Overexpression of human telomerase reverse transcriptase promotes the motility and invasiveness of HepG2 cells in vitro

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Abstract. Recent studies have indicated that telomerase activity promotes cancer invasion and metastasis, but the underlying mechanism remains unclear. Several studies have shown that expression of exogenous human telomerase reverse transcriptase (hTERT) can promote motility and invasiveness among telomerase-negative tumor cells, and inhibition of endogenous telomerase activity can reduce invasiveness in tumor cells. However, whether overexpression of hTERT can further enhance the motility and invasiveness of telomerase-positive tumor cells has yet to be determined. In the present study, we showed that stable overexpression of hTERT can increase telomerase activity and telomere length, which significantly promotes the invasive and metastatic potential of telomerase-positive HepG2 cells but does not affect cell proliferation. Further analysis suggested that enhanced invasiveness and metastasis may act through corresponding upregulation of mRNA and protein expression of matrix metalloproteinase 9 (MMP9) and Ras homolog gene family member C (RhoC). Our study indicated that exogenous expression of hTERT may promote invasiveness and metastasis through upregulation of MMP9 and RhoC.

Introduction

Human telomerase, a ribonucleoprotein enzyme complex, is composed of catalytic component human telomerase reverse transcriptase (hTERT), human telomerase RNA component (RNA template hTERC) and human telomerase associated protein 1 (hTEP1) (1-3). hTERT can catalyze the synthesis of the repeating sequence (TTAGGG)n and maintain telomere length at chromosomal ends using hTERC as a template (4). It counteracts telomere shortening due to each round of cell division and therefore prevents senescence and cellular aging. In line with these conclusions, telomerase activity has been detected in 80-90% of human cancer cases, but it is rare in normal somatic cells. Exceptions include stem cells, reproductive cells and activated lymphocytes. (5-11). It has been shown in numerous studies that activation of telomerase is strongly correlated with tumorigenesis and metastasis; therefore, it may serve as an indicator of prognosis (12-21).

Previous studies have shown that the majority of hepatocellular carcinomas (HCCs) exhibit telomerase activity and that the level of telomerase activity is related to upregulated hTERT expression (22). Early recurrence after hepatectomy is one of the most important factors affecting the prognosis of patients with HCC. Quantitative analyses of telomerase activity suggested that HCC resection patients positive for telomerase activity in non-cancerous liver tissue have a higher rate of recurrence. The relative telomerase activity (RTA) of early recurrent patients is significantly higher than in patients who do not experience recurrence (23). Peripheral blood telomerase activity can be used as a molecular marker for the detection of circulating hepatoma cells in the blood of HCC patients; it also indicates hematogenous micrometastasis (24). Previous studies have consistently shown that telomerase activity may serve as an independent predictor of recurrence after HCC resection (25). These observations led to the hypothesis that telomerase activity may be correlated to invasiveness and metastasis of cancer. To support this hypothesis, recent research data was collected and showed that telomerase can promote the invasion and metastasis of telomerase-negative tumor cells (26-28). By contrast, tumor cell growth, proliferation, invasion and metastasis can be reduced through inhibition of telomerase activity of tumor cells by various methods (29,30). However, to date, few research studies have focused on the mechanisms underlying this process. The issue of whether the motility and invasiveness of telomerase-positive tumor cells can be further promoted by overexpression of hTERT has yet to be addressed.

In the present study, we observed enhanced motility and invasive capacity in telomerase-positive human hepatoma cell line (HepG2) cells through exogenous expression of hTERT gene using a retroviral vector. Our data suggested that changes in motility and invasive capacity may be caused by upregulation of the metastasis-related genes, matrix metalloproteinase 9 (MMP9) and Ras homolog gene family member C (RhoC).
Materials and methods

Cell lines and retroviral vectors. Human hepatoma cell line (HepG2) (ATCC, Manassas, VA, USA) and GP2-293 packaging cell line (Clontech Laboratories, Mountain View, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA) at 37°C in a humidified incubator containing 5% CO2. The retroviral expression vector xlox(gfp) TERT containing coding sequences for hTERT and green fluorescent protein (GFP) was provided by Dr David Ott (Frederick National Laboratory for Cancer Research, Frederick, MD, USA). The control vector xlox-GFP was constructed on the basis of xlox(gfp)TERT in our laboratory. Briefly, the portion of hTERT in xlox(gfp)TERT was removed using EcoRI followed by religation using T4 DNA ligase.

Cell transduction. Retroviral vector stocks were produced by cotransfecting the plasmid encoding VSV-G protein and xlox(gfp)TERT/xlox-GFP vector into the packaging cell line GP2-293 and harvesting virus-containing culture medium 48 h after transfection. These actions were performed as described in the Retroviral Gene Transfer and Expression user manual and instructions from VigoFect (transfection reagent; Vigorous Biotechnology, Beijing, China). A 2:1 ratio of retroviral vector/ VSV-G DNA mixture was used in these experiments. The resulting vector stocks (Virus-hTERT and Virus-GFP) were then used to infect HepG2 cells in the presence of polybrene (8 µg/ml). Two groups of transduced cells were identified and sorted 72 h after transduction using flow cytometry for GFP expression, termed hTERT/HepG2 and GFP/HepG2. Both untransduced HepG2 and GFP/HepG2 were used as the controls and cultured at the same time as the experimental groups.

Real-time PCR for hTERT mRNA. Total RNA was isolated from cells using TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA was then converted to cDNA using oligo(dT) primers and SuperScript III Reverse Transcriptase (Invitrogen Life Technologies). Real-time PCR analysis was performed using the SYBR® Green Real-time PCR Master mix (Toyobo, Japan) and Bio-Rad (Hercules, CA, USA) MiniOpticon real-time PCR system. Primers for human β-actin and hTERT were designed with Primer Express Software Version 3.0 and synthesized by Sangon Biotech Co. (Shanghai, China), and were: β-actin sense, 5'-GGCATACC GACGCACGCAGT-3' (112 bp). hTERT sense, 5'-TGTCAAGGTGGATGTGACGGGC-3' and anti sense, 5'-GAAGGAAGGCTGGAAGAGTG-3' (137 bp); hTERT and β-actin were measured using the SYBR® Green Real-time PCR Master mix (Toyobo, Japan) and Bio-Rad (Hercules, CA, USA) MiniOpticon real-time PCR system. Primers for human β-actin and hTERT were designed with Primer Express Software Version 3.0 and synthesized by Sangon Biotech Co. (Shanghai, China), and were: β-actin sense, 5'-GAAGGAAGGCTGGAAGAGTG-3' (137 bp); hTERT sense, 5'-TGG ACTTCGAGCAAGAGATG-3' and antisense, 5'-GGCATACC GACGCACGCAGT-3' (112 bp).

hTERT protein detection. Expression of the hTERT protein was detected using western blot analysis. Total proteins were isolated from cells of each group. Forty micrograms of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were first incubated with anti-hTERT mAb overnight at 4°C and then with HRP-labeled secondary antibody (Zhong-Shan Golden Bridge BioTechnology, China) for 1 h at RT. The membranes were washed again, treated with chemiluminescence reagent (Amersham Pharmacia Biotech, Amersham, UK), exposed to autoradiography film (Kodak) and developed.

TRAP assay for telomerase activity. Telomerase activity of cells was detected using the telomeric repeat amplification protocol (TRAP) assay with a TRAP kit (Tiangz, Beijing, China). The procedure was performed according to the manufacturer's instructions. Briefly, the cell pellet was resuspended in 200 µl of 1X lysis buffer/106 cells, incubated on ice for 30 min and then centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was collected and the protein concentration was determined using standard procedures (BCA protein assay). The reaction system containing 25 µl TRAP reagent was incubated for 30 min at 30°C and then for 3 min at 93°C, and 1.5 µl TRAP primer mixture and 0.5 µl of Taq polymerase were added to the PCR tube followed by 30 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 60 sec. PCR product (25 µl) was loaded onto a 10% non-denaturing PAGE in 0.5X TBE buffer. Following electrophoresis, the gel was stained using a Rapid Silver Stain kit for Nucleic Acid (Tianzd). Lysis buffer (provided in the TRAP kit) was used as a negative control.

FISH assay for telomere. hTERT/HepG2 and GFP/HepG2 cells ~10 PDs after transduction and untransduced cells were cultured in a 6-well plate to the logarithmic phase. Then, colcemid was added and the cells were incubated for 1 h. Cells were then treated with hypotonic KCl for 60 min at 37°C and fixed in methanol/acetocetic. Fluorescence in situ hybridization (FISH) was performed on metaphase chromosomes with Cy-3 labeled (CCCTAA), PNA probe (Panagen, South Korea) according to the manufacturer's instructions. Digital images were captured for 4.6 diamidino-2-phenylindole (DAPI) and Cy-3. The subsequent quantitative analysis of telomere fluorescence was detected using Leica QWin Pro. version 2.6 (Germany).

In vitro motility assay and invasiveness assay. Cell motility was analyzed using a wound healing assay. Cells (5x105 cells/well) were seeded into a 6-well plate. A scratch wound in the monolayer was created using a sterile 200 µl pipette tip following incubation for 24 h. The detached cells were gently washed with PBS. The cells were incubated with new medium containing 1% FBS and then the distances between the wounds were measured by microscope at 0 and 48 h. These distances were measured using ImageJ. Cell motility was evaluated using the following formula: Cell motility=(distance0 - distance4)/distance0. For invasiveness assays, cells (5x104 cells/well) were suspended in DMEM with no FBS and seeded onto the inner compartment of Matrigel-coated 24-well chambers with a filter membrane containing 8-µm pores (Corning Incorporated, Corning, NY, USA). The outer chamber contained the same medium with 10% FBS. Following incubation for 24 h at 37°C, cells that did not migrate through the pores were removed by gentle scraping of the membrane with a cotton swab. Cells that transversed the membrane were fixed in 70% ethanol and stained with 0.1% crystal violet. The number of cells that invaded the undersurface of the membrane was determined using 5 randomly selected microscopic fields per sample. Data represent the average of 3 wells.
Cell growth curves. Cell proliferation was determined using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions. One hundred microliters of cell suspension (2,000 cells/well) were seeded into the 96-well plate and incubated for 24 h (37˚C, 5% CO₂). CCK-8 solution (10 µl) was added to each well and the cultures were incubated at 37˚C for 90 min. Absorbance at 450 nm was measured using an automatic microplate reader (Tecan Sunrise, Switzerland). The results were plotted as means ± SD of 3 separate experiments having 6 determinations per experiment for each experimental condition.

Real-time PCR for MMP9 and RhoC mRNA. The expression levels of mRNA for MMP9 and RhoC were analyzed using real-time PCR as previously described. Primers for MMP9 and RhoC were designed with Primer Express Software version 3.0 and synthesized by Sangon Biotech Co., and were: MMP9 sense, 5'-GGCGGTGATTGACGACGCT-3' and anti-sense, 5'-CCGTGCTCCGCGACACCAA-3' (126 bp); RhoC sense, 5'-CGGAGCGGAAGCCCCACCAT-3' and antisense, 5'-AGGGACGTAGACCTCCGGAAA-3' (126 bp).

MMP9 and RhoC protein detection. The expression levels of proteins for MMP9 and RhoC were detected using western blot analysis, as previously described.

Statistical analysis. All experiments were performed in triplicate and data are presented as means ± SD of 3 separate determinations. Statistical analysis was performed using one-way analysis of variance (ANOVA). P<0.05 was considered to indicate statistically significant differences. All statistical analyses were performed using SPSS 16.0 software.

Results

Control vector construction and cell transduction. The control vector xlox-GFP was constructed on the basis of xlox(gfp)TERT by removing part of the hTERT in xlox(gfp)TERT with EcoRI and linking it using T4 DNA ligase. The vector was confirmed by digestion with EcoRI and NotI (Fig. 1A). Virus-hTERT and Virus-GFP were produced and used to infect HepG2 cells. Two groups of transduced cells were identified and sorted 72 h after transduction using flow cytometry for GFP expression, hTERT/HepG2 and GFP/HepG2 (Fig. 1B). The following experiments were performed using hTERT/HepG2, GFP/HepG2 and HepG2 cells.
Overexpression of hTERT mRNA and protein in hTERT/HepG2.

The expression of hTERT in all 3 groups of cells was examined using real-time PCR. β-actin served as a loading control for both mRNA and protein analyses. We found hTERT mRNA expression to be markedly higher in hTERT/HepG2 cells (up to 80-fold) than in HepG2 and GFP/HepG2 control cells (Fig. 2A). Elevated hTERT protein levels were also detected in hTERT/HepG2 cells relative to HepG2 and GFP/HepG2 cells (Fig. 2B). There was no significant difference between HepG2 and GFP/HepG2 in either mRNA or protein expression. The relative expression levels of hTERT mRNA in HepG2, GFP/HepG2 and hTERT/HepG2 were 1.00, 1.76 and 81.65 (Fig. 2A). We used this information to generate a stable cell line with high hTERT expression. This line was used for subsequent studies.

hTERT overexpression enhances telomerase activity and telomere length in HepG2 cells. To further examine the effect of hTERT overexpression, TRAP assay was performed to assess telomerase activity in HepG2, GFP/HepG2 and hTERT/HepG2. Our results showed telomerase activity in the hTERT/HepG2 cells to be significantly more pronounced than in the 2 control cells (Fig. 3A). The average length of telomere repeats (indicated by the intensity of telomere fluorescence) at chromosome ends in HepG2, GFP/HepG2 and hTERT/HepG2 was determined using semiquantitative FISH. As in our exper-
iments on elevated telomerase activity, telomere length, here indicated by fluorescence intensity, was significantly longer in hTERT/HepG2 cells than in HepG2 and GFP/HepG2 control cells (Fig. 3B).

Figure 4. Effects of hTERT transduction on cell migration and invasion in HepG2 cells. (A) Images of wounded monolayers of 3 groups of cells were captured at 0 and 48 h after incubation (x40). (B) The mean distance of migration of each group of cells was measured and compared. (C) Representative cell Matrigel-coated invasion assay for each group and images of invaded cells at 48 h. (D) Quantitative evaluation of cell invasion assay. (*P<0.05, relative to HepG2 mean values; #P<0.05, relative to GFP/HepG2 mean values, ANOVA).

hTERT overexpression leads to increased motility and invasiveness of HepG2 cells. Previous studies conducted using tissue samples from certain cancer patients indicated that levels of high telomerase expression and activity are corre-
HTERT promotes the motility and invasiveness of HepG2 cells

Increased telomerase expression leads to enhanced cell motility and invasiveness, suggesting a critical role in tumor progression. Overexpression of hTERT in HepG2 cells led to increased cell motility and invasiveness relative to control cells (Fig. 4). Taken together, our data suggested that telomerase expression enhanced cell motility and invasiveness in vitro and facilitated tumor progression towards metastasis in vivo.

Overexpression in telomerase-positive HepG2 slows down cell proliferation. Telomerase expression may promote cell proliferation and maintain homeostasis in adult tissue. To determine whether this is the case in HCCs overexpressing hTERT, a CCK-8 assay was performed to assess the cell proliferation rate. The speed of cell proliferation among hTERT/HepG2 was significantly slower than among GFP/HepG2 and HepG2 control cells (Fig. 5). However, further studies are required to fully clarify this.

Upregulation of MMP9 and RhoC may be responsible for enhanced motility and invasiveness in HepG2 when hTERT expression is forced. In order to determine the molecular mechanism by which hTERT promotes the motility and invasiveness of HepG2 cells, we assessed the mRNA and protein levels of 2 important molecules involved in cell migration and invasion. We observed marked upregulation of RhoC and MMP9 on both the mRNA and protein levels in hTERT/HepG2 (Fig. 6A and B). Our findings suggested that enhanced cell motility and invasiveness induced by high levels of hTERT in HepG2 cells may have been caused by upregulation of MMP9 and RhoC expression.

Discussion

Aggressiveness is a significant biological characteristic of malignant tumor cells and a leading cause of mortality in patients with cancer. Several studies have demonstrated that telomerase plays an important role in elongating telomeres and maintaining the infinite proliferation ability of telomerase-positive tumor cells. Clinical findings have indicated that telomerase activity may be a prognostic marker of various human malignant tumors, including HCC (23). Sato et al. (31) found relative telomerase levels to be closely correlated with both motility and invasiveness in 13 pancreatic carcinoma cell lines and concluded that the magnitude of telomerase activation may reflect the potential for aggressive behavior within cancer cells. Previous experimental studies regarding the link between telomerase and tumor aggressiveness have focused on the following two phenomena; first, the transfection or transduction of hTERT can promote the motility and invasiveness of telomerase-negative tumor cells. The invasive and metastatic potential of RasG12V/SV40 Large T antigen-transduced bovine cells can be restored by transduction of hTERT (26). The proliferation, adhesion and invasion of telomerase-negative osteosarcoma cell line U2OS was found to be significantly promoted by hTERT transfection (28). Second, cell proliferation, migration and invasion of telomerase-positive tumor cells can be inhibited by inhibition of either hTERT expression or telomerase activity. Anti-TER ribozyme-mediated suppression of mouse telomerase RNA reduced telomerase RNA expression, telomerase activity and telomere length, which significantly reduced the invasiveness and metastatic potential of melanoma cells (32). Knockdown of hTERT siRNA and concurrent treatment with IFN-γ effectively inhibited cell proliferation, migration, and invasion in glioblastoma cells (30). However, to our knowledge, there are no experimental studies on the link between hTERT overexpression and aggressiveness in telomerase-positive tumor cells.
In the present study, we found that hTERT overexpression can also significantly promote the motility and invasive potential of cancer cells, at least in telomerase-positive HepG2 cell lines. This provides further evidence of the relationship between telomerase activity and metastatic potential of tumor cells.

Tumor cell invasion and metastasis are multi-step, complex processes. They involve proteolytic degradation of basement membranes and extracellular matrix (ECM), cell adhesion, migration and angiogenesis. Both MMPs and RhoC have been shown to play important roles in some of these processes. MMPs are a family of zinc-dependent endoproteinasises responsible for degradation of the components of basement membranes and ECM. Overexpression of MMPs has been observed in most malignant tumors. MMP9 expression has been found to be strongly correlated to metastasis of HCC (33,34). Analysis of cell phenotypes expressing dominant-negative Rho or RhoC indicates that RhoC is important in tumor cell invasion (35). RhoC plays a critical role in the movement of tumor cells through regulation of actin cytoskeleton (36,37). RhoC has been shown to be upregulated in various types of tumors, including HCC (38-42). Studies have indicated that RhoC significantly promotes metastasis by augmenting motility and invasion of tumor cells via activation of MMP2 and MMP9 (43,44). In this study, we chose these two key molecules for invasion of tumor cells for our preliminary investigation of the molecular mechanisms by which hTERT promotes motility and invasiveness. We found expression levels of MMP9 and RhoC to be markedly upregulated in hTERT/HepG2 cells at both the mRNA and protein levels. We speculate that upregulation of RhoC and MMP9 may be one of the mechanisms through which hTERT enhances the motility and invasiveness of tumor cells. In this process, there may be complicated regulating networks that control tumor aggressiveness. However, the details of these mechanisms require further study in subsequent experiments.

hTERT is generally considered to be closely related to tumor cell growth and proliferation (45) and it is believed to promote cell growth and proliferation (46-48). However, the few studies that have been performed have shown no significant association between telomerase activity and proliferative index in various tumor tissues (14,15,31,49). Presumably, malignancy and poor outcome among cancer patients may not necessarily be strictly correlated with tumor size or proliferation rate. Other characteristics, such as the degree of differentiation, may be more suitable indicators of malignancy and poor outcome in these patients. In the current study, we showed that cell proliferation was not increased by overexpression of hTERT. Our results also show that cell proliferation is slower in the two transduced groups than in untransduced cells. One possible reason for this may be that forced expression of increased amounts of TERT may cause telomerase-positive cells to acquire some properties and capacities, such as those involving de-differentiation, motility, and invasiveness, but not an increased growth rate. Whatever the reason, the motility and invasiveness of tumor cells were found to have been significantly promoted by overexpression of hTERT, and these were considered indicators of enhanced metastatic potential. In this sense, our results support previous observations that hTERT and telomerase play important roles in the promotion of motility and invasiveness in tumor cells.

The overexpression of hTERT was found to increase the motility and invasiveness of telomerase-positive HepG2 cells. However, whether the depletion of hTERT in these cells can lead to the opposite effects requires further studies. Additional efforts should be made to investigate the universality of our findings with respect to other cancer cell lines.

In conclusion, our findings demonstrate that hTERT overexpression can significantly promote cell migration and invasiveness among human telomerase-positive HepG2 cell lines but does not promote proliferation. Upregulation of metastasis-associated molecules, MMP9 and RhoC, may be one of the most important mechanisms underlying this process. Our study provides experimental evidence of the relationship between telomerase activity and metastatic potential of telomerase-positive tumor cells and also aids in the research into the mechanisms underlying this process. These findings provide a possible explanation of the clinical observation that high telomerase activity predicts poor patient outcome in HCC, particularly in cases of recurrence after HCC resection.

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References


