Optimized construction of MUC1-VNTR$_n$ DNA vaccine and its anti-pancreatic cancer efficacy

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Abstract. Considering mucin 1-variable number tandem repeat (MUC1-VNTR$_n$) as a novel target for pancreatic cancer immunotherapy, the present study aimed to screen and identify the pVAX1-MUC1-VNTR$_n$ DNA vaccine with the strongest immunogenicity. Following construction of a pVAX1-MUC1-VNTR$_n$ plasmid, immature dendritic cells (DCs) were subjected to transfection, and mature DCs were then co-cultured with autologous T-cells. The numbers of cytotoxic T lymphocytes (CTLs) secreting interferon (IFN)-γ were determined using an enzyme-linked immunospot assay, and CytoTox® was also used to examine the MUC1-VNTR$_n$-specific Lethal effect of CTLs on Capan2 cells. Additional *in vivo* experiments in mice were performed to confirm the antitumor effect of the DNA vaccine candidate. The present study successfully constructed the pVAX1-MUC1-VNTR$_n$ plasmid, which expresses the target protein in eukaryotic cells. Additionally, upon uptake of the pVAX1-MUC1-VNTR$_n$ plasmid, the immature DCs differentiated into mature DCs. The levels of the DC surface molecules cluster of differentiation (CD) 80, CD86, human leukocyte antigen-antigen D related, interleukin (IL)-12, IL-17 and IFN-γ were significantly higher, while the levels of IL-10 and IL-14 were lower, in mature DCs of the stimulated groups compared with the immature DCs of the non-stimulated groups (all P<0.01). In addition, the MUC1-VNTR$_6$ and MUC1-VNTR$_9$ groups, in which DCs were capable of activating autologous T-cells, showed increased IFN-γ-producing T-cells compared with the other groups (strong MUC1-VNTR$_1$, weak VNTR$_1$, VNTR$_3$, VNTR$_4$ and MUC1-cDNA groups; all P<0.001). In addition, the Lethal effect of CTLs on Capan2 cells in these two groups was stronger compared with the other groups (all P<0.001). Furthermore, the induced protective and therapeutic immune responses in mouse experiments showed that the pVAX1-MUC1-VNTR$_6$DNA vaccine likely possessed the strongest immunogenicity, and its ability to inhibit panc02-MUC1 tumor growth was superior to other DNA vaccines (P<0.01). The present study provides compelling evidence that pVAX1-MUC1-VNTR$_n$ has the potential to express the target protein in eukaryotic cells, and thatpVAX1-MUC1-VNTR$_6$ was characterized by the strongest Lethal effect in both *in vivo* and *in vitro* experiments.

Introduction

Pancreatic cancer is an aggressive solid malignancy that occurs in the pancreas with clinical symptoms such as abdominal pain, back pain, sallow skin, unexplained weight loss and anorexia (1). In the United States, pancreatic cancer is the most common type of digestive cancer with an incidence of ~44,000 patients diagnosed per year, second only to colorectal cancer (2). With a morbidity rate of 2.8% in male and 3.2% in female patients, pancreatic cancer is the fifth leading cause of cancer-associated mortality in Europe; it is responsible for an estimated 70,000 mortalities annually in the Western world (3). The specific mechanisms underlying the initiation, development and maintenance of pancreatic cancer remain largely unknown, although it is considered that a variety of factors, such as tobacco, alcohol, coffee, diabetes and chronic pancreatitis, and even blood type, may contribute to the pathology of pancreatic cancer (2,4). Pancreatic cancer can also result from overexpression of oncogenes, inactivation of tumor suppressor genes or dysregulation of various signaling proteins (5). Current available treatments for pancreatic cancer are relatively inefficacious, and tumor resection surgery is the only choice that enables the possibility of long-term

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survival (6,7). The high 5-year mortality of pancreatic cancer is a combined consequence of delayed and inaccurate diagnostic techniques, and limited treatment options (4,8). All these clinical difficulties lead to an urgent requirement for an effective and accurate therapeutic tool for pancreatic cancer prevention and treatment.

Several previous studies have shown that mucin 1 (MUC1), a type of epithelial protein, is expressed in numerous malignant hematopoietic cell lines and is overexpressed in malignant tumor tissues, thus being implicated as a potential biomarker for cancers (9,10). Notably, the expression of MUC1 was observed in almost all examined pancreatic cancer cells, which suggests that MUC1 plays an instrumental role in the progression of pancreatic cancer. MUC1 represents a unique type of target that shows better efficacy when combined with immunotherapy rather than radiotherapies or chemotherapies; importantly, it allows specific targeting of the tumor without eliciting damage to adjacent normal tissues (11,12). Variable number of tandem repeat (VNTR) is defined as a variable number of connected repeats of a 20-amino acid sequence in the extracellular domain of MUC1. In normal cells, it is heavily glycosylated at the threonine and serine residues; however, it has underglycosylated VNTR domains that are overexpressed in 90% of pancreatic cancer (13). Previous studies have indicated that MUC1 exhibits abnormally high expression and incomplete glycosylation in pancreatic cancer epithelial cells, with exposed extracellular VNTRs that potentially become a new target for pancreatic cancer immunotherapy (14,15). DNA vaccines are a rapidly deployed next generation vaccination system that mainly contributes to the treatment of human and animal diseases by encoding antigenic proteins that initiate and mediate antibody- and cell-mediated immune responses (16). Increasing evidence suggests that the DNA vaccines of MUC1-VNTR_n may influence cancer prognosis and treatment, although no previous studies have clarified the precise number of the repeat sequences of MUC1-VNTR_n that allows for the optimum immunogenicity (17,18). Therefore, the current study aimed to screen for and identify the MUC1-VNTR_n DNA vaccine with the strongest immunogenicity by constructing different extracellular repeats and assessing their effects *in vitro* and *in vivo*.

Materials and methods

Ethical issues. The present study was approved by the Animal Ethics Committee of the Affiliated Cancer Hospital of Guangzhou Medical University (Guangzhou, China). All experiments were conducted in strict accordance with the established Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Cell lines. The human pancreatic cancer cell strain Capan2 (MUC1-positive), mouse pancreatic cancer cell strain panc02 (MUC1-negative) and cervical carcinoma cell strain HeLa (MUC1-negative) were obtained from the laboratory center of the Affiliated Cancer Hospital of Guangzhou Medical University. The cells were cultured in an atmosphere containing 5% CO_2 at 37˚C. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Construction of the stable panc02-MUC1 cell line expression MUC1 was completed by Ryder Company (Guangzhou, China).

Construction of pVAX1-MUC1-VNTR_n plasmid. The MUC1-VNTR_n gene (one repeat of VNTR encoded 20 amino acids GVTSAPDTRPAPGSTAPPAH) (19) was synthesized and provided by Takara Biotechnology Co., Ltd. (Dalian, China). Two single repeat amino acid sequences of PDTRP were added close to the C-terminus (strong immunogenicity, strong MUC1-VNTR_1) or N-terminus (weak immunogenicity, weak MUC1-VNTR_1) of MUC1-VNTR_n (Table I). The MUC1-VNTR_n target gene sequence was obtained after n repeats of strong MUC1-VNTR_1, and then cloned into pVAX1 (Invitrogen; Thermo Fisher Scientific, Inc.). The MUC1-VNTR_n plasmid and pVAX1 vector were subjected to double enzyme digestion (*Nhe*I and *Kpn*I) at 37˚C for 2 h. The products of digestion were isolated by 1% agarose gel electrophoresis and verified using the UVP imaging system. Subsequently, the ligation reaction was prepared in a 10 μl volume containing: 3 μl VNTR gene, 2 μl pVAX1 vector, 1 μl 10X ligase buffer (Takara Biotechnology Co., Ltd.), 1 μl T4DNA ligase and 3 μl distilled H_2O. This reaction was incubated at 16˚C for 2 h. The ligation reaction product was subsequently transformed into *Escherichia coli* DH5α competent cells. The cells were cultured on Luria-Bertani agar medium containing 50 μg/ml kanamycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) to discriminate between recombinant and non-recombinant cells. The plates were incubated at 37˚C for 16 h. The recombinant plasmids were digested using a double enzyme digest. The digested product was isolated by 1% agarose gel electrophoresis, and then sent for sequencing (Takara Biotechnology Co., Ltd.). The result was compared with the target sequence using BLAST. The HeLa cell strain (MUC1-negative) was transfected with pVAX1-MUC1-VNTR_n. The blank pVAX1 was used as control group. At 48 h following transfection (X-tremeGENE HP DNA Transfection Reagent; Roche Diagnostics, Basel, Switzerland), expression of the target protein MUC1-VNTR_n was examined using western blot analysis. Total protein was isolated from the HeLa cells using a radioimmunoprecipitation assay protein lysis buffer (Biyuntian, Shanghai, China). The concentration of protein was determined using a BCA protein quantification kit (Biyuntian), according to the manufacturer's protocol. A total of 20 μg protein was separated using a 12% (w/v) sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) according to the standard protocol, and then transferred to a polyvinylidene difluoride membrane. The membrane was incubated with the anti-MUC1-VNTR monoclonal antibody (cat. no. VU4H5; Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1,000) at 4˚C overnight. The membrane was subsequently incubated with the secondary goat anti-mouse immunoglobulin G antibody (Boster, China; 1:5,000) at room temperature for 1 h. Subsequently, the experiment results were obtained and analyzed by coloration using the SuperSignal West Pico kit (Thermo fisher Scientific, Inc.), development and fixing of the photographic materials.

Dendritic cell (DC) culture and biological characteristics. Peripheral blood leukocyte suspensions from healthy subjects

were collected from the Guangzhou Blood Bank (Guangzhou, China). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density gradient centrifugation. PBMCs was adjusted to 5×10^6/ml in RPMI-1640 culture medium containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 2 mmol/l L-glutamine and 100 U/ml penicillin-streptomycin, and then incubated at 37°C in 5% CO_2 for 2 h to remove non-adherent cells. Adherent cells were cultured in RPMI-1640 culture medium supplemented with 1,000 U/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and 500 U/ml interleukin (IL)-4, and approximately half of the medium was changed to supplement cell factors every other day. Immature DCs were obtained on the 6th day (control group) and divided into four groups: Group A, in which immature DCs were cultured with GM-CSF and IL-4; group B, in which immature DCs were treated with 1,000 U/ml tumor necrosis factor-α (TNF-α); group C, in which immature DCs were transfected with the $VNTR_1$ plasmid; and group D, in which immature DCs were transfected with the $VNTR_1$ plasmid and treated with 1,000 U/ml TNF-α. At 24 h following induction with TNF-α and plasmid, mature DCs were obtained, and the surface markers, cluster of differentiation CD80, CD86 and human leukocyte antigen-antigen D related (HLA-DR), were analyzed using flow cytometry (Cytomics FC 500MPL) performed by the Flow Cytometry Department of the Sun Yat-Sen Memorial Hospital Laboratory Center (Guangzhou, China). Additionally, the supernatant of DCs was collected to quantify the levels of IL-12, IL-4, IL-17, IL-10 and interferon (IFN)-γ using an ELISA kit (Neobioscience, Shenzhen, China).

Detection of CTLs producing IFN-γ using the enzyme-linked immunospot (ELISPOT) assay. The concentrations of DCs loaded with different $MUC1\text{-}VNTR_n$ antigens ($MUC1\text{-}VNTR_n$ antigens, n=3, 4, 6 and 9) in these four groups were adjusted to 1×10^5/ml. Autologous T-cells from the peripheral blood suspensions were filtrated using nylon wool columns were used as effector cells, and were adjusted to 1×10^6/ml. A total of 100 µl of DCs and T-cells were added to the ELISPOT plate (BD Biosciences, Franklin Lakes, NJ, USA) in the ratio of 1:10. Subsequently, 100 µl culture medium was added to one group of effector cells (3 wells), while 100 µl culture medium containing 2 µg/ml phytohemagglutinin was added to another group of effector cells (3 wells). Cells were then incubated at 37°C with 5% CO_2 for 16 h. The IFN-γ ELISPOT kit (BD Biosciences) was used to detect IFN-γ-producing CTLs according to the manufacturer's protocol. The organization of the groups is presented in Table II.

Lethal effect of CTLs in vitro. Mature DCs loaded with different $MUC1\text{-}VNTR_n$ antigens and autologous T-cells (ratio 1:10) were seeded into a 6-well plate supplemented with 100 U/ml IL-2 culture medium; approximately half of the medium was changed to supplement cell factors every other day. Subsequent to a 5-day culture, the cells were counted using a light microscope. A total of three groups were classified: $MUC1\text{-}VNTR_n$ group; pVAX1 without vector group; and HeLa cells group (negative control group). The organization of the groups is presented in Table II. MUC1-positive Capan2

Table I. Fragments of MUC1-VNTR target gene.

MUC1-$VNTR_1$	Sequence
Strong target gene	GCTAGCGCCACCATGCCGGGCTCCACCGCCCCCCAGCCCACGG*TGTCACCTCGGCCCCGGACACCAGGCCGGCCTGAGGTACC*
Weak target gene	*GCTAGCGCCACCATGCACGGTGTCACCTCGGCCCCGGACACCAGGCCGGCCTCCACCGCCCCCCAGCCTGAGGTACC*

The strong target gene shows strong immunogenicity, as the PDTRP sequence has been added to the N-terminal end of MUC1-VNTR. GCCACC is a Kozak sequence and the italic part refers to MUC1-$VNTR_1$. MUC1, mucin 1; VNTR, variable number of tandem repeats.

Table II. Organization of the groups in the present study.

Determination of IFN-γ-producing CTLs by ELISPOT	Lethal effect of CTLs *in vitro*	Establishment of pancreatic cancer in mice	Treatment of tumor-bearing mice
Empty vector group	Empty vector group	Empty vector group	Empty vector group
Weak VNTR$_1$ group	HeLa cells group	Panc02 control group	Panc02 control group
Strong VNTR$_1$ group	Weak VNTR$_1$ group	Weak VNTR$_1$ group	Weak VNTR$_1$ group
VNTR$_3$ group	Strong VNTR$_1$ group	Strong VNTR$_1$ group	Strong VNTR$_1$ group
VNTR$_4$ group	VNTR$_3$ group	VNTR$_3$ group	VNTR$_3$ group
VNTR$_6$ group	VNTR$_4$ group	VNTR$_4$ group	VNTR$_4$ group
VNTR$_9$ group	VNTR$_6$ group	VNTR$_6$ group	VNTR$_6$ group
MUC1-cDNA group	VNTR$_9$ group MUC1-cDNA group	VNTR$_9$ group	VNTR$_9$ group

Weak VNTR$_1$, single VNTR repetitive sequence with weak immunogenicity; Strong VNTR$_1$, single VNTR repetitive sequence with strong immunogenicity; VNTR$_3$, strong VNTR$_1$ with 3 repeats; VNTR$_4$, strong VNTR$_1$ with 4 repeats; VNTR$_6$, strong VNTR$_1$ with 6 repeats; VNTR$_9$, strong VNTR$_1$ with 9 repeats; MUC1, mucin 1; CTLs, cytotoxic T lymphocytes.

cell strains were collected as the target cells, and the effector cells and target cells were seeded into a 96-well plate with the ratio of 40:1. The Lethal effect of CTLs was examined using the CytoTox Non-Radioactive Cytotoxicity assay kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

Establishment of pancreatic cancer in mice. A total of 120 female C57BL/6 mice were purchased from the laboratory animal center of Sun Yat-Sen University (Guangzhou, China). The experiments were performed in strict accordance with the requirements of biosafety rules and operated under the protocol of the laboratory animal center of Sun Yat-Sen University. The C57BL/6 mice were housed at a controlled temperature of between 20 and 26°C with a relative humidity of between 40 and 60%. The mice were subjected to a 12 h light-dark cycle and were fed and given water 2 times/week. The C57BL/6 mice were randomly divided into eight groups (n=10 per group): i) Empty vector group; ii) panc02 control group; iii) strong VNTR$_1$ group; iv) weak VNTR$_1$ group; v) VNTR$_3$ group; vi) VNTR$_4$; vii) VNTR$_6$ group; and viii) VNTR$_9$ group. The organization of the groups is presented in Table II. The mice in each group were injected into the right anterior tibial muscle with 100 μg/100 μl plasmid DNA inPBS3 times at 2-week intervals. At 1 week following the third injection, the C57BL/6 mice were treated with subcutaneous injection of 5×10^5/100 μl panc02-MUC1 or panc02 cells into the left leg. The tumor size (long and short diameter) was measured every 2-3 days for 4 weeks, and the survival time was recorded.

Treatment of tumor-bearing mice. The second batch of 60 C57BL/6 mice were randomly divided into the same eight groups (n=10 per group). The mice were subcutaneously injected with 5×10^5/100 μl panc02 or panc02-MUC1 cells. On the 4, 8 and 12th days following inoculation of the tumor cells, the mice were administered intramuscular injection of 100 μg/100 μl plasmid in PBS solution. Additionally, the tumor size (long/short diameter) was measured every 2-3 days for 4 weeks to generate the tumor growth curve. The survival time was recorded.

Statistical analyses. Measurement data are expressed as the mean ± standard deviation. The comparison between two independent samples was performed using Student's *t*-test. Additionally, measurement data were compared using analysis of variance, and the Fisher's least significant difference (LSD) test was applied for comparisons among multiple groups. SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Plasmid construction. The present study successfully constructed 7 plasmids containing weak pVAX1-MUC1-VNTR$_1$, strong VNTR$_1$, VNTR$_3$, VNTR$_4$, VNTR$_6$, VNTR$_9$ and pVAX1-MUC1 (empty plasmid). Double digestion products of the plasmids were separated by agarose gel electrophoresis, and the present study was able to confirm the insertion of the target gene fragments

Table III. Dendritic cell phenotype, as determined by flow cytometry.

Treatment group	Phenotype		
	CD80 (P-value)	CD86 (P-value)	HLA-DR (P-value)
Day 6 without stimulation (control group)	10.12±4.15 (reference)	19.25±8.76 (reference)	70.89±8.21 (reference)
Day 7 without stimulation (group A)	16.86±8.05 (P=0.267)	24.85±11.64 (P=0.542)	71.62±8.31 (P=0.919)
TNF-α-stimulated maturation (group B)	49.92±9.36 (P=0.003)	89.68±2.12 (P<0.001)	89.45±2.81 (P=0.021)
Stimulation by the $VNTR_1$ plasmid (group C)	53.87±8.41 (P=0.001)	91.29±3.93 (P<0.001)	90.83±4.91 (P=0.023)
TNF-α and $VNTR_1$ plasmid co-stimulation (group D)	58.63±9.05 (P=0.001)	92.24±3.27 (P<0.001)	93.43±3.62 (P=0.012)

CD, cluster of differentiation; HLA-DR, human leukocyte antigen-antigen D related; TNF-α, tumor necrosis factor-α.
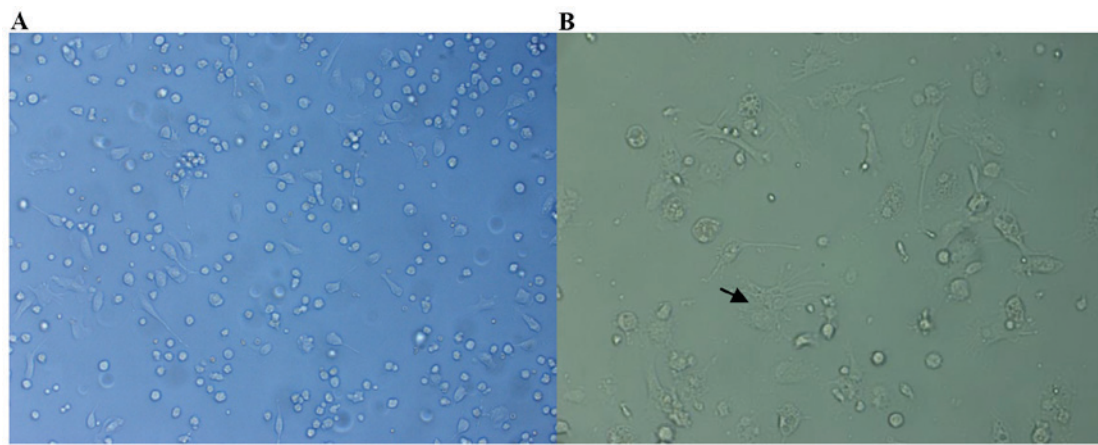

Figure 1. Mature dendritic cells in suspension under an inverted microscope at (A) magnification, x20 and (B) magnification, x40, were larger compared with leukomonocytes, with spinous processes and a number of slender protrusions, as indicated by the black arrow.

into the pVAX1 plasmid vector in reference to the band size at the corresponding position in the gel. Sequencing results of the 7 plasmids were compared with the fragments of the target gene in the Basic Local Alignment Search Tool program (blast.ncbi.nlm.nih.gov/Blast.cgi), and showed a 100% overlap ratio. MUC1-negative HeLa cell lines were transfected with the $pVAX1-MUC1-VNTR_n$ plasmid, and expression of the target protein $MUC1-VNTR_n$ was detected by western blot analysis, confirming that the $pVAX1-MUC1-VNTR_n$ plasmid can be expressed in eukaryotic cells (data not shown).

Cultivation and biological characteristics of DCs. Under an inverted microscope (Fig. 1), mature DCs in suspension were found to be larger compared with leukomonocytes, with a spinous shape and numerous slender protrusions. As shown in Table III, expression of the surface markers CD80, CD86, and HLA-DR in mature DCs, as examined by flow cytometry, exhibited significant differences between the stimulated groups (groups B, C and D) and the control (all P<0.05). However, there was no significant difference among the DC phenotypes without stimulation on days 6 and 7 (P>0.05). Similarly, no significant differences were identified among the mature DC phenotypes subsequent to induction of the stimulated groups (all P>0.05). However, the pairwise comparisons between stimulated and non-stimulated groups showed significant differences (all P<0.05).

The supernatants of immature DCs and cytokine/antigen-induced mature DCs were collected to determine the amounts of secreted IL-12, IL-10, IL-4, IL-17 and IFN-γ using double-antibody sandwich ELISAs, which indirectly reflected the degree of maturation in DCs. As shown in Table IV, there were no significant differences in the levels of IL-12, IL-10, IL-4, IL-17 and IFN-γ between days 6 and 7 without stimulation (all P>0.05). Additionally, no significant differences were found in the levels of IL-12, IL-10, IL-4, IL-17 and IFN-γ between the supernatants of TNF-α-induced, $VNTR_1$ plasmid-stimulated, and TNF-α+$VNTR_1$ plasmid-stimulated groups (all P>0.05), while the pairwise comparisons using the LSD test showed significant differences between the stimulated and non-stimulated groups (all P<0.01).

Determination of IFN-γ-producing CTLs by ELISPOT. As shown in Fig. 2, the $MUC1-VNTR_6$- and $MUC1-VNTR_9$-transfected DCs induced significantly more IFN-γ-producing T-cells compared with the DCs in the other groups ($MUC1-VNTR_6$, 105.2±8.7; $MUC1-VNTR_9$, 93.7±7.3; strong $MUC1-VNTR_1$, 56.4±6.2; weak $MUC1-VNTR_1$, 47.5±5.4; $MUC1-VNTR_3$, 69.3±7.2; $MUC1-VNTR_4$, 71.5±6.7; MUCI-cDNA, 81.2±7.4; empty plasmid, 16.8±4.8; P<0.001). However, there was no significant difference in the number of T-cells between the $VNTR_6$ and $VNTR_9$ groups (P=0.350).

Table IV. Secretion of IL-12, IL-10, IL-4, IL-17 and IFN-γ by dendritic cells, as determined using a double-antibody sandwich enzyme-linked immunosorbent assay.

Treatment group	Factor, pg/ml				
	IL-12 (P-value)	IL-10 (P-value)	IL-4 (P-value)	IL-17 (P-value)	IFN-γ (P-value)
Day 6 without stimulation (control group)	7.18±2.63 (reference)	64.42±3.59 (reference)	49.58±2.84 (reference)	16.38±6.94 (reference)	70.12±3.25 (reference)
Day 7 without stimulation (group A)	5.86±3.54 (P=0.632)	65.23±4.10 (P=0.810)	50.41±2.94 (P=0.743)	16.21±6.86 (P=0.977)	69.25±3.42 (P=0.765)
TNF-α-stimulated maturation (group B)	57.01±12.54 (P=0.003)	14.33±2.39 (P<0.001)	8.29±2.05 (P<0.001)	60.01±5.96 (P<0.001)	218.69±7.51 (P<0.001)
Stimulation by the VNTR$_1$ plasmid (group C)	46.18±9.93 (P=0.003)	13.96±2.77 (P<0.001)	9.17±2.16 (P<0.001)	68.26±7.01 (P<0.001)	224.31±7.61 (P<0.001)
TNF-α and VNTR$_1$ plasmid co-stimulation (group D)	52.47±11.26 (P=0.003)	13.24±2.48 (P<0.001)	9.53±2.44 (P<0.001)	61.95±7.12 (P=0.001)	228.95±7.81 (P<0.001)

IL, interleukin; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.
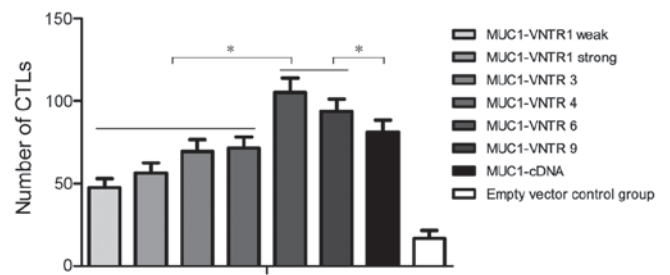
Figure 2. Number of CTLs producing IFN-γ in the different groups, as determined by an enzyme-linked immunospot assay. The MUC1-VNTR$_6$ and MUC1-VNTR$_9$ groups showed increased IFN-γ-producing T-cells compared with the other groups (VNTR$_1$, weak VNTR$_1$, VNTR$_3$, VNTR$_4$ and MUC1-cDNA). *P<0.001. IFN, interferon; CTL, cytotoxic T lymphocyte; MUC1, mucin 1; VNTR, variable number tandem repeat.
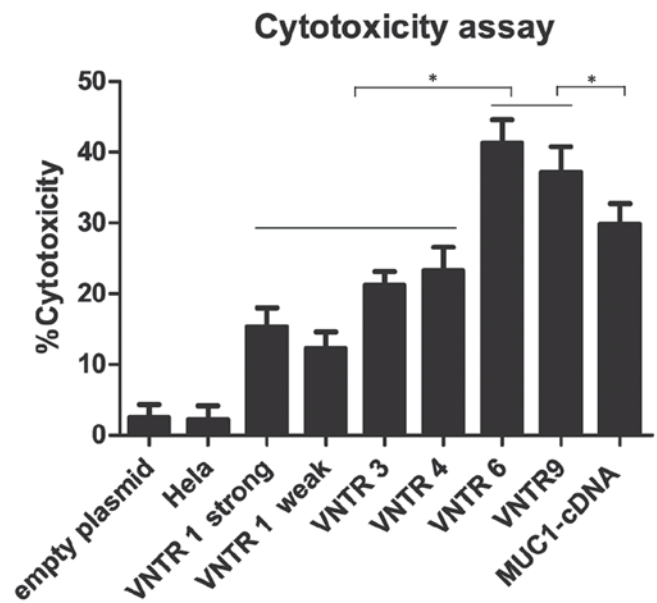
Figure 3. Lethal effect of MUC1-VNTR$_n$-stimulated cytotoxic T lymphocytes on Capan2 cells. VNTR$_6$ and VNTR$_9$ groups exhibited stronger Lethal effects on Capan2 cells compared with the other groups (strong VNTR$_1$, weak VNTR$_1$, VNTR$_3$, VNTR$_4$ and MUC1-cDNA). *P<0.001. VNTR, variable number tandem repeat; MUC1, mucin 1.

pVAX1-MUC1-VNTRn enhances the cytotoxicity of CTLs. As shown in Fig. 3, a stronger Lethal effect on Capan2 cells was observed for the T-cells stimulated with VNTR$_6$ and VNTR$_9$ DCs compared with the other groups (VNTR$_6$, 41.25±3.34%; VNTR$_9$, 37.18±3.61%; strong VNTR$_1$, 15.32±2.71%; weak VNTR$_1$, 12.25±2.35%; VNTR$_3$, 21.24±1.89%; VNTR$_4$, 23.28±3.27%; MUC1-cDNA, 29.85±2.89%; all P<0.001). The Lethal effect of CTLs on Capan2 cells was significantly different between the empty vector group (2.46±1.87%) and all pVAX1-MUC1-VNTR$_n$ DNA vaccine groups (P<0.001). Additionally, there were significant differences in the Lethal effect of CTLs on MUC1-negative HeLa cells between the strong VNTR$_1$ group (2.17±1.98%) and the other groups (all P<0.001).

pVAX1-MUC1-VNTR$_n$ DNA vaccine suppresses panc02-MUC1 tumor growth in vivo. Compared with the empty vector and panc02 control groups, the strong pVAX1-MUC1-VNTR$_1$,
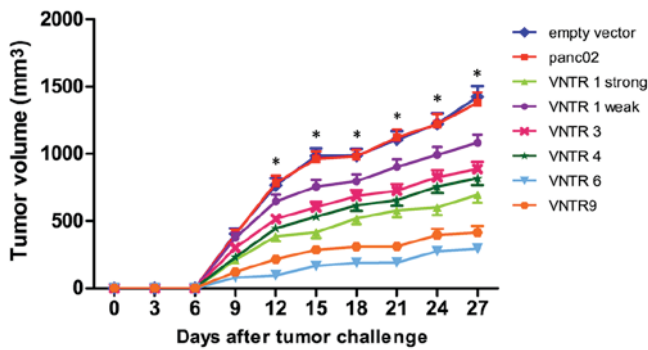
Figure 4. Effect of pVAX1-MUC1-VNTR$_n$ DNA vaccine immunization on panc02-MUC1 tumor growth in mice. The pVAX1-MUC1-VNTR$_6$ DNA vaccine showed a stronger inhibitory effect on panc02-MUC1 tumor growth *in vivo* compared with the strong pVAX1-MUC1-VNTR1, VNTR$_3$, VNTR$_4$ and VNTR$_9$ groups. pVAX1-MUC1-VNTR$_n$ DNA vaccines showed no evident inhibitory effect on panc02 tumor cells, indicating MUC1 specificity. *P<0.001. MUC1, mucin 1; VNTR, variable number tandem repeat.
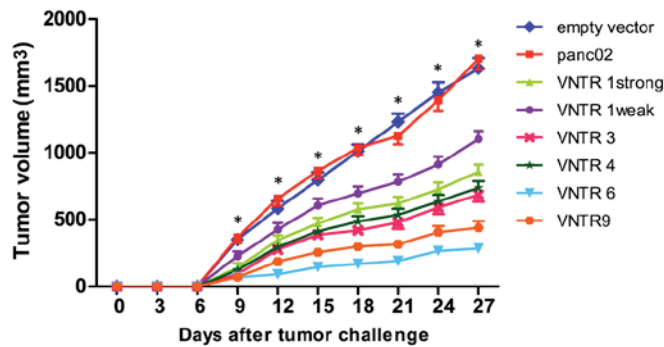

Figure 5. Effect of treatment with thepVAX1-MUC1-VNTR $_n$ DNA vaccines on *in vivo* growth of panc02-MUC1 tumors. The pVAX1-MUC1-VNTR$_6$ DNA vaccine showed a stronger inhibitory effect on panc02-MUC1 tumor growth compared with the strong pVAX1-MUC1-VNTR$_1$, VNTR$_3$, VNTR$_4$ and VNTR$_9$ groups. No evident inhibitory effects on panc02 tumor cells were observed for all pVAX1-MUC1-VNTR$_n$ DNA vaccines, indicating MUC1 specificity. *P<0.001. MUC1, mucin 1; VNTR, variable number tandem repeat.

VNTR$_3$, VNTR$_4$, VNTR$_6$ and VNTR$_9$ groups showed significantly increased inhibitory effects on panc02-MUC1 cell tumor growth from day 12 following tumor challenge (all P<0.001; Fig. 4). In addition, the inhibitory effect of the weak VNTR$_1$ DNA plasmid on panc02-MUC1 tumor growth was significantly milder compared with the other p-VAX1-MUC1-VNTR$_n$ groups (all P<0.05). LSD analysis indicated that the pVAX1-MUC1-VNTR$_6$ DNA vaccine showed a stronger *in vivo* suppressive effect on panc02-MUC1 tumor growth compared with the strong pVAX1-MUC1-VNTR$_1$, VNTR$_3$, VNTR$_4$ and VNTR$_9$ vaccines (all P<0.01), while there was no significant difference in the suppressive effects among the strong pVAX1-MUC1-VNTR1, VNTR$_3$ and VNTR$_4$vaccines (all P>0.05; Fig. 4). pVAX1-MUC1-VNTR$_n$ DNA vaccines showed no detectable inhibitory effects on panc02 tumor cells (Fig. 4), indicating robust MUC1 specificity.

Treatment with p-VAX1-MUC1-VNTRn suppresses panc02-MUC1 tumor growth in tumor-bearing mice. The mice were administered subcutaneous injections of panc02 or

panc02-MUC1 cells and, after 4, 8 and 12 days, received DNA vaccination. As shown in Fig. 5, compared with the empty vector and panc02 negative groups, growth of panc02-MUC1 tumors was significantly inhibited from day 9 in the strong pVAX1-MUC1-VNTR$_1$, VNTR$_3$, VNTR$_4$, VNTR$_6$ and VNTR$_9$ groups (all P<0.001). In addition, the suppressive effect of the weak VNTR$_1$ vaccine on panc02-MUC1 tumor growth was reduced compared with the other p-VAX1-MUC1-VNTR$_n$ groups (all P<0.05). According to the LSD analysis, the pVAX1-MUC1-VNTR$_6$ DNA vaccine showed a stronger inhibitory effect on panc02-MUC1 tumor growth compared with the strong pVAX1-MUC1-VNTR$_1$, VNTR$_3$, VNTR$_4$ and VNTR$_9$ vaccines (all P<0.01), while there was no significant difference among the strong pVAX1-MUC1-VNTR$_1$, VNTR$_3$, VNTR$_4$ and VNTR$_9$ groups (all P>0.05; Fig. 5). No evident inhibitory effects on panc02 tumor cells were found for all pVAX1-MUC1-VNTRn DNA vaccines (Fig. 5), indicating MUC1 specificity.

Discussion

The present study, utilizing MUC1-VNTR$_n$ as a potentially novel target for pancreatic cancer immunotherapy, screened for and identified the pVAX1-MUC1-VNTR$_n$ DNA vaccine with the strongest immunogenicity. As a highly malignant carcinoma with the poorest prognosis of all cancers, pancreatic cancer is one of the leading causes of cancer-associated mortality in the USA (2). Therapeutic modalities used to treat pancreatic cancer include chemotherapy, immunotherapy, surgical resection and radiotherapy; however, the survival outcome for patients with pancreatic cancer remains poor (4,6). As a transmembrane mucin glycoprotein that is aberrantly overexpressed in a variety of adenocarcinomas, including pancreatic cancer, MUC1 provides a reliable target for immunotherapy (20). Importantly, the present results demonstrated successful construction of pVAX1-MUC1-VNTR$_n$, which was shown to enable expression of the target protein in eukaryotic cells. Generally, the peptide backbone of MUC1 is controlled by a region that constitutes 80-200 repetitions of 20-amino-acid repeats (21,22). It is well documented that the MUC1 region, which possesses long branched O-linked carbohydrates, is heavily glycosylated in healthy epithelial cells. By contrast, enhanced expression of MUC1 with heavy glycosylation occurs in unhealthy epithelial cells in the majority of human adenocarcinomas (23). A previous study reported that the majority of pancreatic cancer patients have compromised MUC1-specific immune responses, and are thus unable to inhibit and kill tumor cells (24). The present study presumed that MUC1-VNTR, as a new target for immunotherapy, may effectively enhance the immune response to MUC1-positive tumor cells, eliciting and boosting efficacious antigen-specific immune responses.

Another significant finding in the current study was that co-stimulation of immature DCs with the VNTR$_1$ antigen and TNF-α was able to robustly induce DC maturation, thereby strongly activating the proliferation of allogeneic T-cells due to the high antigen-presenting ability of mature DCs (25). It has previously been reported that DCs, known as a potent type of antigen-presenting cell, are distributed widely throughout the human body. DCs have been identified in the majority of

tissues, including the cornea, heart and brain tissues. However, the total quantity of DCs is estimated to be low in the body, only accounting for 1% of peripheral white blood cells and 0.5% of the total number of spleen cells (26). Previous studies have indicated that DCs are capable of activating T lymphocytes, thereby inducing cellular immune responses including CD4$^+$T helper type 1cells and CD8$^+$T-cells (11,27). In addition, DCs can activate memory B lymphocytes to induce humoral immune responses. Furthermore, DCs are also able to activate natural killer (NK) cells and NKT-cells, which play a unique role in the induction of immune responses (26).

The present study also demonstrated that the amount of IFN-γ-producing CTLs in the MUC1-VNTR$_6$ group and the MUC1-VNTR$_9$ group were significantly increased compared with the other groups. In addition, the Lethal effect of CTLs in these two groups on Capan2 cells was significantly stronger compared with the other groups. The extracellular domain, particularly the VNTR region, of MUC1 undergoes marked alterations in posttranslational modifications when normal cells are transformed into tumor cells, and this may lead to variable interactions with the extracellular environment (27). Previous studies in animal models have demonstrated that CTLs alleviate disease severity and promote viral clearance rather than providing sterilizing immunity (28,29). In addition, CTLs induced by DNA vaccination play a key role in eradicating tumors, because the CTLs can directly kill tumor cells and indirectly elicit IFN-γ-mediated antitumor responses (30). The present study adopted DCs transfected with the MUCI-VNTR DNA vaccine as antigen presenting cells to stimulate autologous T lymphocytes and induce the amplification of MUCI-specific CTLs.

The current study determined that the pVAX1-MUC1-VNTR$_6$DNA vaccine had the strongest Lethal effect in both *in vivo* and *in vitro* experiments, and demonstrated that this vaccine possessed MUC1 specificity. DNA vaccines are reported to be potentially safer compared with traditional vaccines, in addition to their stability and cost effectiveness (31). Additionally, the immune system responses activated by anticancer DNA vaccines effectively prevented the occurrence and recurrence of cancer in patients in a previous study (32). A variety of vaccines utilizing MUC1 as the target antigen were shown to induce MUC1-specific humoral and cellular immunity to varying extents, thereby eradicating MUC1-expressingtumor cells (33). Snyder *et al* (34) indicated that the use of a certain number of tandem repeat sequences in the MUC1-VNTR$_n$ DNA vaccine was able to induce a robust and strong immune response to target and kill pancreatic cancer cells. In addition, Quinlin *et al* (35) found that the peptide vaccine, VNTR$_1$, with its PDTRP sequence adjacent to the C-terminus, was characterized by an increased immunogenicity compared with VNTR$_3$, with its PDTRP sequence adjacent to the N-terminus. In the present study, protective and therapeutic immune response experiments in mice were performed to further assess the immunogenicity of the DNA vaccines. It was found that the ability of the pVAX1-MUC1-VNTR$_6$ DNA vaccine to inhibit panc02-MUC1 tumor growth *in vivo* was significantly superior to that of the pVAX1-MUC1-VNTR$_1$, VNTR$_3$ and VNTR$_9$ DNA vaccines.

In summary, the present study demonstrated that pVAX1-MUC1-VNTR$_n$ has the potential to express target proteins in eukaryotic cells, and that pVAX1-MUC1-VNTR$_6$ was characterized by a strong Lethal effect on MUC1-positive tumor cells in both *in vivo* and *in vitro* experiments.

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