

## Cloning of human melanoma antigen MAGE-A9 and its expression in hepatocellular carcinomas

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Received 5 May 2007

### Abstract

**Objective:** To express the melanoma associated gene MAGE-A9 recombinant protein, obtain the anti-MAGE-A9 monoclonal antibody and to examine the expression of MAGE-A9 in hepatocellular carcinoma specimens. **Methods:** MAGE-A9 cDNA was cloned from human hepatocellular carcinoma tissue by using RT-PCR, and then subcloned into the plasmid pMD18-T. After sequencing, the MAGE-A9 was cloned into the prokaryotic expression vector pBAD/gIII to construct the recombinant expression vector pBAD/g III -MAGE-A9, and was transformed into *E. coli* TOP10. The recombinant MAGE-A9 protein was expressed under induction of L-Arabinose, and was purified through Hitrap column. The anti-MAGE-A9 monoclonal antibody was generated. The expression of MAGE-A9 in hepatocellular carcinoma specimens was examined through ABC assay. **Results:** The cDNA sequence of the cloned MAGE-A9 gene was consistent with the reported sequence. By affinity column and SDS-PAGE, the purified MAGE-A9 fusion protein displayed a band of Mr 35,000, and subsequently the anti-MAGE-A9 monoclonal antibody was obtained. We found that MAGE-A9 expressed in the cytoplasm of positive cells and MAGE-A9 antigen was detected in 8 cases out of 39 (21%) hepatocellular carcinoma specimens. **Conclusion:** MAGE-A9 antigen was expressed in a fair proportion of hepatocellular carcinoma specimens, these patients might be suitable candidates for immune involving antigen, encoded by the MAGE-A9 gene.

**Keywords:** melanoma antigen; MAGE-A9; RT-PCR; gene expression; immunohistochemistry

### INTRODUCTION

The occurrence of hepatocellular carcinoma (HCC) is high in China. However, there is currently no satisfactory treatment against hepatocellular carcinoma. A potentially useful strategy for treating HCC is to develop vaccines against antigens that are commonly expressed on this type of cancer. It has been reported that the specific expression of some MAGE-A genes in hepatocellular carcinoma is high. Therefore, the tumor-specific expression makes MAGE-A genes interesting for their application to anti hepatocellular carcinoma immunotherapy.

In 1991, van der Bruggen *et al.*<sup>[1]</sup> identified the first

melanoma antigen gene (MAGE), MAGE-A1, in a human melanoma cell line. Since then, 12 MAGE-A genes have been isolated and divided into different classes based on X-chromosome location and sequence homology. Genes of the MAGE-A family are expressed in several types of solid tumors but are silent in normal tissues, with the exception of male germline cells, which do not carry HLA molecules<sup>[2]</sup>. Therefore, peptides encoded by MAGE-A genes are strictly tumor-specific antigens that can be recognized by CTL and constitute promising targets for diagnosis and therapy<sup>[3]</sup>. Furthermore immunologically, both humoral and cell-mediated immune responses against MAGE antigens were detected in cancer patients and thus MAGE has been used as a model for cancer immunotherapy<sup>[4-6]</sup>.

MAGE-A9 is of particular relevance in the context of the MAGE-A gene family, which has been mapped

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to chromosome X, and its gene product has been identified as a protein of an apparent molecular weight of Mr 35,000. Since little is known about the expression of MAGE-A9 in hepatocellular carcinomas in the Chinese population, we examined the expression of MAGE-A9 in 39 prognostic parameters of hepatocellular carcinoma.

## MATERIALS AND METHODS

### Tissues, *Escherichia coli* strains, vectors, patient samples, animals

One tumor biopsy specimen collected by surgery from the Tumor Hospital of Jiangsu Province was frozen immediately in liquid nitrogen within 5 minutes, and kept in a liquid nitrogen tank until RNA extraction. Among the other thirty-eight formalin-fixed and paraffin-embedded specimens of hepatocellular carcinoma and their normal counterparts, specimens were obtained from the archives of the Department of Pathology at Jiangsu Province People's hospital. The thirty-nine patients included 36 males and 3 females, aged from 31~74 years old. *Escherichia coli* strains TG1, TOP10, plasmids pMD-18T, pBAD/g III were all kept in our laboratory. 6~8 weeks old female BALB/c mice were then bought from the animal center of Nanjing Medical University.

### Prokaryotic expression cloning in *Escherichia coli*.

Total RNA was extracted from the hepatocellular carcinoma tissue by using Trizol and reverse transcribed. cDNA was amplified by 30 cycles of PCR (Biometra) by using the following primers, embracing the entire reading frame of MAGE-A9 gene and introducing *EcoR* I and *Hind* III restriction, sited at the 5' end of sense primer and the 3' end of antisense primer: P1, 5' CA CAA GCT TCG GAC TCC CTC TTC CTC CTC TCT 3', and P2, 5' CC GGA ATT CGA ATG TCT CTT GAG CAG AGG AGT 3'.

The PCR product thus obtained was digested with *EcoR* I and *Hind* III, and ligated with a similarly restricted pMD-18T simple vector. The recombinant plasmid was transferred into *E.coli* TG1 and sequenced.

The purified plasmid was digested with *EcoR* I and *Hind* III and subcloned into expression plasmid vector pBAD/g III with six-histidine tag and the recombinant plasmid was transferred into *E.coli* TOP10. For the expression of the recombinant protein, a positive clone was cultured in LB medium supplement with ampicillin (50 µg/ml) overnight at 37°C with shaking. This culture was used to inoculate into fresh LB medium and was incubated at 37°C with vigorous shaking to A600 about 0.6. This culture was induced by 4 h treatment with 20% L-arabinose (to final concentration 0.02%) at 37°C

according to the producer's instructions and the amount of recombinant protein expression. Purification of the fusion protein, from the lysate of culture product (by HisTrap affinity columns, Amersham Biosciences) was performed by the following procedures recommended by the manufacturer, taking into account the concentration of imidazole (from 0.2 mol/L to 0.5 mol/L).

12% SDS-PAGE and Western blot was used to test the production and purification of the recombinant protein. SDS-PAGE was performed in the Mini-Protein system (Bio-Rad). After electrophoresis, one of the gels was stained with Coomassie blue to visualize the protein bands. The proteins of the other gel were transferred onto the nitrocellulose membrane by semidry transfer (Trans-Blot; Bio-Rad). Unspecific binding was blocked overnight by incubation in 5% non-fat dried milk powder in PBS with 0.05% Tween 20. The membrane was probed with the mouse anti-His (Merck) primary antibody in blocking solution (1:10000). A peroxidase conjugated goat anti-mouse IgG (Boster) was used as the secondary antibody (1:200) and a DAB detection system was used to reveal specific binding.

### Production of monoclonal antibodies.

BALB/c mice were immunized i.p. at 2-week intervals with 100 µg of purified material, together with Freund's adjuvant, incomplete adjuvant, and in the absence of adjuvant. Four days after the last boost, the animals were sacrificed and fusions were performed as described above. Hybridoma supernatants were screened by ELISA and the purified MAGE-A9 protein was used as the antigen.

The monoclonal antibody was identified by SDS-PAGE and Western blot, monitoring the culture of TOP10, which can express recombinant MAGE-A9 protein, both after and before inducing. The relevant hybridoma supernatant was used as the first step antibody, and a peroxidase labeled goat anti-mouse IgG (Boster) was used as the secondary antibody (1:200).

### Immunohistochemistry.

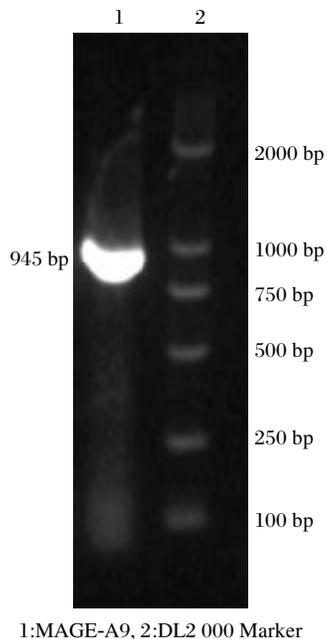
Formalin-fixed, paraffin-embedded hepatocellular carcinoma tissues were stained by the avidin-biotin complex (ABC) method and using the ABC kit (Vector). After antigen retrieval with microwave procedure in 0.01 mol/L sodium citrate buffer (pH 6.0), deparaffinised specimens were incubated in 3% H<sub>2</sub>O<sub>2</sub> for 15 minutes, and normal horse serum was kept for 20 minutes at room temperature. After overnight incubation in anti-MAGE-A9 mAb at 4°C, the specimens were incubated in biotinylated horse anti-mouse IgG for 40 min and avidin-biotin complex for 20 min at 37°C. Color development was performed with diaminobenzidine, followed by counterstaining with hematoxylin.

## RESULTS

### Expression cloning and purification of the MAGE-A9 gene product

The 945 bp MAGE-A9 gene was amplified by 30 cycles of PCR from one hepatocellular carcinoma tissue cDNA by taking advantage of the primers described above (Fig 1).

Then the PCR product was purified, *EcoRI* and *HindIII* restricted, and ligated with the digested pMD-18T vector. The ligation mixture was transformed *in E.coli*



**Fig 1** RT-PCR amplification product of MAGE-A9

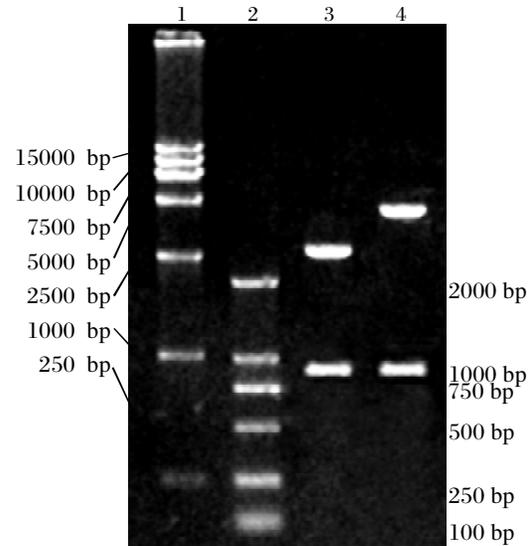
Bacterial lysates were passaged on a metal chelation column and the retained material was eluted at the concentration of imidazole from 0.2 mol/L to 0.5 mol/L and SDS-PAGE run (Fig 4), resulting in a purified product that was used for mice immunization.

### Generation of MAGE-A9-specific monoclonal antibodies

After repeated immunization (as described in the Material and Methods section) fusions were formed, and the hybridoma supernatants were differentially screened on lysates of MAGE-A9 gene-transformed bacterial colonies in a purposely designed ELISA test. The positive rate of ELISA was 100% after three subclones and a stable hybridoma cell line (which secretes anti-MAGE-A9 monoclonal antibody) has been created. The cell line secreting anti-MAGE-A9 monoclonal antibody showed exclusive reactivity on the transformed bacteria (Fig 5).

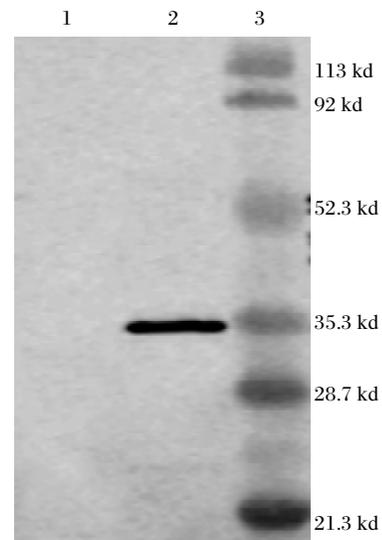
TG1. Positive clones were identified by PCR, plasmids restriction mapped, and colonies were expanded and sequenced. The sequence product matched the published sequence. The plasmid was subsequently transformed into the TOP10 expression host. Positive clones were expanded and tested (Fig 2).

L-arabinose induction resulted in the production of a protein exhibiting an apparent Mr35,000, matching the previously described rMAGE-A9 gene product and identified by Western blot (Fig 3).

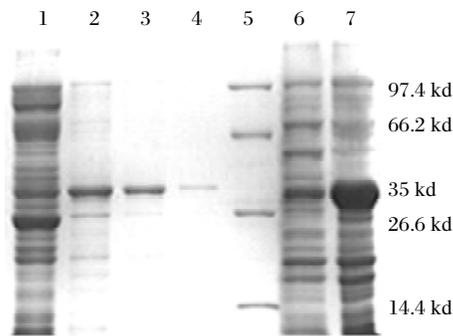


**Fig 2** Restriction mapping of recombinant plasmid pMD18-T-MAGE-A9 and pBAD/g III -MAGE-A9

MAGE-A9 and pBAD/g III -MAGE-A9

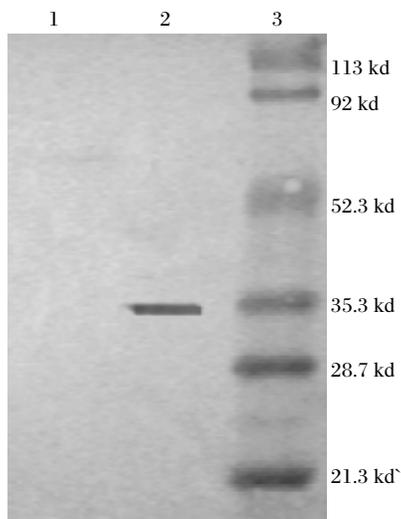


**Fig 3** Identification of the expression MAGE-A9 protein by Western blot using anti-His antibody



1-4:purified MAGE-A9(concentration of imidazole from 0.2 mol/L to 0.5 mol/L), 5:Marker, 6: the recombinant pBAD/g III -MAGE-A9 without L-arabinose induction, 7: the recombinant pBAD/g III -MAGE-A9 with L-arabinose induction

**Fig 4** Expression and purification of MAGE-A9 protein displayed by SDS-PAGE



1:The recombinant pBAD/g III -MAGE-A9 without L-arabinose induction, 2: The recombinant pBAD/g III -MAGE-A9 with L-arabinose induction, 3: Marker.

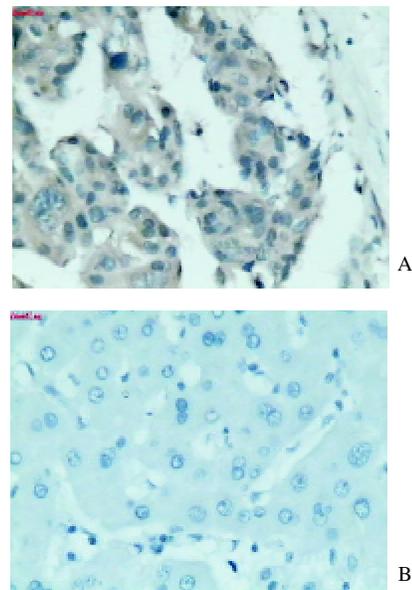
**Fig 5** Identification of anti-MAGE-A9 monoclonal antibody by using Western blot

### Expression of MAGE-A9 genes in hepatocellular carcinoma tissues

Intracellular detection of the MAGE-A9 gene product was attempted in hepatocellular carcinoma tissues, and a clear cytoplasmic reaction was obtained with anti-MAGE-A9 monoclonal antibody(**Fig 6**). Tissues of 39 hepatocellular carcinomas were analyzed for MAGE-A9 by immunohistochemistry. Among 39 hepatocellular carcinomas MAGE-A9 was expressed in 8 cases (21%).

### DISCUSSION

The development of therapeutic antibodies for use in the treatment of human diseases has long been a goal



A:Positively stained; B:Negatively stained(ABC method, × 400)

**Fig 6** Expression of MAGE-A9 protein in hepatocellular carcinoma

for many researchers in the antibody field. Since cytolytic T lymphocytes on a human melanoma cell line were first manipulated by the gene transfection approach to identify the human melanoma antigen-1, (MAGE-A1) gene<sup>[1]</sup>, 11 related MAGE-A genes have since been cloned and characterized. Peptides encoded by MAGE-A genes are strictly tumor-specific antigens<sup>[7-9]</sup> and can be recognized by CTL<sup>[10-12]</sup>, so they are treated as targets for diagnosis and therapy against tumors<sup>[13,14]</sup>. Several immunotherapeutic approaches using MAGE-A proteins or relevant antigenic peptides have been developed, and successful results have been brought to light on a phase I clinical vaccination trial in melanoma patients, using dendritic cells pulsed with antigenic peptides<sup>[15,16]</sup>.

The MAGE-A9 gene is among the MAGE-A family, some genes of which have been highlighted as excellent candidates for cancer vaccine or tumor therapy. Due to the high homology of MAGE-A9 and other MAGE-A family genes, MAGE-A9 may be a target for diagnosis and therapy against tumors. Like other MAGE-A genes, MAGE-A9 has been reported expressed in some malignant tumors such as cutaneous T-cell lymphoma (27%)<sup>[17]</sup>, and its overexpression was found in esophageal adenocarcinoma relative to Barrett's metaplasia on oligonucleotide microarrays<sup>[18]</sup>. However to date, there has been no data relating the expression of MAGE-A9 to hepatocellular carcinomas. So we analyzed the expression of MAGE-A9 in hepatocellular carcinomas and the results of this study may be useful for fundamental data on the expression status of MAGE-A9 gene,

and for the development of antigen-specific immunotherapy of hepatocellular carcinomas.

In this work, we not only extracted the total RNA of MAGE-A9 gene from hepatocellular carcinoma tissue, but cloned and expressed the MAGE-A9 gene. After we produced the specific monoclonal antibody, capable of identifying and localizing intracellularly the MAGE-A9 gene product, we analyzed the expression of MAGE-A9 genes and the presence of their protein products in tissues of 39 hepatocellular carcinomas from patients using immunohistochemical analyses. The basis for the expression of MAGE-A9 in hepatocellular carcinoma is not clear, but has important implications for tumor-specific targeting of hepatocellular carcinoma using monoclonal antibodies.

Several groups have already initiated clinical studies targeting MAGE-A antigens in hepatocellular carcinoma, based on the presence of RNA transcripts for these antigens in tumor cells<sup>[19-21]</sup>. However, for these antigens there are discrepancies between mRNA and protein levels determined by expression analyses, and for immunotherapy, knowledge of the actual antigen presence is essential. Our data suggests that the level of antigenic protein in this tumor may be variable and may affect the results of such approaches. Our study documents the expression of MAGE-A9 antigen in hepatocellular carcinoma at the level of the antigenic protein. The results indicate that the MAGE-A9 gene is expressed in the cytoplasm of positive hepatocellular carcinoma cells and the expressed frequency was 21%(8/39). Although further studies are needed to reveal the function of MAGE-A9 protein, the results suggest that this antigen maybe a useful target for immno-therapy of hepatocellular carcinoma. Identification of additional tumor-specific antigens is warranted to develop widely applicable poly-specific hepatocellular carcinoma vaccines.

In summary, we were successful in finding the expression of the MAGE-A9 gene in hepatocellular carcinoma tissues. However further studies are needed to reveal the function of MAGE-A9 protein, the type of tissue, the cell lines which express MAGE-A9 gene, and the potential of the MAGE-A9 protein to be used as a tumor vaccine in hepatocellular carcinoma.

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