

Original Article

Construction, expression and purification of recombinant HBcAg-MAGE-A3 therapeutic cancer vaccine

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Abstract: Objective: To construct and identify recombinant expression of a therapeutic tumor vaccine with HBcAg as a vector. Methods: PCR primers were designed according to the gene sequences of restriction enzyme sites of recombinant pKK233.2-hepatitis B core antigen (HBcAg) and melanoma-associated antigen 3 (MAGE-A3: 112-120aa). The target fragment of MAGE-A3 was synthesized and cloned to pKK233.2-HBcAg expression vector. The recombinant pKK233.2-HBcAg-MAGE-A3 expression vector was identified by PCR detection followed by enzyme restriction and sequencing. The expression and purification of HBcAg-MAGE-A3 fusion protein at large scale were accomplished by crack bacterial precipitation through repeated freeze-thaw and ultracentrifugation. The recombinant protein vaccine was identified by western-blotting and dot blot. Results: The pKK233.2-HBcAg-MAGE-A3 expression vector was established. PCR and restriction enzyme digestion assays verified the size of the target fragment as predicted. The recombinant expression vector contained the full sequences of HBcAg and MAGE-A3 genes. Western-blotting and dot blotting analyses confirmed purity of recombinant HBcAg-MAGE-A3. Conclusion: Recombinant HBcAg-MAGE-A3 vectors and the methods for purifying therapeutic cancer vaccines were successfully established.

Keywords: HBcAg, MAGE-A3, cancer, vaccine, vector, construction

Introduction

Immune therapy particularly therapeutic cancer vaccine have been intensively studied for the past few years. Therapeutic cancer vaccines include DNA, peptide, cell vaccines [1-5]. Main idea is to develop the vaccines for viral infections and for cancers such as prostate cancer, non-small cell lung cancer, ovarian cancer and pancreatic cancer [5-8]. Molecular weight of epitope peptide is small and vulnerable to protease degradation. Furthermore, single epitope has weak immunogenicity [9, 10]. Therefore, vector or fusion protein are necessary for effective immunization. It is important in choosing an ideal immunological carrier protein and immunization strategy [10, 11].

MAGE-A3 is a member of the melanoma antigen MAGE-A family. It expressed in tumors, not normal tissues, except for testis and pla-

centa. This tumor-specific protein has been found in many tumors, including melanoma, non-small cell lung cancer, hematologic malignancies, gastrointestinal cancer [12, 13]. Currently, cancer vaccines targeting MAGE-A3 have been developed using peptides, DNA and minigenes-expressing adenoviruses expressing [14].

The HBc protein of Hepatitis B virus core antigen (HBcAg) is capable to correctly self-assembling into a natural shape of HBc core particles in the absence of any other viral components [15]. Because of its unique structure and immunological characteristics, it can be used as ideal vaccine vector and adjuvant in the immune response [16-18]. Immune carriers hepatitis B core antigen (HBcAg) has three characteristics. First, as a particulate antigen, it is easy to be up-taken, proceeded, handled and presented by antigen-presenting cell for initiate effec-

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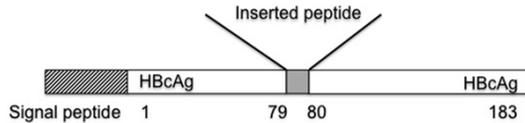


Figure 1. Schematic diagram of the expression plasmids pKK233.2-HBcAg-MAGE-A3. The numbers in the figure refer to HBcAg amino acid positions. In pKK233.2-HBcAg-MAGE-A3, the DNA fragment encoding HBcAg (1-183aa, represented by open box) was placed after signal peptide, and the recognition sequence of restriction enzyme NheI was located at between 79-80aa.

tive humoral and cellular immune responses. Second, it activate B cells directly and T cells indirectly. Therefore, it can serve as either TD or TI antigens. Third, HBcAg 78-83 amino acid residues locate in spike of the apex. As a few amino acid residue of the HBc immune advantage have been deleted, leading to attenuated its own antigenicity of HBcAg and/or enhanced immunogenicity of insertion sequence.

In this study, MAGE-A3 (112-120aa) was chosen as the insertion sequences and HBcAg vector as the vehicle. Through the prokaryotic expression, purification, procuring getting large scale recombinant protein as a vaccine. This therapeutic cancer vaccine will generate effective cellular and humoral immune response against the tumor specific antigen.

Materials and methods

Materials

E.coli HB101 strain and recombinant pKK233.2-HBcAg plasmid was preserved at our laboratory. Restriction endonucleases (NheI), Taq DNA polymerase and T4 DNA ligase were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. DNA extraction and gel DNA purification kits were from Tiangen Biotech (Beijing) Co., Ltd and used following the instructions. The anti-HBcAg antibodies were generously gifted by Dr. Jingli Li. Other antibodies were from Dingguo Biotech (Beijing) Co., Ltd.

Plasmid construction

Recombinant plasmid pKK233.2-HBcAg was extracted. Restriction enzyme digestion was performed using NheI for the recombinant plasmid pKK233.2-HBcAg and MAGE-A3 fragment

(112-120aa: LysValAlaGluLeuValHisPheLeu). T4 DNA ligase was used to clone MAGE-A3 (AAGTTGCCGAACCTGTTCACCTTTCTT) to plasmid pKK233.2-HBcAg to construct recombinant pKK233.2-HBcAg-MAGE-A3 (**Figure 1**). The constructed plasmids were verified by enzyme digestion and PCR and confirmed by sequencing at Beijing Genomics Institute. We referred the construct as aspKK233.2-HBcAg-MAGE-A3.

Expression of HBcAg-MAGE-A3 fusion protein

For the expression of the recombinant protein, *E.coli* HB101 transformed with pKK233.2-HBcAg-MAGE-A3 plasmid were inoculated in a tube containing 10 ml of LB medium supplemented with 100 µg/ml ampicillin, and cultured overnight at 37°C in a shaking incubator (200 rpm) overnight. Next day, 1 ml of culture was transferred to a 100 ml flask containing 50 ml of LB medium supplemented (Amp, 100 µg/ml). The flask was shaken at 37°C until the culture reached an OD of 1.0 read at a wavelength of 600 nm. The 50 ml culture was then transferred into 1 L LB medium containing ampicillin (100 µg/ml). Protein expression was induced by addition of 0.8 mM isopropyl-d-thiogalactopyranoside (IPTG) when the OD≈0.8. After continuing to foster, the cells were harvested by centrifugation when OD = 4.

Purification of HBcAg-MAGE-A3 fusion protein

To purify the recombinant HBcAg-MAGE-A3 protein, the bacterial precipitation were undergone three cycles of freeze-thaw and resuspended in 25% sucrose containing lysozyme solution, and added lysis buffer (EDTA, DOC and Triton-X) and incubated at 37°C for 30 min. After centrifuge, the supernatant were ultracentrifuged through serial sucrose density gradient at 28000 rpm for 4 h. Then the samples were collected. The methods were shown in **Figure 2**.

SDS-polyacrylamide gel electrophoresis and western blotting and dot blotting analysis

The bacteria pellets were suspended in sample loading buffer and boiled for 5 min prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For immunoblotting, peptides of IPTG induced bacteria were separated by SDS-PAGE and then electrotrans-

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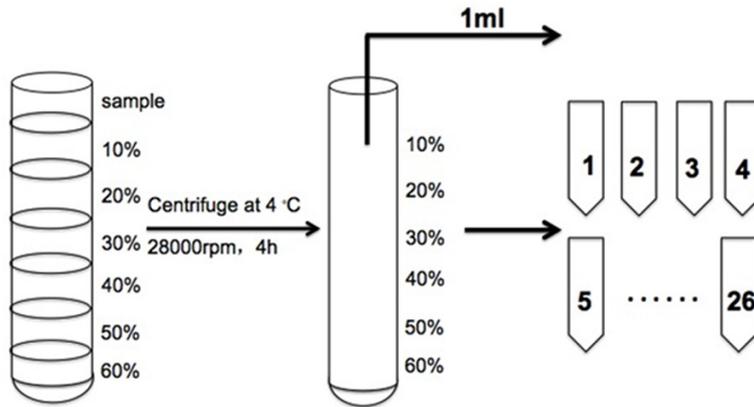


Figure 2. The purification and extraction method of recombinant HBcAg-MAGE-A3 by serial sucrose density gradient centrifugation.

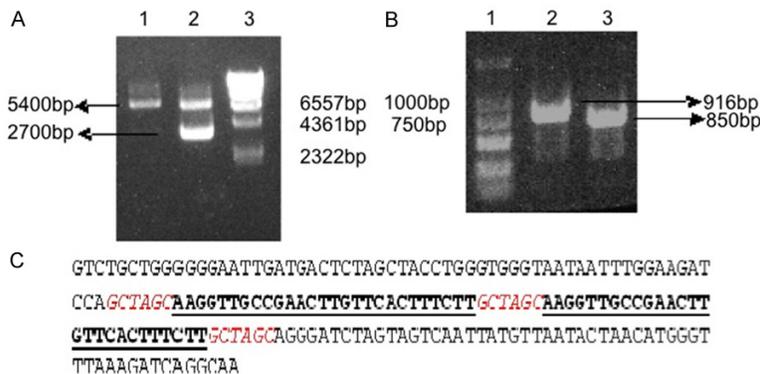


Figure 3. Identification of pKK233.2-HBcAg-MAGE-A3 vector. A: Enzyme products of vector pKK233.2-HBcAg. M1: pKK233.2-HBcAg digested with NheI; M2: Recombinant plasmid pKK233.2-HBcAg; M3: DNA Marker (DL15000). B: PCR amplification results of HBcAg gene. M: DNA Marker (DL2000); M2: PCR products of HBcAg-MAGE-A3 gene; M3: PCR products of HBcAg gene. C: The result of genetic sequencing of HBcAg-MAGE-A3 gene.

ferred onto nitrocellulose filter membrane 0.2 μm with transfer buffer containing 5 mM Tris, 50 mM glycine, 20% (v/v) methanol, 0.1% SDS, pH 8.3. Electrotransfer was carried out at 80 mA for 1.5 h. Then, the membrane was blocked with 3% bovine serum albumin in phosphate buffer saline (PBS) overnight. Protein was detected with anti-HBcAg antibodies (1:1000 of dilution in PBS) at room temperature for 2 h. Then, the membrane was washed with PBS/Tween 20 three times, each 15 min, followed by incubation with goat-anti-G. pig-HRP (1:5000) at room temperature for 2 h. Following three PBS/Tween 20 washing, membrane was incubated with a solution containing 6 mg diaminobenzidine 10 ml PBS containing 5 μl 30% H_2O_2 . For dot blot, the purified proteins were transferred to nitrocellulose membrane. The other steps are the same with western blotting.

Results

Construction of eukaryotic expression vector pKK233.2-HBcAg-MAGE-A3

Recombinant plasmid pKK-233.2-HBcAg was digested by restriction enzyme NheI. Then, it was examined by 1% agarose gel electrophoresis for the presence of HBcAg. The results showed one clear band of 5400 bp (**Figure 3A**). The pKK233.2-HBcAg-MAGE-A3 positive colonies as identified by ampicillin-resistance LB medium were selected for PCR amplification. We observed a clear band of approximately about 916 bp, consistent with the predicted size of HBcAg-MAGE-A3 insert (**Figure 3B**). Direct sequencing confirmed two fragments: 66 bp and 850 bp for 2 MAGE-3 and HBcAg fragments, respectively) (**Figure 3C**).

Extraction and purification of recombinant HBcAg-MAGE-A3 protein

ASDS-PAGE analysis revealed a protein at the molecular weight of slightly smaller than 52 kDa MW as compared the control vector (**Figure 4A**). This is in agreement with the estimated molecular mass for the recombinant protein, corresponding to the dimer of 183aa. Western blotting detection demonstrated the expression of HBcAg-MAGE-A3 protein at expected molecular mass (**Figure 4B**). The purified protein containing the target protein HBcAg-MAGE-A3 protein as documented by Dot blot. These results suggest that HBcAg-MAGE-A3 fusion protein is mainly present in the 30-50% sucrose media (**Figure 5**).

Discussion

Therapeutic cancer vaccines, which trigger cytotoxicity immune response, could be a potential treatment strategy for cancer therapy. Cytotoxic CD8^+ T cells (CTLs) recognize tumor-

HBcAg-MAGE-A3 therapeutic cancer vaccine

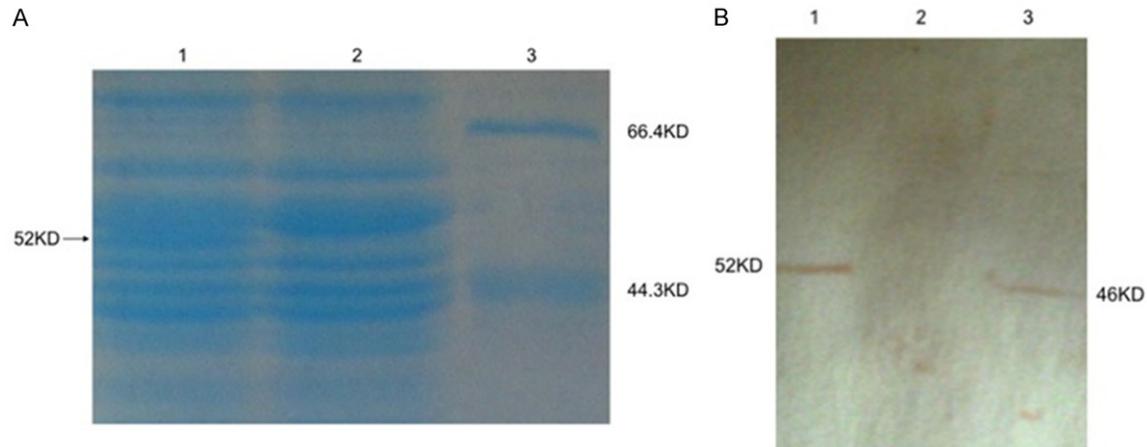


Figure 4. The identification of recombinant HBcAg-MAGE-A3 protein. A: Identification of HBcAg-MAGE-A3 protein expression by SDS-PAGE. M1: the cleaved supernatant of pKK233.2-HBcAg-MAGE-A3 plasmid product; M2: the cleaved supernatant of pKK233.2 plasmid product; M3: protein marker. B: Identification of HBcAg-MAGE-A3 protein expression by western-blotting. M1: the cleaved supernatant of pKK233.2-HBcAg-MAGE-A3 plasmid product; M2: the cleaved supernatant of pKK233.2 plasmid product; M3: protein marker.



Figure 5. The identification of purified recombinant HBcAg-MAGE-A3 protein by dot blot. HBcAg-MAGE-A3 fusion protein distributed mainly in the 30-50% sucrose media.

associated antigens and attack tumor cells which express these antigenic peptides. However, due to ineffective in presenting these epitopes to CTLs in tumor microenvironment, CTLs fail to be activated for combating tumour cells effectively. HBcAg (hepatitis B virus core antigen), as a particulate antigen, has icosahedral structure formed by the core protein subunits, rendering antigen-presenting cells to uptake, process, handle and present antigen effectively, and thus inducing strong CTL response. In the last few decades, the most successful approaches of this scaffold has been the influenza vaccine ACAM-FLU-A, produced by Sanofi Pasteur, and the malaria (*Plasmodium falciparum*) vaccine Malarivax (ICC-1132), produced by Apovia [19]. Because the 78~83 aa

at the amino terminus of HBcAg form a spike tip, the major immunodominant region (MIR), foreign epitopes could be inserted into MIR to generate a fusion peptide. This region was previously reported to be the major B cell epitope of HBcAg. Some studies observed that the short deletion in the MIR diminished the intrinsic immunogenicity of HBcAg and generated strong anthrax-specific immunity. Most importantly, this deletion completely protected mice from a lethal dose anthrax toxin challenge [20]. The VLV expressing HBV core protein (HBcAg) neither induced a CTL response nor protected against challenge [21]. Thus, HBcAg particle is an ideal vector for recombinant antigen.

However, there are some facts that may influence proper HBcAg capsid formation, such as the primary sequence and size of the inserts and the hydrophobicity of inserted peptide [22]. It has been reported that the longest sequence would be more than 200aa, such as GFP. However, principally short inserted peptide may ensure the spike formation and sufficient self-assemble. The presence of cysteines will form disulphide bonds and affect capsid formation. The more hydrophobicity amino acids are inserted, the more effective self-assemble into icosahedral particles will be achieved. In our experiments, the inserted peptide included 2 CTL epitopes (9aa), ensuring to provide enough CTL epitopes to stimulate cellular immune response. The sequence of inserted MAGE-A3 peptide is

LysValAlaGluLeuValHisPheLeu. Among them, Val, Ala, Leu, Phe are all hydrophobicity amino acids, thus influence on capsid self-assemble will not occur.

Though recombinant HBCAg-MAGE-A3 vectors and the methods for purifying therapeutic cancer vaccines were successfully established, the purification methods should be modified to large scale protein production for future applications.

Disclosure of conflict of interest

None.

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