

Melanocyte-specific gene 1 promotes melanoma progression by enhancing the expression of Bcl-2

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Received November 24, 2015; Accepted April 28, 2017

DOI 10.3892/ol.2017.7592

Abstract. Malignant melanoma is a class of highly malignant tumors derived from melanocytes. At present, the dysregulated gene expression involved in the progression of melanoma has attracted much attention. In the present study, the gene expression profile of human melanoma tissue was screened using a cDNA microarray, and it was identified that melanocyte-specific gene 1 (*MSG1*) was significantly overexpressed in melanoma tissue compared with paired nevus tissues. The overexpression of *MSG1* in melanoma was subsequently confirmed using immunohistochemistry in a set of melanoma tissues. It was additionally identified that the overexpression of *MSG1* may promote cell viability and inhibit cell apoptosis in human melanoma A375 cells, thus promoting melanoma progression. Mechanistically, following screening of the expression of apoptosis-associated proteins, *MSG1* was demonstrated to enhance the expression of the apoptosis inhibitor B-cell lymphoma 2 (Bcl-2) to inhibit melanoma cell apoptosis. Therefore, it was concluded that the overexpression of *MSG1* inhibits apoptosis by enhancing Bcl-2 expression in malignant melanoma, thus promoting melanoma progression.

Introduction

Malignant melanoma, a class of highly malignant tumors derived from melanocytes, and is a type of skin cancer with the highest metastasis and mortality rate (1-3). The cure

rate of early melanoma may be >90% when it is surgically resected (1-3), while metastatic malignant melanoma often requires chemotherapy, radiotherapy, targeted therapy, immunotherapy or other kinds of combination therapy. Current therapeutic interventions for metastatic melanoma are not sufficient, and the 5-year survival rate is <20% (1-3). Therefore, understanding the molecular mechanisms of melanoma pathogenesis and identifying novel potential therapeutic targets are of importance for the prevention of malignant melanoma and the development of interventions (4-6).

Identification of the dysregulated genes in cancer tissues is important in the study of cancer biology. Gene expression microarrays have been applied in the high-throughput profiling of gene expression in number of types of cancer (7,8). In malignant melanoma tissues, a set of genes were identified to be dysregulated compared with normal tissues, and were demonstrated to be associated with processes involved in the carcinogenesis and progression of melanoma, including cell growth, cell cycle progression, apoptosis, cell migration and metastasis (9,10). Furthermore, several of these genes were identified to be associated with the prognosis, survival, and responses to chemotherapy of patients with melanoma (11,12). However, identification of dysregulated genes in melanoma tissues and their roles in cancer development remain an ongoing process in the study of melanoma biology.

Melanocyte-specific gene 1 (*MSG1*; also known as Cbp/P30-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1) is a transcriptional cofactor that interacts with CREB-binding protein/p300 and modulates the transcription of a set of downstream genes (13,14). Previous studies have identified that *MSG1* is an important factor in the differentiation and pigmentation of melanocytes. For example, *MSG1* may promote the synthesis of melanin, thus enhancing melanogenesis in melanocytes (15,16). However, the roles of *MSG1* in the carcinogenesis and progression of malignant melanoma require additional investigation and elucidation.

In the present study, in order to determine the dysregulated gene expression present in melanoma, the gene expression profiles of human melanoma tissues were screened using a cDNA microarray, and compared with the expression profiles of nevus tissues. *MSG1* expression was identified to be significantly overexpressed in melanoma tissues. The overexpression

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Key words: melanocyte-specific gene 1, melanoma, cell viability, apoptosis, Bcl-2

of *MSG1* in melanoma was subsequently confirmed using immunohistochemistry (IHC) in a set of melanoma tissues. The present study aimed to further examine the roles of *MSG1* in the carcinogenesis and progression of malignant melanoma cancer biology, so as to elucidate novel molecular mechanisms underlying melanoma development and potential therapeutic targets for treatment.

Materials and methods

Clinical melanoma specimens. Human malignant melanoma tissues and melanocytic nevus tissues (surgically resected and later histopathologically diagnosed as benign) were obtained from patients with melanoma during surgery, and diagnosed by pathological validation. A total of 10 patients with nevus and melanoma tissues examined by pathology and without other skin diseases from the Changhai Hospital (Shanghai, China) were included in the study, including 7 male and 3 female patients with a mean age of 48 (age range, 36–65). Ten nevus and matched melanoma tissue samples were obtained between September 2005 and September 2008. All samples were snap-frozen in liquid nitrogen until examination. All human samples were collected with the written informed consent of the patients, and use of human tissues was approved by the Institutional Research Ethics Committee of the Second Military Medical University (Shanghai, China).

Cell culture and transfection. The human malignant melanoma A375 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (PAA; GE Healthcare, Chicago, IL, USA) with 10% fetal bovine serum (FBS) (GE Healthcare, Chicago, IL, USA), under 37.5°C and 5% CO₂. Cells were transfected with small interfering (si)RNAs using INTERFERin® reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's protocol, and transfected with *MSG1* expressing plasmids (constructed using a pcDNA 3.1 vector) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using JetPEI® reagent (Polyplus-transfection SA) following the manufacturer's protocol, and confirmed by western blot analysis, in order to induce *MSG1* overexpression. The siRNA target sequences for human *MSG1* gene were 5'-UAGCAGCACAUCAUCGAAUA-3' (sense) and 5'-CCC AAUAUUGUCAUUAUUUA-3' (antisense), and the negative control siRNA sequences were 5'-UCUCCGAACGUG UCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGA ATT-3' (antisense). siRNA duplexes were transfected at a final concentration of 10 nM.

cDNA microarray assay. The Affymetrix GeneChip Human Genome U133 Plus 2.0 array assay was performed by Shanghai Bohao Industrial Co., Ltd., Shanghai, China. In brief, 5 µg total RNA samples from melanoma tissues and paired nevus tissues were reverse transcribed into cDNA for use in the microarray, as previously described (17). Hybridization was performed overnight using a micro-circulation pump (Atactic Technologies, Inc., Houston, TX, USA), and images were collected and quantified (17). The differentially detected signals were gathered and presented.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA, sourced from patient tissues, was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RT-qPCR analysis was performed using a SYBR RT-PCR kit (Takara Bio, Inc., Otsu, Japan) and LightCycler (Roche Diagnostics, Basel, Switzerland). The primer sequences for *MSG1* were 5'-GGC GGCACCACCATGTACCCT-3' (sense) and 5'-AGGGGCCGG ACTCGTCATACT-3' (antisense); the primer sequences for *Bcl-2* were 5'-GGTGGGGTCATGTGTGTGG-3' (sense) and 5'-CGGTTTCAGGTACTCAGTCATCC-3' (antisense); and the internal control b-actin sequences were 5'-GGCGGCACCACC ATGTACCCT-3' (sense) and 5'-AGGGGCCGGACTCGTCAT ACT-3' (antisense). The PCR cycle conditions were 95°C 15 sec, 55°C 30 sec, 72°C 30 sec for 45 cycles, and three independent experimental repeats were performed. The relative expression level of gene mRNAs was normalized to that of internal control β-actin by using 2^{-ΔΔC_q} cycle threshold method (18).

Cell viability analysis. The cell viability of transfected A375 cells was examined by the MTT method. Briefly, cells (10,000) were seeded into 96-well plates, cultured in DMEM with 10% FBS, and transfected as described. Control cells were transfected with empty pcDNA 3.1 vectors. At the indicated time points (0, 48 and 96 h), cell culture medium was replaced by fresh medium containing 0.5 mg/ml MTT. Cells were then incubated at 37°C for 2 h, and the MTT-containing medium was then replaced by 0.1 ml of dimethyl sulfoxide to dissolve the formazan (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The absorbance in each well was detected at 570 nm.

Apoptosis analysis. Melanoma cells were transfected for 48 h and cell culture medium was subsequently replaced by serum-free medium (DMEM; PAA; GE Healthcare). At the indicated time points (0 and 48 h), cells were harvested and apoptosis was detected using a Calbiochem® Annexin V-FITC Apoptosis Detection kit (Merck KGaA) according to the manufacturer's protocol, and a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The Annexin V-positive cells were regarded as apoptotic.

IHC. *MSG1* expression in melanoma tissues was examined by IHC. In brief, tissues were fixed with formalin and embedded in paraffin, and then sectioned to make tissue sections (4 µm thick), which were deparaffinized in xylene for 20 min and rehydrated in graded ethanol (95, 85, 75 and 50% for 5 min each). Endogenous peroxidase activity was blocked by a 30 min incubation in 3% H₂O₂ in PBS, and antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) by heating to boil for 5 min. The anti-*MSG1* primary antibody (cat no. ab87978; Abcam, Cambridge, UK) was diluted 1:500 and incubated at 4°C overnight. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (cat no. ab97040; Abcam, Cambridge, UK) in 1:1,000 dilution for use and in incubation at 4°C for 2 h. Immunostaining was visualized using a 3,3'-diaminobenzidine staining kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and analyzed using HistoFAXS system and mean DAB staining intensity was calculated using Histoquest

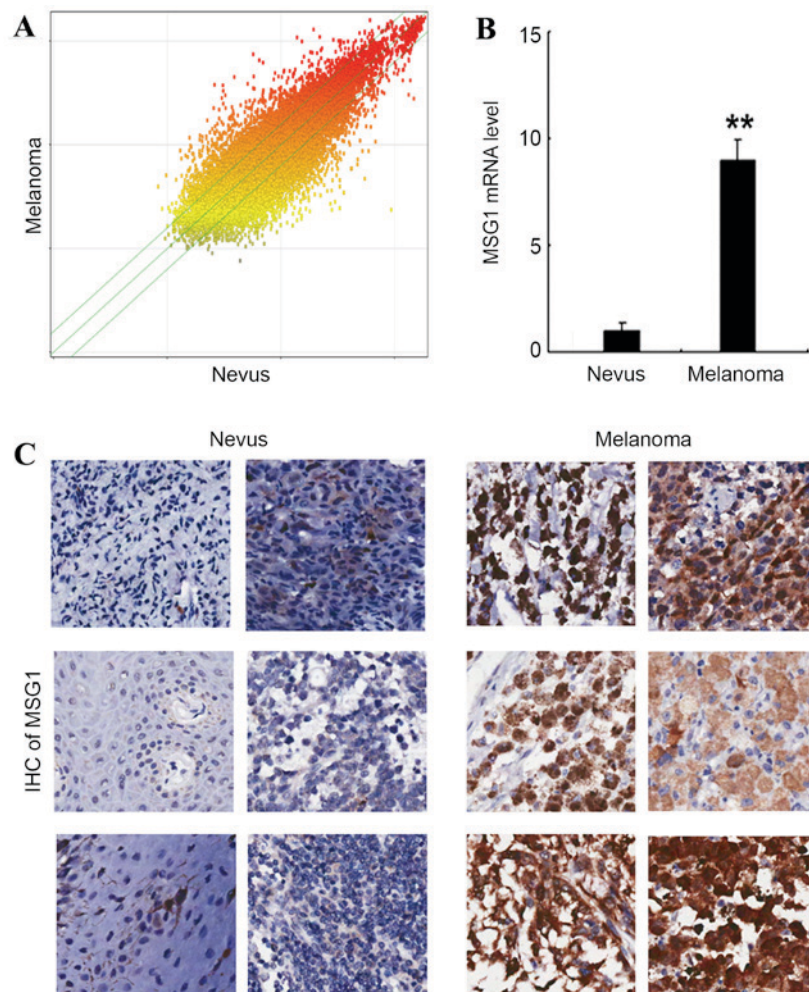


Figure 1. *MSG1* expression is upregulated in malignant melanoma. (A) cDNA microarray analysis of the gene expression profile of human melanoma and paired nevus tissues. The upper left interspace indicates the genes upregulated in melanoma tissues, and the lower right interspace indicates the genes downregulated in melanoma tissues compared with nevus tissues. (B) The expression level of *MSG1* mRNA was confirmed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation ($n=3$); ** $P<0.01$ vs. nevus tissues. (C) IHC staining of *MSG1* in paired melanoma and nevus tissues from 6 out of 10 patients; representative images are shown. Magnification, $\times 40$. *MSG1*, melanocyte-specific gene 1; IHC, immunohistochemistry.

software (both from TissueGnostics, Vienna, Austria), and the images are presented in Fig. 1C.

Western blotting. Cells were lysed using Passive Lysis Buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Protein concentrations were measured using the BCA Protein Assay kit (Takara Bio., Inc.) and equal amounts of extracts ($30 \mu\text{g}$) were subjected to SDS-PAGE (10% gel), transferred onto a polyvinylidene fluoride membrane, and then blotted. The *MSG1* antibody (cat no. ab87978) was purchased from Abcam. B-cell lymphoma 2 (Bcl-2; cat no. CST 2872) and β -actin (cat no. CST 3700) antibodies, all at a 1:1,000 dilution, and horseradish peroxidase-coupled secondary antibodies were purchased from Cell Signaling Technology, Inc. Antibody incubation was performed for 3 h at 4°C . At least 3 replicates were performed. Blocking was performed using tris-buffered saline with Tween-20, with 5% bovine serum albumin for 1 h at 20°C . Imaging was performed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.), and densitometric analysis was performed using Labworks Image Acquisition and Analysis Software (UVP, Upland, CA, USA).

Statistical analysis. Results are presented as the mean \pm standard deviation. Statistical analyses were performed using a Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

***MSG1* expression is upregulated in malignant melanoma.** In order to determine the gene expression profile in malignant melanoma, a cDNA microarray was performed in the melanoma and paired nevus tissues, and the differential gene expression is presented in Fig. 1A. Among the differentially expressed genes, it was identified that the melanocyte differentiation-associated gene *MSG1* was significantly upregulated in melanoma tissue compared with nevus tissue ($P<0.01$; Fig. 1B). Additionally, the upregulation of *MSG1* was confirmed by IHC in the melanoma tissues compared with nevus tissues (Fig. 1C). The results indicated that *MSG1* is upregulated in melanoma, and may potentially participate in melanoma carcinogenesis and progression.

***MSG1* promotes cell viability in melanoma.** As *MSG1* was upregulated in melanoma, the roles of *MSG1* in melanoma

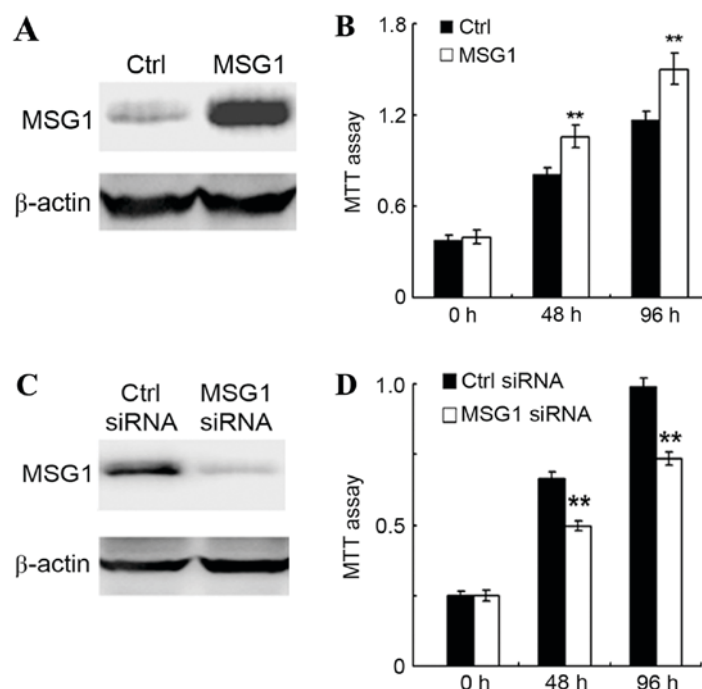


Figure 2. *MSG1* promotes cell viability in malignant melanoma. (A) Increased *MSG1* expression was confirmed by western blotting in A375 cells transfected with *MSG1*-expressing plasmids. (B) MTT cell viability analysis of A375 cells transfected with Ctrl or *MSG1*-expressing plasmids, at the indicated time points post-transfection. (C) Knockdown of *MSG1* expression was confirmed by western blotting in A375 cells transfected with *MSG1*-specific siRNA. (D) MTT cell viability analysis of A375 cells transfected with Ctrl siRNA or *MSG1*-specific siRNA, at the indicated time points post-transfection. Quantified data are presented as the mean \pm standard deviation ($n=4$); representative images are shown for the western blot results. ** $P<0.01$ vs. corresponding Ctrl group at the same time point. *MSG1*, melanocyte-specific gene 1; Ctrl, control; siRNA, small interfering RNA.

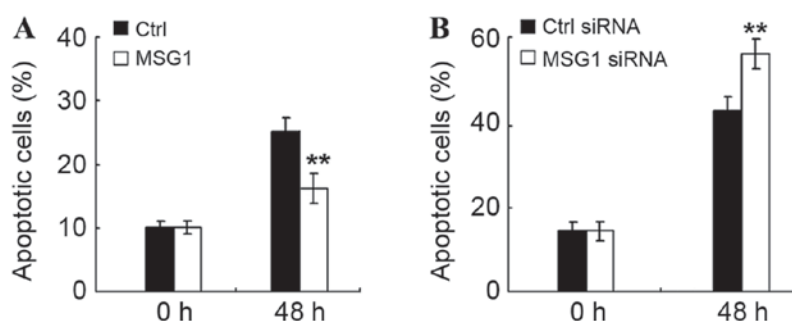


Figure 3. *MSG1* inhibits cell apoptosis in malignant melanoma. Melanoma A375 cells were transfected with (A) Ctrl vectors or *MSG1*-expressing plasmids, (B) Ctrl siRNA or *MSG1*-siRNA as indicated. At the indicated time points post-serum deprivation, cell apoptosis was examined by Annexin V-propidium iodide staining and flow cytometric analysis. The Annexin V-positive cells were regarded as apoptotic cells. Data are presented as the mean \pm standard deviation ($n=4$). ** $P<0.01$ vs. corresponding Ctrl group at the same time point. *MSG1*, melanocyte-specific gene 1; Ctrl, control; siRNA, small interfering RNA.

development were further examined. The cell viability of melanoma A375 cells was examined in control-transfected or *MSG1*-overexpressing cells, revealing that *MSG1* overexpression could significantly increase cell viability in A375 cells ($P<0.01$; Fig. 2A and B). In addition, this result was confirmed by the knockdown of *MSG1* expression. Transfection of the A375 cells with an *MSG1*-specific siRNA significantly decreased the cell viability compared with the control-siRNA-transfected cells ($P<0.01$; Fig. 2C and D). Thus, it was concluded that overexpression of *MSG1* in melanoma may increase cell viability.

***MSG1* inhibits cell apoptosis in melanoma.** In order to determine the mechanism of *MSG1*-promoted cell viability, the effect of *MSG1* on cell apoptosis were additionally examined in melanoma cells. As demonstrated in Fig. 3A, *MSG1*

overexpression significantly inhibited cell apoptosis induced by serum deprivation ($P<0.01$), whereas knockdown of *MSG1* expression significantly promoted the serum deprivation-induced cell apoptosis ($P<0.01$; Fig. 3B). Therefore, *MSG1* may inhibit cell apoptosis of melanoma cells, thus enhancing cell viability and promoting melanoma progression.

***MSG1* enhances anti-apoptotic Bcl-2 expression.** The molecular mechanisms responsible for the inhibition of apoptosis mediated by *MSG1* overexpression were explored. The expression of apoptosis-associated intracellular proteins in *MSG1*-overexpressing A375 cells were screened, and it was identified that the expression of the anti-apoptotic protein Bcl-2 was significantly increased by *MSG1* overexpression at the mRNA ($P<0.01$; Fig. 4A) and protein levels (Fig. 4B).

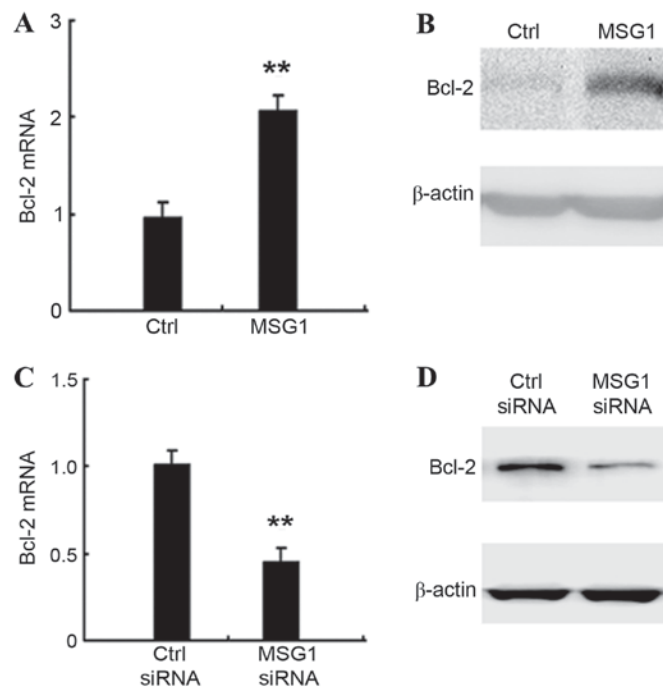


Figure 4. *MSG1* enhances Bcl-2 expression in melanoma. (A and B) Melanoma A375 cells were transfected with a Ctrl vector or *MSG1*-expressing plasmids. *MSG1* (A) mRNA and (B) protein levels were determined using RT-qPCR and western blot analysis, respectively, at 48 h post-transfection. (C and D) Melanoma A375 cells were transfected with Ctrl siRNA or *MSG1*-siRNA. *MSG1* (C) mRNA and (D) protein levels were determined using RT-qPCR and western blot analysis, respectively, 48 h post-transfection. Quantified data are presented as the mean \pm standard deviation (n=4); representative images are shown for the western blot results. **P<0.01 vs. Ctrl group. *MSG1*, melanocyte-specific gene 1; Bcl-2, B-cell lymphoma 2; Ctrl, control; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Furthermore, the knockdown of *MSG1* expression significantly inhibited the expression of Bcl-2 in A375 cells (P<0.01; Fig. 4C and D). Taken together, the results indicate that *MSG1* may enhance the expression of anti-apoptotic Bcl-2, thus inhibiting cell apoptosis and promoting melanoma progression.

Discussion

In the present study, the gene expression profile in human melanoma tissues was screened using a cDNA microarray, and it was identified that the melanocyte differentiation-associated gene *MSG1* was significantly overexpressed in melanoma tissues. It was also identified that *MSG1* may promote malignant melanoma progression by the inhibition of cell apoptosis, which is mediated by enhanced expression of anti-apoptotic Bcl-2. Therefore, these data suggest a novel molecular mechanism for malignant melanoma carcinogenesis and progression, which may suggest potential therapeutic strategies for the treatment of patients with melanoma.

The dysregulation of a set of genes has previously been identified in the carcinogenesis and progression of malignant melanoma, and several of these genes have been demonstrated to be correlated with the survival of melanoma patients (19-22). For example, caveolin-1 has been demonstrated to be upregulated in melanoma tissues, and correlated with melanoma metastasis and prognosis (23). In the present study, the upregulation of *MSG1* was revealed to be associated with melanoma progression (23), but it remains unknown whether high *MSG1* expression in melanoma tissues predicts a poor survival time in patients with melanoma. Our future studies will further examine this issue in a larger cohort of patients with melanoma.

The promotion of cell viability and the inhibition of cell apoptosis are important aspects of cancer biology, and malignant melanoma has demonstrated a set of mechanisms for survival, including the inhibition of apoptosis and immune evasion (24-26). The present study indicated that expression of the important anti-apoptotic protein Bcl-2 is significantly induced by *MSG1* expression, and may contribute to the *MSG1*-mediated inhibition of apoptosis. However, the detailed mechanism responsible for the *MSG1*-induced Bcl-2 expression remains unknown. We hypothesized that *MSG1* may enhance or participate in the initiation of Bcl-2 gene transcription, which requires additional investigation.

In conclusion, the upregulation of *MSG1* expression in melanoma tissues has been identified in the present study, suggesting that *MSG1* may function as an oncogene in the carcinogenesis and progression of malignant melanoma. However, the detailed mechanism responsible for the upregulated expression of *MSG1* in melanoma cells remains unknown. At present, genetic and epigenetic mechanisms have been identified to underlie the dysregulation of genes in cancer biology (19,27-29). We intend to investigate the detailed mechanism of the regulation of *MSG1* expression in melanoma cells, with the aim of elucidating the potential *MSG1*-mediated regulatory loop in melanoma development.

Acknowledgements

The authors would like to thank Dr Hao Zhang and Dr Hao Tang from Changhai Hospital (Shanghai, China) for their extensive assistance in the present project. The present study was supported by the Project of Shanghai Science and

Technology Commission (grant no. 13JC1401403) and the SMMU Start-up Foundation for Youths (grant no. 2013QN08).

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