Antisense oligonucleotide targeting at the initiator of hTERT arrests growth of hepatoma cells

Su-Xia Liu, Wen-Sheng Sun, Ying-Lin Cao, Chun-Hong Ma, Li-Hui Han, Li-Ning Zhang, Zhen-Guang Wang, Fa-Liang Zhu

INTRODUCTION

Human hepatocellular carcinoma (HCC) is a critical disease threatening human health. It is one of the most common malignant tumors worldwide, however there is no effective treatment at present. It is very necessary to explore new methods for the therapy of HCC. The developments in molecular biochemistry have afforded the possibility for this purpose.

Telomerase, a ribonucleoprotein enzyme that synthesizes telomeric DNA, is thought to be necessary for cellular immortality and carcinogenesis[1-3], and consists of human telomerase RNA component (hTERC), human telomerase protein 1 (hTEP1), and human telomerase reverse transcriptase (hTERT). Among them, only the expression of hTERT mRNA is correlated with telomerase[4], and mainly regulates the expression of human telomerase enzymatic activity[5-6]. hTERT is a useful marker for telomerase activation[7,8], and consists of a related group of telomerase enzymatic activity[9-11], which produce tumor cells in malignant human cancers. Therefore, telomerase is the target of anti-tumor drugs research. For example, many scientists declared that ribozyme which cleaved telomerase mRNA and PS-ODNs complementary to the repeat sequence of the mammalian telomere (5’ TTAGGG3’) could arrest tumor cells[12-13] in cell lines. In this study, we observed the inhibitory effect of as-hTERT both in vivo and in vitro.

In HCC, the activity of telomerase played a very important role during the occurrence of HCC and cirrhotic livers[14]. The positive hTERT mRNA was 89.47% in HCC tissues[15]. Though many factors were related to hepatocarcinogenesis[16], infection of HBV and HCV[17] was the most important. Tahara et al[18], discovered that telomerase activity was 100% in HCC tissues with HBV positivity. These studies indicated that there might be a close relationship between HBV infection and telomerase activity during hepatocarcinogenesis. Therefore, in this study, HepG2.2.15 cells, a cell line in which HBV genome was integrated into the chromosome, were the target cells and as-hTERT complementary to hTERT promoter was added to these cells, and the anti-tumor effects and inhibitory action on HBV gene expression of as-hTERT were studied in vitro and in vivo.

MATERIALS AND METHODS

Materials

The oligomers used in this study were prepared by Shenggong Co. The solutions were suspended in sterile phosphate-buffered saline(PBS) and filtered into 1.5 ml sterile Eppendorf tube, and stored at 4 °C until use. AsON used in this study targeted at the promoter area of human hTERT with the sequence 5’ GCC ACG TGG GAA GGG 3’ (-192~-176 site). In this area, there is a potential binding site of upstream stimulating factor, such as pro-oncogene c-myc. The random sequence (5’ TTG CCG AGC GGG GTA 3’ ) was used as control. HepG2.2.15 was used as targeted cells. This cell line was purchased from Beijing Institute of Medical Biology. Fetal calf serum (FCS) and G418 were purchased from GIBCO and Sigma respectively. The quantitative detection kit of telomerase (PCR-ELISA) was purchased from Roche Molecular Biochemicals, Germany.

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Cell culture
The experimental protocols were similar to those as previously described[18-20]. HepG2.2.15 was a hepatoma cell line which was integrated with HBV genome, and could replicate intact HBV DNA and extract HBsAg and HBeAg. The cells were grown in vitro in a 25 ml flask at a concentration of 5×10^6 cells per millilitre in MEM media supplemented with 10% fetal bovine serum containing 380 µg/ml G418[21-22]. The cells were tested for HBsAg and HBeAg and found to replicate HBV DNA. When the cells grew to 75% confluence, they were digested and used for the in vitro and in vivo studies.

MTT assay
The cells were cultured in 60 mm^2 dishes with 2×10^5 cells per dish. The next day as-hTERT and control sequence were added at a concentration of 10 µM. After 72 h incubation, these cells were added 20 µL of MTT per dish. Four hours later, the cells were given 100 µL of DMSO per dish and the absorbance of the sample was measured at 570 nm and 630 nm using an ELISA microtiter plate reader. The value of absorbance at 570 nm and 630 nm was named A570 and A630.

Determination of telomerase activity
Telomerase activity assay was performed according to a polymerase chain reaction-based telomeric repeat amplification protocol as described previously[23-26] using a kit from Roche (Germany). The cells (10^4-10^6 per dish) were harvested after 72 h incubation with as-hTERT and control sequence.

Preparation of extracts from cells
The cells were put in a 1.5 ml Eppendorf tube and pelleted at 3 000xg for 10 min in a refrigerated centrifuge at 4 ºC. Then these cells were washed once in cold PBS. For one tube, 200 µL pre-cooled lysis reagent was added and incubated on ice for 30 min and centrifuged at 10 000xg for 20 min at 4 ºC. A total of 175 µL supernatant was carefully transferred to a fresh Eppendorf tube. These cell extracts were used for the telomerase assay using the telomerase PCR-ELISA procedure.

Telomeric repeat amplification protocol (TRAP reaction)
For each sample, 25 µl reaction mixture was transferred into a PCR tube. Three µl cell extract and sterile water were carefully transferred to a fresh Eppendorf tube. These cell extracts were used for the telomerase assay using the telomerase PCR-ELISA procedure.

Table 1 Protocol of TRAP reaction

| Step               | Time      | Temperature | Cycle number |
|--------------------|-----------|-------------|--------------|
| Primer elongation  | 30 min    | 25 ºC       | 1            |
| Telomerase inactivation | 5 min    | 94 ºC       | 2            |
| Amplification      | 3-32      |             |              |
| Denaturation       | 30 s      | 94 ºC       |              |
| Annealing          | 30 s      | 50 ºC       |              |
| Polymerization     | 90 s      | 72 ºC       |              |
|                    | 10 min    | 72 ºC       | 33           |

Hybridization and ELISA procedure
The product of PCR was aliquoted, denatured, bound to a streptavidin-coated 96 well plate, and hybridized to a DIG-labelled telomeric-repeat specific probe. An antibody to DIG conjugated to peroxidase was subsequently bound to DIG. Then tetramethyl benzidine (TMB) was metabolized, the substrates of the enzyme was added to produce a colored reaction product. The absorbance of the sample was measured at 450 nm using an ELISA microtiter plate reader within 30 min after adding the stop reagent. The value of absorbance at 450 nm was named A450.

Animal studies
BALB/c (nu/nu) mice (weighing approximately 18-20 g) were purchased from the Experimental Animals Center of Chinese Academy of Sciences. The mice were divided into eight teams randomly. These animals were placed into fresh cages with free access to sterile water. All the solutions were filtered into sterile bottles. In order to compare the different curative effects of as-hTERT by different administration routes, the study was divided into three study groups.

Group I: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla and tumor progression was measured daily. Two weeks postinjection, the mice with tumors about 0.5 cm in diameter were selected, and as-hTERT and control sequences were injected into the tumors subcutaneously. These animals were divided into two teams, one team (n=12) was injected as-hTERT 100 µg/mouse/day, the other (n=6) was treated with saline 100 µl/mouse/day. Five days postinjection, the mice were killed by removing the eyeballs and the tumor tissues were removed, and stored in 10% formalin for FCM. These tumor samples were also embedded in paraffin, and sectioned into slices about 5 µm in thickness. The sections were stained with hematoxylin and eosin for histological examination. In this group, the serum were harvested and stored at -20 ºC for detecting HBsAg and HBeAg.

Group II: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla and as-hTERT 100 µg/mouse (n=7) simultaneously, the controls with saline 100 µl/mouse (n=6) and random controls with as-hTERT 100 µg/mouse (n=6), and tumor progression was measured daily.

Group III: Each mouse was injected subcutaneously 5×10^6 cells at the left axilla. One day postinjection, each mouse was injected as-hTERT 100 µg/mouse (n=12), saline 100 µl/mouse (n=8) and random controls with as-hTERT 100 µg/mouse (n=8) at the same axilla. Three weeks postinjection, no tumors were detected in 10 animals treated with as-hTERT. These mice were divided further into another two teams: one team (n=6) was injected subcutaneously as-hTERT at the dosage of 30 µg/mouse at the same place as the first time, the other (n=4) was given saline.

In each group, tumor progression was measured, and observations on animal behavior were recorded daily during the study period.

HBV antigen detection
According to the protocol of antigen detection kit (Lizhu Co. Shenzhen, China), the concentrations of HBsAg and HBeAg in the supernatant were detected using an ELISA microtiter plate reader. The results were illustrated with P/N value (P/N=sample A/negative control A. A stands for the amount of light absorbance).

Statistical analysis
All the data were expressed as the mean ± standard error of the mean (SE). The P values were calculated by ANOVA or exact probability method. A P value less than 0.05 was considered statistically significant.

RESULTS
as-hTERT on activity of telomerase
The results were shown with the value of A570nm. A630nm of as-hTERT treated cells, random sequence control, positive control were 0.42, 1.49 and 1.51 respectively (Figure 1). That of the negative control was 0.08. Telomerase activity was inhibited with the addition of 17-base as-hTERT at a concentration of 10 uM.
Antiproliferation effect of as-hTERT on HepG2.2.15 cells
Seventy-two hours after incubation with the drugs, as-hTERT caused significant (F=251.13, P=0.0001) inhibition of cell growth as shown in Figure 1, but not in random sequence controls and saline controls.

Growth arrest of human hepatocarcinoma in vivo
The drugs were injected into the tumors. After seven days, the peaks of apoptosis of tumor cells treated with as-hTERT, saline control were 21.12% and 7.92% respectively (Figure 2). Inflammation was observed in tissues treated with as-hTERT, but not in controls. The conformation of the animals’ liver was normal.

Alteration of HBV antigen expression
as-hTERT could inhibit HBV expression in vitro and in vivo. In vitro, the inhibition peaks on HBsAg and HBeAg were 76% and 56% respectively, while in vivo, the inhibition peaks were 38% and 73%.

DISCUSSION
Hepatocarcinogenesis is a very complicated event, and many factors were involved in this process, such as activation of oncogenes, inactivation of tumor suppressor genes and hepatitis virus infection[27]. But the most important is the activation of telomerase. Therefore, to inhibit telomerase activity so as to arrest tumor cell growth by antisense technique is of great significance. In most researches, the target gene was hTR, regulatory region of hTERT, the most important region which controlled mainly at the transcriptional level[28]. Among the potential transcription factor binding sites within hTERT promoter region, a typical E-box (CACGTG, -178 to the bHLHZ family of transcription factors (such as oncogene c-myc), was likely to play an important role in hTERT gene transcription[29-31]. As-hTERT could retard tumor cell growth. In the group in which the drugs were injected in combination with HepG2.2.15 cells, tumors were undetectable subcutaneously during the whole study period (10 weeks, 0%, 0/7), while the saline and random sequence controls failed to inhibit tumor growth. In two weeks, tumors were developed in all of the animals in control teams (100%, 8/8). There was a significant difference between as-hTERT treated and control animals (P<0.01).

If the drugs were injected 24 h after the animals were injected with the cells, in three weeks, tumors were detectable in all of the animals in control teams (100%, 8/8), while in team of animals treated with as-hTERT tumors began to appear in two (16.7%, 2/12), and grew very slowly. Furthermore, if the animals without tumor were injected supplementary drugs, subcutaneous tumor was undetectable during the later 6w (0%, 0/6), while in the team of animals without extra addition of the drugs, tumors developed (Figure 3).
to E-box binding site. Therefore, telomerase activity was decreased, telomere was shortened to the crisis point, and cell growth was arrested, and apoptosis was increased. However, asON could not completely inhibit telomerase activity. This result indicated that E-box is one of the cis-elements which regulate telomerase transcription. Furthermore, it is very important to study on cis-element and transcriptional factors regulating telomerase activity.

AsON targeting at the promoter region of telomerase could inhibit hepatoma cell growth of mice model in vivo. Different ways of drugs administration demonstrated different growth inhibitory effects. Apoptosis was detected in tumors injected as-hTERT, but not in cells treated with saline controls. The best growth inhibitory effect on tumor cells was observed in animals injected as-hTERT in combination with the cells. In another team, tumor growth was observed in the mice which were given as-hTERT 24 hours postinjection of HepG2.2.15 cells at the same site, but the tumors grew very slowly. It could not inhibit tumor cell growth completely through this way. However, if the mice were given a smaller dosage of the drug continually, tumor cell growth would be inhibited completely. These results demonstrated that as-hTERT could inhibit tumor cell growth, the earlier the drug were given, the better the effect was. It suggests that as-hTERT can be an effective drug for eliminating the remaining tumor cells after operation.

HBV genome could integrate into the telomerase gene[12,39]. HBV was a potential cis-activation factor which regulated telomerase[40]. The expression of HBV gene could increase telomerase activity[41]. This study demonstrated that as-hTERT could affect the expression of HBV antigen both in vitro and in vivo. The reason may be that as-hTERT inhibited telomerase activity, and cell growth was retarded. Therefore, HBsAg and HBeAg expressions were inhibited. Maybe, there is some relationship between telomerase activity and HBV, which needs to be further clarified.

Furthermore, there was no toxicity on the mice. In summary, we have demonstrated that a short asON which targets at the promoter region of telomerase could inhibit telomerase activity and retarding tumor cell growth both in vitro and in vivo that needs to be further clarified. It suggests that as-hTERT can be an effective drug for suppressing HBV infection.

Inhibition of HBV DNA replication and expression in 2.2.15 HepG2 cells treated with as-hTERT was significantly suppressed. The expression of HBV gene could be suppressed by as-hTERT. This result suggested that as-hTERT can be an effective drug for suppressing HBV infection.

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