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Application of Adenovirus-Mediated Human Telomerase Catalytic Subunit (hTERT) Gene Promoter in Ovarian Cancer Gene Therapy

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Abstract Telomerase is a ribonucleoprotein complex whose function is to add telomeric repeats to chromosomal ends. Telomerase consists of two essential components, telomerase RNA template (hTR) and catalytic subunit (hTERT). hTERT is expressed only in cells and tissues positive for telomerase activity, i.e., tumor and fetal cells. In this report, the possibility of utilization of the hTERT promoter in targeted cancer gene therapy was tested. The hTERT promoter was cloned in the replacement of the CMV promoter, and the HSV-TK gene was subcloned to be controlled by the hTERT gene promoter in the adenovirus shuttle plasmid. Then, the recombinant adenovirus Ad-hT-TK was constructed and was infected into normal and human gynecological cancer cell lines. The selective tumor specific cell death by Ad-hT-TK was identified through these experiments, showing that Ad-hT-TK could be used for targeted cancer gene therapy.

Key words: Telomerase, hTERT promoter, HSV-TK, adenovirus, targeted cancer gene therapy

Although ovarian cancer accounts for only 4% of cancers in women, it is the leading cause of death from gynecological malignancies in Western countries [1]. Since ovarian cancer tends to be asymptomatic until it is in the well-advanced stage, it is difficult to diagnose the disease in early stages.

Specificity of the therapeutic gene expression is one of the most important factors in cancer gene therapy. As a result, targeted cancer gene therapy has a goal to concentrate the target therapeutic gene expression into the specific target tissue, thereby minimizing a secondary effect and maximizing the therapeutic index. That is, if the tumor-specific promoter can actually be used, tumor cells can be controlled. Telomerase is a ribonucleoprotein, which consists of several components. Among these, two components are known for their functions: the RNA component (hTR) and the telomerase catalytic subunit (hTERT). hTR acts as a template for telomere synthesis [4]. The expression of hTR is ubiquitous in all types of human cells, regardless of the status of telomerase activity, but hTERT is expressed only in cells and tissues positive for telomerase activity, i.e., tumor and fetal cells [5, 7, 16]. Recently, the promoter region of cancer specific human telomerase reverse transcriptase was cloned and characterized [6, 12]. The promoter of hTERT was GC rich and lacked of both TATA and CAAT boxes. Interestingly, transient expression assays revealed that transcription of hTERT was significantly activated in cancer cell lines, but repressed in normal primary cells. Over-expression of c-Myc resulted in a significant increase in transcriptional activity of the core promoter [6, 12]. These results showed that hTERT promoter could be used for targeted cancer gene therapy. In this study, the possibility of the utilization of the hTERT gene promoter in targeted cancer gene therapy was investigated by using adenovirus.

Materials and Methods

Cell Culture

Wi-38 (human normal fibroblast) and QBI-293A (Quantum-Appligene, U.S.A.; a human cell line transformed by adenovirus 5 DNA) cells were grown in DMEM (GIBCO BRL, Germany) supplemented with 10% FCS (HyClone, Logan, U.S.A.), penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO_2. NIH:OVCAR-3

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(OVCAR3; ovarian cancer), A2780 (ovarian cancer), and HeLa (cervical cancer) cells were grown in RPMI1640 (GIBCO BRL, Germany) supplemented with 10% FCS, penicillin (50 units/ml), and streptomycin (50 μg/ml) in the presence of 5% CO_2. All cell lines except for QBI-293A were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

RT-PCR Analysis

Total RNA was isolated from each cell lines by using the RNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's protocol and treated with DNase I to avoid any genomic DNA contamination. cDNA was synthesized from 1 μg of RNA by using the SuperScript II reverse-transcriptase assay (GIBCO BRL, Germany) with Oligo$(dT)_{15}$ primer (Promega, U.S.A.). To amplify the cDNA, 2 μl aliquots of cDNA were subjected to 30 cycles of PCR (at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 90 sec). hTR was amplified with an hTR-specific primer set (5'-tttgtc-taaccctaactgagaagg-3' as forward and 5'-tgtgagccgagtcctg-ggtgcacg-3' as backward), which gave rise to 400 bp. hTERT mRNA was amplified with LT5 (5'-cggaagagtctg-gagcaa-3') and LT6 (5'-ggatgaagcggagtcgga-3') [13]. The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

Luciferase Assay

The selective expression of luciferase gene by hTERT promoter in tumor cells was determined by applying the luciferase reporter plasmid by using FuGENE™6 (Roche, Germany) according to the manufacturer's protocol. Briefly, 10^5 cells seeded in a 35-mm tissue culture dish were exposed to transfection mixture containing 2 μg of luciferase reporter plasmid and 0.5 μg of pSV-β-galactosidase control plasmid vector (Promega) for 5 h at 37°C. Then, 3 ml of growth media were added to the cells, followed by incubation for an additional 16 h. The cells were harvested 48 h after the transfection process. Luciferase assays were performed according to the manufacturer's protocols (Promega). Transcriptional activity was measured with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). The Simian virus 40 (SV40) promoter (pGL3-Promoter) was used as a positive control. hTERT promoter plasmid (pGL3-hTERT) was constructed by replacing the SV40 promoter with hTERT promoter. The luciferase activity of pGL3-promoter plasmid in each cell line was considered as 100%. β-Galactosidase assay was also performed with the same cell extracts to standardize for transfection efficiency. All of the data shown in this study were obtained from at least three independent experiments.

Cloning of hTERT Promoter

The hTERT promoter region was amplified by PCR using LA-Taq polymerase (Takara, Japan) on the HeLa genomic DNA. The hTERT forward (5'-gtcgac agtggattcgcgggcacaga-3') and reverse (5'-aagctt agggcttcccacgtgcgcag-3') primers, both introducing *Sal*I and *Hin*dIII sites (underlined in the sequence respectively), were used. This region is the hTERT promoter region from -204 to +56, which contains a core promoter and two E-boxes [12]. PCR was carried out for 35 cycles at 94°C for 1 min, at 66°C for 1 min, and at 72°C for 1 min. The PCR product was cloned into pGEM-T easy vector (Promega) and digested with *Sal*I and *Hin*dIII. Then, it was ligated with *Sal*I and *Hin*dIII digested pXCMVTK and named pXhTERT-TK. Then, this plasmid was digested with *Xba*I and ligated with *Xba*I digested pShuttle (Ad transfer vector), and named pS-hT-TK (Fig. 1). Direct dideoxynucleotide sequencing ensured a correct sequence and direction.

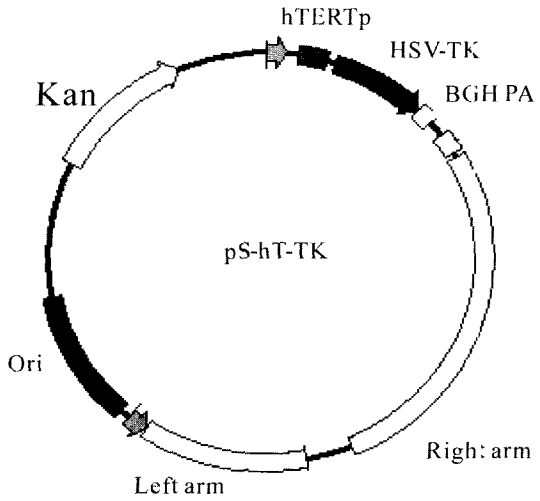

Fig. 1. Adenovirus shuttle plasmid pS-hT-TK containing the hTERT promoter, HSV-TK, and BGH PA (bovine growth hormone polyadenylation site).

Construction of Recombinant Adenovirus Ad-hT-TK

The pS-hT-TK was then co-transformed with pAdEasy-1 (Quantum-Appligene, U.S.A.) into the BJ5183 *E. coli* cells, by using electroporation methods. After making a selection on kanamycin plates, 40 colonies were selected and screened for the recombinants by plasmid size and restriction enzyme analysis. Viral DNAs of 40 candidates were tranfected in QBI-293A cells with FuGENE™6 (Roche, Germany) by following the manufacturer's protocol. This constructed adenovirus vector was named Ad-hT-TK. The constructed adenovirus were then purified from the lysates of infected QBI-293A cells by two rounds of CsCl gradient centrifugation [2], then the titers of the virus stock were determined by using a plaque dilution assay [3]. The recombinant virus were stored at -80°C until used. The negative control vector (Ad5.CMV-Null; Ad-CMV) was purchased from Quantum-Appligene. Ad-CMV is an empty vector, which contains

no coding sequences between the CMV promoter and PA (polyadenylation site).

Adenovirus Infection with GCV Treatment *In Vitro*

Cells (10^5) were plated in six-well plates prior to infection. Ad-hT-TK or Ad-CMV adenovirus was applied in 1 ml of medium at various moi, 24 h later. Cells were incubated at 37°C for 3 h, followed by adding 2 ml of media containing various concentrations of GCV (Ganciclovir; InvivoGen, San Diego, CA, U.S.A.). Cell cultures were replenished every day with a fresh medium containing GCV. All of the data shown in this study were obtained from at least three independent experiments.

RESULTS

Telomerase Activity, and hTR and hTERT Expressions of the Cell Lines

To ensure the presence of the telomerase in experimental cell lines, telomerase activity was assayed with the use of the highly sensitive TRAP assay [8]. As expected, tumor cell lines showed strong telomerase activity, but not normal cell line (Wi-38; Data not shown). To confirm the results of the TRAP assay, hTERT and hTR expressions were analyzed by RT-PCR. As expected, all cell lines expressed hTR, but hTERT mRNA was detected in TRAP assay positive cancer cell lines, except normal fibroblast Wi-38 (Fig. 2). These results showed that telomerase activity and hTERT mRNA expression levels had a close correlation in the experimental cell lines.

Variation of Transcriptional Activity of hTERT Promoter in Tumor or Normal Cells

To demonstrate the variation of transcriptional activity of hTERT promoter in tumor or normal cells, a transient transfection of luciferase reporter plasmid was performed (Fig. 3). Transcriptional activity of pGL3-promoter plasmid

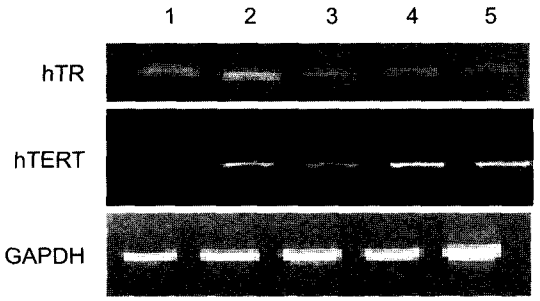

Fig. 2. hTR and hTERT expression of experimental cell lines. The total RNA was isolated and used for RT-PCR in the presence of hTR, hTERT, and GAPDH specific primers.
The PCR products were visualized after 1.5% agarose gel electrophoresis and ethidium bromide staining. 1, Wi-38; 2, HeLa; 3, OVCAR3; 4, A2780; 5, QBI-293A.

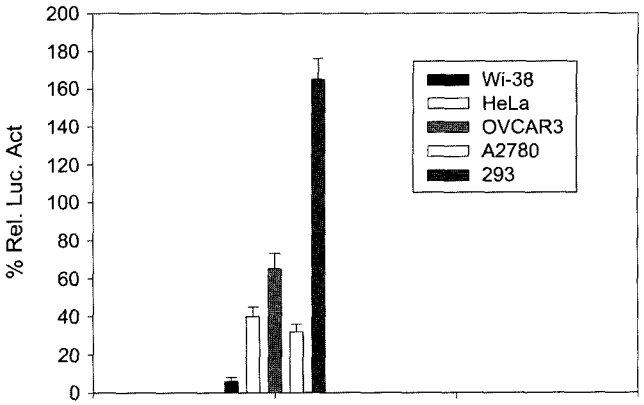

Fig. 3. Specific expression of luciferase gene imparted by the hTERT promoter.
pGL3-hTERT plasmid consisted of hTERT promoter and luciferase gene were introduced into normal fibroblast (Wi-38) and telomerase-positive cell lines (HeLa, OVCAR3, A2780, QBI-293A). Relative luciferase activity was standardized with control plasmid pGL3-promoter transfection. Means from at least three independent experiments are shown; bars, SD.

in each cell line was considered to be 100%. pGL3-hTERT showed a significant transcriptional activity in all tumor cells lines, especially in OVCAR3 cell line with approximately 65% of pGL3-promoter. In contrast, there was no significant transcriptional activity in the telomerase activity negative normal fibroblst cell line (Wi-38). These results showed that tumor cell line had a strong transcriptional activity of the hTERT promoter.

Ad-hT-TK Confers GCV Sensitivity to Tumor Cells but Does Not Affect Normal Fibroblast

To determine the cancer specific cell death by Ad-hT-TK, tumor and normal fibroblast cell lines were infected with Ad-hT-TK with or without GCV (Fig. 4). After 6 days of infection, most of the tumor cells displayed a typical cell death, but not the Wi-38 cells (Fig. 4). In order to show

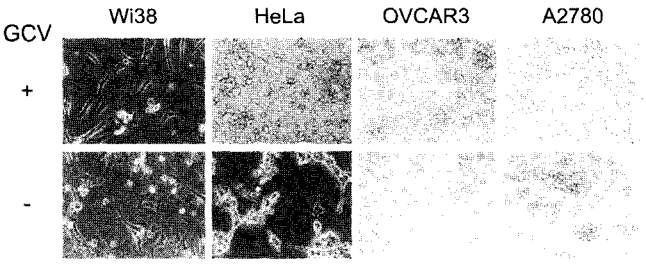

Fig. 4. Ad-hT-TK confers GCV sensitivity to all tumor cells but not normal fibroblast.
Cells (10^5) were seeded in 6-well dishes 24 h prior to infection. Ad-hT-TK was infected into gynecological cancer cell lines and normal fibroblast at a moi of 100 with 30 μM of GCV. Most of the cell death was observed for telomerase-positive cancer cell lines after 6 days of drug administration. No significant effect was observed in a normal fibroblast after drug treatment in comparison to the control cultures. Normal fibroblast Wi-38 grows much more slowly than other telomerase positive cell lines.

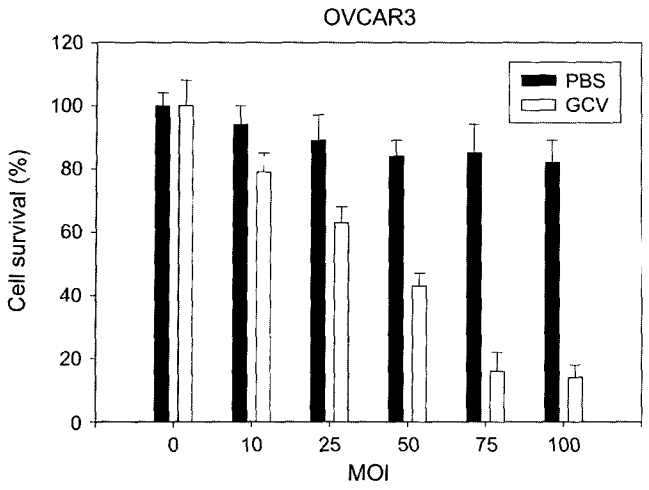

Fig. 5. OVCAR3 cells at increasing moi were exposed *in vitro* to Ad-hT-TK+GCV (10 μM) or PBS, and the number of viable cells was determined by trypan blue exclusion 6 days later.
The OVCAR3 cells were seeded at 10^5 cells in 6-well dishes 24 h prior to infection. Ten μM of GCV alone was not effective in cell growth. Means from at least three independent experiments are shown; bars, SD.

that these effects were multiplicity of infection (MOI) and in a GCV dose-dependent manner, the OVCAR3 cell line was infected with increasing moi of Ad-hT-TK, ranging from 0 to 100 (Fig. 5). Ad-hT-TK with GCV resulted in 86% cell death at an MOI of 100, compared with Ad-hT-TK infected cells treated with PBS alone, in which it did not result in a significant cell death. As shown in Fig. 6, Ad-hT-TK infected OVCAR3 was sensitive to GCV treatment in a dose-dependent manner. Ten μM GCV caused as much as 54% cell death in Ad-hT-TK infectec cells, but only 5- 10% cell death in Ad-CMV or mock infected cells.

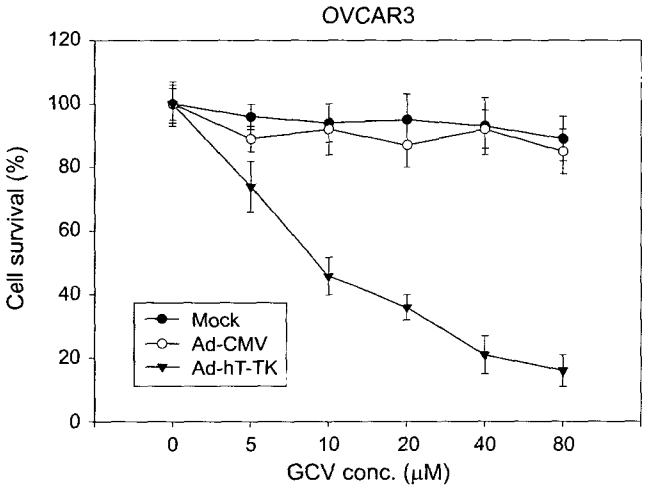

Fig. 6. *In vitro* GCV sensitivity of Ad-hT-TK infected OVCAR3 compared with mock or Ad-CMV infected cells.
The OVCAR3 cells were seeded at 10^5 cells in 6-well dishes 24 h prior to infection. Ad-hT-TK or Ad-CMV was infected into OVCAR3 at a moi of 50. Six days, following GCV treatment, the living cells were measured by using trypan blue exclusion. Means from at least three independent experiments are shown; bars, SD.

DISCUSSION

In cancer gene therapy, restricted expression of the therapeutic gene in tumor is very important. If the therapeutic gene is expressed in all cells, the therapeutic gene will affect tumor as well as normal cells. In fact, by using the tumor specific promoter system will actually solve this problem. However, true tumor specific promoters are rare, and often, these promoters are useful only in particular types of cancers that they have been derived.

There are two kinds of telomerase-associated cancer gene therapy for telomerase-positive tumors [10]. First, tumor cell growth will be inhibited by direct inhibition of telomerase activity, resulting in apoptotic cell death or growth arrest [14, 15]. Telomerase consists of two major components, the RNA template (hTR) and catalytic subunit (reverse transcriptase, hTERT). hTR was considered to be good therapeutic targets [14,15]. The other is the telomerase-specific cancer gene therapy by using telomerase promoter as a means to directly kill the telomerase-positive tumors.

RT-PCR analysis showed that the RNA component of telomerase (hTR) is ubiquitously expressed in most somatic cells, whereas hTERT expression is specific for telomerase-positive cancer cells, but not in normal somatic tissue [5, 7, 16]. The mechanisms of how to control hTERT transcription are not clearly known. It is known that c-Myc has an important role in positive regulation of hTERT gene activation and telomerase activation. Previously, c-Myc level was shown to directly influence the telomerase activity [11]. Telomerase activity in human leukemic cell lines is inhibited by antisense c-Myc oligonucleotide [9]. In hTERT promoter region, there are two typical E-boxes (CACGTG, - 165 to - 160 and 42 to 48), which are known to be potential binding sites of the c-Myc oncoprotein [12]. However, downstream E-box (42 to 48) does not have an additive or synergistic effect [6, 12]. In this study, the hTERT promoter region was selected as -204 to +56, which contained a core promoter and two E-boxes.

To investigate the possibility of utilization of the hTERT promoter in targeted gene therapy, adenovirus vector was constructed expressing HSV-TK that was controlled by the hTERT promoter for the induction of specific telomerase positive cancer cell death. First, to know the existence of the telomerase, telomerase activity was assayed with hTR and hTERT RT-PCR. The result showed that tumor cell lines had a strong telomerase activity and specific expression of hTERT, but not human normal fibroblast (Wi-38) (Fig. 2). Second, adenovirus vector containing the HSV-TK gene under the control of hTERT promoter was constructed, and these virus vectors were introduced

into normal fibroblast and gynecological cancer cell lines. The GCV sensitivity of Ad-hT-TK infected telomerase-positive gynecological cancer cell lines was much greater than that of the mock or Ad-CMV infected cell lines (Figs. 4, 5, 6). These results support the possibility of utilization of hTERT promoter for gynecological cancer gene therapy.

hTERT has an important role in tumorigenicity of normal cell to tumor cell, and the hTERT promoter is specifically activated in tumor cells. If we can utilize the hTERT gene promoter, we can precisely control tumor cells efficiently.

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