Expression of WTX gene in hepatocellular carcinoma and cell lines and its clinical significance

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Abstract: Objective: Wilms’ tumor gene on the X chromosome (WTX) was reported to be a tumor-suppression gene for various cancers. The purpose of this study was to detect the expression and significance of WTX in hepatocellular carcinoma (HCC) and cell lines. Methods: The expression of WTX at mRNA and protein level was evaluated by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, respectively. The results were performed using the LSD and Dunnett multiple comparison test. Results: WTX expression in HCC cell lines was obviously lower than that in hepatocellular cell line both at mRNA (P=0.001 for all) and protein level (P<0.05 for all). Relative to the value of adjacent non-cancerous tissues, the WTX gene expression level in HCC tissues was lower (P=0.001). A further analysis indicated that WTX expression was obviously correlated with TNM stage, differentiation and lymph node metastasis (P<0.05 for all). Conclusions: This study indicates that WTX may be associated with the progression of HCC and be a molecular target for gene therapy in HCC.

Keywords: Tumor-suppressor gene, WTX, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent cancer and is the third reason of cancer-related deaths worldwide [1, 2]. The characteristics of HCC are rapid progression, poor prognosis and frequent recurrence [3]. The incidence rate of HCC in China accounts 50% of the global cases and the morbidity of hepatitis B is high in China [2]. Hepatitis B virus (HBV) infection and then development of liver cirrhosis can lead to HCC at last [4]. Besides HBV infection, hepatitis C virus (HCV) infection and alcohol intake are also recognized as the major causes of HCC [2]. Moreover, HCC is a cancer with the poorest prognosis among common malignant tumors [5]. Although, survival of HCC patients was improved according to surgery, chemotherapy and radiotherapy, the long-term survival, recurrence, metastasis and prognosis remain unsatisfactory [6]. Therefore, the development of improving the survival of patients after surgery is a crucial tissue.

A special tumor-suppressor gene and new X chromosome gene named Wilms’ tumor gene on the X chromosome (WTX) was discovered when studied Wilms’ tumor [7]. Of note, a β-catenin destruction complex forming with WTX protein, β-catenin, AXIN1, β-TrCP2 and APC, promoted the degradation of β-catenin to regulate Wnt/β-catenin signaling [8]. Scheel et al. mentioned that the mutations of WTX gene might damage the β-catenin destruction complex thus cause the process of carcinogenesis in colorectal cancers [9]. It was consistent with that WTX was a tumor-suppressor gene. In addition, previous studies suggested that WTX mutation was rare in gastric, colorectal, hepatocellular carcinomas and acute leukemias [10, 11]. It may correlate with the deletions of WTX. It indicated that the deletions and mutations of WTX gene might lead to tumorigenesis and suggested that WTX might be a novel molecular target for the therapy of HCC.

To investigate the effect of WTX in the progression of HCC, we observed the mRNA expression level of WTX in HCC tissues and adjacent non-cancerous tissues in this study, and we also analyzed the correlation between WTX expression and the clinical characteristics of HCC.
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Table 1. The association between WTX expression and clinical character in HCC patients

<table>
<thead>
<tr>
<th>Clinical factors</th>
<th>Case (n)</th>
<th>WTX expression (mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>0.56</td>
</tr>
<tr>
<td>≤48</td>
<td>26</td>
<td>0.0170 ± 0.0141</td>
<td></td>
</tr>
<tr>
<td>&gt;48</td>
<td>14</td>
<td>0.0252 ± 0.0092</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.815</td>
</tr>
<tr>
<td>Male</td>
<td>32</td>
<td>0.0201 ± 0.0146</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
<td>0.0189 ± 0.0020</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>I-II</td>
<td>13</td>
<td>0.0343 ± 0.0023</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>27</td>
<td>0.0129 ± 0.0100</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td>0.014</td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>0.0257 ± 0.0121</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>0.0156 ± 0.0123</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>0.010</td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>0.0095 ± 0.0138</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>32</td>
<td>0.0225 ± 0.0117</td>
<td></td>
</tr>
</tbody>
</table>

patients. Moreover, to find the potential roles of WTX in HCC, we investigated the expression of WTX at mRNA level and protein level in hepatocellular cell line and hepatocellular cancer cell lines.

Materials and methods

Patients and cell lines

Tumor tissues and adjacent non-cancerous tissues were obtained from 40 HCC patients, who had determined histopathological diagnosis by the First Affiliated Hospital of Nanchang University. All patients did not receive chemotheraphy, radiotherapy or biotherapy before collecting specimens. The specimens were immediately frozen by liquid nitrogen and then stored at -80°C. The detail information of patients was shown in Table 1. Informed consent was given by all patients and the current study was approved by Medical Research Ethics Committee. The tumor differentiation of HCC was assessed by Edmondson-Steiner method [12].

Hepatocellular cell line L02 (HL-7702) and four hepatocellular cancer cell lines, MHCC-97L, MHCC-97H, HepG2 and SMMC-7721, were used in this study. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO₂.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of tissues and cell lines was isolated using RNAiso Plus (Takara) according to manufacturer's recommendations. The first strand-cDNA was synthesized with Prime Script™ RT Reagent Kit (Takara) and PCR was performed with 2× Taq Master Mix Kit. The templates for GAPDH and WTX PCR mixture were same. The GAPDH gene was used as an endogenous control and the primer pairs designed by Primer premier 6.0 were as followed, WTX: forward-5’-TCCCCTTCCCCCTCTATACTG-3’, and reverse-5’-CATGGTCATAGGAGGTATGC-3’ (172 bp), GAPDH: forward-5’-CAGGGCTGCTTTTAACTCTG-3’, and reverse-5’-GATTGGAGGGATCTCGT-3’ (199 bp). The PCR products of WTX and GAPDH were analyzed on 2% agarose gels. The intensities of WTX electrophoretic bands were compared to GAPDH and evaluated by Image J Software.

Western blot

The cells were cultured in 6-well plates and then disrupted in RIPA lysis buffer which contained PMSF. The protein concentration was examined by BCA Protein Quantification Kit (CWBIO, Beijing, China). The samples were separated by SDS-PAGE. After transferring to PVDF membranes, the membranes were incubated in 5% BSA blocking buffer for 1 h. Then primary antibody (rabbit anti human WTX, diluted 1:1000, Abgent) was added and membranes were incubated overnight at 4°C. The membranes were washed and incubated with secondary antibody (rabbit anti human β-actin, diluted 1:1000) for 1 h and then the bands were detected on X-ray films. The protein bands were scanned by Image J Software and the expression of WTX protein was assessed compared to β-actin.

Statistical analysis

Statistical analysis was performed by SPSS 19.0 software and all data represented mean ± standard deviation (SD). Continuous variables were evaluated by Student t test. Multiple comparisons among different groups were performed using the LSD and Dunnett multiple comparison test. For all tests, the P<0.05 was considered significance.
Results

Expression of WTX at mRNA level in cell lines

The mRNA expression of WTX was determined in different human cell lines by RT-PCR. The result showed that compared to the value of human hepatocellular cell line L02, WTX expression value was obviously lower in human hepatocellular cancer cell lines, MHCC-97L, MHCC-97H, HepG2 and SMMC-7721 (P<0.001 for all, Figure 1).

WTX expression at protein level in cell lines

The expression of WTX at protein level was evaluated in different human cell lines using western blot. It indicated that WTX protein level of human hepatocellular cancer cell lines was lower than that in human hepatocellular cell line (P<0.05 for all, Figure 2). It was consistent with the result of WTX expression at mRNA level.

Expression of WTX at mRNA level in human HCC tissues and adjacent non-cancerous tissues

WTX expression at mRNA level was assessed in HCC tissues and adjacent non-cancerous tissues of patients by RT-PCR. Our result confirmed that the expression of WTX was significantly downregulated in HCC tissues than that in adjacent non-cancerous tissues (P<0.001, Figure 3).

Association between WTX expression and clinical characteristics in HCC patients

To investigate the correlation between WTX expression and clinical factors, detail information was evaluated using Student t test. The result suggested that the mRNA expression level of WTX in I-II stage was obviously higher than that in II-IV stage (P=0.001). Meanwhile, there was significant correlation between WTX expression and tumor differentiation, Lymph node metastasis (P<0.05 for all). Moreover, the expression of WTX was not significantly associated with age and gender (P>0.05 for all).

Discussion

Previous studies reported that there were at least three carcinogenesis pathways, including p53, Rb and Wnt/β-catenin signaling, involved in pathogenesis of HCC [13, 14]. There was increasing evidence revealed that the aberrant activation of Wnt/β-catenin signaling was correlated with carcinogenesis, progression of tumor and invasion [15-17]. It was important to block aberrant Wnt/β-catenin signaling for suppression of tumor cells proliferation. In this pathway, β-catenin acted a crucial role in activation of Wnt/β-catenin signaling. Normally, β-catenin was quickly degraded though β-catenin complex to suppress the development...
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One component of this complex was WTX protein. In the present study, we found WTX was associated with HCC and its expression in human hepatocellular cancer cell lines was lower than that in hepatocellular cell line. However, the expression of WTX in tumor was reported rarely.

WTX gene (also called AME-R1, APC membrane recruitment protein 1) encodes a protein of 1135 amino acids, which has interaction with APC (adenomatous polyposis coli) protein [19]. APC is a tumor-suppressor gene with multifunction that negatively regulates Wnt signaling. There were studies implicating the invalidation of APC gene caused liver tumorigenesis by activating the Wnt/β-catenin pathway [20]. However, mutation of APC was important but not enough to account for accumulation of β-catenin in human HCC [21]. Meanwhile, WTX protein could form a complex to degrade β-catenin [8]. Moreover, WTX gene in cells caused apoptosis and suppressed the colony formation [7]. Subsequent studies revealed that Wnt receptor low-density lipoprotein receptor-related protein-6 (LRP6) phosphorylation was an important step in Wnt/β-catenin signaling [22]. As mentioned above, over-expression of AMER1 (WTX) played a role in activation of Wnt signaling at the LRP6 level, whereas the loss and inhibition of function indicated WTX negatively regulated Wnt signaling pathway by inducing degradation of β-catenin [8, 22-24]. Therefore, WTX had a dual functional role in Wnt pathway. However, the major role of WTX
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was tumor-suppression gene in cells and tissues.

It found that there were small deletions and point mutations of the WTX gene in Wilms' tumor [7]. Due to WTX gene located in the X chromosome, compared to the classical biallelic “two-hit" inactivation of other tumor-suppressor gene, the mutations of WTX gene was probably "one-hit" in sporadic Wilms tumor [7]. It applied to the hemizygous deletions and mutations both evaluated in male and female, because one X chromosome in female was inactive during the growth [7]. It demonstrated the WTX gene would play an important role in HCC via Wnt signaling pathway. However, there were little studies on expression and transcription of WTX in HCC.

In our study, the human hepatocellular cancer cell lines, HepG2 and SMMC-7721 had no metastatic potential, MHCC97L had lowly metastatic potential and MHCC97L had highly metastatic potential [25, 26]. The expression of WTX at mRNA level and protein level was not obviously different among hepatocellular cancer cell lines. It suggested that the WTX expression had no correlation with the metastatic potential of human hepatocellular cancer cell lines.

The WTX gene expression in HCC tissues was significantly lower compared with that in adjacent non-cancerous tissues. The studies about WTX expression were not enough to confirming this result. In current, lots of studies proved that WTX expressed abnormally in Wilms tumors and acute leukemias. In the study of Ruteshouser, mutations in WTX were probably about 1/3 of Wilms tumors [27]. However, it was rare in other tumors [10, 11]. It was also possible that the deletion of WTX gene caused WTX inactivation in colorectal or hepatocellular or gastric carcinoma [10]. Moisan et al. showed that WTX deletion caused neonatal lethality and somatic overgrowth in mice [28]. These results might provide evidence to indicate WTX was a tumor-suppressor gene.

In conclusion, the expression of WTX is lower in HCC cell lines, as well as in HCC tissues. WTX may be correlated with progression of HCC, which can act as a molecular target for gene therapy in HCC. Further studies are still required.

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Disclosure of conflict of interest

None.

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