectal cancer cells[21,29]. Tcf factors have also been reported as tumor inducers which aberrantly activate their target genes, now known as the c-myc gene and cyclin D1 gene, in many types of cancer[30-32]. It has been reported that c-myc gene and cyclin D1 gene had a high expression level in HCC and were implicated in tumor progression and metastasis with the unclear mechanism[33-39]. Therefore, it is important to elucidate the internal link between APC/-catenin/Tcf pathway and liver cancer. Our present studies showed that the level of hTcf-4 expression in cancer tissues was much higher than that in para-cancerous tissues and normal liver tissues (P<0.001). Moreover, we found that hTcf-4 expression was closely correlated with the integrity of tumor capsule and intrahepatic metastasis of HCC but not with serum AFP levels, liver cirrhosis degree and tumor size, suggesting that hTcf-4 expression was associated with invasion and metastasis of HCC. This may be due to interaction between Tcf and -catenin. Huber et al. reported that the complex of Tcf and -catenin in the nuclear binds to the -catenin gene promoter and down-regulates -catenin gene transcription[40]. On the other hand, loss of -catenin expression can contribute to the up-regulation of Tcf/ -catenin pathway in human cancers[41]. As a result, the role of -catenin in cell-cell adhesion is reduced, which may contribute to the metastatic potential of tumor cells.

With regard to the relationship between Tcf-4 mutations and tumor, Duval et al[42] reported that 50 % of human MSI-H (high frequency microsatellite instability) colorectal cell lines and 39 % of MSI-H colorectal primary tumors were found to have a 1-bp deletion in an (A)9 repeat within the coding region of this gene. The (A)9 repeat normally codes for several isoforms that could serve as modulators of Tcf-4 transcriptional activity. The deletion of one nucleotide in this repeat could change Tcf-4 transactivating properties by modifying the respective proportions of the different isoforms. In addition, one frameshift mutation in the -catenin binding domain (exon 1), one missense mutation in exon 4 and six nonsense or frameshift mutations localized in the 3' part of the gene were detected in a series of 24 colorectal cancer cell lines[43]. The latter alterations interfered with the Tcf-4 capacity to interact with COOH-terminal binding protein that was implicated in the repression of the Tcf family transcriptional activity. As a result, the Tcf-4 transcriptional activity was enhanced. This indicated that the mutation of Tcf could be an important event during colorectal carcinogenesis by modifying Wnt signaling. In our experiment, we used PCR-SSCP-silver staining analysis to detect mutation of hTcf-4 exons 1, 4, 9 and 15 from human liver cancer tissues. The sensitivity of this method is generally high and greater than 80 % of mutations in most DNA fragments of 300 bp or shorter can be detected since variation of DNA sequence often results in a shift in electrophoretic mobility, which is believed to be caused by sequence-dependent alteration in the tertiary structure of single-stranded DNA. We found SSCP variants in exon 15 of 2 HCC cases (6.25%) only, of which one was complicated by portal vein thrombosis and the other originated from the tumor tissue without metastasis. It seemed that, unlike in colorectal tumor, hTcf-4 mutation may play less important role in HCC and was irrelevant to invasion and metastasis of HCC. Therefore, the enhanced transcriptional activity of hTcf-4 due to aberrant mRNA expression may be the key to HCC occurrence and development. Further study on structure and function of hTcf-4 and its interaction with oncogenes should contribute to clarification of the mechanism of liver carcinogenesis and may provide the theoretical principle for the gene therapy.

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