Expression of Twist Gene in Primary Liver Cancer*

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Summary: In order to investigate the possibility of overexpression of Twist in primary liver cancer (PLC), the Twist expression was detected by using immunohistochemical analysis and RT-PCR assay in 45 patients with PLC. Control tissues were obtained from 9 patients with liver hemangioma. It was found that in 36 (80.0%) out of 45 PLC patients, cancerous regions showed positive cytoplasm and nucleus staining for Twist with a diffuse pattern. In noncancerous adjacent areas and control liver tissues, the expression of Twist was 57.8% and 22.2% respectively. The results of RT-PCR assay revealed that the expression of Twist was stronger in the cancerous tissues than that in the noncancerous adjacent tissues. It was suggested that the expression of Twist was up-regulated in PLC, which play an important role in the progression of PLC.

Key words: Twist; primary liver cancer

Primary liver cancer (PLC) is one of the most common cancers, with more than one million fatalities occurring annually worldwide[1]. Multiple risk factors are associated with the disease etiology, with the highest incidence in patients with chronic hepatitis B virus, alcohol consumption, exposure to dietary aflatoxin B1, hereditary liver disease or liver cirrhosis of any etiology[2–3]. However, the molecular mechanisms causing the development and progression of HCC remain unclear. Twist is a transcription factor. Recently reports showed Twist is overexpressed in gastric cancer, breast carcinoma, and rhabdomyosarcoma[4–6]. It was found that up-regulation of Twist was associated with PLC. In the present study, the expression of Twist was detected by using immunohistochemistry and RT-PCR assay in PLC.

1 MATERIALS AND METHODS

1.1 Patients
Among 45 patients with PLC, 44 patients had chronic HBV infection. Their mean age was 48 years (range 23–78). There were 39 males and 6 females respectively. All patients were confirmed pathologically. Tumor was less than 5 cm in diameter in 11 patients and more than 5 cm or with intrahepatic metastasis in 34. The serum AFP was positive in all cases detected by radio-immunoassay (RIA). Serum concentrations of AFP were above 400 µg/L in 40 patients. Control liver tissues were obtained from 9 patients with liver hemangioma.

1.2 Immunohistochemistry
To demonstrate the presence of Twist in the liver tissues, each fresh specimen was cut at −20°C with the cryostat (4 µm thick), mounted on sialinized adhesion microscope slides, dried overnight at room temperature, fixed with cold acetone at −20°C for 10 min and chloroform for 20 min. Anti-human Twist mouse monoclonal antibody (TRIzol reagent) was used. The immunohistochemical test was performed according to the manufacturer’s instructions. In the negative controls only primary antibodies were substituted for. The cells positive for the Twist expression had brown particles in cytoplasm and nuclei in the tissues of PLC. For Twist analysis, 10 fields were randomly selected and counted at a magnification of 200. Twist staining was evaluated semi-quantitatively on the basis of the percentage of positive cells, and classified as follows: diffusely positive (+++) when positive cells comprised more than 50% of the total cells; moderately positive (++), positive cells 16%–50%; weakly positive (+), positive cells 10%–15%; and negative (-), positive cells less than 10%. Histopathologic examination was performed by a senior pathologist without prior knowledge of the Twist expression.

1.3 RNA Extraction and RT-PCR
Total RNA was extracted with Trizol reagent (Gibco, USA) according to the manufacturer’s instructions. Reveres transcription of RNA was carried out with RT-PCR system (Invitrogen, USA). The cDNA was synthesized according to the manufacture’s instructions. Twist gene was amplified by upstream primer 5′- ccg agg cct aga tgt cat tgtt-3′ and the downs stream 5′- tct aga cgg cag gtc agg tcc acc -3′ under the PCR reaction conditions of 94°C for 3 min, 94°C for 45 s, 55°C for 40 s, 72°C for 50 s, 35 cycles; final extension at 72°C for 8 min.. The PCR product was analyzed with 1% agarose gel, stained with ethidium bromide, and photographed under UV light. As an internal control, GAPDH was amplified by upstream primer 5′- ccg ggc gcc ctg gca aat tcc atg gca -3′ and the downs stream 5′- tct aga cgg cag gtc agg tcc acc -3′ under the PCR reactions. The relative expression levels were generated by comparing the density to the controls and indicated underneath each gel. Measurements were repeated twice to ensure reproducibility of the results.
1.4 Statistical Analysis
Statistical analysis was performed with SPSS 10.0 software. All data were compared by q-test or \( \chi^2 \)-test. A \( P \) value less than 0.05 was considered statistically significant.

2 RESULTS

2.1 Immunohistochemical Staining
In 36 out of 45 PLC patients, cancerous regions showed positive cytoplasmin and nucleus staining for Twist in a diffuse pattern (fig. 1A). Parts of membrane of PLC cells showed stronger positive reactions (fig. 1B). In contrast to the cancerous areas, in noncancerous adjacent areas and control tissues, the expression of Twist was 57.8% and 22.2% respectively (table 1).

![Fig. 1 Immunohistochemical staining of Twist in PLC tissues](image1)

Table 1 Expression of Twist protein in Cancerous, adjacent and control liver tissues

<table>
<thead>
<tr>
<th>Groups</th>
<th>( n )</th>
<th>Positive</th>
<th>Negative</th>
<th>Positive rate</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancerous tissues</td>
<td>45</td>
<td>36</td>
<td>9</td>
<td>80.0%</td>
<td>0.0227*</td>
</tr>
<tr>
<td>Adjacent tissues</td>
<td>45</td>
<td>26</td>
<td>19</td>
<td>57.8%</td>
<td>0.1130</td>
</tr>
<tr>
<td>Control liver tissues</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>22.2%</td>
<td>0.0005***</td>
</tr>
</tbody>
</table>

* Cancerous tissues vs Adjacent tissues \( x^2=5.184 \);
** Adjacent tissues vs Control liver tissues \( x^2=2.501 \);
*** Adjacent tissues vs Control liver tissues \( x^2=12.008 \)

2.2 Expression of Twist mRNA
Twist mRNA in 30 cases of PLC was detected by RT-PCR analysis (fig. 2). The results showed that freshly aspirated liver tissues expressed the gene coding for Twist because RT-PCR generated a DNA fragment corresponding to the predicted length, 244 bp, of the Twist amplification product. In each tissue sample, all GAPDH amplified products were of 598 bp. Twist mRNA in PLC was positive in 25 out of 30 cases (83.3%), and in noncancerous adjacent and control tissues 73.3% and 33.3%, respectively. The result revealed that the expression of Twist was stronger in the cancerous tissues than that in the noncancerous adjacent and control tissues (table 2).

![Fig. 2 Detection of Twist mRNA in cancerous and adjacent tissues by RT-PCR](image2)

Table 2 Comparison of \( A \) values of Twist mRNA among cancerous, adjacent and control liver tissues

<table>
<thead>
<tr>
<th>Groups</th>
<th>( A ) values of Twist mRNA (32±e)</th>
<th>( P ) values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancerous tissues</td>
<td>0.621±0.327</td>
<td>0.000*</td>
</tr>
<tr>
<td>Adjacent tissues</td>
<td>0.490±0.231</td>
<td>0.00006</td>
</tr>
<tr>
<td>Control liver tissues</td>
<td>0.344±0.204</td>
<td>0.037</td>
</tr>
</tbody>
</table>

* Control liver tissues vs Cancerous tissues; 6 Adjacent tissues vs Cancerous tissues; 7 Control liver tissues vs adjacent tissues

3 DISCUSSION
Twist was originally identified in Drosophila as a protein involved in establishing dorsoventral polarity. It belongs to the basic-helix-loop-helix (bHLH) family of transcription factors and is quite similar in the bHLH carboxy-terminal domains. In mammals, Twist contributes to morphogenesis of the cranial neural tube and limb development. The gene maps to 7p21 and mutations in the gene have been reported in Saethre–Chotzen form of craniosynostosis. Although the function of Twist is not fully clear at the molecular level, Twist has been indicated as a potential oncogene interfering with p53-related pathways leading to preventing myc-induced apoptosis in mouse embryonal fibroblasts. Twist was also found to be involved in the suppression of differentiation and protection of apoptosis through inhibition of p21Waf1 via both p53-dependent and -independent pathways. The antiapoptotic function of Twist has been shown through modulating NFκB pathways, and loss of Twist expression leads to sensitization to TNFα-induced apoptosis. These lines of evidence strongly indicate the positive role of Twist in protection against cell death. Recent studies on human cancer indicated that Twist was associated with oncogenic properties. A report showed that 50% of the rhabdomyosarcoma cases had high levels of Twist, although only 15 cases were analyzed. Another study on 28 cases of gastric carcinomas showed that 39% of them (11 out of 28) had increased Twist expression compared to the nonmalignant tissues. In our study on 45 patients with PLC, immunohistochemistry revealed that most of Twist was expressed in the cytoplasm and nucleus (fig. 1A) and some of them expressed on the membrane of the tumor cells (fig. 1B). It was evidenced that the increase of Twist expression was associated with the expression of Twist in the membrane of tumor cells. In summary, the results in this study suggested that Twist was commonly expressed in PLC. Twist may play multiple roles in the formation and progression of PLC.