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# Establishment of a Tumor-bearing Mouse Model Stably Expressing EGFP Labeled Human MUC1 VNTRs

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**Abstract** Two eukaryotic vectors expressing 9 tandem repeats of human MUC1(VNTR), VR1012-VNTR, and pEGFP-VNTR, were constructed by cloning VNTR gene into VR1012 and pEGFP, respectively. VNTR stably expressing murine Lewis lung carcinoma(LLC) cell line($VNTR^+$ LLC) was established by Lipofectamine-mediated transfection of pEGFP-VNTR into LLC cells. The EGFP expression was observed under a fluorescent microscope and VNTR expression in $VNTR^+$ LLC cells was confirmed by means of Western blotting. A syngenic graft tumor model was generated by subcutaneous injection of $VNTR^+$ LLC cells into C57/BL6 mice and tumor size increased rapidly with time and in a cell number dependent manner. VNTR mRNA expression in the tumor formed was confirmed by RT-PCR. After the third immunization mice were challenged subcutaneously with $5\times10^5$ $VNTR^+$ LLC cells, a significant reduction of subcutaneous tumor growth was observed in the groups immunized with VNTR plasmid DNA compared with that in the groups immunized with the vector DNA alone. Thus, the suppression of subcutaneous tumor was antigen-specific. This model is useful for the development of tumor vaccines targeting MUC1 VNTRs.

**Keywords** MUC1 VNTR; Tumor antigen; Tumor model; Tumor vaccine

## 1 Introduction

MUC1 is a transmembrane protein of type I containing a variable number(20—120) of tandem repeats(VNTRs) of a 20 amino acid sequence in its extracellular domain. The VNTRs have the sequence $(VTSAPDTRPAPGSTAPPAHG)_n$. In normal cells VNTRs are heavily glycosylated, up to 70% carbohydrate by weight, at threonine and serine residues, while malignant cells contain underglycosylated VNTR domains, and VNTR is overexpressed in 90% of all adenocarcinomas included in breast, lung, pancreas, prostate, stomach, colon, and ovary. The overexpression of MUC1 and underglycosylation of its VNTRs makes hypoglycosylated and nonglycosylated VNTR-derived epitopes attractive targets for immunodiagnostics and immune intervention[1—3]. Currently there has been a widespread interest in the development of tumor vaccines[4—6].

To test the efficacy of vaccines targeting MUC1, it is important to establish a preclinical tumor model expressing MUC1. In this study, we established the models of Lewis lung carcinoma cell(LLC) line and a subcutaneous tumor in syngenic C57BL/6 mice stably expressing enhanced green fluorescent protein(EGFP) labeled nine tandem repeats of human MUC1 VNTR and showed that VNTR DNA vaccination succeeded in suppressing the growth of VNTR-expressing tumor in mice. This result would provide a basis for further studies in vaccines targeting MUC1 VNTRs.

## 2 Experimental

### 2.1 Vectors, Bacterial Strains, Cell lines and Animals

pGEM-Teasy and VR1012 vectors were purchased from Promega. pEGFP-N3 vector, a kind of enhanced green fluorescent protein(EGFP) labeled vector, was purchased from Clonetech. *E. coli* Top10, Lewis lung carcinoma(LLC) cell line, was preserved in our laboratory. Female C57BL/6 mice, 6 to 8-week-old, were purchased from Beijing Academy of Military Medical Science.

### 2.2 Restriction Enzymes and Major Reagents

The mAb against Human MUC1 VNTR was

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purchased from BD pharmingen. The secondary alkaline phosphatase-conjugated anti-mouse antibody was purchased from Sigma. Restriction enzymes and T4 DNA ligase were purchased from TaKaRa Biotechnology. Taq DNA polymerase was purchased from New England Biolabs. DNA gel extracting kit was purchased from Shanghai Shenggong. RNAse inhibitor, TRIzol reagent, SuperScript™ First-Strand Synthesis System kit, and Lipofectamine™ 2000 were purchased from Invitrogen Life Technologies.

### 2.3 Constructions of Recombinant Expression Vectors VR1012-VNTR and pEGFP-VNTR

A pair of primers, respectively, encompassing the enzyme sites of Sal I and XhoI were designed based on the cDNA sequence[7] of human MUC1 VNTR(m). The sequences were 5'-GTCGACCACGGCGTCA CCTCTGCCCCAGACACCAGGCCGG-3'(m sense) and 5'-CTCGAGAGCTGGAGGAGCAGTGGAACC CGGGGCCGGCCTGGTGTCTG-3'(m antisense), respectively. One repeat of MUC1 VNTR(m) was synthesized by PCR. The PCR product was cloned into vector pGEM-T Easy(pGEMT-m). The fragment m released from pEGMT-m by codigestion with SalI and XhoI was then cloned into the SalI site of the plasmid pEGMT-m, thus forming a new plasmid pEGMT-2m. In this way, ligations were then performed repeatedly and pGEM-VNTR containing 9 repeats of MUC1 VNTRs was constructed. The constructed VNTR gene was subcloned into the expression vector, VR1012 and pEGFP-N3, at the SalI site to create new plasmids VR1012-VNTR and pEGFP-VNTR. Sequences of the two constructs were further analyzed with restriction enzymes and direct sequencing.

### 2.4 Cell Culture and Transfection

Murine Lewis lung carcinoma(LLC) cells were maintained in Roswell Park Memorial Institute(RPMI) 1640, supplemented with 10%(volume fraction) heat-inactivated fetal bovine serum, 2 mmol/L *L*-glutamine, 1 mmol/L sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% $CO_2$ at 37 °C.

Transfection was performed with Lipofectamine$_{TM}$ 2000 kit according to the manufacturer's instructions. Plasmids, pEGFP-VNTR and pEGFP-N3, were extracted and liposome-DNA mixture was made up in advance, respectively. Negatively controlled cells were transfected with vector pEGFP-N3 alone and vacant controlled untransfected cells were set up. After incubation for 8 h, the medium was replaced by the same fresh medium. Forty-eight hours after transfection, the cells were examined by means of fluorescence microscopy in the presence of EGFP positive cells. For selection of EGFP transductants, the cells were cultured in a selective medium containing 800 μg/mL of G418 for 7 days or until the mock transfected cells died. Cells expressing the EGFP gene were maintained in the selected medium containing 200 μg/mL G418. LLC clones expressing EGFP were isolated with cloning cylinders by trypsin-EDTA and were transferred and amplified by conventional culture methods.

### 2.5 Western Blotting

Cells stably transfected with expression plasmid carrying VNTR(VNTR$^+$ LLC) or empty expression plasmid(P$^+$ LLC) and untransfected cells(LLC) were harvested by trypsin-EDTA and washed twice with ice-cold phosphate-buffered saline(PBS). The cell pellets were lysed in ice-cold lysis buffer. After 30 min at 4 °C, cleared lysates were centrifuged at 12000 r/min for 15 min. The supernatant was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk for 2 h and incubated overnight at 4 °C with anti-MUC1 VNTR antibody for detection of VNTR protein expression. The blots were developed using alkaline phosphatase-conjugated secondary antibody colorimetric detection.

### 2.6 Preparation of Cells for Injection

Prior to inoculation into mice, VNTR$^+$ LLC cells in an exponential growth phase were trypsinized and then transferred to warm PBS. Cells are centrifuged for 5 min at 1000 r/min and resuspended again in warm PBS. After checking cell viability with the trypan blue-dye exclusion test, the cells were counted and adjusted, respectively, to $1.0\times10^7$/mL or $5\times10^6$/mL for a 100 μL injection of $1.0\times10^6$ or $5\times10^5$ cells.

### 2.7 *In Vivo* Tumor Generation

Cells were kept fresh to minimize the time for