A novel hTERT promoter–driven E1A therapeutic for ovarian cancer

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Abstract

Currently, an effective gene therapy strategy, which not only retains cancer-specific expression but also limits toxicity, has yet to be developed for ovarian cancer. Mounting reports over the years have shown that human telomerase activity is significantly elevated in cancer cells compared with normal cells. In this study, we evaluated the human telomerase reverse transcriptase (hTERT; T) promoter and showed that it can direct target gene expression preferentially in ovarian cancer cells. However, its promoter (T) activity is much lower than that of cytomegalovirus (CMV), a commonly used nonspecific promoter. To overcome this problem, we have integrated the T promoter into our recently developed VP16-Gal4-WPRE integrated systemic amplifier (VISA) system and dramatically enhanced transgene expression. In addition, to further develop this cancer-specific promoter gene expression system into an applicable therapeutic vector, we expressed E1A (an adenoviral type 5 transcription factor that possesses anticancer properties) through this novel VISA platform. We showed that the T-VISA system specifically targeted the expression of E1A to ovarian cancer cells at a level greater than or comparable with the commonly used CMV promoter, yet remained nearly silent in normal cells, thus making this a suitable gene therapy construct. By using this cancer-specific promoter that limits target gene expression in normal cells/tissues, potential toxicity induced by the CMV promoter would be prevented. More importantly, we showed significant anti-tumor activity with much less toxicity in animal models through i.v. delivery of T-VISA-E1A:liposomal nanoparticles, suggesting a promising role of T-VISA-E1A for ovarian cancer treatment under a gene therapy setting. [Mol Cancer Ther 2009;8(8):2375–82]

Introduction

As the fifth leading cause of cancer deaths among women, ovarian cancer has the highest mortality rate of all gynecologic cancers, with ~15,520 deaths and 21,650 new cases in the United States estimated in 2008 (1). Unfortunately, despite the improvements in cytoreductive surgery and combination chemotherapy, advanced-staged patients who do not respond to therapy or those who recur have limited options to effectively treat this disease. Moreover, prognosis remains unfavorable especially when patients are in the advanced stages (III/IV) at the time of diagnosis with the 5-year survival rate for late-staged patients at below 25% (2,3). Thus, it is crucial that more effective and/or alternative therapeutic strategies are developed to treat ovarian cancer.

One of the ongoing but less developed areas of cancer treatment is gene therapy, which has the potential to significantly improve therapeutic outcomes (4–7). Similar to many existing anticancer therapies, however, gene therapy lacks specificity and the ability to specifically target transgene expression within ovarian cancer cells, attaining maximal antitumor activity with limited toxicity to surrounding normal cells. To address this issue, a system might be devised that utilizes an ovarian cancer–specific promoter to drive expression of the target gene only in cancer cells. In fact, some studies have explored this possibility (8–12), although an ideal promoter that is highly active and specific for ovarian cancer cells has yet been identified.

To find a such a promoter, we searched the literature as well as the Serial Analysis of Gene Expression database and identified three potential promoter candidates: (a) human telomerase reverse transcriptase (hTERT; T; ref. 12); (b) EphA2 (13), and (c) ceruloplasmin (10). Of the three promoters, we showed that T has the potential to be used for transcriptionally targeted gene therapy for ovarian cancer.
inherent to ovarian cancer cells. The T promoter is ideal because its activity has been linked to cancer and has been detected in many invasive cancers but repressed in normal somatic tissues or benign tumors (14, 15). Although telomerase activity is absent in normal somatic cells, >85% of human cancers showed high telomerase activity (14, 16, 17). A number of studies have shown that most ovarian carcinoma specimens exhibit detectable telomerase activity, but little activity has been found in benign tumors or normal ovary (14, 18–20). Interestingly, analysis of the T activity (21). Therefore, the T promoter might be used to drive a therapeutic gene that kills ovarian cancer cells.

Although the T promoter is active in ovarian cancer cells, its activity is generally <5% of that of the CMV promoter, which would not be suitable for gene therapy (7, 22–25). To enhance T promoter activity, we linked it to a VP16-GAL4-WPRE integrated systemic amplifier (VISA) expression vector, which was recently developed to amplify activity of a cancer-specific promoter (26). In particular, when a pancreatic-specific promoter was incorporated into the VISA vector carrying a therapeutic gene, it effectively induced cancer cell–specific killing in a pancreatic cancer model without significant toxicity compared with its CMV-driven counterpart (26). Furthermore, therapeutic gene expression under the VISA system resulted in a significant increase in expression compared with the nonspecific and ubiquitous CMV promoter, making this T-linked VISA vector a powerful tool for efficient production of a suitable target gene that is specifically expressed in cancer cells.

In this study, we have chosen E1A (an adenoviral type 5 transcription factor that possesses anticancer properties) as our therapeutic target gene for ovarian cancer treatment. Briefly, the adenovirus 5 E1A gene is the first viral gene expressed in cells after adenovirus infection and is a well-known transcription factor (27, 28). Although adenoviral type 5 E1A has been classified as an immortalization oncogene because of its ability to immortalize cells, it is incapable of transforming cells by itself. Instead, numerous reports have shown that E1A associates with various anticancer activities (29–31), prompting its evaluation in multiple clinical trials (4, 32–34). Moreover, E1A expression inhibits angiogenesis, induces apoptosis through a bystander effect (35), and activates a protein phosphatase 2a tumor suppressor that triggers a feed-forward mechanism that enhances chemotherapy-induced cancer cell death (36–38). By driving expression of E1A through systemic gene delivery under an ovarian cancer–specific promoter, primary and metastatic tumors could be targeted with minimal toxicity for normal cells. Here, we have shown that T-VISA-E1A specifically targeted ovarian cancer cells and significantly reduced tumor growth in the SKOV3.ip1 mouse model, suggesting that this gene should be evaluated in clinical trials.

**Materials and Methods**

**Constructs**
The T promoter (-380 to +60, relative to the start site; ref. 39) was amplified by PCR using genomic DNA extracted from LNCaP cells as template. The PCR fragment containing the T promoter was subcloned into pCRII-TOPO (Invitrogen) to generate pCRII-TOPO-T. After verification by DNA sequencing, the fragment containing the T promoter was digested with KpnI and XhoI and subsequently inserted into the corresponding sites in pGL-3 basic (Promega) to obtain plasmid pGL3-T-Luc. Plasmids pGL3-EphA2-Luc, pGL3-Cerul-Luc, and pGL3-CMV-Luc containing the EphA2, ceruloplasmin, and CMV promoter, respectively, driving expression of the firefly luciferase gene were described previously (10, 26). Incorporation of the two-step transcriptional amplification (TSTA) system was done by subcloning the HindIII/NotI-blunted T fragment of pCRII-TOPO-T into the MscI/Nhel-blunted site of pGL3-TSTA-Luc (provided by Dr. M. Carey, UCLA School of Medicine, Los Angeles, CA; ref. 40). Finally, construction of pGL3-T-TSTA-Luc-WPRE or pGL3-T-VISA-Luc was carried out by insertion of the NotI/BglII fragment containing the WPRE sequence of pGL3-Luc-WPRE into the same site of pGL3-TSTA-Luc.

A 135 bp E1A fragment was amplified by PCR from pE1A-Neo plasmid using primers (P1.E1A.BII, 5′-TCCGA-GATCTGGCCGGCATGGACATTTATCTGCCACGGAG GTG-3′, and P2.E1A.Nh, 5′-GGCGTTCAGCCTCGGTATTACACCTATGCGCTGGG GCC-3′) and substituted for the luciferase coding sequence in pGL3-T-VISA-Luc to produce pGL3-T-VISA-E1A. Construction of pUK21-T-VISA-E1A was done by subcloning the T-VISA-E1A fragment of pGL3-T-VISA-E1A into NotI and SalI sites of pUK21 (41). A control plasmid pUK21-T-VISA was generated by deletion of the Nhel/BglII fragment from pUK21-T-VISA-E1A. To obtain pUK21-CMV-E1A, the 135 E1A fragment was amplified by PCR from pE1A-Neo plasmid using primers E1A.P3, 5′-TCGA-AAATCGAGCTGCTGTCCACGAAGGT-T-3′, and E1A.P4, 5′-GAAAGGTACCTTCGGAAGAG-3′, and subsequently inserted into the HindIII/EcoRI sites of pUK21-CMV-BikDD by ligation (42).

**Cell Lines and Cell Culture**
The human ovarian cancer cell lines OVCA420, OVCA432, SKOV3.ip1, MDAH2774.c10, and normal lung fibroblasts WI-38 were grown in DMEM/F12 supplemented with 10% fetal bovine serum (Invitrogen). Other human ovarian cancer cell lines, including OVCA3 and HeyA8, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. SKOV3.ip1 was established in our laboratory, as previously described (43). NOE99, NOE115, and NOE119 were primarily cultured normal ovarian epithelial cells at the time of surgery and were maintained in 1:1 of Medium 119 and MCDB 105 (Sigma-Aldrich) with 10% fetal bovine serum and 10 ng/mL epidermal growth factor (Sigma). All patients provided informed consent with institutional review board approval of all protocols.

**Luciferase-Expressing Stable Cell Line**
SKOV3.ip1 cells were cotransfected with 4 μg of pGL3-β-actin promoter–luciferase plasmid and 1 μg pBabe empty vector containing a puromycin-resistance gene using electroporation. The stable clone, SKOV3.ip1-Luc, was generated by selection with 0.5 mg/mL puromycin.
Luciferase Reporter Assay

To determine promoter activities, cells were transiently cotransfected by N-[1-(2,3-dioleoyloxy)propyl]-NNN-trimethylammoniummethyl sulfate:cholesterol liposomes (42, 44, 45) complexed with 1 μg plasmid and 0.1 μg pRL-TK (internal control) in 12-well plate, as previously described (26). After incubation at 37°C with 5% CO₂ for 48 h, cells were lysed using passive lysis buffer, and luciferase expression was detected by the Dual-Luciferase Reporter Assay kit according to the commercial protocol (Promega).

Plasmid Preparation for Animal Injections

Endotoxin-free plasmids were purified by Qiagen EndoFree Mega Plasmid kit (Qiagen) according to a commercial protocol. Using a chromogenic Limulus amoebocyte clotting assay kit (QCL-1000, BioWhittaker), the endotoxin level was determined to be <10 endotoxin units/mg of DNA. The DNA/N-[1-(2,3-dioleoyloxy)propyl]-NNN-trimethylammoniummethyl sulfate:cholesterol complexes were prepared as previously described (26) and administered in mice through tail-vein injections.

Ovarian Cancer Animal Model

To determine the antitumor effect of CMV-E1A and TV-E1A in vivo, 40 athymic female BALB/c nu/nu mice, 4 to 6 wk of age per group, were hosts for human orthotopic xenograft tumors. All mice were housed in a specific pathogen-free environment in compliance with institutional policy, and all animal procedures have been approved by the appropriate institutional review boards. Luciferase-expressing SKOV3.ip1 cells (3 × 10⁶ cells) were injected into peritoneum of the mice. Seven days after inoculation, mice were noninvasively imaged by In vivo Imaging System (Xenogen) to assess tumor growth and randomly assigned to one of three groups. Each group (n = 12) of mice were treated with 100 μL of DNA:liposome complexes that contained 15 μg of pUK21-T-VISA-E1A, pUK21-CMV-E1A, or pUK21-T-VISA (control) administered through tail-vein injection twice per week for three consecutive weeks and imaged once per week for a total of 6 wk before each treatment. Tissues were obtained and terminal deoxyxynucleotidyl transferase-mediated dUTP end nick labeling assays were carried out as previously described to determine the percentage of apoptotic cells (26).

Tissue Distribution of Luciferase Expression

To collect tumors and organs, mice were killed by cervical dislocation. Tissue specimens from tumors and heart and lungs were harvested and homogenized (46). A luminometer (Lumat LB9507, Berthold Technologies) was used to measure the luciferase activity from each of the cell lysate, and the protein concentration was determined as described previously (46). The luminescence results were reported as relative light units per milligram of protein.

Statistical Analyses

Survival rates were analyzed by log-rank test (Mantel-Cox test). All other data were analyzed using Student’s t test.

Results

The T Promoter Is Specifically Expressed in Ovarian Cancer Cells

To identify an ovarian cancer–specific promoter, we searched for genes that are overexpressed in ovarian cancer because of up-regulation of gene transcription such that their promoter activity would be higher in ovarian cancer cells than in normal cells. Using the Serial Analysis of Gene Expression Database and searching the literature, we found three potential candidates: T (12), EphA2 (13) and ceruloplasmin (ref. 10; Fig. 1A). We tested the activities of these three promoters in a panel of ovarian cancer cell lines (OVCAR3, SKOV3.ip1, OVCA420, and MDAH2774.c10) and normal cells (normal ovarian epithelial cells NOE99, NOE115, NOE119, and lung fibroblasts WI-38) using the Dual-Luciferase assay and found that T had the highest promoter activity in the ovarian cancer cell lines. Most importantly, the promoter remained inactive in normal cells (Fig. 1B), suggesting that the T promoter would be an excellent choice as an ovarian cancer–specific promoter to express target gene that can be applied to gene therapy.

VISA Enhances the Activity of the T Promoter While Retaining Its Specificity

While T was significantly more active in ovarian cancer cells compared with normal cells, its activity was in general <5% of the commonly used CMV promoter. Therefore, to enhance the efficiency of the promoter, we incorporated the T promoter into a powerful VISA system that we had

Figure 1. T, EphA2, and ceruloplasmin promoters are active in ovarian cancer. A, schematic of the promoter-driven luciferase report plasmids. B, a panel of ovarian cancer cell lines, normal ovarian epithelial cells NOE99, NOE115, and NOE119), and fibroblasts (WI-38) were transiently cotransfected with plasmid DNA indicated and pRL-TK, the internal control. At 48 h following transfection, dual luciferase ratio was measured as the ratio of luciferase normalized to the Renilla luciferase internal control. The data are means of four independent experiments. Bar, SD.
previously developed (26) and created the T-VISA expression vector (Fig. 2A). Under the VISA expression platform, the T promoter activity in ovarian cancer cells was dramatically increased to a level comparable or even higher than that of the CMV promoter. However, in normal cells, the activity of T-VISA remained virtually silent compared with that of the CMV promoter (Fig. 2B and C). Thus, by controlling target gene expression under the T promoter linked to the VISA system, we not only maintained cancer-specificity but also achieved superb expression that exceeded the nonspecific CMV promoter.

**T-VISA Is Specifically Targeted to Ovarian Tumors**

*in vivo*

Because we determined that T-VISA was highly specific in ovarian cancer cells *in vitro*, we asked next whether this specificity also occurs *in vivo* by using an ovarian cancer orthotopic mouse model. We evaluated the promoter activity by detecting luciferase reporter gene expression after i.p. inoculation of SKOV3.ip1 ovarian cancer cells and tail-vein i.v. injection of the plasmid DNA:liposome complexes carrying either CMV-Luc, T-VISA-Luc, or a control vector. As shown in Fig. 3A, mice that received CMV-Luc exhibited significant luciferase activity in the lungs, which is the first organ it reaches following tail-vein injection, whereas T-VISA-Luc:liposome complexes expressed luciferase primarily at the site of tumor inoculation and not in the lungs. To further confirm targeted-expression of T-VISA-Luc, we sacrificed mice, harvested the corresponding tissues/organs, and imaged them for luciferase expression (Fig. 3B). Contrary to the CMV-Luc vector, T-VISA-Luc exhibited high expression in the abdominal area and in tumor but not in the lungs or heart. In addition, when luciferase expression of T-VISA-Luc was measured in a panel of normal tissues, we found that their luciferase activities were significantly lower than in the tumor (Fig. 3C). In another animal model using the HeyA8 cell line, we also showed the specificity of T-VISA-Luc showing a significant amount of luciferase signals in the abdominal but not in the thoracic area, which contrasts with CMV-Luc (Supplementary Fig. S1A). In addition, T-VISA-Luc targeted not only the primary tumor transplant but also metastases within the liver. As shown in Supplementary Fig. S1B, when we harvested the liver, we detected luciferase signals of tumor metastasized to the liver only in mice that were treated with T-VISA-Luc but not those that received CMV-Luc. Thus, consistent with our *in vitro* data, as well as the SKOV3.ip1 animal model described above, we showed the superiority of T-VISA-Luc over CMV-Luc in cancer-specific target gene expression.

**E1A Expression Under T-VISA Inhibited Ovarian Cancer Cell Growth**

Because the T-VISA expression vector can selectively express target genes in cancer cells but not in normal cells, we engineered a therapeutic T-VISA system that drives expression of E1A (T-VISA-E1A; Fig. 4A). Among the eight ovarian cancer cell lines, we showed that T-VISA-E1A inhibited cell growth *in vitro* at least as effectively as CMV-E1A. However, under the CMV promoter, expression of E1A resulted in a significant decrease in cell survival of normal cells that were otherwise unaffected by T-VISA-E1A (Fig. 4B). Therefore, by expressing E1A under the T promoter, we showed that only cell growth of cancer cells but not normal cells is inhibited, which is crucial to establish T-VISA-E1A as a potential therapeutic agent for treating ovarian cancer.

**T-VISA-E1A Has Significant Antitumor Effect Against Ovarian Cancer in Human Orthotopic Xenograft Mouse Model**

To test potential antitumor effect of T-VISA-E1A, we inoculated ovarian cancer cells, SKOV3.ip1-Luc, in nude mice and treated them with either T-VISA-E1A or the control vector. We found that, just after two treatments, mice that received T-VISA-E1A inhibited tumor growth as indicated by relatively low luciferase signals, whereas those treated with control vector continued to show increase in tumor growth (Fig. 5A). While T-VISA-E1A continued to inhibit tumor growth after six injections, mice that were treated with control vector continued to develop tumor. In addition, T-VISA-E1A treatment significantly prolonged...
survival in mice compared with those that were treated with the control vector (Fig. 5B). We further isolated tissue samples from tumor, lung, and liver of the treated mice and showed by terminal deoxynucleotidyl transferase–mediated dUTP end nick labeling assay that only in the tumor but not the lungs or the liver did we observe apoptosis (Fig. 5C). Thus, this further supports the tumor specificity of the T-VISA-E1A vector in vivo. In addition, mice that were administered with the T-VISA-E1A vector showed more potent apoptosis compared with the control vector, as indicated by the percentages of apoptosis shown in Fig. 5D.

As T-VISA produces little gene expression in normal cells/tissues, we expected that T-VISA would produce fewer side effects and less toxicity than a CMV promoter-based vector. To document the safety of injected T-VISA
DNA:liposome complexes, we measured systemic toxicity at a dosage of 50 μg per mouse. Indeed, we showed that all mice survived following injection of T-VISA-E1A, whereas 10% of mice died after receiving CMV-E1A (Fig. 6A). Moreover, when we measured the alanine aminotransferase level to evaluate the presence of liver toxicity, we showed that the alanine aminotransferase level was significantly increased in mice treated with CMV-E1A compared with those injected with control vector but not in mice that were treated with T-VISA-E1A (Fig. 6B).

**Discussion**
Telomerase (T) has become an interesting and important target for cancer therapeutics because positive telomerase expression, which allows tumors to maintain their telomeres leading to long-term survival, is present in most cancer types (47). In addition, as little telomerase is expressed in normal cells, use of its promoter to drive a target gene could provide cancer-specificity and thus minimize expression in normal cells. Several reports have explored this possibility using viral vectors to express target gene under the
T promoter (48). Use of nonviral vectors would, however, be ideal because viral vector-mediated gene therapy can produce serious complications (49, 50). In this study, we developed an innovative vector that combined the T promoter with a powerful nonviral expression vector called VISA (26). The VISA system can (a) selectively express target gene in ovarian cancer cells under the T promoter; (b) amplify target gene without the use of viral promoter such as CMV; and (c) eliminate the risks that are associated with using viral-mediated gene delivery.

In addition, we used the T-VISA vector in attempt to develop a novel treatment for ovarian cancer and showed that T maintained cancer specificity whereas VISA boosted expression of the target gene in in vitro and in vivo models. In vitro in normal ovarian epithelial cells, T-driven transgene expression was notably absent. In vivo in human orthotopic xenograft mouse models, we showed that the T-VISA vector targeted primary tumor (SKOV3.ip1), as well as metastatic sites (HeyA8), without any expression in the lungs/heart. When we applied this T-VISA system for expression of E1A, which has antitumor activity, it successfully inhibited tumor growth in mouse model. Thus far, we have shown that the use of T-VISA-E1A vector as a potential therapeutic option to combat ovarian cancer.

We selected E1A as the therapeutic gene, which has been known to induce bystander effect (35), as well as sensitizes cancer cells to chemotherapeutic agent (38), and was developed into a phase I/II trial entitled “A Phase I/II Randomized Study on Intraperitoneal tgDCC-E1A and Intravenous Paclitaxel in Women with Platinum-Resistant Ovarian Cancer.” Now that we are able to overcome the limitation of efficient gene expression through the use of nonviral vector, T-VISA-E1A would undoubtedly be an excellent candidate for future development into clinical trial and could have potential impact on ovarian cancer treatment pending additional trials to validate this approach. Because human telomerase is known to be active in other cancer types, it is possible to expand our T-VISA system to additional cancer models such as breast and prostate by expressing the appropriate therapeutic gene to target cancer cells without risking surrounding normal cells/tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References


T-VISA-E1A promotes cancer cell death

In the article by Xie and colleagues on T-VISA-E1A promotes cancer cell death in the August issue of Molecular Cancer Therapeutics, a sentence in the first paragraph of page 2376 was incomplete. The complete sentence appears here.

Interestingly, analysis of the T promoter showed that its activity correlated with the telomerase activity (21).

Reference
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