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• BASIC RESEARCH •

Hepatitis B virus X gene induces human telomerase reverse transcriptase mRNA expression in cultured normal human cholangiocytes

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Abstract

AIM: To study the transcriptional regulation of human telomerase reverse transcriptase (hTERT) mRNA in normal human cholangiocytes (HBECs) after hepatitis B virus X (HBx) gene transfection and to elucidate the possible mechanism of HBV infection underlying cholangiocarcinoma.

METHODS: HBECs were cultured *in vitro* and co-transfected with a eukaryotic expression vector containing the HBx coding region and a cloning vector containing coding sequences of enhanced green fluorescent protein (EGFP) using lipid-mediated gene transfer. The transfection efficiency was determined by the expression of EGFP. The expressions of hTERT mRNA and HBx protein in HBECs were detected by RT-PCR and immunocytochemical stain, respectively.

RESULTS: The transfection efficiencies were about 15% for both HBx gene expression plasmid and empty vector. No hTERT mRNA was expressed in HBECs when transfected with OPTI-MEM medium and empty vector, but a dramatic increase was observed for hTERT mRNA expression in HBECs when transfected with HBx expression vector. HBx protein was only expressed in HBECs when transfected with HBx expression vector.

CONCLUSION: HBx transfection can activate the transcriptional expression of hTERT mRNA. Cis-activation of hTERT mRNA by HBx gene is the primary mechanism underlying the proliferation, differentiation and tumorigenesis of biliary epithelia.

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INTRODUCTION

Telomeres make up the ends of chromosomes of eukaryote and progressively shorten with each cell cycle. Critically short telomeres induce cellular senescence and death[1]. Telomere lengths become stabilized by the activation of telomerase in most tumor cells, highly proliferative cells and human somatic cells. The activation of telomerase is a crucial step in tumorigenesis and cellular senescence[2]. The most important catalytic protein subunit of telomerase ribonucleoprotein is hTERT whose expression parallels telomerase activity[3]. It is known that hTERT expression is regulated mainly at the transcriptional level and that the core promoter of hTERT encompasses numerous transcription factor binding sites. All these factors, which regulate hTERT promoter region individually or coordinately, comprise a complex regulation system[4]. A recent study has shown that HBV DNA integration locates upstream to the hTERT promoter and that HBV enhancer can cis-activate the transcriptional expression of hTERT gene in hepatocarcinoma cell lines[5]. HBx gene also activates the expression of telomerase[6]. All these findings provide a new mechanism of HBV in liver carcinogenesis. There is a prominent expression of HBx protein in tissues of both intrahepatic[7,8] and extrahapatic cholangiocarcinomas[9]. So far, it is not clear whether HBV infection involves in the tumorigenesis of cholangiocarcinoma and if HBx gene can regulate the expression of telomerase gene. In order to determine the possible correlation of HBV infection and cholangiocarcinogenesis, we transfered HBx gene into human normal cholangiocytes (HBECs) and assayed the expression of hTERT mRNA by RT-PCR.

MATERIALS AND METHODS

Cell and culture

HBECs, isolated from normal human bile ducts[10], were kindly provided by Dr. Ludwik K. Trejdosiewicz (ICRF Cancer Medical Research Unit, St James's University Hospital, Leeds, UK). HBECs were maintained as adherent monolayers in "HBEC medium" comprising a 1:1 mixture of Ham's F12 and DMEM (Gibco BRL®), supplemented with 50 g/L fetal bovine serum (Gibco BRL®), 5 ng/mL epidermal growth factor (Intergen Company), 0.4 μg/mL hydrocortisone hemisuccinate, 2 nmol/L triiodothyronine and 5 μg/mL insulin (all from Sigma) and 10 ng/mL human recombinant hepatocyte growth factor (R&D Systems,). Cells were seeded in 25 cm^2 tissue culture flasks and propagated at 37 ℃ in a humidified atmosphere of 550 mL/L CO_2 in air and monolayers passaged approximately once a week at or before confluence by incubation in trypsin-versene for approximately 5 min until the cells were shrunken. Trypsin activity was quenched by addition of fresh medium containing FBS and cells were seeded at 1/2 split ratio.

Plasmids and transfection

The plasmids pcDNA3, pCMV-X and pEGFP were the gifts from Professor Xiao-Dong Zhang (Institute for Molecular Biology, Nankai University, China). The empty pcDNA3 vector, a eukaryotic expression vector, was used as negative control. pCMV-X was constructed by inserting the entire HBx

coding region (HBV nucleotides 1 372-1 833 465 bp) into the *Eco*RI/*Eco*RV sites of the pCDNA3 vector[11]. Cloning vector pEGFP carried an enhanced green fluorescent protein (EGFP) gene that was cloned between the two MCS of the pPD16.43. EGFP encoded by pEGFP could emit bright green fluorescence in eukaryotic cells. All the plasmids contained ampicillin resistance genes for propagation and selection in *E.coli*.

Transient transfection of plasmids into HBECs was performed using Lipofectamine (Gibco BRL®) according to the protocol recommended by the manufacturer. The day before transfection, cells were trypsinized and seeded in 6-well plates. On the day of transfection, cells reached 60% confluence. 2.9 μg pcDNA3 DNA or pCMV-X DNA and 0.1 μg pEGFP DNA were diluted with OPTI-MEM medium (Invitrogen) and then mixed with Lipofectamine. A total of 1 mL transfection medium was added to the cells after the cells were washed one time by OPTI-MEM medium. The cells were incubated at 37 ℃ in a humidified atmosphere of 50 mL/LCO_2 in air for 3 h. After 3 h incubation, the transfection medium was replaced with fresh complete medium containing serum and the cells were incubated for another 36 h. Then the cells were harvested and extracted. The expression of transfected gene was examined by immunocytochemistry. The cells transfected with OPTI-MEM medium were used as blank control and transfected with pcDNA3 vector as empty vector control, co-transfected pEGFP as a marker for transfection efficiency[12].

RT-PCR of hTERT mRNA

Total cellular RNAs were extracted from different groups by TRIzol reagent (Gibco BRL®). A 2 μg of extracted RNA was reverse transcribed into cDNA first-strand with 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) and 1 μg of oligo $(dT)_{15}$ primer (Promega) in a final volume of 25 μL of enzyme buffer for 60 min at 42 ℃. hTERT cDNA analysis was performed by PCR amplification of a 145 bp fragment using primer pairs 5'-CGGAAGAGTGTCTG GA GCAA-3' (sense) and 5'-GGATGAAGCGGAGTCTGGA-3' (antisense) as described previously[13]. A 320 bp fragment of GAPDH gene was amplified as an internal control. The primers for GAPDH were 5'GGAAGCTTGTCATCAATGG 3' (sense) and 5'CTGTGGTCATGAGTCCTTC 3'(antisense). PCR was performed with 5 μL of cDNA first-strand in a 50 μL reaction mixture containing 2 mmol/L $MgCl_2$, 1 mmol/L dNTPs, 0.4 μmol/L of each primer, and 2.5 units of *Taq* DNA polymerase (Promega). The reaction mixture was heated at 94 ℃ for 5 min, then 33 cycles of PCR were performed. Each cycle included denaturation at 94 ℃ for 40 s, annealing at 60 ℃ for 40 s and extension at 72 ℃ for 90 s. A 10 μL PCR products was assessed by 15 g/L agarose eletrophoresis and ethidium bromide staining (0.5 μg/mL), visualized under ultraviolet light and analyzed by NIH Image software.

Immunocytochemistry

Cells in different groups were cultured on coverslips and fixed with acetone and methanol. Detection of HBx protein expression in the transfected cells was performed by the ultrasensitive immunochemistry kit (Maixin Company, Fuzhou, China) according to the manufacturer's instructions. Rabbit anti-human HBx polyclonal antibody (1:800) was provided by Dr. Wen-Liang Wang (The 4th Military Medical University, Xi'an, China).

RESULTS

Transfection efficiency

Under fluorescence microscope, EGFP can only be observed in the HBx gene transfected cell cultures and the cells of empty vector control (Figure 1). There was no such green fluorescence in the cells of the blank control. Transfection efficiency was estimated by counting the percentage of EGFP-expressing cells in at least 3 fields of vision under fluorescence microscope. For the HBx gene transfected cell cultures and empty vector control, the transfection efficiency was about 15%.

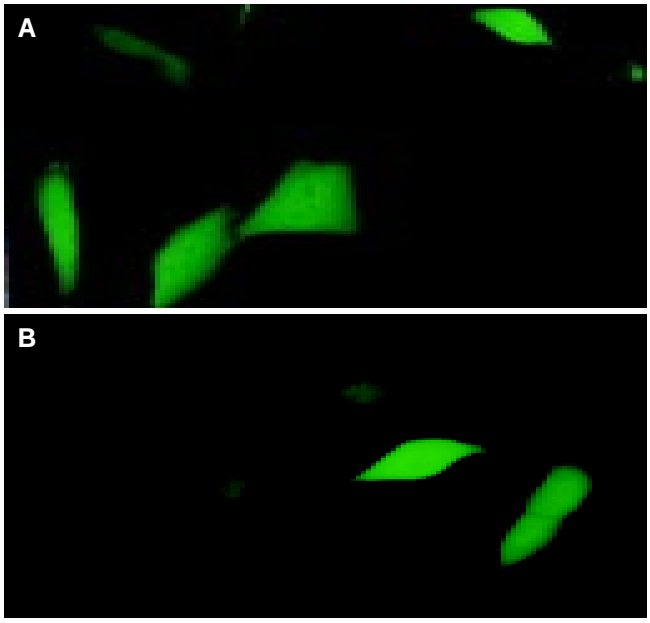

Figure 1 Transfection efficiency evaluated by EGFP-expressing cells in the HBx gene transfected cell cultures and empty vector control. pEGFP 0.1 μg and pCMV-X (or pcDNA3) 2.9 μg were co-transfected into HBECs by Lipofectamine. Thirty-eight hours after transfection. EGFP-expressing cells were visible under fluorescence microscope in the HBx gene transfected cell cultures (A) and empty vector control (B), but not in the blank control (Under fluorescence microscope ×200).

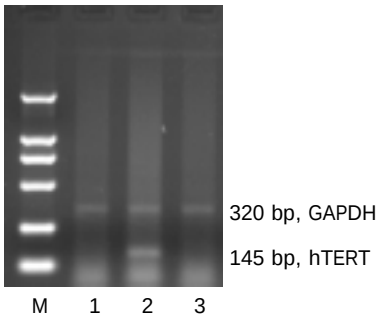

Figure 2 Analysis of hTERT mRNA expression by RT-PCR. RT-PCR was performed on total RNA extracted from HBECs transfected with OPTI-MEN medium (1), pCMV-X (2) and pcDNA3 (3), respectively. M: DL2000 Marker.

hTERT mRNA expression

To examine the effects of HBx gene on hTERT transcription, pCMV-X expression vector was co-transfected with pEGFP into HBECs. The expression of human GAPDH RNA in all of the samples was quantitatively measured and used as an internal control. After RT-PCR analysis, a prominent level of hTERT transcript was detected in the HBx gene transfected cell cultures. In contrast, as shown in Figure 2, hTERT mRNA of HBECs in the blank control and empty vector control were undetectable. The relative expression level of hTERT mRNA was determined by measuring band intensities of both hTERT transcript and GAPDH transcript and calculating the ratio of hTERT to GAPDH. As shown in Figure 3, after transferred with HBx

gene, the HBECs exhibit more distinct hTERT mRNA expression in contrast to those transfected with blank control and empty vector.

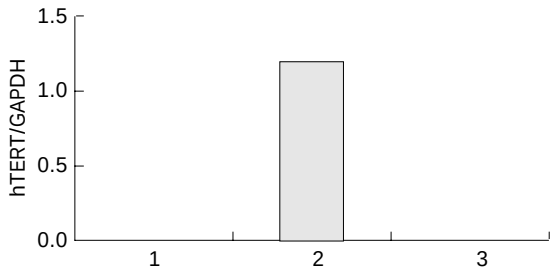

Figure 3 Quantitative and relative changes of hTERT mRNA expression analyzed by NIH Image software. Dramatic expression of hTERT mRNA was observed in HBECs when transferred with pCMV-X vector (lane 2), but there was no hTERT mRNA expression in HBECs when transferred with OPTI-MEM medium (lane 1) and empty vector (lane 3).

HBx protein expression in HBECs

HBx protein expression in transferred HBECs was identified by immunocytochemistry. Positive signals could be observed sporadically in the HBx gene transfected cell cultures (Figure 4B). As for the HBECs in the blank control and empty vector control, there are no such observable positive signals (Figures 4A, C).

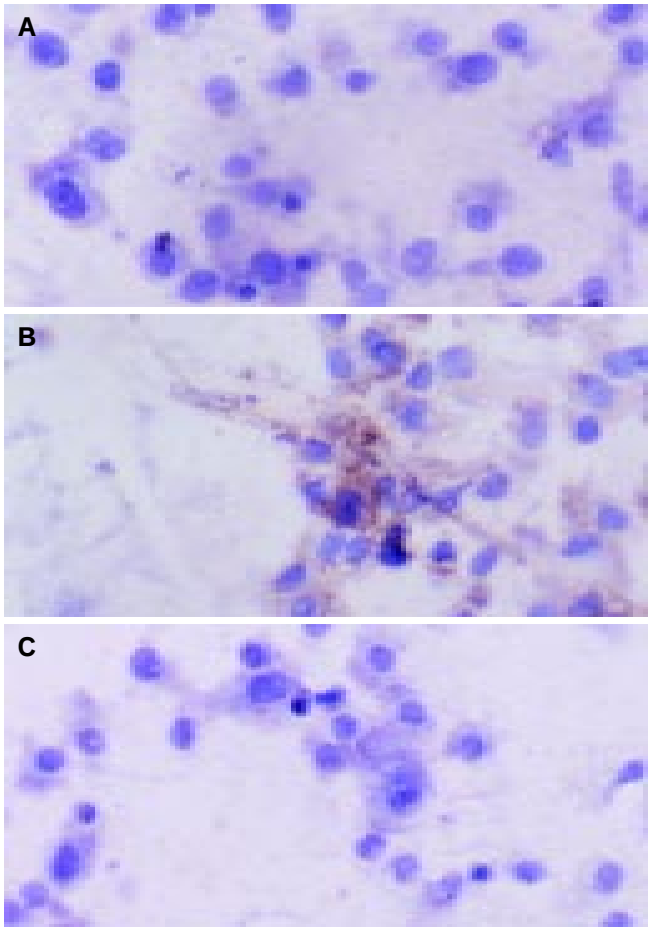

Figure 4 HBx protein expression in transferred HBECs assayed by ultrasensitive immunocytochemistry. The blank vector and OPTI-MEN transfected HBECs showed no expression of HBx protein (A, C), but pale brown positive signals scattered in pCMV-X vector transferred HBECs (B). Immunocytochemistry (S-P methods, ×200).

DISCUSSION

HBECs were isolated from normal human bile duct epithelia and cultured *in vitro*. As no retroviral transduction with SV-40 large T antigen cDNA was performed, the cells were not immortalized. They were really primary culture cells and would only grow for a few passages before becoming senescent, the cells simply stopped dividing and died eventually. Though there was expression of telomerase endogenous genes (such as the telomerase RNA component gene) in this kind of finite cell lines, telomerase was inactivated as the expression of hTERT was repressed. The inhibition of hTERT expression originated from the presence of numerous transcription factors in the core promoter of hTERT. Transcriptional repressors such as p53[14,15], Mad1[16,17] and myeloid-specific zinc finger protein 2[18], could specifically inhibit the transcriptional expression of hTERT mRNA in normal human somatic cells. Our results confirmed this hypothesis. When we transferred the empty vector and OPTI-MEN medium into the HBECs, we could not assay the expression of hTERT mRNA. Dramatic hTERT mRNA expression in HBECs transfected with HBx gene showed that HBx gene could cis-activate transcriptional expression of hTERT gene. An early study also showed that normal human cells could restore the telomerase activity in the presence of other oncogenic viruses[19]. It has been reported that HBV genome was integrated into the promoter region of hTERT both in HuH-4 human hepatocellular carcinoma-derived cell line[5] and in liver tumor tissues[20]. The integration of HBV enhancer upstream of the hTERT promoter cis-activated hTERT gene transcription in HuH-4 cells[5]. It is known that the up-regulation of telomerase activity could be observed in hepatocellular carcinoma cell line HepG2 after transferred with X gene[6]. Together with our results, this was a most important demonstration of transcriptional regulation of telomerase gene through HBx gene in carcinogenesis of both human hepatocarcinomas and cholangiocarcinomas. The precise mechanism of such an action is still unknown.

It has been well known that HBx protein encoded by HBx gene, is a potential oncogenic factor and mainly acts as a transcriptional co-activator involving in multiple gene regulation and signaling pathway[21]. Up-regulation of telomerase gene expression may be another major role of HBx at the stage of carcinogenesis[22]. Our early study found that higher expression levels of HBx protein and mRNA could be assayed in the tissues of cholangiocarcinomas[9,23]. Based on our present finding that HBx protein expression could be detected in HBECs transfected with HBx gene, we could suggest that HBV infection and its genome integration might involve in the pathogenesis of cholangiocarcinomas[24]. HBx protein, which has long beed studied as the major causative factor for hepatocarcinogenesis[25,26], might still play more important role in the carcinogenesis of cholangiocarcinomas than other proteins encoded by other genes of HBV[27]. As for the mechanism for HBx protein' carcinogenic action, we deduced there should be a binding site for HBx protein in the core promoter region of hTERT gene although this motif has not been identified. Another notion derived from our study is that HBx protein may recruit some cis-action elements (such as c-Myc[28], AP-2[29]) or repress other factors (like p53[30], E2F1[31]) in mediating the transcriptional regulation of hTERT gene. When exogenous HBx gene was transferred into HBECs, HBx protein translation was achieved in some of HBECs. It is the expression of HBx protein in the transfected cells that be responsible for cis-activation of hTERT mRNA directly or indirectly.

In summery, HBECs do not show the expression of hTERT mRNA and a dramatic high expression of hTERT mRNA can be observed in HBECs transfected with HBx gene. The cis-activation of hTERT gene by HBx is the primary mechanism

underlying proliferation, differentiation and tumorigenesis of biliary epithelia.

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