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• LIVER CANCER •

Expression, purification and serological analysis of hepatocellular carcinoma associated antigen HCA587 in insect cells

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Abstract

AIM: In order to assess hepatocellular carcinoma associated antigen HCA587 as a potential target for immunotherapy, the Bac-to-Bac expression system was used to express recombinant protein HCA587 in insect cells.

METHODS: The cDNA encoding HCA587 gene was cloned into donor vector pFasBacHtb and recombinant pFasBac Htb-587 was transformed into competent cells DH10Bac. Recombinant Bacmid-587 was transfected into Sf9 insect cells using CELLFECTIN, Recombinant HCA587 protein was produced in Sf9 insect cells after infection with recombinant baculovirus, and was purified using Ni-NTA resin. Sera from HCC patients were also screened using recombinant protein HCA587.

RESULTS: The molecular weight of the recombinant protein HCA587 expressed in insect cells was approximately 43kd. Western blot results proved the recombinant protein HCA587 had the similar antigenicity with its native counterpart. Serological analysis told that the rate of seroreactivity to HCA587 was not high in HCC patients.

CONCLUSION: The recombinant protein HCA587 was successfully expressed and purified using Bac-to-Bac expression system. It paved the way for generation of specific antibody and investigation of immunohistochemical analysis and immune responses of HCC in the future.

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INTRODUCTION

The recent developments in the molecular characterization of human tumors and a better understanding of tumor immunology have led to the identification of different kinds of tumor-associated antigens[1-5]. Of these antigens, Cancer/testis antigens (CT antigens) have played important roles as targets for cancer vaccine development because of their characteristic expression pattern in cancer and testis[6-9]. Many promising results have been achieved in tumor immunotherapy using peptides derived from CT antigens[10-13]. Identifying new CT antigens and evaluating their possible application in the clinic have become a hot spot in this field.

Since hepatocellular carcinoma (HCC) is one of the most pernicious cancers in China, we have adopted serological analysis of recombinant cDNA expression library (SEREX) method and successfully identified a number of novel HCC genes encoding immunogenic proteins. Of these, hepatocellular carcinoma associated antigen HCA587 was identified as one novel CT antigen which was predominantly expressed in HCC and other types of cancers, but not in normal tissues except testis[14]. Studies on HCA587 may play important roles in transformation, metastasis, diagnosis and immunotherapy of HCC.

The bac-to-bac baculovirus expression system is an eukaryotic gene expression system which allows the rapid and efficient generation of recombinant baculovirus DNAs by site-specific transposition in *E.coli*, rather than homologous recombinant in insect cells[15,16]. High level heterologous gene expression are often achieved compared to other eukaryotic expression systems, and most of the expressed proteins were shown to have the similar functions as their authentic counterparts. In the present studies, we utilized the Bac-to-Bac system to express the recombinant protein HCA587 in *Spodoptera frugiperda* (sf9) cell lines. The HCA587 protein was then purified using Ni-NTA resin, and the anti-HCA587 antibodies were screened in sera from 81 HCC patients.

MATERIALS AND METHODS

Expression system, insect cells and sera

The Gibco BRL BAC-TO-BAC Baculovirus expression system consists of the transposing vector pFasBacHTb, CELLFECTIN reagent and Max Efficiency DH10Bac competent cells which contain Bacmid (baculorivus shuttle vector plasmid) and helper plasmid to be used to generate recombinant Bacmids. *Sf9* insect cells were cultured at 27 ℃ in SF-900 SFM (Cell culture media and reagents were Gibco BRL brand). All sera of HCC were collected from Peking University teaching hospitals with the agreements of HCC patients.

Amplification and DNA sequencing of gene HCA587

The oligonucleotide primers specific for gene HCA587 were designed and synthesized by Sangon biotechnology company (P1: 5' ATCGGATCCCCTCCCGTTCCAGGCGT 3', P2: 5' ACTAAGCTTTCACTCAGAAAAGGAGAC 3'). The cDNA produced from normal testis was amplified as template. The PCR products were cloned into pGEM-T-easy vector and sequenced with T7 and SP6 primers by the dideoxy chain termination method using the BigDye Terminator cycle sequencing kit and an ABI PRISM automated DAN sequencer.

Construction of pFasBac HTb-587 plasmid

pFasBac HTb donor plasmid and pGEM-T-easy-587 were prepared by digesting with restriction enzymes BamHI and HindIII. The fragments of interests were purified and recovered from gel using clontech DNA purification system. After ligated

by T4 DNA ligase, the ligation mixture were transformed into DH5α competent cells. The recombinant plasmid pFasBac HTb-587 was identified by restriction endonuclease digestion.

Generation of recombinant bacmid DNA

Recombinant pFasBac HTb-587 plasmids were transformed into Max Efficiency DH10Bac competent cells, and the gene of interest was transposed into Bacmid through lacZ gene disruption. White clones, containing the recombinant Bacmids were selected on Luria agar plates with 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluogal, and 40 μg/ml IPTG. After 36 h incubation at 37 ℃, High-molecular-weight DNA was isolated from the overnight cultures as described in the manual. PCR analysis was used to verify successful transposition to the recombinant Bacmid with M13/pUC primes.

Transfection of Sf9 cells with recombinant bacmid-587

The minipreparations of recombinant Bacmid DNA were transfected into *Sf9* insect cells using CELLFECTIN reagent. For each transfection, 9×10^5 cells were seeded in a 6-well plate and allowed to attach for at least 1 h. The Lipid reagent and Bacmid DNA were diluted separately into 100 μl of SF-900 SFM without antibiotics, then combined to form lipid-DNA complexes. The lipid-DNA complexes were diluted to 1 ml with SFM and laid over the washed Sf9 cells. The cells were incubated for 5 h at 27 ℃, rinsed, and incubated for another 72 h. Recombinant baculovirus were harvested from supernatant and titrated by viral plaque assay. The expression of recombinant protein HCA587 was analyzed by western blot.

Expression and purification of HCA587 from Sf9 insect cells

The recombinant baculovirus were amplified from the suspension cultures of *Sf9* cells at MOI of 0.1. We analyzed the effects of several factors in various combinations on the level of protein expression, the factors were: adherent or suspension cultures in different densities; multiplicity of infection (MOI) and recombinant virus replication time. Since the expressed recombinant protein contained 6×histidine tag at N-terminal, it was purified using Ni-NTA resin conveniently according to the manufacturer' s instructions. SDS-PAGE was performed to analyze the purified protein from the infected cells.

Serological analysis of recombinant HCA587 protein

To analyze the anti-HCA587 antibody in sera of patients, Western blot method was used to screen the reactivity of recombinant HCA587 to sera from 81 HCC patients. The serum was diluted 1/250 as primary antibody, and rabbit-anti- human IgG, conjugated to AP, were used as secondary antibodies. In this assay, the negative control was the serum from healthy volunteers.

RESULTS

Identification of recombinant pFasBacHTb-587 and Bacmid-587

The fragments of gene HCA587 were amplified by PCR using specific primers (Figure 1) and sequenced to ensure the correctness of the ORF. Restriction endonuclease digestion was performed to verify the correct insertion of the gene HCA587 in the recombinant pFasBacHTb-587. The gel electrophoresis in 1 % agarose showed 1.2kb of HCA587 and 4.8kb of pFasBacHTb donor plasmid (Figure 2).

The Bacmid DNA is >135kb. Verification of the insertion of the gene HCA587 in recombinant Bacmid-587 is difficult using classical restriction endonuclease digestion analysis. So PCR was used to confirm the recombinant Bacmid-587. The pUC/M13 amplification primers are directed at sequences on either side of the mini-attTn7 site with the lacZ α-complemention region of the Bacmid. Amplification products from transposition of recombinant bacmid-587 generated a band of 3.5kb (lane1,2) while amplification of the non-recombinant Bacmid plasmid generated a 300bp band (lane3,4) (Figure 3).

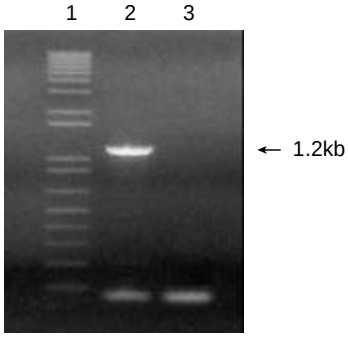

Figure 1 PCR amplified products of HCA587. (Lanes 1: 1 kb DNA marker; lane2: 1.2 kb fragment of HCA587; lane3: negative control).

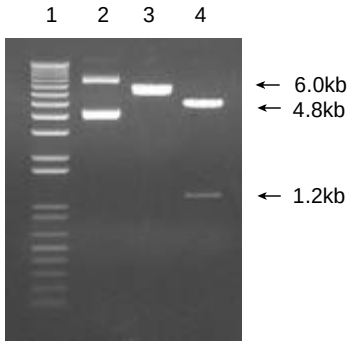

Figure 2 Recombinant pFasBac Htb-587 vector digested by BamHI and HindIII. (Lanes1: 1kb DNA marker; lane2: recombinant plasmid; lane3: digested by BamHI; lane4: digested by BamHI+HindIII).

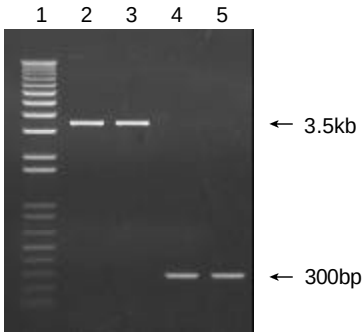

Figure 3 PCR identification of recombinant Bacmid-587. (lane 1: DNA marker; lane 2-3: recombinant Bacmid-587; lane 4-5: blank bacmid).

Transfection of Sf9 cells and amplification of recombinant virus

Recombinant Bacmid-587 was isolated from overnight cultures and transfected into insect cells sf9 with CELLFECTIN reagents. Infected and uninfected sf9 cells can be distinguished by morphology. Uninfected cells continued to divide and form a confluent monolayer while infected cells stopped dividing and enlarged (data not shown).

The Bacmid-587 transfected cells were collected and

analyzed for recombinant protein expression by western blot. Figure 4 showed a specific expressed protein band at 43kd as expected, while no specific band apperared in Sf9 cells without transfection (lane 3). Viral plaque assay showed the viral titer could reach 5×10^8 pfu/ml after amplifying the primary virus in *Sf9* suspension cell culture.

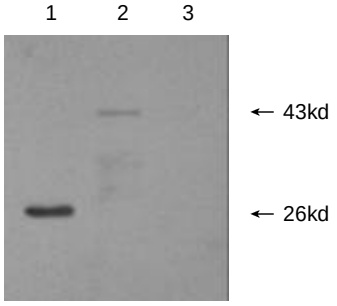

Figure 4 Western blot analysis of recombinant protein expression in sf9 insect cells after transfection. (lane 1: positive control; lane2: Sf9 cell transfected with bacmid-587; lane 3: blank Sf9 cells).

SDS-PAGE analysis of purified HCA587 protein

The optimal conditions varies to express different proteins. Recombinant HCA587 protein was expressed at the highest level when 1×10^6 *Sf9* cells/ml were infected with an MOI of 5 and harvested after 96 h of replication. After purification with chromatography using Ni-NTA resin, the purified protein, infected and uninfected *Sf9* cells were lysed directly in SDS-loading buffer and boiled for 5 min. All samples were cleared by centrifugation and analyzed on 12.5 % acrylamide gels, which was stained by Coomassie blue and scanned by a densitometer to visualize the purity of purified protein. Figure 5 showed that the recombinant protein with 43kd was more than 90 % purity.

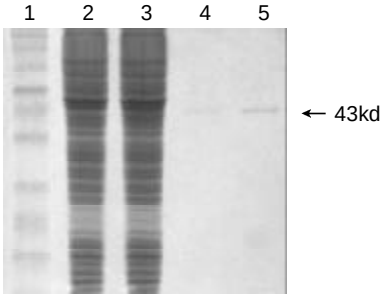

Figure 5 SDS-PAGE analysis of recombinant HCA587. (lane 1: protein marker; lane2: sf9 lysate; lane3: infected sf9 lysate; lane4-5: purified HCA587 protein).

Identification of antigenicity of the recombinant protein HCA587

In order to know if the recombinant protein HCA587 has the similar antigenicity with its native counterpart, the serum containing antibodies which recognize native HCA587 was used as primary antibody in western blot analysis. Figure 6 showed that the recombinant protein HCA587 was able to react with the specific antibody in serum (lane 2) while no reactivity was seen in negative control (lane 3), indicating it' s similar functions with natural counterpart.

Reactivity of allogeneic HCC sera to recombinant protein HCA587

To determine the anti-HCA587 antibody produced in HCC patients, Sera collected from 81 allogeneic HCC patients were screened to test their reactivities with the recombinant protein HCA587 expressed from *Sf9* insect cells. The positive frequency of antibody response in HCC patients was not high, with only 2 positive of 81 patients (Figure 7, negative results not shown). This low late of serological reactivities was also shown in other CT antigens[24].

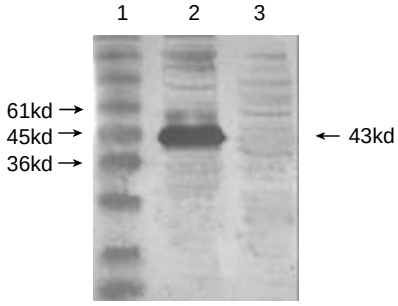

Figure 6 Western blot of recombinant HCA587 reacting with positive serum. (lane 1: protein marker; lane 2: positive serum of HCC; lane 3: negative control).

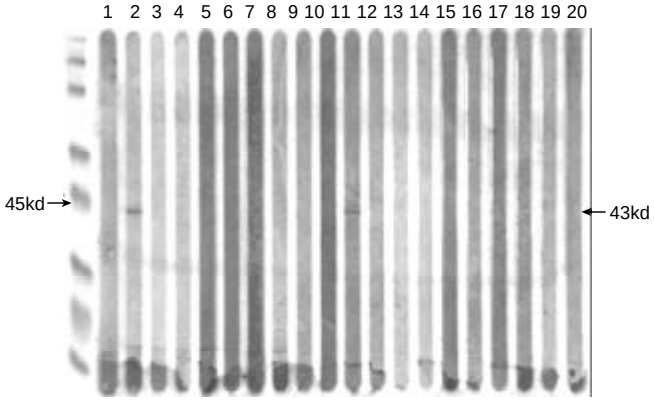

Figure 7 Serological analysis of HCC patients with recombinant protein HCA587. (lane 2 and 11: positive; others: negative; left: protein marker).

DISCUSSION

HCA587 was one of the SEREX-defined CT antigens identified from HCC patients[14]. The protein of HCA587 was immunogenic and capable of inducing an antibody response as it was cloned by serologic method from the sera of HCC patients. In view that the SEREX-defined NY-ESO-1 can induce a CTL response and CTL-defined MAGE-1 has also been identified by SEREX[17,18], this suggests that HCA587, as a CT antigen, may possibly contain both B and T epitopes and can elicit CTL responses. Because CTLs represent a major arm of the immune response against cancer, the elicitation of a specific CTL response against tumor Ags is one of the main aims of current immunotherapy trial. Therefore, it is crucial to define $CD8^+$ T cell epitopes in HCA587 protein, which may determine its potential vaccine candidates for HCC immunotherapy. In addition, the mRNA expression rate of CT antigens can reach 70 %(14/20) in liver cancer tissues from HCC patients but not in normal liver tissue or cirrhosis, indicating its potential functions in tumorigenesis[14]. Current knowledge about this expression pattern is mainly based on RT-PCR analysis, not based on protein levels[19-21], So immunohistochemical analysis of HCA587 antigen expression in HCC tissues is very necessary. Expression and purification of HCA587 protein makes both CTL-mediated responses and immunlohistochemical study become possible.

Bac-to-Bac baculovirus expression system was developed which allows rapid and efficient generation of recombinant baculovirus. With this system, recombinant virus DNA isolated from selected colonies is not mixed with parental virus which eliminate the need for multiple rounds of plaque purification. High-titer virus are produced from the initial transfection too. These features reduce the time to identify and purify recombinant virus from 4 to 6 weeks to 7 to 9 days. In the present study, The cDNAs of the HCA587 were subcloned into pFasBacHTb donor vector at the BamHI and HindIII sites. The recombinant bacmid-587 was constructed by transposing a mini-Tn7 element from a pFasBacHTb donor plasmid to the mini-*att*Tn7 attachment site on the bacmid when the Tn7 transposition functions are provided in *trans* by a helper plasmid in DH10Bac competent cells. After transfecting the recombinant bacmid-587 to Sf9 cells, we successfully expressed and purified the recombinant protein HCA587 which contained 333 amino acids with 6 his tags at its N-termianl, which makes the purification procedure more convenient with its high affinity to Ni-NTA resin[22,23].

Western blot analysis showed that the recombinant protein HCA587 has the similar immunological reactivity with its natural counterpart (Figure 6). It was used as antigen to screen the generation of anti-HCA587 antibody in serum from HCC patients. The results proved that HCC patients was able to develop humoral immune response to HCA587 antigen, but the frequency is not high (2/81). This low rate of seroreactivity to allogeneic sera is similar to some other CT antigens, either defined by a $CD8^+$ CTL response (MAGE-1)[24] or by SEREX (SSX-2)[25]. In a survey of humoral responses of cancer patients against recombinant tumor antigens, it was indeed shown that seroreactivity to MAGE gene products is uncommon, and sera from 234 cancer patients showed antibodies to MAGE-1 in 3, to MAGE-3 in 2, and to SSX-2 in 1 patient[24]. Since many members of MAGE family have been shown to be recognized by CTL from cancer patients[26-30], it would be important to investigate possible HCA587 epitopes recognized by $CD8^+$ and $CD4^+$ cells. In conclusion, our experiments pave the way for further study of HCA587 for the development of tumor vaccine and clinical tumor diagnosis.

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