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Original Article

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

Table 1. The association between *WTX* expression and clinical character in HCC patients

Clinical factors	Case (n)	*WTX* expression (mean ± SD)	*P*
Age			0.056
≤48	26	0.0170±0.0141	
>48	14	0.0252±0.0092	
Gender			0.815
Male	32	0.0201±0.0146	
Female	8	0.0189±0.0020	
TNM stage			0.001
I-II	13	0.0343±0.0023	
III-IV_A	27	0.0129±0.0100	
Tumor differentiation			0.014
High	17	0.0257±0.0121	
Low	23	0.0156±0.0123	
Lymph node metastasis			0.010
Yes	8	0.0095±0.0138	
No	32	0.0225±0.0117	

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 chemotherapy, 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% CO_2.

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'-TCCCCTTTCCCTCTATACTG-3', and reverse-5'-CATGGTCATAGGAGGTATGC-3' (172 bp), *GAPDH*: forward-5'-CAGGGCTGCTTTTAACTCTGGT-3', and reverse-5'-GATTTTGGAGGGATCTCGCT-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.

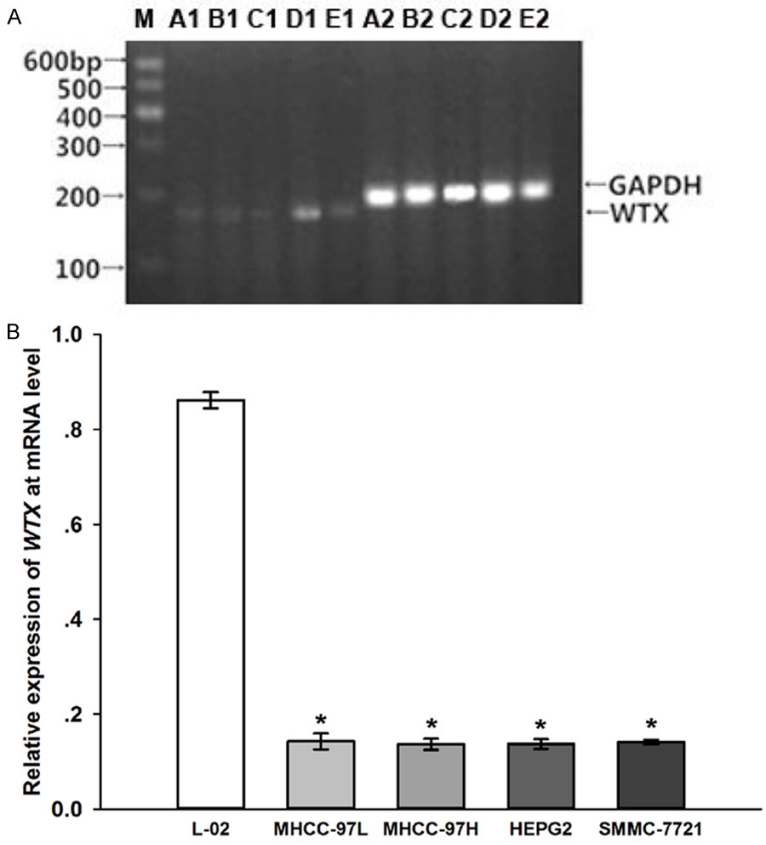

Figure 1. The expression of *WTX* mRNA in different cell lines. A. RT-PCR analysis, Lane M: DNA size marker; Lanes A1-E1: *WTX* expression in L02, MHCC-97L, MHCC-97H, HepG2 and SMMC-7721 cell lines; Lanes A2-E2: *GAPDH* expression in L02, MHCC-97L, MHCC-97H, HepG2 and SMMC-7721 cell lines. B. Semi-quantitative analysis, the *WTX* gene in HCC cell lines was lower compared with that in hepatocellular cell line (P=0.001 for all). *: P<0.05.

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 down-regulated 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_A 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

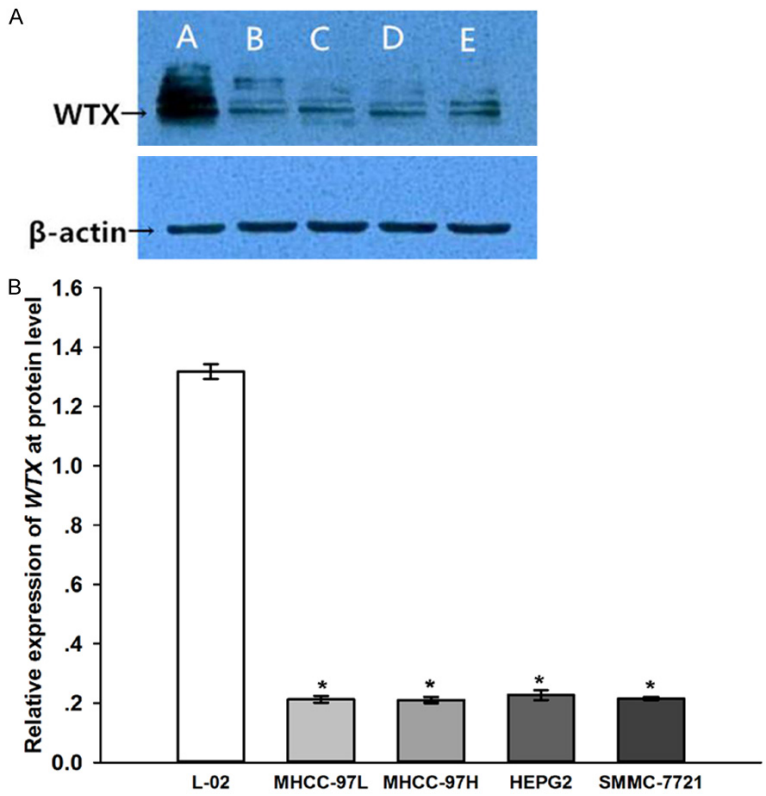

Figure 2. The expression of *WTX* protein in different cell lines. A. Western blot, Lane A: L02; Lane B: MHCC-97L, Lane C: MHCC-97H, Lane D: HepG2 and Lane E: SMMC-7721. B. Semi-quantitative analysis, relative to the value of hepatocellular cell line, the *WTX* protein expression value of HCC cell lines was lower ($P<0.05$ for all). *: $P<0.05$, compared to the protein expression of WTX in L02 cell line.

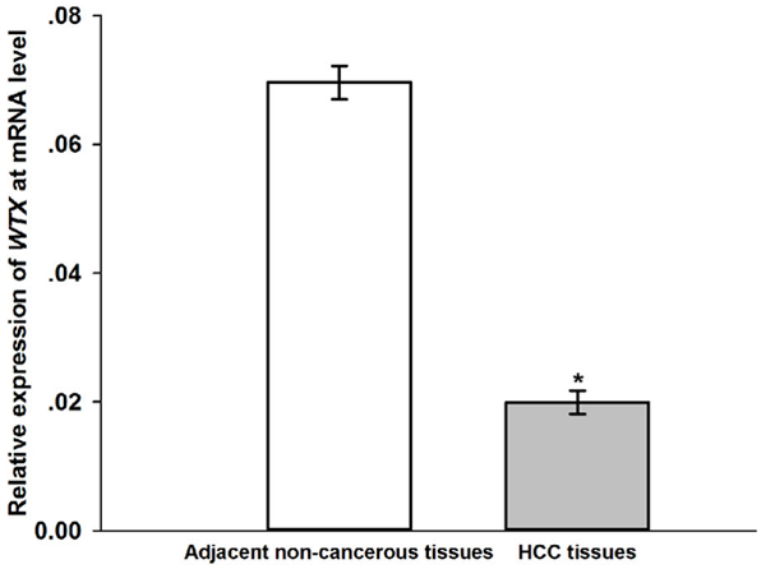

Figure 3. *WTX* mRNA expression in HCC tissues and adjacent non-cancerous tissues (*$P<0.05$ indicated the significant difference relative to adjacent non-cancerous tissues). The expression level of *WTX* was down-regulated in HCC tissues compared to that in adjacent non-cancerous tissues ($P=0.001$).

and progression of tumor [8, 18]. 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 *AMER1*, 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 an 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*

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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