

Cancer-testis antigen HCA587/MAGEC2 interacts with the general transcription coactivator TAF9 in cancer cells

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Abstract. Hepatocellular carcinoma-associated antigen 587/melanoma antigen gene (HCA587/MAGEC2) is a cancer-testis antigen, which is highly expressed in various types of tumors, but not in normal tissues with the exception of male germ-line cells. HCA587/MAGEC2 has been previously recognized as a tumor-specific target for immunotherapy; however, its biological functions have been relatively understudied. To investigate the function of HCA587/MAGEC2, the amino acid sequence of HCA587/MAGEC2 was analyzed by bioinformatics and it was demonstrated that HCA587/MAGEC2 contains a 9-amino acid transactivation domain which may mediate the interaction of most transcription factors with TATA-box binding protein associated factor 9 (TAF9), a general transcription coactivator. Co-immunoprecipitation experiments revealed that HCA587/MAGEC2 interacted with TAF9 in transfected 293T and in A375 melanoma cells endogenously expressing HCA587/MAGEC2, and confirmed the endogenous interaction of HCA587/MAGEC2 and TAF9 within cells. Endogenous HCA587/MAGEC2 and TAF9 were demonstrated to be co-localized principally in the nucleus of tumor cells using immunofluorescence. Glutathione-S-transferase pull-down experiments demonstrated that HCA587/MAGEC2 interacts with TAF9 directly and the conserved region in the TAF9 may be crucial for HCA587/MAGEC2 binding. The present study demonstrated that the cancer-testis antigen HCA587/MAGEC2 directly interacted with TAF9, which may provide novel information for identifying the oncogenic functions of HCA587/MAGEC2 in tumor cells.

Introduction

Hepatocellular carcinoma-associated antigen 587 (HCA587) was identified by serological analysis of a recombinant cDNA expression library from hepatocellular carcinoma (HCC) in our previous study (1) and the sequence of the HCA587 gene was identical to that of MAGEC2, a member of melanoma antigen gene (MAGE) family (2). The MAGE family of proteins is divided into two classes based on their expression pattern, the type I MAGEs include MAGEA, MAGEB and MAGEC protein subfamilies. The type I MAGE proteins are cancer-testis (CT) antigens and their expression is restricted to the undifferentiated spermatogenic cells or trophoblast lineage cells; however, they may be aberrantly expressed in various types of human cancer (3,4). The type II MAGEs, such as MAGE-D, MAGE-E, MAGE-F and MAGE-G are expressed in various normal tissues (5). Type I and type II MAGE proteins share a conserved domain termed the MAGE homology domain (4).

Type I MAGE CT antigens have long been considered to be potential immunotherapy targets due to their restricted tissue expression (6-10) and the phase I/II trials of MAGE-based immunotherapy exhibited encouraging results (11-14). Previous studies have demonstrated that high MAGE expressions are associated with a poor clinical prognosis, increased tumor growth and metastases (15-19). It has been previously reported that the nuclear expression of MAGEC2 in prostate cancer is an independent predictor of recurrence following radical prostatectomy (20). In addition, multiple MAGE family proteins have been demonstrated to form complexes with certain RING domain proteins with specificity in cells, including MAGE-A2/C2-transcription intermediary factor 1- β , MAGE-B18-ligand of numb protein x1 and MAGE-G1-non-structural maintenance of chromosomes element 1 complexes (21). A recent study demonstrated that high expression of MAGEC2 in cancer cells is associated with amoeboid movement and increased tumor metastasis by interacting with signal transducer and activator of transcription 3 (STAT3) and inhibiting proteasomal degradation of tyrosine phosphorylated STAT3 (22). To further investigate the biological function of CT antigen HCA587/MAGEC2 within tumor cells, the amino acid sequence of HCA587/MAGEC2 was analyzed by bioinformatics and the results indicated that HCA587/MAGEC2 contains a 9-amino acid transactivation domain (9aa TAD), which is common to the transactivation domains of a number of

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diverse yeast and animal transcription factors. It has been previously reported that the majority of transcription factors (VP16, P53, heat shock factor protein 1, nuclear factor- κ B, NFAT1) interact with the general transcription coactivator TAF9 via the 9aa TAD (23,24). Interaction of HCA587/MAGEC2 and TAF9 was demonstrated by co-immunoprecipitation and Glutathione-S-Transferase (GST) pull-down, and the conserved region (CR) in the TAF9 was determined to be required for the interaction of HCA587/MAGEC2 with TAF9. Identification of HCA587/MAGEC2 protein interactions may further elucidate the function and regulation of HCA587/MAGEC2.

Materials and methods

Gene constructs. The cDNA encodings of HCA587/MAGEC2 and TAF9 were generated by reverse-transcription polymerase chain reaction (RT-PCR) using cDNA from A375 human malignant melanoma cells (ATCC, Manassas, VA, USA) as templates. Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and first strand cDNA was generated using the Reverse Transcription kit (Promega Corporation, Madison, WI, USA). Primer sequences were forward, 5'-GCGTCGACGCCACCATGCCTCCCGTTCCAGGCGTT-3' and reverse, 5'-GAATGCGGC CGTCACTCAGAAAAGGAGAC for HCA587/MAGEC2; forward, 5'-GCGTCGACAATGGAGTCTGGCAAGACG GCT-3' and reverse, 5'-ATAAGAATGCGGCCGCTTACA GATTATCATAGTCAT-3' for TAF9. The PCR conditions for both HCA587/MAGEC2 and TAF9 were 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 90 sec. The aforementioned cDNA was subcloned into expression vectors pRK-FLAG and/or pRK-hemagglutinin (HA) (provided by Professor Jun Zhang, Peking University), and the constructed plasmids were transfected into mammalian cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for expression of HCA587/MAGEC2 and TAF9 respectively. cDNA encoding TAF9 (residues 1-73), TAF9 (residues 74-147), TAF9 (residues 148-278) were generated by PCR (M3001; Promega Corporation) using TAF9 full-length cDNA plasmids as templates. Primer sequences were forward, 5'-GCGTCGACAGAGTCTGGCAAGACGGCTTCT-3' and reverse, 5'-ATAAGAATGCGGCCGCTCATGCCAATCGCA CATCATCT-3' for TAF9 (residues 1-73); forward, 5'-GCGTCG ACAATCCAGTGCCGCGCTGATCA-3' and reverse, ATA AGAATGCGGCCGCTCACCGCGGGACTGTTATTC for TAF9 (residues 74-147); forward, 5'-GCGTCGACATTAAGT GTTGGTTTACAGTTAC-3' and reverse, 5'-ATAAGAATGCGG CCGCTTACAGATTATCATAGTCAT-3' for TAF9 (residues 148-278). The PCR conditions for all the fragments were 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. For the glutathione S-transferase (GST)-fusion protein expression, cDNAs encoding full-length and truncated TAF9 were ligated into PGEX-4T vector (GE Healthcare, Chicago, IL, USA).

Co-immunoprecipitation and western blotting. 293T cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). The expression vectors pRK-FLAG-HCA587 and/or pRK-HA-TAF9

(1 μ g/ml respectively) were co-transfected into 293T cells, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were harvested 48 h following transfection, washed with PBS, lysed with IP-buffer (20 mM Tris-HCl, 150 mM NaCl, 1% TritonX-100 and 1 mM EDTA) supplemented with a protease inhibitors cocktail (Roche Diagnostics, Indianapolis, IN, USA), and the protein concentration of the cell extract was determined by BCA protein assay. Lysates were cleared by centrifugation at 16,000 \times g for 10 min at 4°C, pre-cleared with empty beads and subjected to immunoprecipitation with mouse monoclonal anti-HA antibody (M180-3; 1:2,000; MBL, Nagoya, Japan), mouse monoclonal anti-FLAG antibody (F1804; 1:5,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or an equal amount of normal mouse immunoglobulin (Ig)G (I5381; Sigma-Aldrich; Merck KGaA) at 4°C overnight, followed by adding protein A-Sepharose (GE Healthcare) for additional 4 h. For endogenous co-immunoprecipitation, A375 cells were lysed with IP-buffer and protein concentration was determined as described above, and subjected to immunoprecipitation with rabbit polyclonal anti-HCA587 antibody (TC-1; 1:200; 2 mg/ml prepared by our laboratory) (25) or normal rabbit IgG (I5006; 1:200; 2 mg/ml; Sigma-Aldrich; Merck KGaA). The immunoprecipitates were subsequently washed with IP-buffer for 1 h at 4°C. Complexes were released from the protein-A-sepharose by boiling for 5 min in 2X SDS loading buffer, subjected to 10% SDS-PAGE (50 μ l sample/lane), transferred to NC membranes, blocked with PBS-5% skimmed milk at room temperature for 1 h, and detected using anti-HA (M180-3; 1:2,000), anti-FLAG (F1804; 1:5,000), anti-HCA587 (McAb, LX-CT10.5; 1:1,000; provided by Professor Boquan Jin, Fourth Military Medical University) (26), or anti-TAF9 (ab169784; 1:1,000; Abcam, Cambridge, MA, USA) antibodies, followed by adding anti-mouse (W4021; 1:8,000; Promega Corporation) or anti-rabbit (W4011; 1:10,000; Promega Corporation) antibodies conjugated to horseradish peroxidase. Immunoreactive bands were analyzed with an enhanced chemiluminescence kit (BF06053; Beijing Biodragon Immunotechnologies Co., Ltd., Beijing, China.)

Immunofluorescence. A375 cells were grown directly on glass coverslips for 24 h and then fixed with 3.7% formaldehyde in PBS for 5 min at room temperature, and further permeabilized by 100% methanol for 5 min at -20°C. Following 1 h blocking in PBS-5% skimmed milk at room temperature, cells were incubated overnight with the following antibodies: Anti-HCA587/MAGEC2 mouse IgG (LX-CT10.5; 1:500), anti-TAF9 rabbit IgG (sc98825; 1:100; Santa Cruz Biotechnology, Inc.), normal mouse IgG or normal rabbit IgG (equal amount of antibodies) were used as negative controls. Following washing with PBS, cells were incubated for 1 h at room temperature with FITC-conjugated anti-rabbit IgG or rhodamine-conjugated anti-mouse IgG (ZF0311 and ZF0313 respectively; 1:200; OriGene Technologies, Inc., Beijing, China). Hoechst 33342 (Sigma-Aldrich; Merck KGaA) staining was performed for 5 min at room temperature to visualize the cell nucleus. Images were captured and analyzed using a confocal microscope.

GST pull-down assay. Recombinant human HCA587/MAGEC2 protein was prepared by Crown Bioscience, Inc., (Beijing,

| | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 | C11 | C12 | |
|---------|----|----|----|----|----|----|----|----|----|-----|-----|-----|--------------------------|
| 135 143 | + | - | + | - | + | + | + | + | + | + | + | + | Wrong match-SSFTYTLDE |
| 139 147 | - | + | + | - | + | + | + | + | + | + | + | + | Poor match-YTLDEKVAE |
| 147 155 | + | + | + | | + | - | + | + | + | + | + | + | Wrong match-ELVEFLLK |
| 166 174 | + | + | - | | + | - | + | + | + | + | + | + | Wrong match-EMLMIVIKY |
| 227 235 | + | + | - | | - | - | + | + | + | + | + | + | Wrong match-NSLLIILS |
| 228 236 | + | + | - | | - | - | + | + | + | + | + | + | Wrong match-SLLIILSV |
| 246 254 | + | + | + | + | + | + | + | + | + | + | + | + | Perfect match-EEVI WEVLN |
| 247 255 | + | + | + | + | + | + | + | + | + | + | + | + | Perfect match-EVI WEVLNA |
| 272 280 | + | + | + | + | + | + | + | + | + | + | + | + | Perfect match-ELLTKVWVQ |
| 363 371 | + | - | + | - | - | + | - | + | + | + | + | + | Wrong match-SVMSSNVSF |

Figure 1. Identification of 9aa transactivation domain in hepatocellular carcinoma-associated antigen 587/melanoma antigen gene, C1-C12: Refinement criteria for 9aa transactivation domain prediction (24). Amino acids of the same group are allowed as substitutions. aa, amino acids.

China) (27). GST-TAF9 (full-length), TAF9 (residues 1-73), TAF9 (residues 74-147) and TAF9 (residues 148-278) were expressed in bacterial host BL21(DE3) by induction with 1 mM isopropyl-L-thio- β -D-galactopyranoside (Sigma-Aldrich; Merck KGaA), and were purified with glutathione Sepharose 4B beads (GE Healthcare) according to the instructions of the manufacturer. A total of 10 μ g glutathione Sepharose-linked GST or GST-fusion protein was incubated with the HCA587/MAGEC2 protein in 200 μ l GST binding buffer (PBS, 0.1% NP-40, 5 mM dithiothreitol and protease inhibitors cocktail) with rotation overnight at 4°C. The beads were then washed for 1 h at 4°C and bound proteins were separated by 10% SDS-PAGE (30 μ l sample/lane), transferred to NC membranes, blocked with PBS-5% skimmed milk at room temperature for 1 h, and detected with anti-HCA587 (LX-CT10.5; 1:1,000), anti-GST (1:2,000; 10000-0-AP; ProteinTech Group, Inc., Chicago, IL, USA), or anti-TAF9 (sc98825; 1:1,000; Santa Cruz Biotechnology, Inc.) antibodies, followed by adding anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (W4021 and W4011; Promega Corporation) at room temperature for 1 h. Immunoreactive bands were analyzed with enhanced chemiluminescence kit as described above.

Results

HCA587/MAGEC2 contains a 9aa TAD. To determine the biological function of HCA587/MAGEC2, the aa sequence of HCA587/MAGEC2 was analyzed by bioinformatics. Based on the prediction algorithm provided by Piskacek *et al* (<http://www.at.embnet.org/9aa>) (24), the present study demonstrated that HCA587/MAGEC2 contains 3 perfectly matched 9aa TAD sequences which are located at aa 246-254, 247-255 and 272-280, respectively. The results of the 9aa TAD analysis are presented in Fig. 1.

Interaction of HCA587/MAGEC2 with TAF9 in transfected 293T cells. Most proteins with a 9aa TAD are known to bind the general transcriptional cofactor TAF9. The presence of the 9aa TAD sequence in HCA587/MAGEC2 led to the investigation of whether HCA587/MAGEC2 interacts with TAF9. 293T cells were co-transfected with FLAG-tagged HCA587/MAGEC2 and HA-tagged TAF9, and the cellular

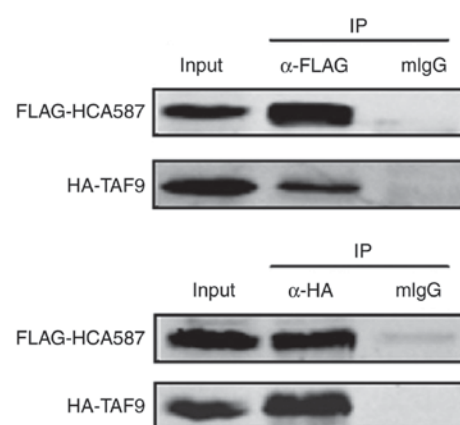


Figure 2. Co-immunoprecipitation assay for the interaction between HCA587/MAGEC2 and TAF9. Plasmids encoding FLAG-HCA587/MAGEC2 and HA-TAF9 proteins were co-transfected in 293T cells. The cell lysates are immunoprecipitated with anti-FLAG mAb, anti-HA mAb or mouse control IgG. The precipitates were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies. The result demonstrated that overexpressed HCA587/MAGEC2 and TAF9 may interact with each other. Input corresponds to 10% of the total cell lysate. TAF9, TATA-box binding protein associated factor 9; HCA587/MAGEC2, hepatocellular carcinoma-associated antigen 587/melanoma antigen gene; mAb, monoclonal antibody; HA, hemagglutinin; mIgG, mouse immunoglobulin G.

lysates were immunoprecipitated with anti-FLAG or anti-HA monoclonal antibodies followed by immunoblotting with anti-HA or anti-FLAG antibodies. The results demonstrated that HCA587/MAGEC2 was co-immunoprecipitated with TAF9 (Fig. 2), suggesting that HCA587/MAGEC2 interacts with TAF9 in transfected 293T cells.

Subcellular co-localization of HCA587/MAGEC2 and TAF9. The subcellular co-localization of endogenous HCA587/MAGEC2 and TAF9 was examined in A375 human melanoma cell line expressing endogenous TAF9 and HCA587/MAGEC2 using double immunofluorescence staining. As presented in Fig. 3, HCA587/MAGEC2 and TAF9 were co-localized in the nucleus of A375 cells.

Interaction of HCA587/MAGEC2 and TAF9 in vivo. To confirm the endogenous interaction of HCA587/MAGEC2 with TAF9 within the tumor cells, a co-immunoprecipitation

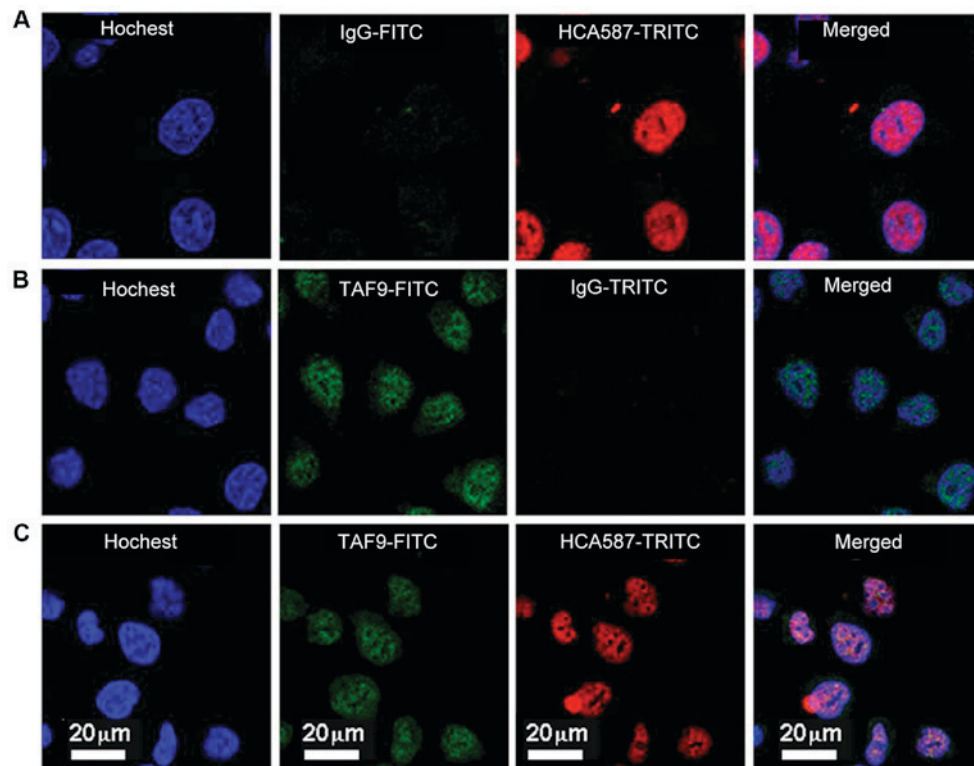


Figure 3. HCA587/MAGEC2 co-localizes with TAF9 to the nucleus of A375 cells. (A) The cellular distribution of endogenous HCA587/MAGEC2 in A375 cells detected by immunofluorescence using anti-HCA587 antibody. (B) The cellular distribution of endogenous TAF9 in A375 cells. (C) The cellular distribution of endogenous HCA587/MAGEC2 and TAF9 in A375 cells were analyzed using anti-HCA587/MAGEC2 and anti-TAF9 antibodies and visualized using FITC and TRITC labeled secondary antibodies, respectively. The merged image demonstrates the signals of anti-HCA587/MAGEC2 (red), anti-TAF9 (green) and Hoechst for DNA staining (blue) altogether. TAF9, TATA-box binding protein associated factor 9; HCA587/MAGEC2, hepatocellular carcinoma-associated antigen 587/melanoma antigen gene; TRITC, tetramethylrhodamine; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

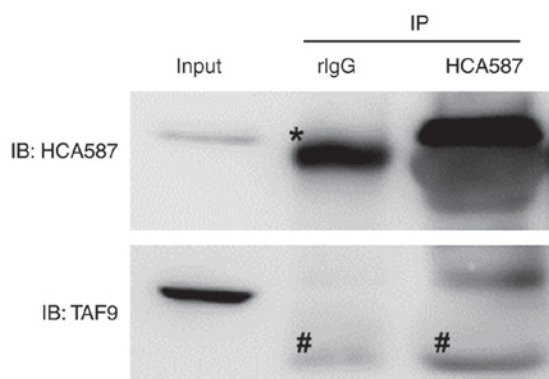


Figure 4. The endogenous interaction of HCA587/MAGEC2 with TAF9 in A375 cells was detected by co-immunoprecipitation assay. Lysates from A375 cells were immunoprecipitated with anti-HCA587 antibody or control rabbit IgG followed by immunoblotting with anti-HCA587 or anti-TAF9 antibodies. *Indicates Ig heavy chain, #indicates Ig light chain. Input corresponds to 10% of the total cell lysate. TAF9, TATA-box binding protein associated factor 9; HCA587/MAGEC2, hepatocellular carcinoma-associated antigen 587/melanoma antigen gene; rIgG, rabbit immunoglobulin G.

assay was performed in the A375 human melanoma cell line, which endogenously expresses HCA587/MAGEC2 and TAF9 molecules. Cellular lysates from A375 cells were immunoprecipitated with the anti-HCA587/MAGEC2 antibody and TAF9 was detected in HCA587/MAGEC2 immunoprecipitates (Fig. 4), indicating that endogenous HCA587/MAGEC2 interacts with endogenous TAF9 within tumor cells.

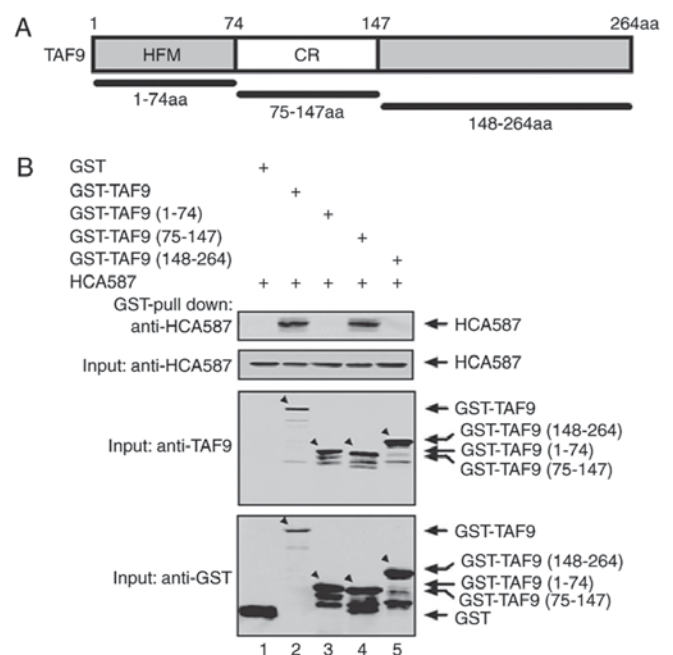


Figure 5. HCA587/MAGEC2 interacts with the CR domain of TAF9 *in vitro*. (A) Domain structure of the full-length TAF9. (B) GST pull-down assay demonstrated that HCA587/MAGEC2 bound specifically to full-length TAF9 and TAF9 CR (aa 75-147). Input lane represents 10% of the proteins used for the pulldown assay. The arrows indicate the band positions of TAF9 full-length and TAF9 truncated mutants. TAF9, TATA-box binding protein associated factor 9; HCA587/MAGEC2, hepatocellular carcinoma-associated antigen 587/melanoma antigen gene; GST, glutathione-S-transferase; CR, conserved region; aa, amino acids; HFM, histone fold homology.

In vitro mapping of the interactive domains between HCA587/MAGEC2 and TAF9. *In vitro* GST pull-down assays were used to confirm the interaction between HCA587/MAGEC2 and TAF9, and to determine the respective interactive domains. To map the interactive domains of TAF9 responsible for its HCA587/MAGEC2 binding, a series of GST-TAF9 truncated mutants, the histone fold homology domain (HFD) GST-TAF9 (residues 1-74), the conserved region (CR) TAF9 (residues 75-147) and the C terminal region TAF9 (residues 148-278), were generated and used as bait in respective pull-down assays with HCA587/MAGEC2 (Fig. 5A). An interaction was observed between HCA587/MAGEC2 and TAF9 (full-length; Fig. 5B; lane 2); however, not with GST alone (Fig. 5B; lane 1). Only the CR of TAF9 [GST-TAF9(75-147)] was able to bind HCA587/MAGEC2 effectively (Fig. 5B; lane 4). GST-TAF9 (residues 1-74) and GST-TAF9 (residues 148-278) demonstrated no binding to HCA587/MAGEC2 (Fig. 5B; lanes 3 and 5). These results demonstrated that TAF9 directly binds HCA587/MAGEC2 through its CR domain.

Discussion

HCA587/MAGEC2 is a CT antigen expressed in a wide variety of malignancies, including hepatocellular carcinoma, melanoma, bladder cancer, head and neck cancer, breast cancer and lung cancer (25,28). Previous studies focused primarily on identifying the potential of HCA587/MAGEC2 as a target for tumor immunotherapy. HCA587/MAGEC2 has been previously demonstrated to be immunogenic and induce spontaneous antibody and T cell immune responses in cancer patients with HCA587/MAGEC2-expressing tumors (28-30). To the best of our knowledge, 9 CTL and 4 Th epitopes have been identified by the present authors and others (30-32). Previous studies demonstrated that MAGEC2 expression in cancer cells correlated with their metastatic potential (18,22,33,34).

In the present study, TAF9 was identified as a binding partner for HCA587/MAGEC2. TAF9 and HCA587/MAGEC2 were co-localized in the nucleus and were co-immunoprecipitated from transfected 293T cells. In addition, the endogenous interaction of HCA587/MAGEC2 with TAF9 within the tumor cells was demonstrated by co-immunoprecipitation assay. TAF9 is a TATA-binding protein associated factor shared by two transcription co-activator complexes, TFIID and Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (35). A genomic synthetic genetic array analysis using a temperature-sensitive allele of *TAF9* indicated that *TAF9* interacts genetically with >100 genes, including genes involved in the mediator complex, chromatin modification/remodeling complexes, regulators of transcription elongation and key genes for cell cycle control at the G1/S transition, as well as genes involved in cell polarity, cell integrity and protein synthesis (36). Synthetic interactions usually identify a functional connection between the genes involved. The genetic interactions of TAF9 revealed a significant functional association of TAF9 with regulators of transcription elongation, initiation, cell cycle progression and growth control. It was previously demonstrated by microarray analysis that TAF9 is required for the expression of ~60% of the yeast genome (37). TAF9 is important because it is present in both

TFIID and SAGA and it is one of several histone-fold TAFs critical for the maintenance of the structural integrity of these complexes (38-40). However, the biological functions of TAF9 in tumorigenesis and development of cancer remain to be elucidated. Nault *et al* (41) reported that upregulated expression of TAF9 was associated with a poor prognosis for patients with hepatocellular carcinoma treated by resection. In addition, TAF9 was demonstrated to interact with Gli and regulated Gli activity, and treatment with an inhibitor that interferes with the Gli/TAF9 interaction suppresses tumor growth (42). In the present study, the binding of TAF9 with HCA587/MAGEC2 was demonstrated and HCA587/MAGEC2 was previously reported to promote tumor metastasis by binding with STAT3 (22); however, whether the interaction of TAF9 with HCA587/MAGEC2 is also associated with tumor metastasis requires further investigation.

TAF9 contains several domains: A highly conserved N-terminal HFD, important for interaction with the H4-like TAF6, another conserved region, with DNA binding activity and a long less-conserved C-terminal tail (43). The findings of the present study demonstrate that TAF9 directly binds HCA587/MAGEC2 through its CR domain, suggesting that HCA587/MAGEC2 may involve in TAF9 binding with DNA to regulate certain genes transcription.

The present study has provided considerable evidence that HCA587/MAGEC2 interacts with TAF9 in melanoma cells, further studies on the interaction between HCA587/MAGEC2 and TAF9 may potentially provide additional information on the role of HCA587/MAGEC2, or TAF9, in the development and progression of cancer.

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