

RESEARCH ARTICLE

Luciferase Assay to Screen Tumour-specific Promoters in Lung Cancer

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Abstract

Objective: Specific promoters could improve efficiency and ensure the safety of gene therapy. The aim of our study was to screen examples for lung cancer. **Methods:** The firefly luciferase gene was used as a reporter, and promoters based on serum markers of lung cancer were cloned. The activity and specificity of seven promoters, comprising CEACAM5 (carcinoembryonic antigen, CEA), GRP (Gastrin-Releasing Peptide), KRT19 (cytokeratin 19, KRT), SFTPB (surfactant protein B, SP-B), SERPINB3 (Squamous Cell Carcinoma Antigen, SCCA), SELP (Selectin P, Granule Membrane Protein 140kDa, Antigen CD62, GMP) and DKK1 (Dickkopf-1) promoters were compared in lung cancer cells to obtain cancer-specific examples with strong activity. **Results:** The CEACAM5, DKK1, GRP, SELP, KRT19, SERPINB3 and SFTPB promoters were cloned. Furthermore, we successfully constructed recombinant vector pGL-CEACAM5 (DKK1, GRP, SELP, KRT19, SERPINB3 and SFTPB) contained the target gene. After cells were transfected with recombinant plasmids, we found that the order of promoter activity from high to low was SERPINB3, DKK1, SFTPB, KRT19, CEACAM5, SELP and GRP and the order for promoters regarding specificity and high potential were SERPINB3, DKK1, SELP, SFTPB, CEACAM5, KRT19 and GRP. **Conclusion:** The approach adopted is feasible to screen for new tumour specific promoters with biomarkers. In addition, the screened lung-specific promoters might have potential for use in lung cancer targeted gene therapy research.

Keywords: Luciferase assay - lung cancer - tumor-specific promoter - gene therapy - tumor biomarker

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Introduction

Over the past 10 years, lung cancer has become the leading cause of cancer death in worldwide. More than 7,000 people died from lung cancer in Taiwan in 2010 and more than 150,000 people died from lung cancer in US in 2010 according to the new statistics (Jemal et al., 2010; Shieh et al., 2013). Since the overall survival of lung cancer has improved only minimally over the past four decades, novel therapeutic approaches are needed (Siegel et al., 2013).

Suzuki et al. have been found that combination of genomic modification to achieve tumor-selective replication and capsid modification to enhance infectivity yields more potent oncolytic adenoviruses for use in cancer treatment (Suzuki et al., 2001). What's more, tumor-specific promoters (TSPs) regulated target genes can be specifically expressed in cancer cells. Therefore, TSPs could greatly increase the therapeutic efficacy and security of gene therapy for cancer which is considered a breakthrough in the field.

It has been reported that transfection of NSCLC (non-small cell lung cancer) cells with both human NIS (sodium iodide symporter) and TPO (thyroperoxidase) genes resulted in an increase in radioiodide uptake and retention and enhanced tumor cell apoptosis (Huang et al., 2001). An adenovirus mediated transgene expression system of the hNIS (human sodium iodide symporter) under the control of hTERT (human telomerase reverse transcriptase) promoter has the potential to become an effective wide-spectrum yet highly specific anti-cancer strategy (Shi et al., 2010). The study of Tadashi et al. showed the possibility of gene therapy using the cell type-specific promoter of CEA (carcinoembryonic antigen) gene against CEA-producing adenocarcinoma of the lung and adenovirus carrying the NIS gene linked to the CEA promoter induced high levels of tumor-specific radioiodide accumulation in HCT 116 cells (Osaki T, 1994; Scholz et al., 2005). Survivin promoter is a cancer-specific promoter for various cancers which may be useful controlling gene expression in cancer cells and in cancer gene therapy (Bao et al., 2002; Chen, 2004). Reciprocal binding of

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CTCF (CCCTC binding factor) and BORIS (Brother of the Regulator of Imprinted Sites) to the NY-ESO-1 (a member of the cancer–testis family of antigens) promoter mediates epigenetic regulation of this CT (cancer/testis) gene in lung cancer cells which may be a novel strategy to augment immunogenicity of pulmonary carcinomas (Hong JA, 2005). Protein expression is regulated by genes, and gene transcription and replication are subject to a number of complex regulatory elements (Levine and Tjian, 2003; Kelly and Scarpulla, 2004). Since promoter is the most representative element, screening for specific promoters could improve efficiency and ensure the safety of gene therapy. Therefore, the aim of our study was to screening more specific promoters for lung cancer marker genes.

In this study, six cell lines were transfected by recombinant plasmids which were verified by agarose gel electrophoresis. Furthermore, the activity and specificity of seven promoters, including CEACAM5 (carcinoembryonic antigen, CEA) promoter, GRP (Gastrin-Releasing Peptide) promoter, KRT19 (cytokeratin 19, KRT) promoter, SFTPB (surfactant protein B, SP-B) promoter, SERPINB3 (Squamous Cell Carcinoma Antigen, SCCA) promoter, SELP (Selectin P, Granule Membrane Protein 140kDa, Antigen CD62, GMP) promoter and DKK1 (Dickkopf-1) promoters were compared to obtain lung cancer-specific promoters with strong activity. We anticipate that the results of this study could be benefit for exploring the mechanism and gene therapy for the lung cancer.

Materials and Methods

Cell lines and culture

Human lung cancer cell lines, including A549, H446 and H460, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 units/mL of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B and maintained at 37°C in a humidified 5% CO₂ atmosphere. Cells in good condition were seeded in 6-well cell culture plate.

Extraction of DNA genomes and concentration determination

When the cell fusion rate was over 90%, the genome DNA was extracted from each cell line using the Universal Genomic DNA Extraction kit Ver.3.0 (Takara Shuzo Co.) according to the manufacturer's instructions. Furthermore, the concentrations of extracted DNA genomes were determined by measuring the ultraviolet (UV) absorbance employing a UV visible spectrophotometer (Thermo Scientific, USA).

Candidate promoters cloning and verification

The cutting sites of restriction enzymes (Fermentas) identified by the software of primer 5.0 version (Primer, Canada) were provided in Table 1. The sequences of cloned promoters were verified by gel electrophoresis detection. Briefly, mixtures in centrifuge tubes were centrifuged in a portable centrifuge (microOne Mini Personal Centrifuge, Tokyo) for 5 s. Then, agarose gel

electrophoresis detection was conducted on PowerPac Basic electrophoresis apparatus (BIO-RAD) after incubating all the tubes in a water bath for 2 h at 37°C.

Vector plasmids extraction and verification

The domesticated laboratory strain *E. coli* DH5α were grown in LB (Luria broth) medium supplemented with 40 µg/ml ampicillin (HyClone, Logan, UT) when required for selection of plasmids. Vector plasmids were extracted from *E. coli* DH5α harboring the pGL3, sAAV-CMV-Luc and pRL-TK plasmid (Promega, USA) by using the Plasmid Mini Preparation Kit (Beyotime, China). Then the pGL3 plasmid vector, pRL-TK co-transfection vectors and dsAAV-CB-EGFP plasmids were digested for verification. Briefly, the reactants were blended and centrifuged in a portable centrifuge for 5 s. Next, agarose gel electrophoresis detection was performed on PowerPac Basic electrophoresis apparatus (BIO-RAD) after incubating in a water bath for 2 h at 37°C.

Construction of recombinant plasmids

Restricted DNA products verified by agarose gel electrophoresis and plasmids were recovered using a gel extraction kit (Beyotime, China) according to the manufacturer's instructions. The mixture system with the volume of 10 µl, including 2 µl recovered plasmid (50-100 ng), target gene fragments (molar ratio of linear vector molecule DNA to exogenous DNA molecule was between 1:1 and 1:10) and double- distilled water (ddH₂O) were incubated in a water bath for 6 min at 45°C to melt and reanneal the cohesive ends. And the mixture was rapidly transferred to an ice bath for 3 min. Then, the volume was raised to 20 µl by addition of 1 µl 10 × T4DNA ligase Buffer, 0.5 µl of 5 U/µl T4 DNA Ligase (Takara Shuzo Co.), ddH₂O and incubated at 14 - 16°C overnight.

Recombinant plasmids transfection

Plasmids were purified using a QIAGEN Plasmid Midi Kit (Qiagen) and detected by agarose gel electrophoresis. Then, highly purified plasmid DNA was used for cell transfection. The vectors loading firefly luciferase and Renilla luciferase reporter gene were used to transfect lung cancer cells. Renilla luciferase reporter gene and firefly luciferase reporter gene were served as control and experimental reporter gene, respectively. All transfection experiments for cell lines were carried out in triplicate.

Luciferase luminescence detection

Cells were washed twice by 1 × PBS of pH 7.0 after the culture medium was removed. Then, lysates were prepared

Table 1. The Restriction Enzyme Cutting Sites

Promoter	Enzymes	Single restriction sites (bp)
GRP	TaqI	533/675
CEA	SmaI	485/430
SP-B	KpnI	293/775
KRT	SmaI	395/788
CMV	XbaI	409/182
DKK	MluI	882/167
GMP	KpnI	157/787
SCCA	HindIII	263/530

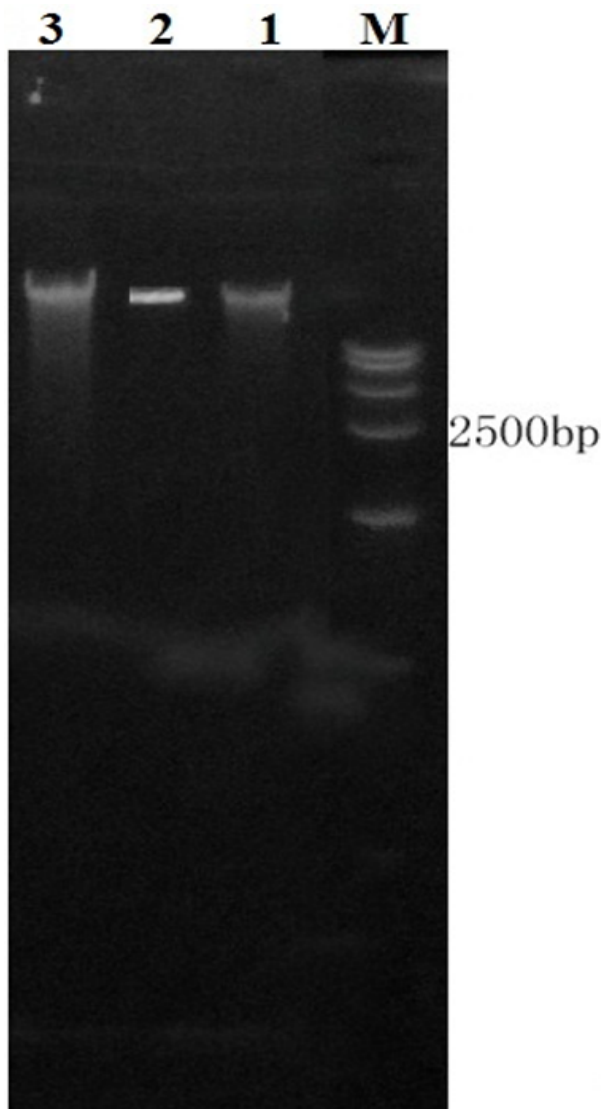


Figure 1. Gel Electrophoresis Photograph Showing the Extracted Genome DNA from Each Cell Line. Lane 1: H446 genome DNA; Lane 2: A549 genome DNA; Lane 3: H460 genome DNA; Lane M: DNA marker DL15000

by applying 250 μ l of 1 \times PLB (Passive Lysis Buffer) and scraping for 15min at room temperature. A 20 μ l aliquot of cell lysate was mixed with 50 μ l of Luciferase Assay Reagent II (LAR II). Total 50 μ l of Stop & Glo Reagent was then added to the luminometer tube to quench the firefly luciferase (fLuc) reaction and simultaneously activate the renilla luciferase (rLuc) reaction. Renilla luciferase activity was measured immediately with a FB12-Berthold luminometer (Berthold Technologies, Bad Wildbad, Germany) interfaced with a computer was used to trace the luminescence emission over a 12 second period (2 s: pre-read delay, 10 s: numerical reading) in determination. Data are expressed as relative luciferase activity of duplicate experiments and relative luciferase activity was calculated as the ratio of firefly luciferase activity to renilla luciferase activity.

Statistical analysis

All in vitro experiments were done thrice, and the in vitro experimental data were presented as the mean \pm SD and analyzed by Student's t test for statistical

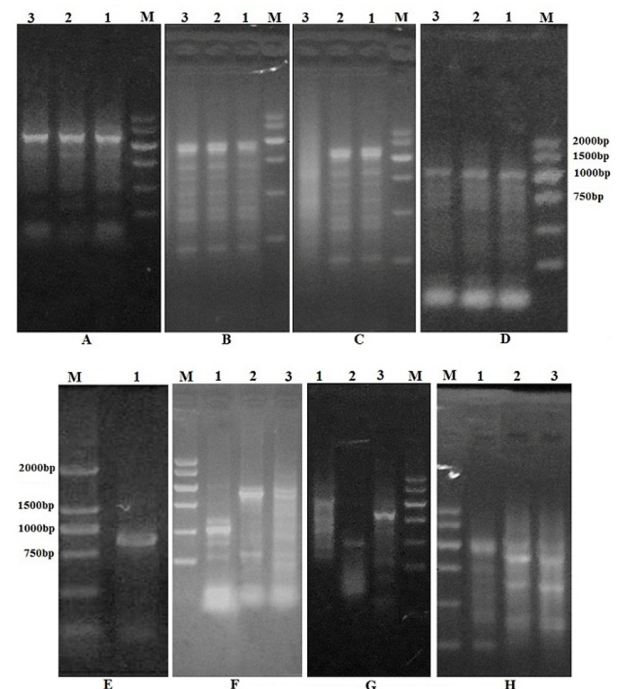


Figure 2. Gel Electrophoresis Image of the PCR Products Showing the PCR Result of Promoter with DNA Genome as Template. A: GRP promoter; B:CEA promoter; C: KRT promoter; D: SP-B promoter; E: CMV promoter; F: GMP promoter; G: SCCA promoter; H: DKK promoter. Lane 1: H446 genome DNA; Lane 2: A549 genome DNA; Lane 3: H460 genome DNA; Lane M: DNA marker V

significances. A *P*-value of less than 0.05 was considered statistically significant.

Results

DNA genomes of human lung cancer cell lines

The electrophoresis photograph showed DNA genomes extracted from A549, H460 and H446 were larger than 15000bp which indicated that the extracted DNA genomes were of high quality (Figure 1). The concentrations of genomic DNA extracted from A549, H460 and H446 were 108 ug/ml, 89 ug/ml and 98 ug/ml, respectively.

PCR products of Promoters

The DNA genomes extracted from A549, H446, H460 cells were used as templates, and PCR cloning products of promoters were detected by agarose gel electrophoresis (Figure 2).

For PCR products of promoters, a clear 1200bp band of GRP promoter, a clear 900bp band of CEA promoter, a clear 1100bp band of KRT promoter, a clear 1078bp band of SP-B promoter, a clear 600bp band of CMV promoter, a clear 1000bp band of GMP promoter, a clear 750bp band of SCCA promoter, a clear 1000bp band of DKK promoter could be observed.

Vector plasmids extraction and verification

The obtained competent state of Escherichia coli cells with high activity were used for the subsequent transformation of recombinant plasmids. The agarose gel electrophoresis images of pGL3-Basic Vector and pRL-TK

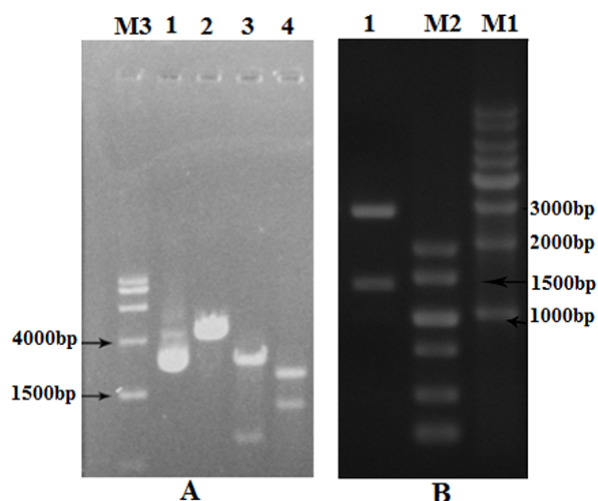


Figure 3. The Agarose Gel Electrophoresis Images of pGL3-Basic Vector and pRL-TK Vector. (A) Lane 1: pGL3-Basic Vector; Lane 2: pGL3-Basic Vector digested with HindIII; Lane 3: pGL3-Basic Vector digested with BglIII; Lane 4: pGL3-Basic Vector digested with SacI and XbaI; Lane M3: DL15000 DNA marker; (B) Lane 1: pRL-TK Vector digested with NotI and EcoRI; Lane M1: 1kb DNA Marker; Lane M2: DNA marker V

Vector were shown in Figure 3. The results indicated that the vector plasmids extracted from *E. coli* DH5 α were correct.

Recombinant plasmid

The recombinant plasmid including pGL3-GRP, pGL3-CEACAM5, pGL3-KRT19, pGL3-SFTPBP, pGL3-CMV, pGL3-SELP, pGL3-DKK1 and pGL3-SERPINB3 were identified by agarose gel electrophoresis (Figure 4). The images proved that all the plasmids were recombinant plasmids.

The expression activities of candidate promoters in cell lines

The relative luciferase activity of seven candidate promoters in six kinds of transfected cells (A549, H460, H446, 7721, HeLa and 293) were shown in Figure 5. The differences in relative luciferase activity among the candidate promoters were observed with the CMV promoter as control. The order about promoter activity from high to low was SERPINB3, DKK1, SFTPBP, KRT19, CEACAM5, SELP and GRP. The specificity and high potential of promoters from high to low order were SERPINB3, DKK1, SELP, SFTPBP, CEACAM5, KRT19 and GRP. Therefore, the screened new promoters, such as SERPINB3, DKK1, SFTPBP and KRT19, have potential application in gene therapy of lung cancer.

Discussion

Appropriate promoter selection allows for improved gene expression in the respiratory system, such as human polyubiquitin C (UbC) promoter which is one of the lung-specific endogenous promoters (Gill et al., 2009). In this study, seven promoters of lung cancer marker genes including SERPINB3, DKK1, SFTPBP, KRT19,

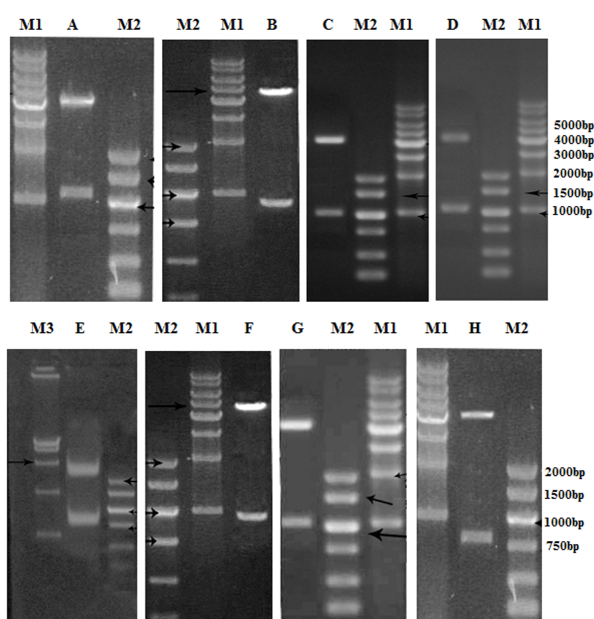


Figure 4. Identification of pGL3-GRP, pGL3-CEACAM5, pGL3-KRT19, pGL3-SFTPBP, pGL3-CMV, pGL3-SELP, pGL3-DKK1 and pGL3-SERPINB3. Lane A: pGL3-GRP digested with KpnI and HindIII; Lane B: pGL3-CEACAM5 digested with SacI and HindIII; Lane C: pGL3-SFTPBP digested with SacI and HindIII; Lane D: pGL3-KRT19 digested with KpnI and HindIII; Lane E: pGL3-CMV digested with SacI and XhoI; Lane F: pGL3-SELP digested with SacI and xhoI; Lane G: pGL3-DKK1 digested with KpnI and HindIII; Lane H: pGL3-SERPINB3 digested with SacI and XhoI; Lane M1: 1kb DNA Marker; Lane M2: DNA marker V; Lane M3: DL15000 DNA marker

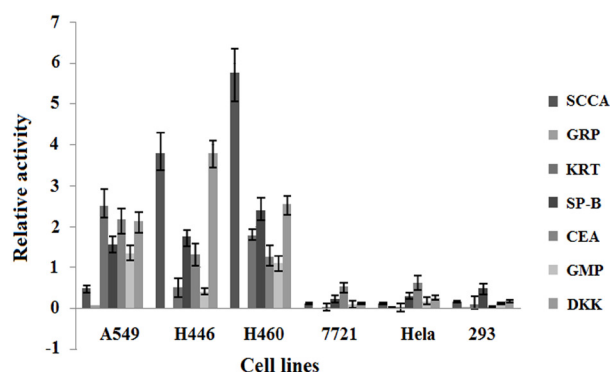


Figure 5. The Differences in Relative Luciferase Activity among the Candidate Promoters in A549, H460, H446, 7721, HeLa and 293 cells. SCCA: SERPINB3, Squamous Cell Carcinoma Antigen; GRP: Gastrin-Releasing Peptide; KRT: KRT19, cytokeratin 19; SP-B: SFTPBP, surfactant protein B; CEA: CEACAM5, carcinoembryonic antigen; GMP: SELP, Granule Membrane Protein; DKK: DKK1, Dickkopf-1

CEACAM5, SELP and GRP 7 promoters were cloned. The comparison results of activity and specificity of these seven promoters showed that the screened promoters, such as SERPINB3, DKK1, SFTPBP and KRT19, have the potential to be used in the gene therapy for lung cancer.

Studies have shown that direct injection of DNA into tumors can lead to transduction of a significant proportion of the tumor cells (Stewart et al., 1992; Plautz et al., 1993) and that use of a tissue-specific promoter can achieve essentially tumor-specific expression of the

injected gene (Vile and Hart, 1993). The main method of screening a promoter is to clone promoters according to the sequences of promoters searched in the human genome. The promoters of biological serum markers, including alpha-fetoprotein (AFP) and phosphatidylinositol proteoglycans-3 (GPC-3) in liver cancer, human kallikrein 2 (HK2) and prostate specific antigen (PSA) in prostate cancer, pancreatic cancer germ resistant (POA) and mucin 1 (MUC1) in pancreatic cancer and neuron-specific enolase (NSE) in lung cancer, have been reported (Qiao J, 2002; Greenberg and Lee, 2007; Johnson et al., 2010; Ma et al., 2010; X Cao, 2011). Therefore, it might be a feasible approach to screen new tumor specific promoters by tumor biomarkers.

SERPINB3, a member of the serpin superfamily, are fundamental for the control of proteolysis. Idiopathic pulmonary fibrosis (IPF) lung samples wherein more extensive fibrosis was observed in patients with higher SERPINB3/B4 expression (Calabrese et al., 2008). Furthermore, it has confirmed that SERPINB3 plays role in inhibiting inflammation and favoring epithelial proliferation with increased TGF- β secretion and thus the likelihood of consequent fibrogenesis (Lunardi et al., 2011). DKK1, a secreted protein, controls Wnt signaling by binding the lung resistance-associated protein coreceptor and sterically blocking Wnt binding to the receptor complex (Bafico et al., 2001; Mao et al., 2001). Serum DKK1 concentrations were significantly higher in patients with lung cancer than in patients with other malignant tumors or benign lung diseases and healthy controls (Sheng et al., 2009).

SFTPb is a small, hydrophobic peptide that plays an important protective role in the lung (Tokieda et al., 1999). A critical level of SFTPb production is needed to prevent lung disease, and individuals with even one SFTPb mutation limiting SFTPb production may be at risk for lung disease if other factors further decrease SFTPb production (Yin et al., 2013; Yin et al., 2013). Even partial deficiency of SFTPb gene may result in susceptibility to lung injury, suggesting that optimal SFTPb levels are necessary for the maintenance of lung function (Clark et al., 1997; Melton et al., 2003). SFTPb are known to be tissue-specific markers expressed in normal and lung adenocarcinoma (Khor et al., 1997). Of the 134 lung cancer patient blood samples, 84.3% expressed at least one of the three tumor markers, including tumor specific antigen 9 (TSA-9), Keratin 19 (KRT-19), and Pre-progastrin-releasing peptide (Pre-proGRP). A significant correlation was observed between the number of positive markers and disease stage and progression. Positivity of more than one marker predicted a poor response to therapy and short survival time in non-small cell lung cancer patients (Liu et al., 2008). CEACAM5 is carcinoembryonic antigen-related cell adhesion molecule 5, and CEACAM5 showed high expression in lung tumor tissue (Benlloch et al., 2009). D'Cunha et al. have analyzed the expression of CEACAM5 in 232 lymph nodes from 53 patients with stage I non-small cell lung cancer. In this study, 90.5% of primary tumor samples were positive for CEACAM5 mRNA expression, and 57% of the patients had at least one CEACAM5-positive lymph node and

would theoretically be upstaged to stage II (D'Cunha et al., 2002).

In our study, promoters corresponding to the tumor markers of lung cancer described above were screened systematically and the sequences of promoters can be stable obtained for further gene targeted therapy after constructed into plasmid vectors. Therefore, screening novel and tumor-specific promoters based on the tumor biomarkers could promote the research development of target gene therapy.

Studies about tumor-specific promoter have opened up a new and effective way for the efficiency and safety issues of cancer gene therapy. However, some limitations of tumor-specific promoter such as transfection efficiency and stable and long-term gene expression are still not resolved (Podolska et al., 2012). What's more, the potentially risks of tumor-specific promoters need to be further confirmed, and the optimization and modification of the promoters are very complicated with high technical requirements. Therefore, it is worthy for us to screen more significant tumor-specific promoters and explore more effective optimization program, as well as application methods of promoters.

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