

Construction of a fusion expression plasmid containing the *G250* gene and *human granulocyte-macrophage colony stimulating factor* and its significance in renal cell carcinoma

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Received August 23, 2010; Accepted December 22, 2010

DOI: 10.3892/ol.2010.230

Abstract. This study aimed to construct a eukaryotic expression plasmid containing the *G250/MN/CA IX (G250)* and *human granulocyte-macrophage colony stimulating factor (hGM-CSF)* genes, and to detect the expression of these proteins *in vitro* by recombinant plasmids in eukaryotic cells. *pORF-hGM-CSF* and *pcDNA3.0-G250* were used as the template to amplify *G250* and *hGM-CSF* by routine polymerase chain reaction (PCR). The two PCR products were cloned into the eukaryotic vector *pVAX1*, in order to construct a recombinant plasmid *pVAX1-G250-hGM*, and the plasmid was transfected into human embryonic kidney 293 cells. The protein expression was then determined by immunocytochemistry, atomic force microscopy, ELISA and Western blotting. DNA sequencing showed that the cloned *G250* and *hGM-CSF* sequences were consistent with the reported Gene Bank ones. Moreover, a high expression was noted following recombinant plasmid transfection of the *G250* and *hGM-CSF* proteins. Thus, the eukaryotic expression vector *pVAX1-G250-hGM* containing *G250* and *hGM-CSF* was constructed, allowing for the investigation of the anti-*G250* antigen vaccine and immune response mechanisms of biological immunotherapy in renal cell carcinoma.

Introduction

Renal cell carcinoma (RCC) is a common malignant tumor of the urinary system. One out of nearly a million individuals succumb to the disease each year worldwide and the incidence is on the increase (1). RCC patients frequently present with subclinical disease and 20-30% of patients are admitted (2).

However, traditional radical nephrectomy in RCC in the early stage has a positive effect, which is not the case in advanced stage and metastatic RCC. Simultaneously, RCC is not sensitive to radiotherapy and chemotherapy (3). Since RCC is a highly immunogenic tumor, advances in molecular and immune biology allow for the potential use of tumor vaccines as immune therapy (4). *G250/MN/CA IX* (MN antigen receptor/carbonic anhydrase-9) is one of the tumor markers that possess favorable tumor specificity (5). The specific expression of *G250/MN/CA IX* in RCC renders it a key target for cancer diagnosis and treatment. In this study, a eukaryotic expression vector, containing the *G250/MN/CA IX* and *human granulocyte-macrophage colony stimulating factor (hGM-CSF)* genes, was constructed and transfected into human embryonic kidney 293 (HEK 293) cells. The fusion protein expression and immunoreactivity were then detected to establish anti-renal cell carcinoma vaccines for studies based on the *G250* gene.

Materials and methods

Materials. *E. coli* Top 10, vector *pVAX1*, recombinant plasmid *pORF-hGM-CSF* and *pcDNA3.0-G250* were obtained from the Department of Microbiology and Immunology, Medical College, Jinan University, Guangzhou, China. *Ex-Taq* DNA polymerase, *T4* DNA ligase, restriction enzymes *Xba*I, *Hind*III and *Kpn*I, 1 kb DNA marker, 100 bp DNA marker, plasmid mini and gel extraction kits were from Takara Bio Inc., Japan. The target gene sequencing analysis was from Shanghai Sangon Biological Engineering Technology and Services Company, China. Lipofectamine-2000 and Opti-MEM were from Invitrogen Corporation (Carlsbad, CA, USA), and the immunohistochemical kit was from Zhongshan Company, China. The ELISA kit was purchased from R&D Inc., USA, and the *G250* antibody was from Abcam Inc., USA.

Construction and identification of recombinant plasmids. Based on the CDS sequence of the *G250* gene in Gene Bank (NCBI: BC014950), primer 5.0 was used to design the primers 5'-GCAAGCTTTTCCAATGCACGTACAG-3' (forward) and 5'-TCGGGTACCGGCTCCAGTCTC-3' (reverse) with the appropriate restriction endonuclease sites and omission of the

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Key words: renal cell carcinoma, *G250/MN/CA IX* gene, human granulocyte-macrophage colony stimulating factor

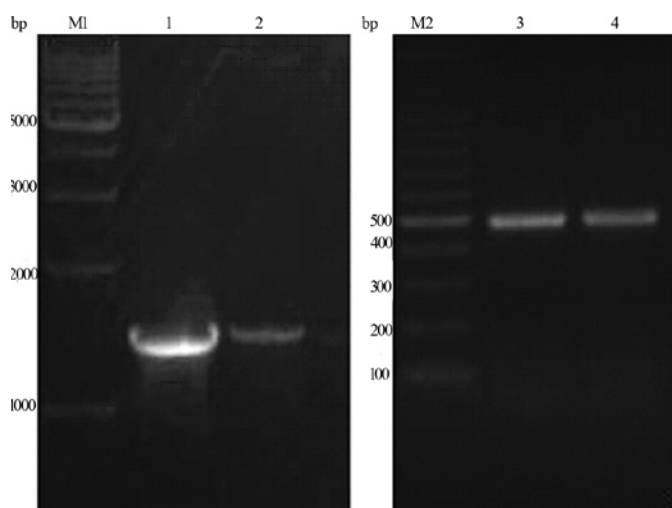


Figure 1. Electrophoresis of the PCR product. M1: 1 kb DNA ladder marker; lanes 1 and 2: PCR product of the *G250* gene; M2: 100 bp DNA ladder marker; lanes 3 and 4: PCR product of the *hGM-CSF* gene.

termination codon which was used to amplify *G250* (Fig. 1). The PCR fragments and plasmid *pVAX1* were digested by *KpnI* and *HindIII*. The cleaved products were ligated using *T4* DNA ligase at 16°C overnight. The ligated products were transformed into the competent *E. coli* Top 10, and antibiotic selection and the restriction endonuclease assay (Fig. 2) were used to screen and identify positive clones. DNA sequencing analysis was performed using Sanger dideoxy chain termination.

The coding sequences of the *hGM-CSF* fragments were synthesized by PCR from *pORF-hGM-CSF* using specific primers: 5'-TATGGTACCGGATCAGGAGGTTCTATGTGGCTGCAGAGCCT-3' (forward) and 5'-GGGTCTAGATATCATGTCGAGCTAGCGAATTC-3' (reverse), which were cloned into the *KpnI* and *XbaI* sites of *pVAX1-G250* using standard cloning techniques (Fig. 1). The recombinant plasmids were purified and double digested with *KpnI* and *XbaI* (Fig. 2). The procedure involving the sequence analysis of the recombinant plasmids was terminated by the Shanghai Bio-Engineering Company and recombinant plasmid *pVAX1-G250-GM* was successfully constructed.

Cell transfection. HEK 293 cells were digested with 0.25% trypsin and diluted to $1-4 \times 10^5$ cells/ml. The cells were plated in 6-well plates with 2 ml medium per well. When the cells achieved 60-70% confluence, 4 μ g purified plasmid was transfected to the prepared cells using 8 μ l lipofectamine-2000 reagent. After 48 h, the living cells were examined directly and photographed under an inverted fluorescence microscope.

Immunocytochemistry staining. Non-transfected cells were regarded as the blank comparison and *pVAX1*-transfected cells as the negative comparison. Immunocytochemistry staining was performed according to the manufacturer's instructions and mouse anti-*G250* antibody was used as the primary antibody (Fig. 3).

Atomic force microscopy. After being dried, samples were immunoblotted, visualized using DAB chromogen and scanned by atomic force microscopy (AFM) (AutoProbe CP

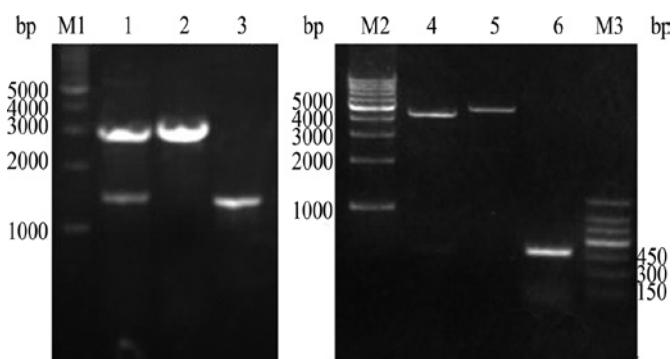


Figure 2. Restriction map of recombinant plasmids. M1: 1 kb DNA ladder marker; lane 1: *pVAX1-G250/HindIII + KpnI*; lane 2: *pVAX1/HindIII + KpnI*; lane 3: PCR product of *G250*. M2: 1 kb DNA ladder mark; lane 4: *pVAX1-G250-GM/KpnI + XbaI*; lane 5: *pVAX1-CAIX/KpnI + XbaI*; lane 6: PCR product of *hGM-CSF*; M3: 150 bp DNA ladder marker.

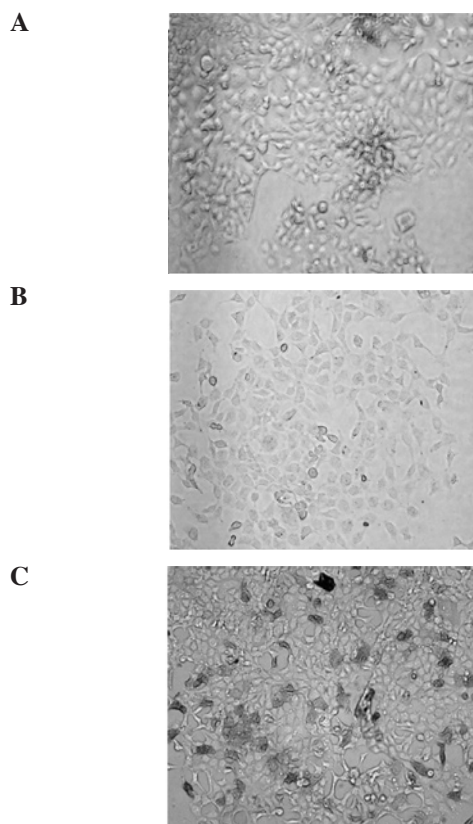


Figure 3. Identification of the protein expression in HEK 293 cells by immunocytochemistry staining (magnification, $\times 100$). (A) Negative group (non-transfected). (B) Negative group (transfected *pVAX1*). (C) Positive group (transfected *pVAX1-G250-GM*).

Research, ThermoMicroscopes Inc., USA). The samples were placed in XY scanning stage, and then the monitor was used to locate the scanning area of the sample and scanning image. The test was conducted using 100 μ m scanners, UL20B silicon probe, and a force constant of 2.8 N/m. Equipment configuration software (ThermoMicroscopes pro-scan image processing software version 2.1) was used for image data acquisition and processing. Images were smoothed to eliminate the scan direction and ensure a low level of background noise (Fig. 4).

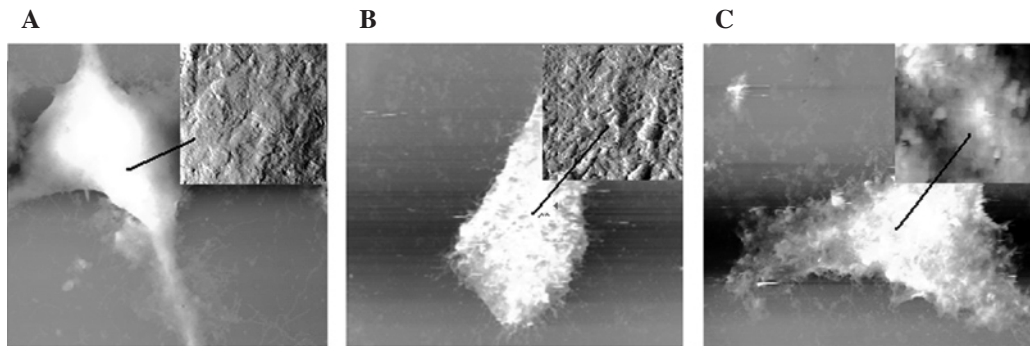


Figure 4. Atomic force microscopy. (A) Normal HEK 293 cells. (B) HEK 293 cells transfected with *pVAXI*. (C) HEK 293 cells transfected with *pVAXI-G250-GM*.

Table I. Expression value of hGM-CSF protein by ELISA.

Group	24-h hGM-CSF (pg/ml)	48-h hGM-CSF (pg/ml)
<i>pVAXI</i>	53.97±0.53	54.10±0.79
<i>pVAXICAIX-hGM</i>	482.47±5.86a	513.36±4.45a

ELISA. Double-antibody sandwich ELISA was used to detect the hGM-CSF protein level. The results were shown as the $\bar{x} \pm s$ and the significant level of difference between the values was analyzed using SPSS 13.0 software. $P < 0.05$ was considered to be statistically significant (Table I, Fig. 5).

Western blotting. A Western blot analysis of the fusion proteins was performed according to the standard method. The purified protein was separated on 120 g/l SDS-PAGE and transferred to a nitrocellulose membrane. Anti-G250 antibody at 1:1,000 dilution was used as the primary antibody to detect the G250 protein. The blots were developed using the ECL method with HRP-labeled anti-goat IgG at a dilution of 1:6,000 (Fig. 6).

Results

PCR products of G250 and hGM-CSF genes. Results of an agarose gel electrophoresis assay showed that the size of the specific PCR amplification products were ~1.48 and 0.48 kb for the *G250* and *hGM-CSF* genes, respectively. The results were in agreement with the anticipated fragment.

Recombinant plasmid detection by restriction enzyme digestion. *pVAXI-G250* was double digested by *KpnI* and *HindIII*. Results of an agarose gel electrophoresis assay showed the two fragments to be ~3.0 and 1.5 kb for the *G250* and *hGM-CSF* genes, respectively. *pVAXI-G250-GM* was double digested by *KpnI* and *XbaI*, and the two fragments were found to be ~4.5 and 0.48 kb for the *G250* and *hGM-CSF* genes, respectively, were noted.

Sequencing identification. The recombinant plasmid *pVAXI-G250-GM* was examined by sequencing. The results showed that it was identical to the reported *G250* gene sequence (NCBI accession: BC014950) and the *hGM-CSF* gene sequence (NCBI accession: M10663).

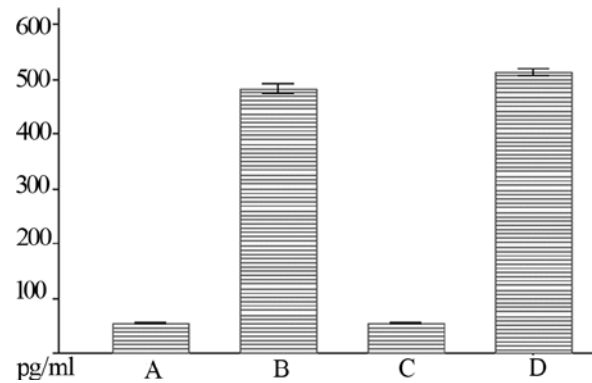


Figure 5. Expression value of hGM-CSF protein by ELISA. (A) Transfected *pVAXI* (time, 24 h). (B) Transfected *pVAXI-G250-GM* (time, 24 h). (C) Transfected *pVAXI* (time, 48 h). (D) Transfected *pVAXI-G250-GM* (time, 48 h).

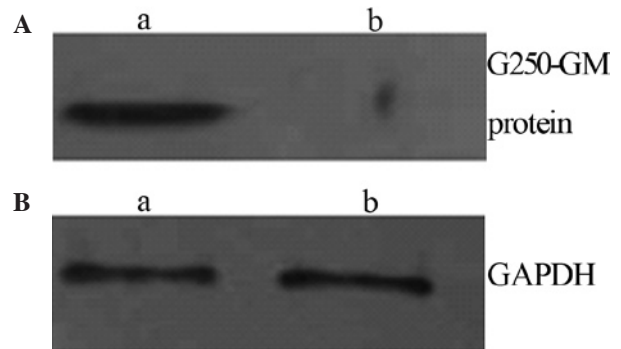


Figure 6. Western blot analysis of protein expressed in HEK 293 cells. Lysates of HEK 293 cells transfected with: (A-a) recombinant plasmid *pVAXI-G250-GM*; (A-b) blank plasmid *pVAXI*; (B-a) recombinant plasmid *pVAXI-G250-GM* and (B-b) blank plasmid *pVAXI*.

Identification of fusion protein by immunocytochemistry staining. Immunocytochemistry staining results showed that the expression of the G250 protein was negative in the transfected *pVAXI* group. However, in the *pVAXI-G250-GM*-transfected group the expression of G250 protein was positive (Fig. 3).

Atomic force microscopy. Normal HEK 293 cells had a smooth surface, with a rich-pseudopod extension and a cell height

that reached 1,250 μm . When transfected with *pVAX1*, the cell surfaces became rough and the cell height was 1,161 μm . *pVAX1-G250-GM* was transfected into cells and the cell height became significant ($\sim 7,450$ nm).

ELISA analysis. The expression values of the hGM-CSF protein of the *pVAX1* control group were 53.97 ± 0.53 and 54.10 ± 0.79 pg/ml ($\bar{x} \pm s$, $n=3$), whereas the expression values of the hGM-CSF protein of the *pVAX1-G250-GM*-transfected group were 482.47 ± 5.86 and 513.36 ± 4.45 pg/ml. The results were considered to be statistically significant ($P < 0.05$).

Western blotting. The proteins which bound to the G250 antibody were detected following recombinant plasmid *pVAX1-G250-hGM* transfection into the HEK 293 cells.

Discussion

RCC is a highly immunogenic tumor that induces the host immune response. RCC primary tumors and their metastasis were found to be relatively stable over time. The tumors occasionally spontaneously regressed, indicating that immune mechanisms play a key role in RCC. *G250/MN/CA IX*, a good tissue-specific RCC-associated antigen, has been identified and cloned from a variety of RCC cell lines. This antigen has 459 amino acids lying in the plasma and nuclear membranes, with a molecular weight between 58 and 54 ku (6). Clear cell carcinoma of the kidneys, and the majority of other types of RCC, express *G250* antigen. Approximately 88% of tumor metastases also express the *G250* antigen (7), which regulates cell proliferation in hypoxic conditions. *G250* possesses HLA-A2.1-restricted epitopes (8), and the *G250* transduction in peripheral blood mononuclear cells produced cytotoxic T cells, which inhibited the tumor cells expressed in the growth of *G250*. For these reasons, *G250* is a key target for RCC immunotherapy (9).

In recent years, increasing importance has been attached to gene therapy as adjuvant treatment in DNA vaccination. As a preponderant adjuvant, *hGM-CSF* has the ability to activate endotheliocytes and macrophagocytes through a variety of mechanisms. It also regulates the amount of and function of antigen-presenting cells and enhances the cytoactive level of cytotoxic T lymphocyte and natural killer cells to strengthen the immune level. *hGM-CSF* is considered to be an adjuvant therapy that assists in the preparation of DNA vaccination and more favorable experimental results (10,11). Tani *et al* (12) prepared *hGM-CSF* gene-modified autologous tumor cells from type IV tumors and used gene-modified tumor cells to treat RCC patients. The results showed that the vaccine induced a tumoral immune response, indicating that it was able to enhance the anti-tumor effect. Simultaneously, *hGM-CSF* combined with autologous tumor cells increased the number of lymphocytes, resulting in slow disease progression and extended patient survival (13).

Concerning safety requirements, we used vector *pVAX1*, which is approved by the FDA and can be applied to the human body. Linkers were added when the upstream primers were designed in order to amplify *hGM-CSF*. To produce *G250* protein, the linkers were added between the fusion proteins and the hGM-CSF fold in natural three-dimensional structures while maintaining intrinsic immunogenicity. To assess the

character of the fusion proteins, we transfected the recombinant plasmids into HEK 293 cells using the liposome transfection method and detected the proteins with immunocytochemistry staining and Western blotting. The results showed that cells transfected with recombinant plasmids in immunocytochemistry expressed protein, whereas the control group had no significant change in color. This expression shows that *G250* protein is immunoreactive. Immunocytochemistry usually uses conventional light or electron microscopy. These classical methods lack dynamic, three-dimensional and single-molecule measurement ability. This study utilized AFM, a novel surface imaging technique that detected nanometer positioning resolution, in order to scale antigen-antibody complex positioning, qualitative, quantitative and three-dimensional display on the membrane surface. AFM detects, not only single-molecule single atoms, but also specific biological molecules or non-specific binding between the three-dimensional morphology of the dynamic process of change. Cells transfected with recombinant plasmid ultrastructures exhibit surface roughness and the height changes significantly, due to the recombinant plasmid which, since it contains *G250*, binds with the antibody, thereby reducing the interaction between the cells. Notably, the use of ELISA has shown an accurate expression of hGM-CSF fusion protein.

In conclusion, we constructed a eukaryotic vector containing *G250* and *hGM-CSF* and detected the expression of the two proteins. However, further investigations regarding immunogenicity and safety of the vaccine are required, as well as examination of the vaccination of the anti-*G250* antigen and its immune response mechanism of biological immunotherapy in RCC.

Acknowledgements

This study was supported by the Key specialist of Guangdong Provincial 11th Five-Year Plan.

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