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A novel therapeutic vaccine composed of a rearranged human papillomavirus type 16 E6/E7 fusion protein and Fms-like tyrosine kinase-3 ligand induces CD8$^+$ T cell responses and antitumor effect

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ABSTRACT

The development of cervical cancer is mainly caused by infection with high risk genotypes of human papillomavirus, particularly type 16 (HPV16), which accounts for more than 50% of cervical cancer. The two early viral oncogenes, E6 and E7, are continuously expressed in cervical cancer cells and are necessary to maintain the malignant cellular phenotype, thus providing ideal targets for immunotherapy of cervical cancer. In this study, a novel vaccine strategy was developed based on a rationally shuffled HPV16 E6/E7 fusion protein, the addition of Fms-like tyrosine kinase-3 ligand (Flt3L) or the N domain of calreticulin (NCRT), and the usage of a CpG adjuvant. Four recombinant proteins were constructed: m16E6E7 (mutant E6/E7 fusion protein), rm16E6E7 (rearranged mutant HPV16 E6/E7 fusion protein), Flt3L-RM16 (Flt3L fused to rm16E6E7), and NCRT-RM16 (NCRT fused to rm16E6E7). Our results suggest that Flt3L-RM16 was the most potent of these proteins in terms of inducing E6- and E7-specific CD8$^+$ T cell responses. Additionally, Flt3L-RM16 significantly induced regression of established E6/E7-expressing TC-1 tumors. Higher doses of Flt3L-RM16 trended toward higher levels of antitumor activity, but these differences did not reach statistical significance. In summary, this study found that Flt3L-RM16 fusion protein is a promising therapeutic vaccine for immunotherapy of HPV16-associated cervical cancer.

1. Introduction

Globally, cervical cancer has become the fourth most deadly cancer of women [1]. Infection with human papillomavirus (HPV) is a major risk factor for the development of cervical cancer [2]. Currently, two types of prophylactic HPV vaccines, Gardasil (Merck) and Cervarix (GlaxoSmithKline), are commercially available [3]. Prophylactic vaccines could prevent HPV virus from entering into the cervical epithelium [4,5], but are ineffective against pre-existing HPV infections [6]. Thus, there is an urgent need to develop effective therapeutic HPV vaccines [7,8].

There are over 170 different types of HPV [9], which can be broadly broken down into low-risk and high-risk types [10]. HPV type 16 (HPV16) accounts for more than 50% of cervical cancer cases worldwide [2]. HPV oncoproteins E6 and E7 respectively down regulate p53 and pRb tumor suppressor proteins [11,12]. Furthermore, E6 and E7 are continuously expressed within cervical cancer cells and are necessary to maintain the malignant state of the cell [13–15]; thus, they provide ideal targets for immunotherapy of cervical cancer [16]. To augment HPV E6/E7-specific CTL responses, various types of therapeutic vaccines have been developed, such as viral or bacterial vector vaccines [17,18], peptide vaccines [19–21], protein vaccines [22], DNA vaccines [23–25], chimeric virus-like particle vaccines [26–28], adoptive transfer of tumor-specific T cells [29], and dendritic cell (DC)-based vaccines [30,31]. However, these therapeutic vaccines have been limited by inadequate antigen-specific T cell immune responses. Therefore, further research is needed to develop a more potent therapeutic vaccine capable of initiating very robust antigen-specific cell immune responses.

The use of DC stimulatory molecules has been developed to increase the immunogenicity of protein vaccines since DCs are

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the most potent antigen-presenting cells and are the central players for mediating cancer immunotherapy [32]. Fms-like tyrosine kinase-3 ligand (Flt3L) has been identified as an important cytokine for accelerating DC proliferation [33,34]. Early studies also showed the systemic administration of Flt3l to be effective in reducing tumor growth and inducing a tumor infiltration by DCs and CTLs [35]. The addition of Flt3l to tumor-specific antigens seems to produce stronger antigen-specific and longer-lasting immune responses [36]. In DNA vaccines, Flt3L has been fused to HPV E6/E7 antigens and has been shown to enhance cellular and antitumor immunity [37–39].

Calreticulin (CRT) is an abundant 46 kD multifunctional protein with roles in calcium homeostasis, molecular chaperoning and cell adhesion located in the endoplasmic reticulum (ER) [40,41]. Emerging evidence suggests that CRT also plays a crucial role during tumor development. The CRT protein is composed of three domains, the N domain, P domain, and C domain, and the amino acid sequence of the N domain is extremely conserved in all CRTs [42]. The N domain (residues 1–180) of CRT (abbreviated NCRT) has been reported not only to take part in chaperone functions of full-length CRT [43], but also to control the growth of endothelial cells and inhibit angiogenesis [41,44,45].

This study aimed to find a more efficient therapeutic protein vaccine. Here, we investigated whether or not the rearranged mutant HPV16 E6/E7 fusion protein (rm16E6E7) could generate stronger antigen-specific T cell responses than mutant HPV16 E6/E7 fusion protein (m16E6E7). We also tested if linking Flt3l or NCRT to rm16E6E7 (forming Flt3l-RM16 and NCRT-RM16, respectively) would enhance the potency of rm16E6E7 protein vaccines. The results may lead to improved methods for treating HPV16-associated cervical cancer through antigen-specific immunotherapy.

2. Materials and methods

2.1. Recombinant vector construction

The mutant HPV16 E6 gene (F47R, L50G, C63G, and C106R for E6 protein [18,46–48]), mutant HPV16 E7 gene (Y23G, C24G, Y25G, C58G, and C91G for E7 protein [49,50]), Flt3L gene, and NCRT gene were each codon optimized using an *Escherichia coli* System and were synthesized by Genecreat (Wuhan, China) to clone into the *pUC57* plasmid. Using *pUC57-E6* and *-E7* as templates, the m16E6E7 fusion gene was synthesized by overlap PCR including full-length mE6 and mE7 genes (Fig. 1). The arrangement of HPV16 E6 and E7 was intentionally shuffled by successively combining N-terminal E6/E7 and C-terminal E6/E7 [51,52] together to generate a 16E6N-16E7N-16E6C-16E7C recombination protein called rm16E6E7 (Fig. 1).

Next, m16E6E7 and rm16E6E7 were cloned into the Nde I/Hind III site of *pET26b* to generate *pET26b-m16E6E7* and *pET26b-rm16E6E7*. NCRT-RM16 (NCRT fused to rm16E6E7) and Flt3l-RM16 (Flt3L fused to rm16E6E7) fusion genes were respectively synthesized by overlap PCR from *pUC57-NCRT*, *pUC57-Flt3l*, and *pET26b-rm16E6E7* plasmids (Fig. 1) and then cloned into the Nde I/Xho I site of *pET28a*.

2.2. Expression, purification, and characterization of recombinant proteins

The recombinant proteins were expressed in *E. coli* strain BL21 (DE3). All engineered bacterial cultures were grown to an optical density at 600 nm (OD_{600}) of 0.6–0.8 at 37 °C in LB medium supplemented with 50 μg/mL kanamycin, and protein expression was then induced with 0.4 mM IPTG for 16 h at 16 °C. Bacteria (20 g) were harvested by centrifugation and re-suspended in 100 mL of PBS (pH 7.4) containing 1 mM EDTA and 1 mM PMSF and then lysed by a high-pressure homogenizer. Lysates were centrifuged at 15,000 ×*g* for 30 min at 4 °C to obtain supernatant (soluble protein) and pellet (inclusion body) fractions. The inclusion bodies, which contained the recombinant proteins, were washed with solution I (50 mM Tris-HCl, 50 mM NaCl, pH 8.5) and then by solution II (50 mM Tris-HCl, 50 mM NaCl, 2 M urea, 1% TritonX-100, pH 8.5). The inclusion bodies were then dissolved in 30 mL of solution III (50 mM Tris-HCl, 50 mM NaCl, 6 M urea, 50 mM DTT, 0.5% SDS, pH 8.5) and gradient dialyzed with solutions containing different urea concentrations (50 mM Tris-HCl, 50 mM NaCl, pH 8.5 with 3, 1, or 0 M urea) and finally dialyzed extensively with PBS (pH 8.0). The target recombinant protein was obtained after being processed with an endotoxin removal spin column (Thermo, Massachusetts, USA).

SDS-PAGE was used to determine the purity of the recombinant proteins. Concentrations of the recombinant proteins were measured by BCA assay. All purified recombinant proteins were identified by western blot analysis using an anti-his tag antibody (Thermo).

2.3. Cell lines and mice

Female C57BL/6 mice aged 6–8 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). All animals were maintained under specific-pathogen-free conditions, and all procedures were performed according to approved protocols and in accordance with recommendations for the proper care of laboratory animals.

TC-1 tumor cells were purchased from ATTC and grown in RPMI 1640 (Gibco, Massachusetts, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) at 37 °C in 5% CO_2.

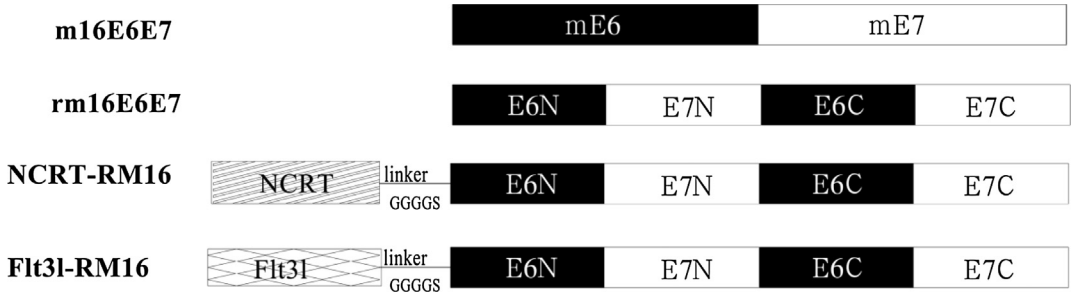

Fig. 1. Diagram depicting the composition of the various recombinant protein constructs used in the study. The m16E6E7 and rm16E6E7 fusion gene were cloned into pET26b. NCRT-rm16E6E7 and Flt3l-rm16E6E7 fusion gene were cloned into pET28a. Mutant E6/E7 fusion protein: m16E6E7; rearranged mutant HPV16 E6/E7 fusion protein: rm16E6E7; NCRT fused to rm16E6E7: NCRT-RM16; Flt3L fused to rm16E6E7: Flt3L-RM16.

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