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Preparation and characterization of a high-affinity monoclonal antibody against human epididymis protein-4

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ABSTRACT

Human epididymis protein-4 (HE4) may serve as a putative biomarker for the early diagnosis, therapy and especially prognosis of ovarian, lung and breast cancer. Detection and targeting of HE4 using the anti-HE4 antibody could be one of the effective strategies for the cancer diagnosis and treatment in clinical practice. In this study, a high-efficiency expression system was established to purify recombinant HE4. We obtained high purity HE4 in 400 mg quantity from 1 L culture supernatant of HEK293F cells. CCK-8 and cell cycle assays indicated that the purified recombinant HE4 protein could promote SKOV3 cell cycle and proliferation at the concentration of 0.1 mg/L. Furthermore, an anti-HE4 high-affinity monoclonal antibody 9C3 (ka = 8.1×10^6 1/MS, kd = 4.4×10^{-5} 1/S, KD = 5.5×10^{-12} M) was prepared using hybridoma technique and analyzed by surface plasmon resonance technology using this HE4 protein. Differential Scanning calorimeter (DSC) analysis showed that 9C3 had a commendable thermal stability with the T_m value of 73 °C. Analyses of western blot, immunohistochemistry and immunofluorescence showed that the 9C3 was highly specific to HE4 in human cancer cells and tissues. In conclusion, our study designed a method to prepare human recombinant HE4 with high yield and generated a high-affinity anti-HE4 monoclonal antibody that might have potential for basic research and clinical application.

1. Introduction

Ovarian cancer is a malignant tumor in female reproductive system that has the highest death rate among the three major gynecological malignancies [1]. In recent years, about 70% of ovarian cancer patients were diagnosed with peritoneal carcinomatosis at advanced stages [2]. Therefore, biomarkers for early detection of ovarian cancer are very important for patients to receive appropriate and potentially curative treatment. Gynecological ultrasonography and serum ovarian cancer-related tumor marker CA125 (CA125) are the traditional methods to screen the ovarian cancer [3,4]. However, due to lack of specificity and sensitivity of CA125, the false positive rate is high. It is imperative to develop effective early diagnosis biomarker [5,6]. Human epididymis protein-4

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(HE4), a small secreted glycoprotein, was found to be a novel tumor biomarker, and has been studied extensively for the past few years [7,8]. The diagnostic accuracy of adnexal masses using HE4 alone and in combination with CA125 was improved compared with CA125 alone, especially in early ovarian cancer detection [9].

HE4 was first identified from the human epididymis skin cells in 1991, and its functions were associated with sperm development and natural immunity [10]. HE4 was highly expressed in ovarian cancer tissues, but not in the adjacent tissues [11]. Furthermore, HE4 level was significantly higher in ovarian cancer patients' serum compared to normal human serum [12–15]. HE4 as an early diagnostic biomarker of ovarian cancer was approved by European Union and United States FDA and implemented into clinical applications in 2008 [16,17]. Moreover, HE4 as a sensitive histological marker is also highly expressed in lung cancer, breast cancer and pancreatic cancer, which broadens its application in the early diagnosis, therapy and prognosis of these cancers [9,18–23].

The HE4 gene is located on chromosome 20q12-q13.1, including 5 exons and 4 introns [24–26]. Human HE4 protein has a highly conserved WAP domain that consists of 4 disulfide core regions formed by 8 cysteines. The loop in the WAP domain can insert into the protease activity region inhibiting protease activity [27]. However, the biological functions and pathological significance of HE4 in cancers need to be further studied.

Since the first monoclonal antibody was approved in 1986, monoclonal antibodies have been successfully applied to clinical cancer diagnosis and therapy [28]. Screening and preparing a high affinity antibody associated with disease biomarker can be an effective tool for early diagnosis and treatment in clinical practice. Hybridoma technique is a traditional and efficient method for producing a large amounts of identical antibodies since 1975 [29]. High purity and sufficient quantity of antigen is the basis for screening and preparation of target antibodies using hybridoma technique. Human HE4 protein is a small secreted and disulfide bonds containing glycoprotein, so *Escherichia coli* was not a proper expression system for HE4 preparation [30]. HE4 expressed by *P. pastoris* with high-mannose glycosylation presented lower biological activity than its complex-glycosylated form. Although HE4 fused with IgG1 Fc showed its biological activity, the redundant 26 kDa Fc is not suitable for industrial application and is not an ideal antigen for antibody preparation [31].

In this study, human recombinant HE4 was expressed and purified with 6 × His tag at the C terminus via a mammalian expression system. The yield of recombinant HE4 reached 400 mg/L, which had good biological activity. Furthermore, the human recombinant HE4 protein was used as antigen to prepare anti-HE4 antibody using hybridoma techniques. A high-affinity anti-HE4 monoclonal antibody named 9C3 (KD = 5.5 × 10^{-12} M) was developed as assessed by Biacore. DSC analysis showed that 9C3 had a commendable thermal stability with the T_m value of 73 °C. In addition, the 9C3 showed high specificity for the antigen in human cancer cells and tissues. Our study established a high yield method to purify the human recombinant HE4 protein and produce a high-affinity anti-HE4 monoclonal antibody which deserves further research for the HE4-based cancer diagnosis and therapy in clinics.

2. Materials and methods

2.1. Materials

The human HE4 cDNA, plasmid vector pTT5, *E. coli* DH5α and HEK293 cells were supplied by AtaGenix. All reagents for molecular cloning, including PrimeSTAR® Max DNA Polymerase, DNA Ligation Kit Ver.2.1 and restriction enzymes were purchased from Takara. DNA Gel Extraction kit and Plasmid Miniprep kit were from Omega Bio-Tek. FectoPRO® DNA transfection kit was from Polyplus-Transfection and Serum-free medium was products of GIBCO. cOmplete™ His-Tag Purification Resin was from Sigma-Aldrich. HRP-conjugated goat anti-mouse IgG antibody was self-produced by AtaGenix. Animal experiments were done in AtaGenix's specific pathogen-free Animal House and were conducted in accordance with the Animal Care and Use Committee of Sichuan University. Biacore X100 (GE Healthcare), and Auto VP-capillary DSC (GE Healthcare) were performed in accordance with the operating regulations of State Key Laboratory of Biotherapy, Sichuan University.

2.2. Cloning and expression of human HE4

To obtain the secreted HE4 protein, *HE4* gene was ligated into the *Eco*RI/*Hin*dIII sites of pTT5 vector with a His tag on the C-terminal end and full length of human HE4 cDNA with its natural signal was amplified. After amplification, the HE4 fragments and pTT5 vector were double-digested by *Eco*R1 and *Hin*dIII and then ligated. The ligation mixture was transformed into *E. coli* DH5α (AtaGenix), and the positive clones were sequenced.

900 mL of HEK293 cell suspension at 1 × 10^6 cells/mL were prepared on the day before transfection. The cells were cultured overnight at 37 °C, 125 rpm and 5% CO2 levels. Next day, 500 μg HE4 plasmid, diluted into 100 mL of serum-free medium, was mixed with 750 μL of pure FectoPRO® DNA transfection reagent, and incubated at 25 °C for 10 min after homogenization. The 100 mL FectoPRO®/DNA transfection mix were added to the cells, then cultured until harvest and purification.

In order to screen the best expression condition, 1 mL cell culture solution including culture medium and cells were collected on day 4, day 5 and day 6 to analyze the expression level of HE4. To analyze the expression location of HE4, each cell culture solution was separated into cell culture medium, cell lysis supernatant and cell lysis precipitate. The concentration of target protein in culture medium is often low. To detect the target protein clearly by SDS-PAGE, HE4 was enriched from culture medium by His-Tag Purification Resin which could effectively increase the concentration of target protein and remove the other impurity proteins. The detailed steps are as follows. 1 mL each cell culture medium (Lane 2–4) was incubated with 20 μL His-Tag Purification Resin on a roller for 20 min at room temperature. The supernatant was abandoned, and the His-Tag Purification Resin was resuspended into 150 μL 1 × loading buffer (50 mM Tris pH 6.8, 1% SDS, 0.1% bromophenol blue, 10% glycerol, 1% β-mercaptoethanol). The resin mixture was heated at 95 °C for 10 min and centrifugated to collect the supernatant. 10 μL supernatant of each sample was loaded into the SDS-polyacrylamide gel.

2.3. Purification of the recombinant protein

After harvest, the 1 L culture medium was concentrated to 90 mL, and divided into two parts for purification in 50 mL centrifuge tubes. 5 mL cOmplete™ His-Tag Purification Resin was put into each centrifuge tube and washed with 25 mL of wash buffer (PBS, pH 7.4). 45 mL concentrated cell culture was poured into the centrifuge tube, and mixed on a roller for 20 min at room temperature. After centrifugation, the supernatant was collected for detection, while the resin was incubated with 45 mL wash buffer on the same roller for 20 min The supernatant was collected again, and the resin was successively eluted with 10 mL Elution buffer 1 (PBS, 30 mM imidazole, pH 7.4) for one time, 10 mL elution buffer 2 (PBS, 100 mM imidazole, pH 7.4) for two times, and 10 mL elution buffer 3 (PBS, 400 mM imidazole, pH 7.4) for six times by the same methods. The supernatant of each step was attained for