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Cloning of mouse telomerase reverse transcriptase gene promoter and identification of proximal core promoter sequences essential for the expression of transgenes in cancer cells

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Abstract. Telomerase is a ribonucleoprotein complex, whose function is to add motif-specific nucleotides to the end of chromosomes. Telomerase consists of three major subunits, the telomerase RNA template (hTR), the telomerase-associated protein (TEP1) and telomerase reverse transcriptase (TERT). TERT is the most important component responsible for the catalytic activity of telomerase and a rate-limiting determinant of the activity. Telomerase activities were at high levels in approximately 90% of mouse cancers or tumor-derived cell lines through TERT transcriptional up-regulation. Unlike human telomerase, telomerase activity exists in colon, liver, ovary and testis but not in brain, heart, stomach and muscle in normal mouse tissues. In this study, we prepared 5' truncations of 1086 bp fragments upstream of the initiating ATG codon of the mTERT gene to construct luciferase reporter gene plasmids, and transfected these plasmids into a normal mouse cell line and several cancer lines to identify the core promoter region essential for transcriptional activation in cancer cells by a luciferase assay. We constructed a eukaryotic expression vector of membrane-expressing staphylococcal endotoxin A (SEA) gene driven by the core promoter region of the mTERT gene and observed if the core promoter region could express the SEA gene in these cancer cells, but not in normal cells following transfection with the construct. The results showed that the transcriptional activities of each fragment of the mTERT gene promoter in the cancer cell lines Hepa1-6, B16 and CT26 were higher than those in NIH3T3 cells, and the proximal 333-bp fragment was the core promoter of the mTERT gene in the cancer cells. The proximal 333-bp fragment was able to make the SEA express on the surface of the cancer cells, but not in NIH3T3 cells. It provides a foundation for cancer targeting gene therapy by using the mTERT gene promoter.

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Introduction

Telomere is a specialized structure at the end of chromosomes and essential for maintaining the stability of the eukaryote genome (1). Telomerase adds motif-specific nucleotides to the end of chromosomes to maintain the length of the telomere and is composed of three major subunits. The telomerase RNA component provides the template for telomere repeat synthesis (2,3), the telomerase-associated protein (TEP1) binds to telomerase RNA and coordinates assembly of telomerase holoenzyme (4,5), and the most important component responsible for the catalytic activity of telomerase is telomerase reverse transcriptase (TERT) (6,7).

Previous studies have shown that TERT is expressed in most malignant tumors but not in normal human tissues and the expression of TERT is closely associated with telomerase activity, whereas two other components are constitutively expressed in both tumors and normal tissues (8,9). These findings indicate that TERT is a rate-limiting determinant of catalytic activity of telomerase and the expression of telomerase is tightly controlled in normal human cells. Reports of telomerase activities in mouse tumors indicated that telomerase activities were at high levels in approximately 90% of mouse cancers or tumor-derived cell lines through TERT transcriptional up-regulation (10). Unlike human telomerase, in the normal mouse tissues, telomerase activity exists in colon, liver, ovary, and testis but not in brain, heart, stomach, and muscle (11-13). The promoter regions of human TERT gene have been cloned and their activities in cancer cells characterized. In the mouse, although the core promoter of TERT gene responsible for basal transcriptional activity in muscle cells has been identified (14), little work has been carried out with mTERT gene promoter in mouse cancer cells. Therefore, the activity of mTERT gene promoter in cancer cells is not yet clear.

Pericuesta *et al* (15) generated the construct mTERT-GFP using the mTERT gene promoter of 1, 2 or 5 kb upstream of the first ATG of the open reading frame of mTERT gene to promote the expression of EGFP. In their transgenic model

no fluorescent expression of the mTERT-EGFP construct could be identified in adult tissues. This suggests that although telomerase activity exists in colon, liver, ovary, and testis, there is a tight repression system of mTERT gene promoter in adult mouse tissues in physiological conditions.

Previous studies (15,16) have confirmed that the principal transcriptional regulation site is present in the 1 k-mTERT gene promoter. In this study, 5' truncations of 1086 bp fragments upstream of the initiating ATG codon of mTERT gene were prepared to construct luciferase reporter gene plasmids, and tested by a luciferase assay to identify the core promoter region essential for transcriptional activation in cancer cells. Then, we constructed a eukaryotic expression vector of membrane-expressing staphylococcal endotoxin A (SEA) gene driven by the core promoter region of mTERT gene and observed if the core promoter region could make SEA gene express in these cancer cells, but not in normal cells after transfection with the constructs.

Materials and methods

Vectors, bacterial strains, and cell lines. The eukaryotic expression vectors pGL3-Basic, pGL3-Control and pRL-CMV from Promega Corporation (USA) and *Escherichia coli* DH5α were maintained in our laboratory. The recombinant plasmid pMD-18-SEA containing SEAtm gene was previously constructed by us (17). Mouse hepatoma cell line Hepa1-6, melanoma cell line B16, colon cancer cell line CT26 and fiberblast cell line NIH3T3 were kindly provided by Professor Yanfang Sui from the Fourth Military Medical University (Xian, China). All the cell lines were maintained in Roswell Park Memorial Institute RPMI-1640 (Gibco-BRL, Gaithersburg, MD, USA) or high-glucose Dulbecco's modified Eagle's medium (DMEM), which were supplemented with 10% fetal bovine serum and penicillin/streptomycin in an atmosphere of 5% CO_2 chamber at 37ºC.

Cloning of the promoter region of mTERT gene and construction of luciferase reporter gene plasmids. The luciferase reporter gene plasmids were generated using the available sequence of 1086 bp of the promoter of mouse mTERT gene (NCBI GeneBanck; accession number AF 121949). Ten fragments of the mTERT gene promoter of 1086, 904, 715, 514, 333, 282, 229, 184, 144 and 80 bp, upstream of the first ATG of the open reading frame of mTERT gene, were amplified by PCR from total DNA of Hepa1-6 cells. The amplified products were digested with *Xho*I and *Nco*I, and cloned into pGL3-Basic vector upstream of the luciferase gene, respectively, to promote the expression of luciferase. The presence of the inserted fragment was confirmed by restriction enzyme digestion and gel electrophoresis. All constructs were confirmed by DNA sequencing. The constructs were named as pGL3-mTERT1 to pGL3-mTERT 10, indicating the construct with 1086, 904, 715, 514, 333, 282, 229, 184, 144, and 80 bp of the mTERT gene promoter, respectively.

Luciferase assays. Three tumor cell lines Hepa1-6, B16, CT26 and one fiberblast cell line NIH3T3 were used in this study. Transient transfection of luciferase reporter plasmids was performed with lipofectamine 2000 (Invitrogen, USA) according to the protocol recommended by the manufacturer. For better comparison among cell lines with different transfection efficiencies, the pGL3-control plasmid, which has the firefly luciferase gene under the transcriptional control of SV40 enhancer/promoter, was also transfected into each cell line and used for normalization of the activities shown by the mTERT gene promoter-luciferase construct. The control plasmid, pRL-CMV containing the Renilla reniformis luciferase gene under the transcriptional control of CMV enhancer/promoter, was cotransfected with the mTERT gene promoter-luciferase constructs to standardize for transcription efficiency. The level of firefly luciferase activity was normalized to that of Renilla reniformis luciferase activity for each transfection. Briefly, the day before transfection, $0.5\text{-}2\times10^5$ cells/ well were seeded in a 24-well tissue culture dish and were exposed to transfection mixtures containing 0.8 μg of the luciferase reporter plasmids described above and 0.04 μg of pRL-CMV control vector (Promega) in 0.6 ml growth medium for 5 h at 37°C, and then the growth medium was changed by 3-ml fresh growth medium. The cells were harvested 48 h after the transfection and assayed by the dual-luciferase reporter assay system (Promega, WI), using a luminometer (EG&G Berthold, Germany) to identify the core promoter region of mTERT gene. All of the data shown in this study were obtained from at least three independent experiments.

Construction of the eukaryotic expression vector of SEA gene driven by mTERT gene core promoter. Membrane-expressing SEA (named as SEAtm) gene from pMD18-SEAtm was subcloned into *Bgl*II and *Xba*I site of pGL3-mTERT5 to replace the luciferase gene and was driven by the core promoter region mTERT gene. The construct was named as pGL3-mTERT-SEA. The presence of the inserted fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

RT-PCR. The expression of SEA mRNA in Hepa1-6, B16, CT26 and NIH3T3 cells was detected after transfection with pGL3-mTERT-SEA. Briefly, the cells were plated at a density of 5×10^5 cells/well in 6-well culture plates 24 h before transfection. After 48 h transfection with lipofectamine 2000, the cells were harvested and total RNA was isolated using the guanidine isothiocyanate method. The RT-PCR primers, 5'-ATGAAAAAAACAGCATTTACA-3' (sense) and 5'-TTATACAGGGCGTACACTTTC-3' (antisense), were constructed according to the SEAtm cDNA. First-strand cDNA was synthesized with 1 μg of total RNA using a first strand cDNA synthesis kit (Life Technologies, Inc., Gaithersburg, MD) in the presence of 1.6 μg of oligo-(dT)n primer in a final volume of 25 μl. After denaturation for 5 min at 94°C, 1 μl of reaction product was amplified by PCR for 25 cycles (94°C for 30 sec; 58°C for 30 sec; 72°C for 1 min). The amplified products were separated by electrophoresis on a 1% agarose gel electrophoresis and visualized with ethidium bromide. The DNA sequences of RT-PCR products were confirmed at least once by DNA sequencing and were found to be identical to the corresponding sequence of SEAtm cDNA (data not shown). Each RT-PCR was performed three times with independent

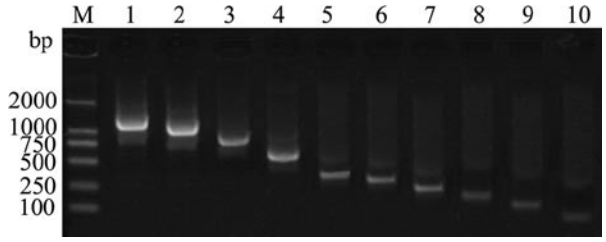

Figure 1. Gel electrophoresis of PCR products of different mTERT gene promoter fregments. Ten fragments of the mTERT gene promoter of 1086, 904, 715, 514, 333, 282, 229, 184, 144 and 80 bp, upstream of the first ATG of the open reading frame of mTERT gene, were amplified by PCR and named as mTERT1 to 10, respectively. All PCR products were analyzed by electrophoresis in a 1.0% agarose gel containing 0.5 lg/ml ethidium bromide. M, DL2000 DNA marker; lanes 1-10, PCR products of mTERT1 to 10, respectively.

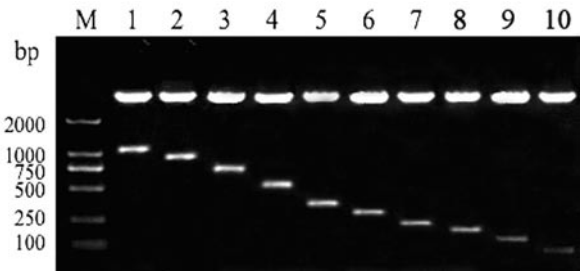

Figure 2. Identification of luciferase reporter gene plasmids by endonuclease digestion. The luciferase reporter gene plasmids pGL3-mTERT1 to 10 were digested with *Xho*I and *Nco*I, and then visualized in a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide. M, DL2000 DNA marker; lanes 1-10, pGL3-mTERT1 to 10 digested with *Xho*I and *Nco*I, respectively.

preparations of RNA. As an internal control, RT-PCR of β-actin was performed for all RNA samples, using the PCR primers, 5'-AGCAAGAGAGGCATCCTCAC-3' (sense) and 5'-AGCTCGTAGCTCTTCT CCAG-3' (antisense).

Antibodies. Rabbit anti-SEA polyclonal antibody (Abcam, USA), FITC-conjugated donkey anti-rabbit IgG (Biolegend, USA) were used in this study.

Confocal microscopy analysis. The expression of SEA on the surface of cells was visualized by *in situ* indirect immunofluorescent staining. Hepa1-6, B16, CT26 and NIH3T3 cells were seeded at a low density onto glass coverslips and grown for 24 h. After 48 h transfection with pGL3-mTERT-SEA, the cells were rinsed with PBS, fixed with 4% paraformaldehyde for 30 min at 4ºC, incubated with a rabbit anti-SEA polyclonal antibody, then washed three times with washing buffer, incubated with PE-conjugated donkey anti-rabbit IgG. Following washing three times, coverslips were mounted onto glass slides with buffered glycerol with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI). Coverslips were mounted onto glass slides. Fluorescence distribution was analyzed using a laser confocal scanning microscope.

Results

Cloning of the promoter region of mTERT gene and identification of luciferase reporter gene plasmids. Ten fragments of the mTERT gene promoter of 1086, 904, 715, 514, 333, 282, 229, 184, 144 and 80 bp, upstream of the first ATG of the open reading frame of mTERT gene, were amplified by PCR and named as mTERT1 to 10, respectively. The products were visualized after 1.0% agarose gel electrophresis and ethidium bromide staning. The result is shown in Fig. 1. The PCR products were cloned into pGL3-Basic with the multiple cloning sites of *Xho*I and *Nco*I to construct pGL3-mTERT1 to 10 with *Xho*I and *Nco*I, respectively. Restriction endonuclease digestion analysis of the constructs are shown in Fig. 2. The sequences of mTERT1 to 7 mTERT gene promoter fragments are consistent with the corresponding sequence in GenBank, and have 100% homogeneity.

Transcriptional activity of mTERT gene promoter fregments in cancer and normal cells. To examine the transcriptional activity of mTERT in normal and cancer cells, a transient expression assay was first performed using cancer cell lines and a normal cell line. Luciferase reporter plasmids containing a 1086 bp region upstream of the initiating ATG codon and its trunctions were constructed and transfected into cancer cell lines Hepa1-6, B16, and CT26, as well as the normal cell line NIH3T3, and the cell lysates were tested in luciferase assays, respectively. As shown in Fig. 3, the transcriptional activity of each fragment of mTERT gene promoter in tumor cells was higher than that in NIH3T3 cells, the proximal 333-bp fragment conferred peak transcriptional activity, equivalent to 62-101% of activity in control reporter plasmids (pGL3-Control) driven by SV40 enhancer/promoter. The levels of transcription varied among types of cancer cells. Transcriptional activities of proximal 229-, 184-, 144- and 80-bp fragments were very low, almost equivalent to that in pGL3-Basic.

Identification of the eukaryotic expression vector of SEA gene driven by mTERT gene core promoter. The SEAtm gene was subcloned into pGL3-mTERT5 with *Bgl*II and *Xba*I to replace luciferase gene, and was driven by the core promoter of mTERT gene. The construct was named as pGL3-mTERT-SEA. Restriction endonuclease digestion analysis of pGL3-mTERT-SEA and the construction map of pGL3-mTERT-SEA are shown in Fig. 4. Two segments were digested from pGL3-mTERT-SEA.

Expression of SEA mRNA in tumor and normal cells. The expression of SEA mRNA in Hepa1-6, B16, CT26 and NIH3T3 cells was detected by RT-PCR after transfection with pGL3-mTERT-SEA. The amplified products were visualized by electrophoresis on a 1% agarose gel with ethidium bromide, and typical results are shown in Fig. 5. SEA mRNA expressed in Hepa1-6, B16, CT26 tumor cells, but not in normal cell NIH3T3.

Expression of SEA on the surface of tumor and normal cells. Hepa1-6, B16, CT26 and NIH3T3 cells were transiently transfected with pGL3-mTERT-SEA. After 48 h of transfection, SEA expressing on the surface of cells was stained by indirect immunofluorescence, and was visualized

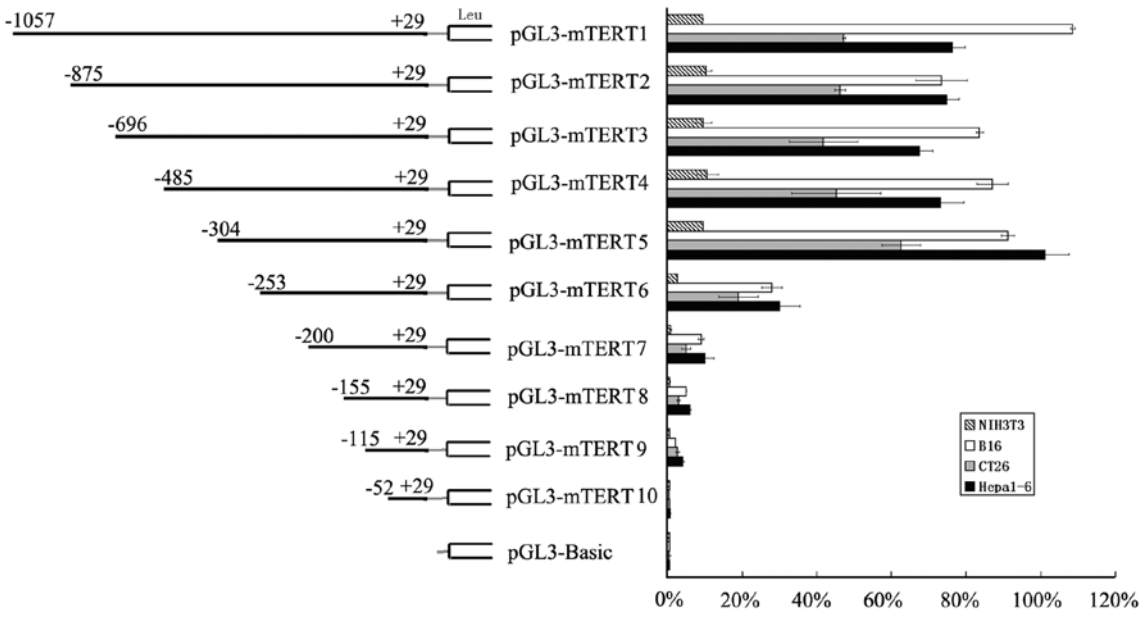

Figure 3. Luciferase assay to identify the regions required for the mTERT gene promoter activity in different cells. NIH3T3, B16, CT26 and Hepa1-6 were transfected with a series of luciferase reporter constructs containing the 52-flanking DNA of the mouse TERT gene. The positions of the bases are indicated relative to the mTERT transcription initiation site. For each transfection, the firefly luciferase activity was normalized with the Renilla reniformis luciferase activity by the cotransfected pRL-CMV. The luciferase activity of pGL3-Control plasmid was normalized to 1, and the relative luciferase activity is shown. The standard deviations (SD) of the means are indicated by the error bars. The relative activity of each construct is expressed as a ratio to the activity of pGL3-Control respectly in different cell lines. The means from at least three independent experiments are shown for each construct.

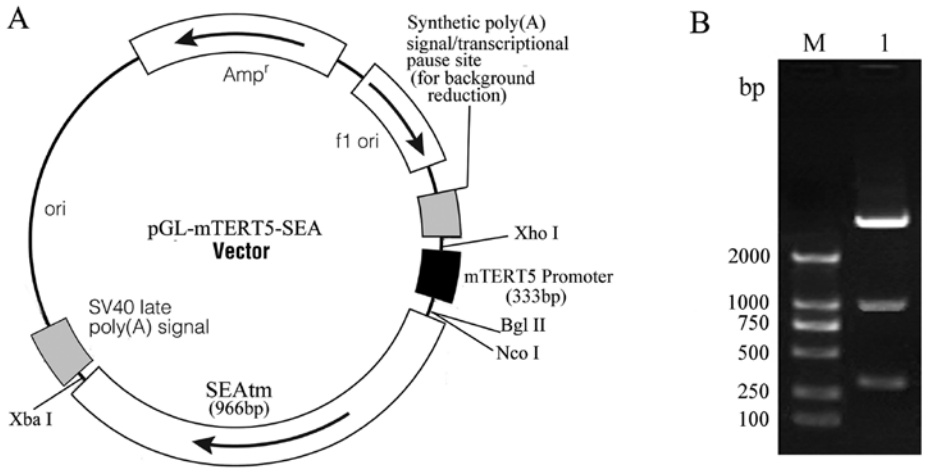

Figure 4. (A) Schematic representation of the eukaryotic expression vector of SEA gene. (B) Gel electrophoresis analysis of the recombinant plasmids digested with restriction endonucleases. The recombinant pGL3-mTERT-SEA plasmid was digested with *Xho*I, *Bgl*II and *Xba*I, and digested products were analyzed by electrophoresis in a 1.0% agarose gel containing 0.5 μg/ml ethidium bromide. M, DL2000 DNA marker; lane 1, the recombinant pGL3-mTERT-SEA vector digested with *Xho*I, *Bgl*II and *Xba*I.

by laser confocal microscopy. Non-transfected Hepa1-6, B16, CT26 and NIH3T3 cells were used as control. The SEA were expressed on the surface of Hepa1-6 B16, CT26 cells, but not in NIH3T3 cells and control cells by indirect immunofluorescence (Fig. 6).

Discussion

In cancer gene therapy, the restricted expression of a targeted gene in the tumor is important. If the targeted gene is expressed in all cells, it will affect both tumor and normal cells. Recent research suggests that use of the tumor specific promoter system solves this problem. Targeted cancer gene therapy has the aim of concentrating the target therapeutic gene expression into the specific target tissue. Then it can minimize side effects and maximize the efficacy of the therapy. Hence, the feasibility of using hTERT promoter to induce tumor specific transgene expression in cancer gene therapy is unsurprising. There are several examples of the use of hTERT promoter in targeted cancer gene therapy by adenovirus (18,19). Telomerase activities were at high levels in approximately 90% of mouse cancer models or tumor-derived

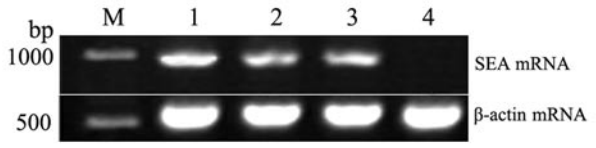

Figure 5. Gel electrophoresis analysis of SEA mRNA expression in Hepa1-6, B16, CT26 and NIH3T3 cells after transfection with pGL3-mTERT-SEA. M, DL2000 DNA Marker; lane 1, SEAtm mRNA expression in Hepa1-6; lane 2, SEAtm mRNA expression in B16; lane 3, SEAtm mRNA expression in CT26; lane 4, SEAtm mRNA expression in NIH3T3.

cell lines through TERT transcriptional up-regulation (10), but in the normal mouse, telomerase activity only exists in colon, liver, ovary, and testis (11,12). It is not known if the mTERT gene promoter can be used as a tumor-specific promoter, and we thus cloned the mTERT gene promoter fragments and analysed their transcriptional activities in tumor cells and normal cells.

A previous study showed that there is a tight repression system of mTERT gene promoter in adult mouse tissues in physiological conditions. The principal transcriptional regulation site is present in the 1 k-mTERT gene promoter. Therefore, in our study, 5' truncations of 1086 bp fragment upstream of the initiating ATG codon of mTERT gene were prepared and tested by a luciferase assay for the transcriptional activities. The luciferase assay showed that the transcriptional activities of each fragment of mTERT gene promoter in Hepa1-6, B16 and CT26 cells were higher than that in NIH3T3 cells, and the proximal 333-bp fragment conferred peak transcriptional activity. The RT-PCR and confocal microscopic analysis results showed that the proximal 333-bp fragment could make SEA gene express on the surface of tumor cells, but not in NIH3T3 cells. These results indicated that the proximal 333-bp fragment upstream of the first ATG in the open reading frame of mTERT gene was the core promoter in the cancer cells and essential for the transgene expression on the surface of cancer cells, but not in NIH3T3 cells. The mTERT gene promoter can be used as a tumor-specific promoter.

mTERT gene expression was tightly regulated by transcriptional factors Sp1, Sp3, and c-Myc (8,14). Previous results showed that the cis-elements in the mTERT core promoter, two GC-boxes (GC-143 and GC-105) bound to Sp1 and Sp3, and an E-box (E-201) bound to c-Myc were very important in mTERT transactivation in mouse. Decreased binding of Sp1, Sp3, and c-Myc to three cis-elements in the mTERT core promoter were well correlated with the down-regulation of mTERT gene expression during muscle cell differentiation. In mTERT gene transcription, both Sp1 and Sp3 act as positive regulators. In mouse, high levels of Sp1 expression have been found in spermatids, T cells in thymus, epithelial cells, and

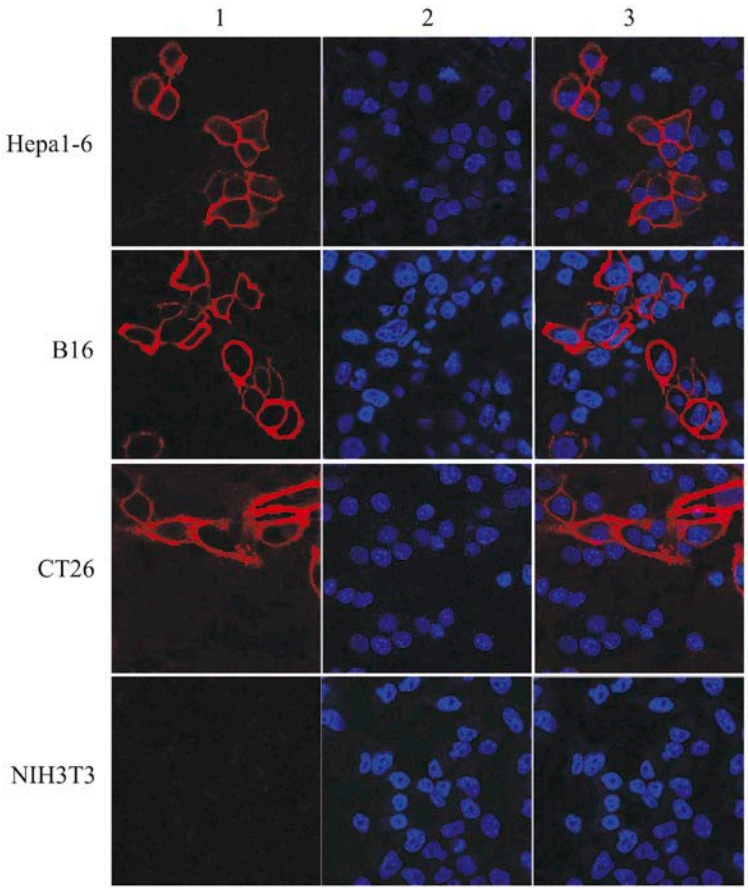

Figure 6. Laser confocal microscopic analysis of the expression of SEA on Hepa1-6, B16, CT26 and NIH3T3 cells after transfection with pGL3-mTERT-SEA (x630). 1, The SEA antigen expressed on the cells [Phycoerythrin (PE) labeled]; 2, the nuclei stained by [4',6-diamidino-2- phenylindole 2HCL (DAPI)]; 3, the merged image of SEA and nuclei.

hematopoietic cells, whereas Sp1 expressions in heart, skeletal muscle, and smooth muscle cells of stomach have been shown to be low (20). Telomerase activities are not detected in the brain, heart, stomach, and muscle in mouse (12). The different expression levels of Sp1 are consistent with tissue-specific expression of telomerase activity in adult mouse. Different extents of Sp1 and Sp3 expression exist among different cell types. C-Myc, a transcription factor encoded by a proto-oncogene, is suppressed during myogenic differentiation through post-transcriptional mechanisms (21-24). In human, c-Myc binds to the hTERT gene promoter and plays a critical role in the regulation of hTERT expression (25-27). Similar relationships between mTERT and c-Myc were observed during the differentiation of mouse erythroleukemia cells and mitogen-stimulated lymphocyte proliferation. These findings suggest that there is a potential link between increased c-Myc and up-regulation of mTERT in normal proliferating and transformed cells (8,26). Our results showed that transcriptional activities of the same fragment of mTERT gene promoter had different activity in different cancer cells and might be related to the extent of c-Myc, Sp1 and Sp3 expression among different cell types.

In summary, this study suggests that the transcriptional activities of each fragment of mTERT gene promoter in Hepa1-6, B16 and CT26 cells are higher than that in NIH3T3 cells, and the proximal 333-bp fragment was the core promoter of mTERT gene in cancer cells. The proximal 333-bp fragment was able to make the SEA express on the surface of the cancer cells, but not in NIH3T3 cells. It provides the foundation for cancer targeting gene therapy by using the mTERT gene promoter.

Acknowledgements

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