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Cancer–testis antigen HCA587/MAGE-C2 interacts with BS69 and promotes its degradation in the ubiquitin–proteasome pathway

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ABSTRACT

HCA587, also known as MAGE-C2, belonging to the MAGE gene family which is characterized by a conserved MAGE Homology Domain, is active in various types of tumors and silent in normal tissues except in male germ-line cells. The biological function of HCA587 is largely unknown. To analyze it, we attempted to identify protein partners of HCA587. We immunopurified HCA587-containing complex from HEK293 cells and identified BS69, a potential tumor suppressor, as an associated protein by mass spectrometry, and the following Immunoprecipitation and GST pull-down assays confirmed HCA587 interaction with BS69. Interestingly, overexpression of HCA587 promoted ubiquitination and the proteasomal degradation of BS69 whereas knockdown of endogenous HCA587 increased the protein level of BS69. Consistent with a functional role for BS69 in negatively regulating LMP1-induced NF-κB activation, overexpression of HCA587 resulted in a significant enhancement of LMP1-induced IL-6 production. These data indicate that HCA587 is a new negative regulator of BS69.

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1. Introduction

HCA587 was firstly identified by serological analysis of recombinant cDNA expression library from hepatocellular carcinoma (HCC) in our laboratory [1]. The sequence of the HCA587 gene was identical with that of MAGE-C2, a member of type I MAGE family of cancer–testis (CT) antigens [2]. HCA587/MAGE-C2 is highly expressed in tumors of various histological types, including hepatocellular carcinoma, melanoma, sarcoma, lung cancer, bladder cancer and breast cancer, but it is silent in normal adult tissues except in male germ-line cells [2–4]. HCA587/MAGE-C2 is an ideal target for cancer immunotherapies due to its specific expression, and extensive efforts have gone into identifying CTL epitopes and developing cancer vaccines [5–8].

Recent studies on the biological function of type I MAGE CT antigens revealed that they play an active role in tumorigenesis and cancer cell viability [9,10]. And more recently, Doyle et al. reported that multiple MAGE family proteins could bind really interesting new gene (RING) domain proteins with specificity, and they enhance p53 degradation in an ubiquitin–proteasome dependent pathway [11].

To investigate further the function of HCA587, we attempted to search for novel binding partners of HCA587 utilizing the approach of immunoprecipitation (IP) coupled with mass spectrometry (MS). Using this approach, we identified BS69 as a binding partner of HCA587. BS69 was originally identified as an adenoviral early region 1A (E1A)-interacting protein and shown to inhibit the E1A-mediated transcription [12]. Further analysis indicated that BS69 is a potential tumor suppressor that is frequently deleted in human cancers and leukemia and that represses transcription through proto-oncogene products [13,14]. Recent evidence indicated that BS69 interacts with the EBV-encoded latent membrane protein 1 (LMP1) and negatively regulates both canonical and non-canonical NF-κB activation mediated by LMP1 [15,16]. LMP1 is well known to be an oncogenic protein, which is expressed in many EBV-associated tumor cells and is responsible for most of the altered cellular growth properties. Here we report that we identified BS69 as a HCA587-binding protein and investigated the functional significance of interaction between HCA587 and BS69.

2. Materials and methods

2.1. Constructs and antibodies

The coding sequence of human HCA587 was amplified using PCR and subcloned into the SalI/NotI sites on the pRK-FLAG and

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pRK-HA vectors. The plasmid pcDNA3-FLAG-BS69 was kindly provided by Zhenguo Wu (Hong Kong University of Science and Technology, HKUST), and we subcloned it into the SalI/NotI sites on pRK-HA and pGEX-4T-2 vectors. The expression vectors of LMP1 and pCMV-(HA-Ub)$_4$ were obtained from Jun Wan (ShenZhen-PKU-HKUST Medical Center) and Yue Xiong (University of North Carolina), respectively.

The anti-HCA587 mAb (clone LX-CT10.5) [17] was provided by Professor Boquan Jin (Forth Military Medical University, Xi'an, China). The anti-HCA587 polyclonal antibody was prepared by our lab. Antibodies specific for GAPDH, FLAG and HA were obtained from MBL (Nagoya, Japan). Anti-BS69 antibody was from Santa Cruz. Antibodies specific for LMP1 and GST were purchased from Proteintech (Chicago, IL). Nonspecific mouse or rabbit IgG (Sigma) was used as a control for immunoprecipitation. Goat anti-Mouse/Rabbit IgG-HRP was obtained from Promega (Madison, WI), and IRDye 800CW Goat anti-Mouse/Rabbit IgG was purchased from Li-Cor Biosciences (Lincoln, NE).

2.2. Cell culture, transfections, and siRNAs

HEK293, HeLa, and A375 cells were maintained in DMEM supplemented with 10% fetal bovine serum. HEK293 cells were transfected using VigoFect (Vigorous Biotechnology), and HeLa or A375 cells were transfected using Lipofectamin 2000 (Invitrogen) according to the manufacturer's recommendations. The siHCA587#1 (5′-CAAUUGAUACCGCAGAUGATT-3′), siHCA587#2 (5′-GGAAAUACCUUCUCCCUUATT-3′), siBS69 (5′-GAAGUUAUGAAGAGUUCAATT-3′), and non-specific control (siNC, 5′-UUCUC CGAACGUGUCACGUTT-3′) were synthesized at Guangzhou RiboBio Co. Ltd. (Shanghai, China).

2.3. Immunoprecipitation (IP) coupled with mass spectrometry

Immunoprecipitation was performed with FLAG Tagged Protein Immunoprecipitation Kit (Sigma) using HEK293 cells transfected with FLAG-tagged HCA587 following the manufacturer's instructions. Briefly, FLAG-HCA587-transfected HEK293 cells were lysed, bound to anti-FLAG-M2-Agarose Affinity Gel, eluted with 3× FLAG peptide, separated by SDS–PAGE, and stained with silver. Protein bands of interest were excised, in-gel proteolyzed, and identified by MALDI-TOF-MS.

2.4. Quantitative reverse transcription-PCR analysis

Total RNA was isolated from cultured cells using the TRIzol reagent (Invitrogen), and subjected to reverse transcription with Oligo (dT) and M-MLV Reverse Transcriptase (Promega). The resulting cDNA was subjected to real-time PCR using GoTaq qPCR Master Mix kit (Promega) and Illumina Eco Real-time PCR system. Primer sequences were 5′-TGAGCAAGCTGACATTGCGAGG-3′ and 5′-TGGTGGTGGCCAAAGAAGCG-3′ for BS69, 5′-GGCCCTGAGGAAGAACTGAG-3′ and 5′-TGAGATCCAACAGGCCTTGAC-3′ for HCA587, 5′-CTCCACAAGCGCCTTCGGTC-3′ and 5′-AGGGCTGAGATGCCGTCGAG-3′ for IL6, and 5′-GACAGTCAGCCGCATCTTCTT-3′ and 5′-CCAATACGACCAAATCCGTTGAC-3′ for GAPDH. The abundance of transcripts of interest was normalized against that of GAPDH as an internal standard.

2.5. GST pull down assay

GST and GST-tagged BS69 proteins were expressed in bacterial host BL21 (DE3) by induction with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Bacteria were collected by centrifugation and successively lysed in a 100 mM NaCl, 20 mM Tris, pH 8.0, 1 mg/ml lysozyme, 1% TritonX-100 buffer supplemented with protease inhibitors, sonicated, and cleared by centrifugation. Supernatants were incubated with glutathione Sepharose 4B beads (GE) according to the instructions of the manufacturer. Approximately 10 μg glutathione Sepharose linked GST or GST-fusion protein was incubated with recombinant HCA587 protein prepared by Crown Bioscience, Inc [8] in 1 ml of GST binding buffer (10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 100 mM KCl, 5 mM EDTA, 5% glycerol) with rotation overnight at 4 °C. The beads were washed 5 times with cold PBS solution, and bound proteins were separated by SDS/PAGE and blotted with anti-HCA587 or anti-GST antibodies.

2.6. In vivo degradation assay

HEK293 cells were co-transfected with HA-HCA587 and HA-BS69. At 48 h post-transfection, cells were treated with DMSO or MG132 (20 μM) for 8 h before addition of cycloheximide (CHX: 50 μg/mL). Cell lysates were harvested at the indicated times after CHX treatment and levels of BS69 protein were analyzed by Western blotting. Band intensities were quantitated and each of them was normalized against individual level of GAPDH.

2.7. Immunoprecipitation (IP) and Western blotting

Protein lysates prepared with IP buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) TritonX-100 and 1 mM EDTA plus protease inhibitor mixture (Roche)] were pre-cleared with protein A-Sepharose beads (GE Healthcare) and then incubated with indicated antibody or control IgG overnight at 4 °C. Protein A-Sepharose beads were added to each samples and further incubated for 2 h, and the beads were washed 5 times with IP buffer plus 500 mM NaCl. Bound proteins were released from beads by boiling for 5 min in SDS–PAGE sample buffer followed by immunoblotting with appropriate antibodies. GAPDH antibody was used to verify equivalent total protein. Immunoreactive bands were visualized with enhanced chemiluminescence or infrared imaging working with Odyssey Imager (Li-Cor, Lincoln, NE).

2.8. Ubiquitination assay in vivo

HEK293 cells were co-transfected with expression constructs of HA-Ub, FLAG-BS69, and GFP-HCA587 or GFP. At 40 h post-transfection, cells were treated with MG132 (20 μM) for 8 h and then the cells were lysed in buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% (v/v) TritonX-100 and 1 mM EDTA plus protease inhibitor mixture]. The detergent-solubilized cell lysate was incubated with the anti-HA or anti-FLAG antibodies overnight followed by incubating with protein A-Sepharose beads for 2 h at 4 °C. The IP beads were washed five times and bound proteins were analyzed by immunoblotting.

2.9. Detection of IL-6 in the culture supernatants

Concentration of IL-6 in culture supernatants from siRNA and/or plasmid transfected HeLa cells were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Dakewe Biotechnology, Beijing, China).

2.10. Statistical analysis

Results are presented as the mean ± SD. Comparisons between groups were made by unpaired Student's t test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. HCA587 binds with BS69

In search of novel HCA587-associated proteins, lysates from HEK293 cells transfected with plasmid encoding FLAG-HCA587 were immunoprecipitated with anti-FLAG antibody, and bound proteins were eluted with FLAG peptide and resolved by SDS/PAGE. Mass spectrometric analysis identified the presence of BS69 and TRIM28 in the HCA587-containing protein complex (Fig. 1A). The interaction between HCA587 and TRIM28 has been reported previously [10,11]. To confirm an in vivo interaction between HCA587 and BS69, HEK293 cells were co-transfected with expression vectors encoding FLAG-HCA587 and HA-BS69 fusion proteins and then the whole cell lysates were immunoprecipitated with anti-FLAG, anti-HA antibodies or control mouse IgG. As shown in Fig. 1B, BS69 was detected in immunoprecipitates with the anti-FLAG antibody, but not control IgG. Alternatively, FLAG-HCA587 was detected in immunoprecipitates with the anti-HA antibody. Moreover, the interaction between HCA587 and BS69 is direct, as GST pull-down experiments revealed that recombinant HCA587 protein strongly binds to GST-BS69 (Fig. 1C and D). Taken together, these data indicate that HCA587 and BS69 interact in vivo and in vitro.

3.2. HCA587 regulates BS69 stability in cells

To determine the functional significance of the physical interaction between HCA587 and BS69, we examined the effect of HCA587 on the steady-state level of BS69 protein. Western blotting analysis of the cellular lysates of A375 cells transfected with HCA587 specific siRNAs using anti-BS69 antibody revealed that the level of endogenous BS69 protein was significantly increased (Fig. 2A). The increased BS69 protein expression in HCA587-depleted cells was not a result of BS69 mRNA synthesis, as real-time quantitative PCR measurements indicated that HCA587 knockdown did not result in an increased BS69 mRNA level in A375 cells (Fig. 2B). This result suggests that HCA587 may regulate the expression of BS69 at a post-transcriptional level. To examine whether HCA587 promotes proteasomal degradation of BS69 protein, we transfected HEK293 cells with the expression vector for BS69 with or without HCA587 and then the cells were incubated with a proteasome specific inhibitor, MG132. As shown in Fig. 2C, the HCA587-mediated decrease of BS69 protein was completely suppressed when MG132 was added. To further analyze the effect of HCA587 expression on BS69 turnover, we did a cycloheximide (CHX) assay. As shown in Fig. 2D, the results indicated that overexpression of HCA587 in HEK293 cells shortened the half-life of BS69 protein and this process was blocked by MG132

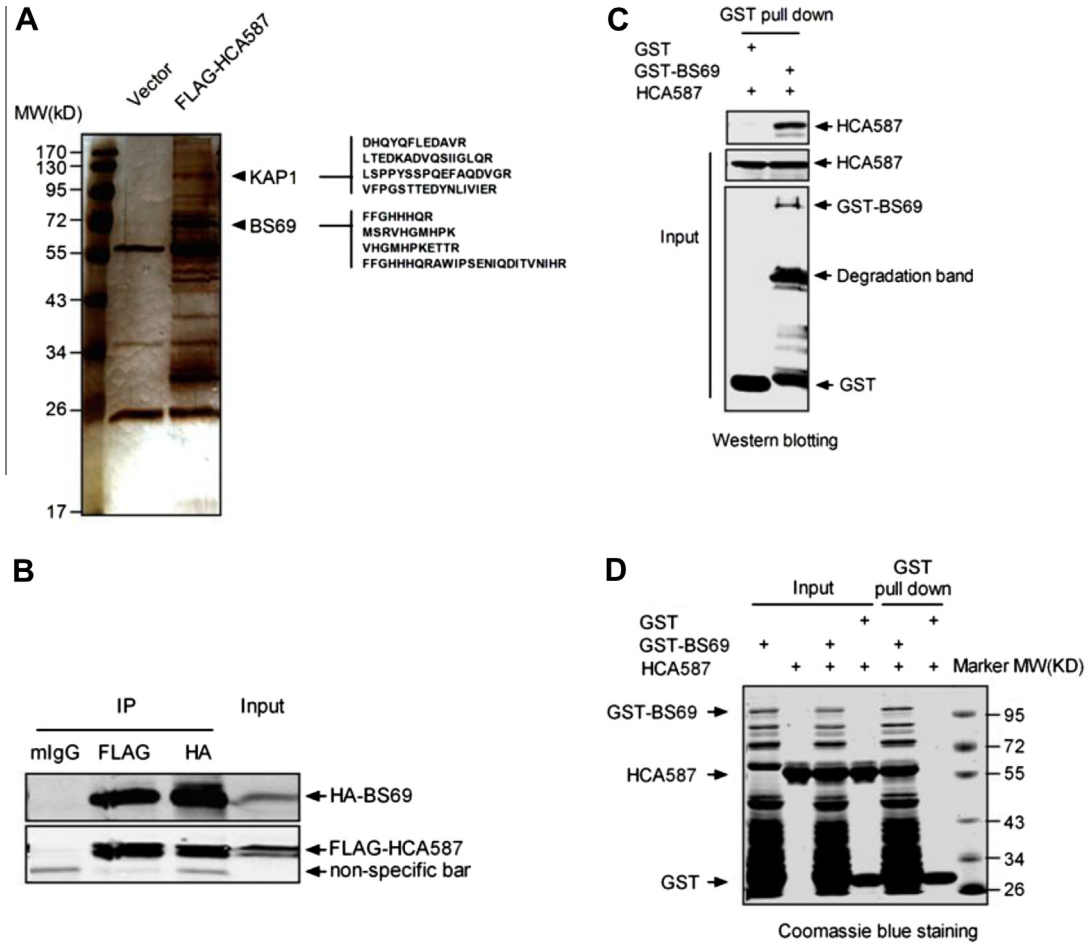

Fig. 1. HCA587 interacts directly with BS69. (A) HCA587 binds endogenous BS69 in vivo. Lysates from HEK293 cells transfected with pRK-FLAG-HCA587 were immunoprecipitated with anti-FLAG-M2-Agarose Affinity Gel. Bound proteins were eluted with 3× FLAG peptide, resolved on 10% SDS–PAGE with silver staining, and analyzed by mass spectrometry. The identified peptides are listed. (B) HCA587 is associated with BS69. Lysates from HEK293 cells co-transfected with FLAG-HCA587 and HA-BS69 were immunoprecipitated with anti-FLAG, anti-HA antibodies or control mouse IgG followed by immunoblotting with anti-HA or anti-FLAG antibodies. (C and D) HCA587 interacts with BS69 in vitro. GST-pull down experiment was performed with purified HCA587 protein and GST-fused BS69 or GST. Proteins were detected with Western blotting by anti-HCA587 or anti-GST antibodies (C) and Coomassie blue staining (D).

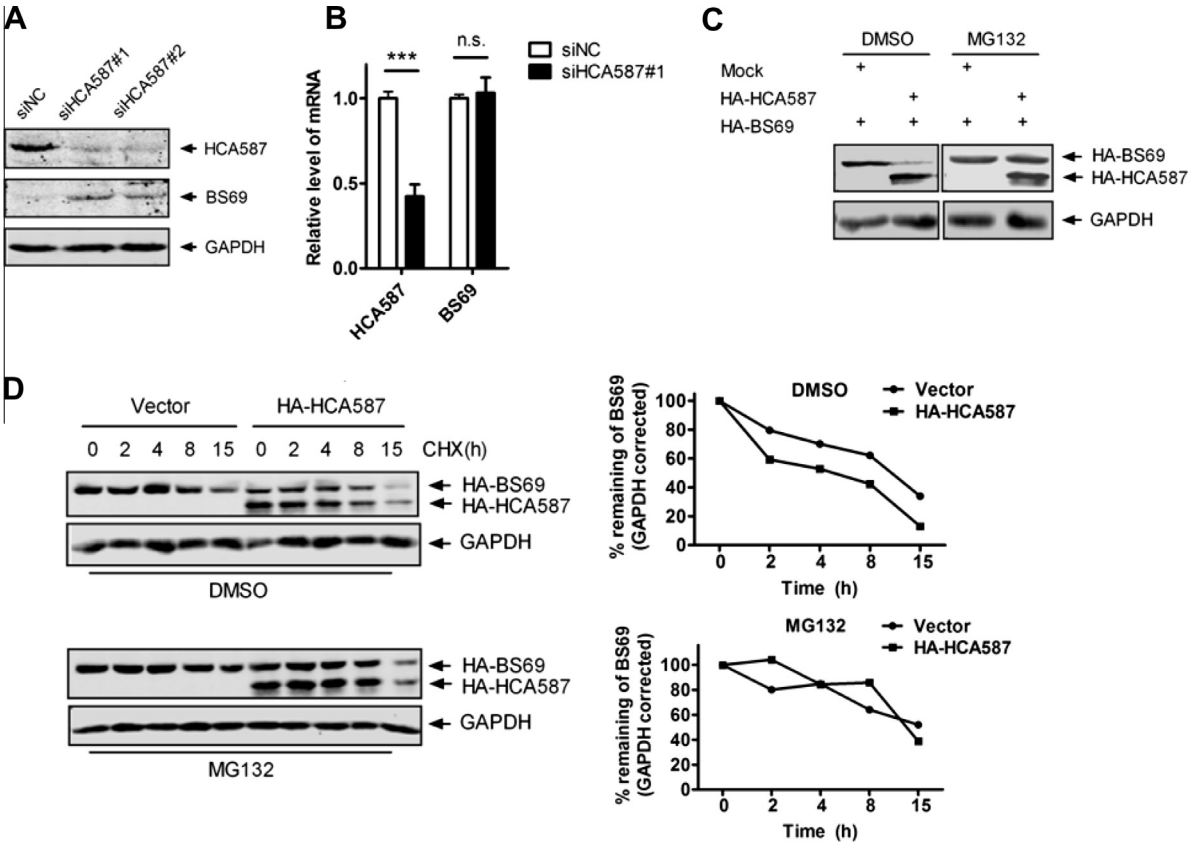

Fig. 2. HCA587 regulates BS69 stability. (A) Downregulation of HCA587 stabilizes BS69. A375 cells were transfected with a control siRNA (siNC) or HCA587-specific siRNAs (siHCA587#1 and siHCA587#2), and the levels of BS69 expression in the transfected cells were analyzed by immunoblotting. The expression of HCA587 was detected with anti-HCA587 mAb. (B) HCA587 does not alter BS69 mRNA levels. Total RNA extracted from above-described A375 cells were analyzed for BS69 and HCA587 expression by real-time quantitative PCR. The data were normalized to GAPDH. Each bar represents the mean ± SD for triplicate experiments. $^{***}P < 0.001$; n.s., $P > 0.05$. (C) HCA587 promotes proteasomal degradation of BS69 protein. HEK293 cells were co-transfected with HA-BS69 and HA-HCA587. Forty-eight hours after transfection, the cells were treated with vehicle (DMSO) or MG132 (20 μM) for 8 h before cells were collected for Western blotting analysis. (D) HCA587 decreases BS69 half-life. HEK293 cells were co-transfected with HA-BS69 and HA-HCA587 or vector. Forty-eight hours after transfection, cells were treated with vehicle (DMSO) or MG132 (20 μM) for 8 h before addition of cycloheximide (CHX: 50 μg/mL). Cells were then harvested for Western blotting analysis at the indicating time after CHX treatment. Quantitation was done by densitometry and expressed as signals of BS69/GAPDH.

treatment, supporting an involvement of the 26S-proteasome pathway. These findings strongly suggest that HCA587 regulates BS69 stability in proteasome-dependent manner.

3.3. HCA587 enhances BS69 ubiquitination

To support the mechanism of BS69 regulation by HCA587 in an ubiquitin–proteasome pathway, we next examined whether HCA587-mediated BS69 degradation is a consequence of BS69 ubiquitination. To this end, HEK293 cells were co-transfected with plasmids encoding FALG-BS69, HA-Ub, and GFP-HCA587 or GFP followed by treating with MG132. Immunoprecipitation of the cellular lysates with anti-FLAG antibody and immunoblotting with anti-HA antibody detected strong BS69 ubiquitination under expression of HCA587 (Fig. 3A). Reciprocal immunoprecipitation in HEK293 cells with anti-HA antibody and immunoblotting with anti-FLAG antibody detected increased levels of ubiquitinated species of BS69 under overexpression of HCA587 (Fig. 3B). These data suggest that HCA587 promotes ubiquitination of BS69 in vivo.

3.4. Biological relevance of HCA587-dependent regulation of BS69

Recent reports on exploring biological function of BS69 indicated that BS69 interacts with LMP1 and negatively regulates LMP1-mediated NF-κB activation [15,16]. To investigate the biological consequences of HCA587-mediated BS69 degradation, we analyzed the effects of HCA587 on the production of IL-6, a pro-inflammatory cytokine induced by NF-κB activation. As shown in Fig. 4, overexpression of HCA587 in HeLa cells resulted in a significant enhancement of LMP1-induced IL-6 mRNA expression and synthesis of IL-6 protein, which is consistent with knockdown of BS69 with BS69-specific siRNA, and further analysis revealed that BS69 is required for HCA587 to regulate LMP1-induced IL-6 production since HCA587 failed to do so in the absence of BS69. These data indicate that HCA587 can regulate LMP1-induced NF-κB activation by promoting the degradation of BS69.

4. Discussion

Cancer–testis antigen HCA587/MAGE-C2 has long been studied as a target for cancer immunotherapy because its expression is restricted to cancer and germ line cells and a proportion of cancer patients presents with immune response against HCA587/MAGE-C2 antigen [3,6,18]. Recently, HCA587/MAGE-C2 expression has been identified as a potent predictor of sentinel lymph node metastasis in primary melanoma and an independent predictor of recurrence in prostate cancer, however, the mechanism behind these association have not been completely determined [19,20]. The biological function of HCA587/MAGE-C2 within tumor cells has been relatively understudied.

In the present study, we searched for novel HCA587-interacting proteins using IP coupled with MS strategy. We identified BS69 as a

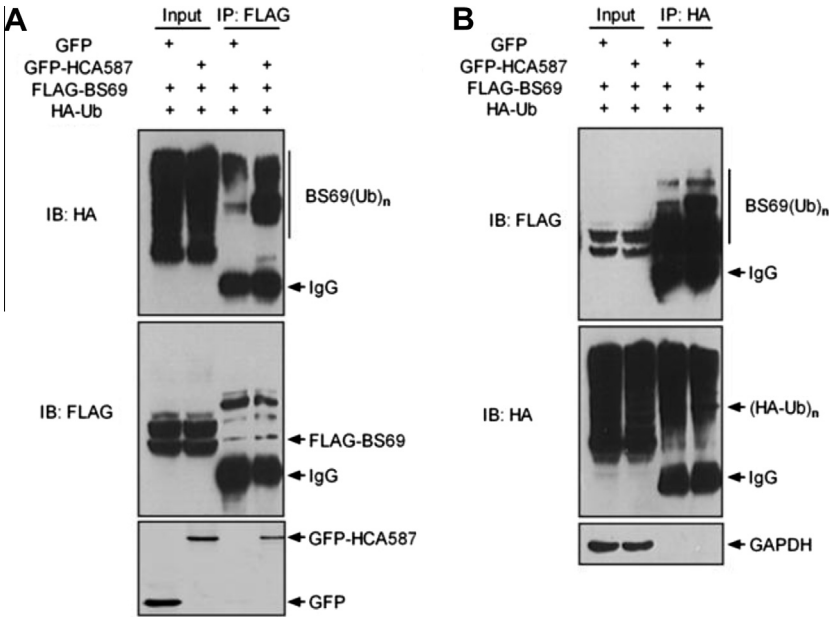

Fig. 3. HCA587 promotes BS69 ubiquitination. (A) HEK293 cells were co-transfected with the indicated plasmids. Forty hours after transfection, cells were treated with MG132 (20 μM) for 8 h before cellular extracts were prepared for co-immunoprecipitation assays with anti-FLAG antibody followed by immunoblotting with anti-HA antibody. (B) The same cell lysates as prepared in (A) were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-FLAG antibody.

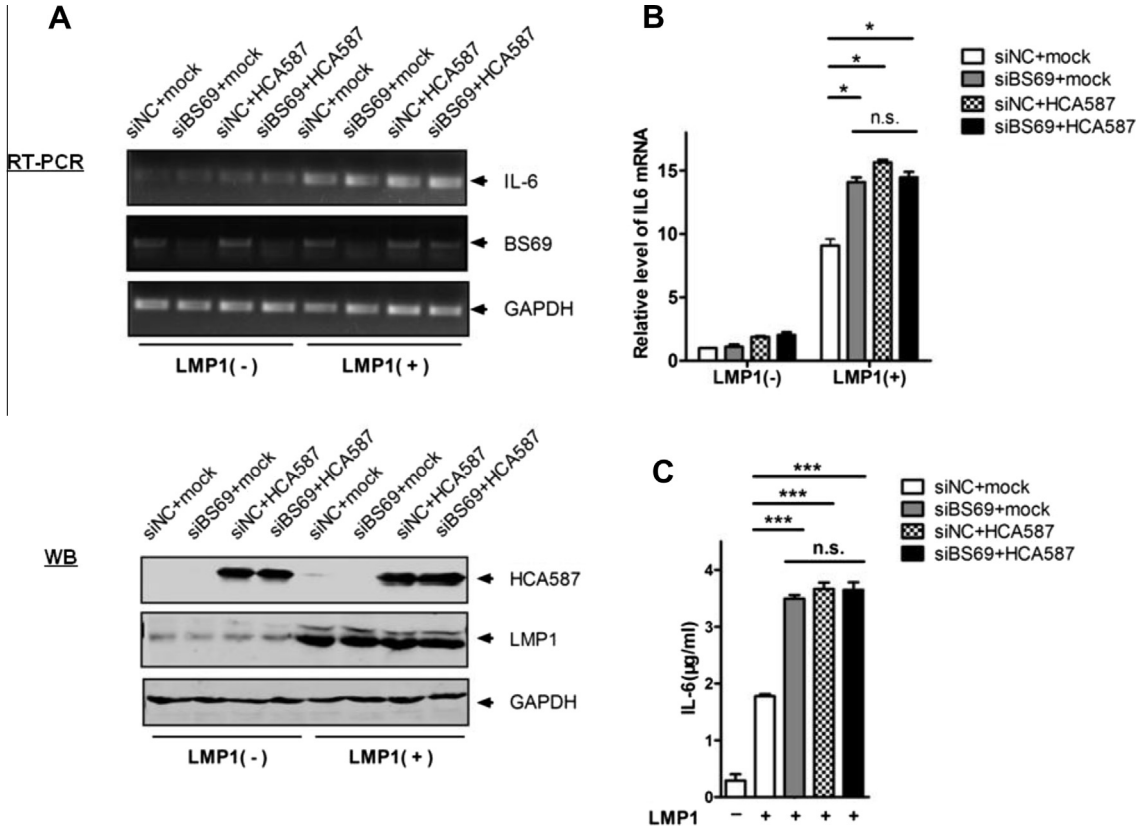

Fig. 4. Overexpression of HCA587 enhances LMP1-induced IL-6 production. (A) HeLa cells were transfected with a control siRNA (siNC) or BS69-specific siRNA (siBS69). The cells were then transfected with expression vectors for LMP1 and HCA587. At 36 h after transfection, total RNA were extracted and analyzed for IL-6 and BS69 mRNA expression levels by RT-PCR analysis. An aliquot of each total cell lysate was also analyzed by immunoblotting with anti-HCA587 and anti-LMP1 antibodies. (B) IL-6 mRNA expression levels described in (A) were analyzed by real-time quantitative PCR. Each bar represents the mean ± SD for triplicate experiments. $^{*}P < 0.05$; n.s., $P > 0.05$. (C) The culture supernatants described in (A) were analyzed for IL-6 with ELISA. Each bar represents the mean ± SD for triplicate experiments. $^{***}P < 0.001$; n.s., $P > 0.05$.

new binding partner of HCA587 and showed that HCA587 physically interacts with BS69 in vitro and in vivo and promotes degradation of BS69 in an ubiquitin- proteasome-dependent manner.

BS69 was first identified as a nuclear protein that interacts directly with adenoviral oncoprotein E1A and strongly inhibits E1A mediated transactivation [12]. It has recently been shown that

BS69 interacts with EBV-encoded LMP1 and inhibits LMP1-mediated NF-κB activation [15,16]. We found that overexpression of HCA587 results in a significant increase in LMP1-induced IL-6 production, a target gene activated by NF-κB transcription, which is consistent with the result of decreased BS69 levels. These data suggest that HCA587 can regulate the biological function through interaction with BS69.

The genomic locus encoding BS69 was mapped to chromosome 10p12.48 [21], a region frequently deleted in human cancer [22], implying that BS69 may have tumor suppressor-like properties. Consistent with this, a more recent report indicated that the copy number variations (CNVs) of BS69 were significantly associated with hematological malignancies [23]. Further studies indicated that BS69 acts as a transcriptional repressor in association with a variety of transcription factors such as c-Myb, Ets2, as well as the BRCA2 repressor EMSY [14,24,25]. Interestingly, all the three BS69-interacting transcription factors were found to be closely associated with the tumorigenic process. It has been reported that high expression of c-Myb was associated with oncogenic activity and poor prognosis in several human cancers including T-cell leukemia, acute myelogenous leukemia, colorectal tumors, and adenoid cystic carcinomas [26–28], increased expression of Ets2 was associated with initiation and progression of various cancer types [29–31], and the amplification of EMSY contributed to the initiation and progression of breast cancer [32–34]. These suggest that BS69 may act as a tumor suppressor by down-regulating transcription factors that have oncogenic potential. Therefore, the degradation of BS69 by HCA587 expression would increase the activities of these oncogenic proteins.

Regulation of BS69 stability has not been reported at present. This is the first study reporting that cancer–testis antigen HCA587 promotes ubiquitin-dependent degradation of BS69. Our data suggest that HCA587/MAGE-C2 may exert its oncogenic activity by negatively regulating the stability of tumor suppressor BS69. Further studies are required to investigate mechanisms of HCA587-mediated down-regulation of BS69.

Author contributions

Y.Y. and Y.Z. designed the research; J.H. performed the research; R.S. and Y.L. analyzed the data; J.H. and Y.Y. wrote the paper.

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