



HMGA1 exacerbates tumor progression by activating miR-222 through PI3K/Akt/MMP-9 signaling pathway in uveal melanoma

Ying Cheng, Tongjie Cheng, Yuqing Zhao, Yi Qu*

Department of Geriatrics, Qilu Hospital of Shandong University No. 107, Wenhuxi Road, Jinan 250012, China



ARTICLE INFO

Keywords:

HMGA1
microRNA-222
PI3K/Akt/MMP9
Uveal melanoma

ABSTRACT

High-mobility group A1 (HMGA1), an architectural transcription factor, participates in different human tumors' biological progression. HMGA1 overexpression is associated with malignant cellular behavior in a wide range of cancers but the underlying mechanism remains poorly illuminated. In this study, we showed PI3K/Akt/MMP9 pathway activity could be positively regulated by HMGA1 using western blotting, real-time polymerase chain reaction (RT-PCR) and immunochemistry both in vitro (C918 and MUM-2B cell lines) and in vivo (xenograft mouse model). Later, MiRTarBase was used to identify the relationship between HMGA1 and miR-222-3p, we found miR-222 is positively regulated by HMGA1. Moreover, the proliferation and migration of UM cells significantly increased in the miR-222 mimics group and decreased in the miR-222 inhibitor group detected by the Annexin V-FITC apoptosis detection kit, CCK-8 and scratch wound-healing. The p-PI3K, p-Akt and MMP9 expressions were elevated in UM cells transfected with miR-222 mimics, and suppressed in the miR-222 inhibitor group. Together, our study highlights that HMGA1 acts as a pivotal regulator in UM tumor growth, proposing a critical viewpoint that HMGA1 expedites progression through the PI3K/Akt/MMP9 pathway and oncogenic miR-222 in UM.

1. Introduction

Uveal melanoma (UM) is the most common primary intraocular tumor in adults, the life expectancy of UM patients is mainly affected by distant metastasis. The prognosis will be poor if the metastasis is detected, usually with an average 5–8 months survival range [1]. Unfortunately, almost 50% UM patients have subclinical metastases at the time of diagnosis [2,3]. Therefore, it is necessary to identify novel target molecules to promote early diagnosis, prognosis prediction and therapeutic strategies for UM.

High-mobility group AT-hook1 (HMGA1) protein is the crucial contributor in the assembly of transcriptional factors and cofactors to form enhanceosomes [4]. HMGA protein binds to the minor groove of AT-rich DNA sequences via AT-hooks in the N-terminal region. Therefore, HMGA1 could alter the transcriptional activity and regulate the chromatin structure in multi-genes. HMGA1 overexpression has been found in different human malignancies, such as colorectal cancer, ovarian cancer, prostate cancer and lung cancer [5–8]. Our previous study demonstrated that high expression level of HMGA1 was associated with adverse clinical outcomes in UM patients [9], but the underlying molecular mechanism of HMGA1 in UM progression remains largely unknown.

MicroRNAs (miRNAs) are the small noncoding RNAs with important regulative roles in a wide range of pathologic and biologic processes. There is a large body of evidence stating that miRNAs may induce the tumor cell proliferation and inhibit cell death, leading to tumor development and progression by activating or inhibiting key genes [10–12], as illustrated by the negative correlation between miR-200a/miR-363 and YY1 expression in Burkitt's lymphoma [13] and the potential diagnostic biomarkers of miR-196 family in head and neck cancers [14]. In particular, aberrant miRNA expressions, including miR-508, miR-326 and miR-222, have been found in patients with UM through several integrated computational approaches, these miRNAs may be regarded as prognostic biomarkers in clinic to address high-risk patients towards more aggressive therapeutic strategies [15–17].

MiR-222, encoded in tandem from a gene cluster located on X chromosome, contains identical seed sequences and are highly conserved in vertebrates [18]. Under physiological conditions, they play roles in essential processes, such as angiogenesis, vessel wound healing, atherosclerotic and vascular aging [19,20]. However, abnormal expression level of miR-222 has been extensively studied in many human tumors, including lung cancer, breast cancer, liver cancer, cervical cancer, glioma and multiple myeloma [21–23]. Some studies reported that HMGA1 could promote cell proliferation by targeting miR-222 in

* Corresponding author at: Department of Geriatrics, Qilu Hospital of Shandong University No. 107, Wenhuxi Road, Jinan 250012, China.
E-mail address: yiquen@sdu.edu.cn (Y. Qu).

Table 1
Detailed information of miRNA-3p sequence and primers.

	Sequences (5'-3')	Annealing temperature (°C)	Extension temperature (°C)	Product size (bp)
HAS-MiR-222 mimics	F-AGCUACAUCUGGCUACUGGGU R-CCAGUAGCCAGAUGUAGCUUU			
Mimics negative control	F-UUCUCCGAACGUGUCACGUTT R-ACGUGACACGUUCGGAGAATT			
HAS-MiR-222 inhibitor	F-ACCCAGUAGCCAGAUGUAGCU			
Inhibitor negative control	F-CAGUACUUUUGUGUAGUACAA			
HMGA1	F-TTCTCTAAGGAGCAGGTGGAA R-CGCATTGTGCTACCAGCG	58	72	149
PI3K	F-AGCCGGAAGACTACACGCT R-GGTCAGGTGAGGGGTC AAC	60	72	122
Akt	F-AGCGACGTGGCTATTGTGAAG R-GCCATCATCTTGAGGAGGAAGT	60	72	96
MMP9	F-GCACTGCAGGATGTCATAGG R-ACGACGTCTCCAGTACCGA	59	72	128
β-actin	F-TTGCCGACAGGATGCAGAA R-GCCGATCCACCGAGTACT	60	72	101

lung cancer and cervical cancer, leading to repression of PPP2R2A expression and activation of Akt signaling [21,22]. The underlying mechanism of HMGA1 and miR-222 in UM has not yet been fully understood.

Previous studies have reported that the PI3K/Akt/MMP-9 pathway is a distinct downstream pathway of HMGA1 [22,24], which is vital for tumor initiation and progression in the microenvironment [25,26]. Matrix metalloproteinase-9 (MMP-9) degrades the basement membranes and exposes cryptic peptide epitopes in the extracellular matrix, associating with tumor dissemination [27]. It is known that epigenetic mechanisms, including non-coding RNAs, DNA methylation and histone modifications, have emerged as key regulators in MMP-9 expression [28,29]. MiR-132 and miR-212 have been reported to reduce MMP-9 expression experimentally through modulating collagen remodeling [30]. Conversely, miR-21, miR-373 and miR-520 are characterized as oncogenic miRNA, contributing to MMP-9 gene transcription [31,32]. Accordingly, dysregulation of miRNA plays an essential role in MMP-9 expression, which in turn is involved in cancer progression and clinical prognosis. In current study, we hypothesized that HMGA1 could enhance miR-222 by regulating PI3K/Akt/MMP-9 pathway in UM, exploring the molecular mechanism underlying of UM progression mediated by HMGA1.

2. Materials and methods

2.1. Animals and ethics statement

The present study conformed to the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. The protocols were approved by the Shandong University Qilu hospital. The BALB/c nude mice were purchased from Vitalriver (Beijing, China).

2.2. Cell culture and transfection

Human uveal melanoma cells (C918 and MUM-2B) were obtained from Chinese Academy of Sciences Cell Bank (Shanghai, China) on June 5th, 2017. C918 and MUM-2B cells were cultured by RPMI 1640 with 10% fetal bovine serum (FBS) at 37 °C in the culture box with 5% CO₂. The culture medium was changed after 48 h. The subculture was digested when the fusion degree had reached 80%. C918 and MUM-2B cells were authenticated by DNA profiling occasionally.

The lentivirus (LV)-HMGA1-RNAi (32762-1) and negative control (NC) lentivirus (CON077, hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) were purchased from the GeneChem Corporation (Shanghai, China) with the 8E+8 transduction units (TU)/ml. The C918 and MUM-2B were plated into 6-well plates at a density of 5×10^4 cells/well and grown to 30–40% confluence after incubated at 37 °C for 24 h, then the

lentivirus infected the UM cells at a multiplicity of infection (MOI) of 10. The cells were cultivated in enhanced infection solution for 12 h. The cell culture medium was then replaced by normal medium containing serum. After infection for 72 h, cells were placed under the fluorescent microscope to observe infection efficiency, then the 2-week puromycin (2 µg/ml) was used for screening. The cells were collected for cell transplantation and RNA/protein extraction. The UM cells were divided into three groups: the cells in the blank control group (normal group) were normally cultured without any handling, the cells in the NC group were transfected with NC lentivirus, and the HMGA1-down group was transfected with LV-HMGA1.

MiR-222-3p mimics, miR-222-3p inhibitor and NC oligonucleotides were obtained from GenePharma (Shanghai, China). Cells were seeded in 12-well plates at a density of 10^5 cells/well one day prior to transfection. When the cells were grown to a density of 70%–80% confluence, EndoFectin™ Max transfection agent (GeneCopoeia, San Diego, CA, USA) was used to respectively transfect miR-222-3p mimics, miR-222-3p inhibitor and two corresponding NC groups (all 100 nM) according to the manufacturer's protocol for 48 h. MiR-222-3p mimics/NC and miR-222-3p inhibitor/NC sequences were shown in Table 1.

2.3. Apoptosis assay

For the detection of cell apoptosis, Annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, C1062, Shanghai, China) was used as described by the manufacturer's instruction. Briefly, the C918 and MUM-2B were collected by centrifugation at 1000 r/min for 5 min and washed twice with PBS. The cells were gently resuspended in 195 µl Annexin V binding buffer, and then incubated with 5 µl Annexin V-FITC/10 µl propidium iodide (PI) for 15 min in the dark. The pictures of apoptotic cells were captured by fluorescence microscope.

2.4. Cell proliferation viability assay

A total of 2×10^3 UM cells per well were plated into 96-well plates, with 200 µl of culture medium and 20 µl of Cell Counting Kit-8 (CCK-8, Beyotime, China) were added into each well. Each experiment group has five parallel wells. CCK-8 is a kind of yellow solution that can be reduced to orange by active cells, whose absorbance is directly proportional to cell number. After 3.5 h of incubation, the OD value of the liquid in each well was measured by a microreader (Bio-Rad 680) at the wavelength of 450 nm. The cell number was proportional to the OD value.

2.5. Scratch wound-healing assay

The cells were cultured into 6-well plates at a density of 3×10^5 cells/well. After being cultured for 24 h at 37 °C in an incubator with 5% CO₂, the cells were then transfected with miR-222 mimics, miR-222 inhibitor and NC. A straight-line scratch was made on the bottom of the cell culture plate using a sterile 200- μ l yellow pipette tip 5 h post-transfection. Fresh and complete media were added, and the wound healing ability was observed for 24 h. Images were captured every 8 h. The distance migrated by the cell monolayer to close the wounded area during this time period was measured. Results were expressed as a migration index, the distance migrated by miRNA mimics or inhibitor treated relative to the distance migrated by NC treated cells.

2.6. UM xenograft mouse model

Ten BALB/c nude mice were divided into two groups (6-week-old female, 5 mice per group) and subcutaneously injected with 100 μ l of 5×10^6 C918 cells to the posterior flank respectively, which were previously stably transfected with LV-HMGA1 or NC lentivirus. When the tumor mass became palpable (usually at day 7 after injection), the tumor volume was measured with a caliper three times per week using the formula: tumor volume (mm^3) = (length \times width²)/2. At day 28, mice were sacrificed, and the tumor volume and the size were calculated. Tumor tissues were extracted and processed for subsequent RT-PCR, western blot and immunohistochemistry (IHC) analyses.

2.7. Western blot analysis

Total protein was extracted from UM cells or tumor tissues from BALB/c nude mice with RIPA lysis buffer (Solarbio, Beijing, China). The tissue lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were separated for further analysis. Protein samples (20 μ g protein/lane) were separated using 10% SDS-PAGE and then transferred onto PVDF membranes (Merck Millipore, Billerica, MA, USA). The membrane was blocked in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20 for 2 h at room temperature and then incubated overnight with primary antibodies: HMGA1 (ab129153, 1:10000, Abcam, Cambridge, MA, USA), PI3K (AF6241, 1:1000, Affinity Biosciences, Jiangsu, China), p-PI3K (AF3241, 1:1000, Affinity Biosciences), Akt (4691, 1:1000, CST, Beverly, MA, USA), p-Akt (4060, 1:2000, CST), MMP-9 (ab38898, 1:1000, Abcam), β -Actin (PR-0255, 1:300, ZSGB-BIO, Beijing, China). After the membranes were washed with PBS, horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody was applied for 1 h at room temperature. After rinsing, the proteins were detected by enhanced chemiluminescence (Merck Millipore). The protein levels were quantified by densitometry and normalized to the corresponding β -Actin level.

2.8. Real-time PCR

The total RNA from UM cells or tumor tissues from BALB/c nude mice were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA then underwent reverse transcription using the Prime Script RT Master Mix kit (Takara, Shiga, Japan) followed by analysis using real-time PCR (RT-PCR) with the SYBR Green PCR Master Mix (TaKaRa) on Roche LightCycler 480 system. The PCR conditions were 30s at 95 °C for denature, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30s. Relative expression of HMGA1, PI3K, Akt and MMP-9 were calculated by the comparative cycle threshold (CT) method using the expression of β -Actin as the reference for mRNA. The $2^{-\Delta\Delta Ct}$ method was used for analysis. The specific primers applied for RT-PCR reaction are shown in Table 1.

2.9. Immunohistochemistry

The immunohistochemical staining of HMGA1 was performed as previously described [33]. Briefly, IHC was performed on paraformaldehyde-fixed paraffin sections of tumor tissue from nude mice. HMGA1 antibody was used in IHC with streptavidin peroxidase-conjugated method. The immunostaining results were scored as the percentage of cells staining positive as follows: 0 for < 1% of cells, 1 for 1%–25% of cells, 2 for 26%–50% of cells, 3 for 51%–75% of cells and 4 for > 75% of cells.

2.10. Statistical analysis

The data were provided as the mean \pm standard error of the mean (SEM). All statistical analyses were performed with Student's *t*-test (two groups) or one-way ANOVA (three or more groups) using the SPSS software (IBM SPSS statistics 21; SPSS Inc., Chicago, IL). The statistical charts were made by GraphPad Prism 5 software (GraphPad, CA, USA). A *P*-value < .05 was considered statistical significance.

3. Results

Expression of HMGA1 is downregulated by LV-HMGA1 in C918 and MUM-2B.

After transfection of lentivirus into C918 and MUM-2B for 48 h, fluorescence microscopy was used to observe cell transfection efficiency marked by enhanced green fluorescent protein (EGFP). The fluorescence showed a high transfection efficiency of ~90% in C918 (Fig. 1A-1D) and ~80% in MUM-2B (Fig. 1E-1H). Then the protein and RNA were extracted to detect the expression level of HMGA1 after 72 h of lentivirus transfection, the results of HMGA1 expression were shown in Fig. 1I-K checked by western blot and RT-PCR. The results demonstrated that HMGA1 expression has been effectively downregulated by LV-HMGA1 in C918 and MUM-2B comparing with NC and blank group (all *P* < 0.001).

Effects of HMGA1 inhibition on PI3K/Akt/MMP-9 pathway in UM cells.

We used western blot and RT-PCR to test whether HMGA1 regulates PI3K/Akt/MMP-9 pathway in UM cells. After 72 h of lentivirus transfection, the RNA and protein of C918 and MUM-2B were collected. The western blot results showed the p-PI3K, p-Akt and MMP-9 expressions of C918 and MUM-2B cells in the LV-HMGA1 group were significantly decreased compared to those in the NC group (all *P* < .001), indicating that HMGA1 could target the PI3K/Akt/MMP-9 pathway in UM (Fig. 2A-C). We further used RT-PCR to reconfirm the mRNA level of this signaling pathway, the results demonstrated MMP-9 expression declined in the LV-HMGA1 group (*P* < .05), which in line with the western blot. The total mRNA of PI3K and Akt showed no difference between two groups (Fig. 2D-E).

MiR-222 increases proliferation and migration of UM cells.

In order to investigate the biological function of miR-222 in C918 and MUM-2B, we regulated the miR-222 expression using miR-222 mimics and inhibitor. The proliferation of UM cells was detected using the Annexin V-FITC apoptosis detection kit and CCK-8 assay, the migration function of UM cells was monitored by scratch wound-healing assay.

The occurrence of apoptosis was obtained by double staining of the cultures with propidium iodide (PI) and annexin V-FITC, a protein that binds with high affinity to phosphatidylserine, which is translocated from the inner to the outer membrane leaflet early in the apoptotic process. C918 and MUM-2B cells represented a similar result, a majority of cells were at an early stage of apoptosis (annexin V-positive, green fluorescence), only a small part of cells were at the late apoptotic stage (double positive staining, green and red fluorescence). MiR-222 mimics induced cells showed less annexin V-positive cells comparing to the NC group, UM cells interfered by miR-222 inhibitor showed more annexin

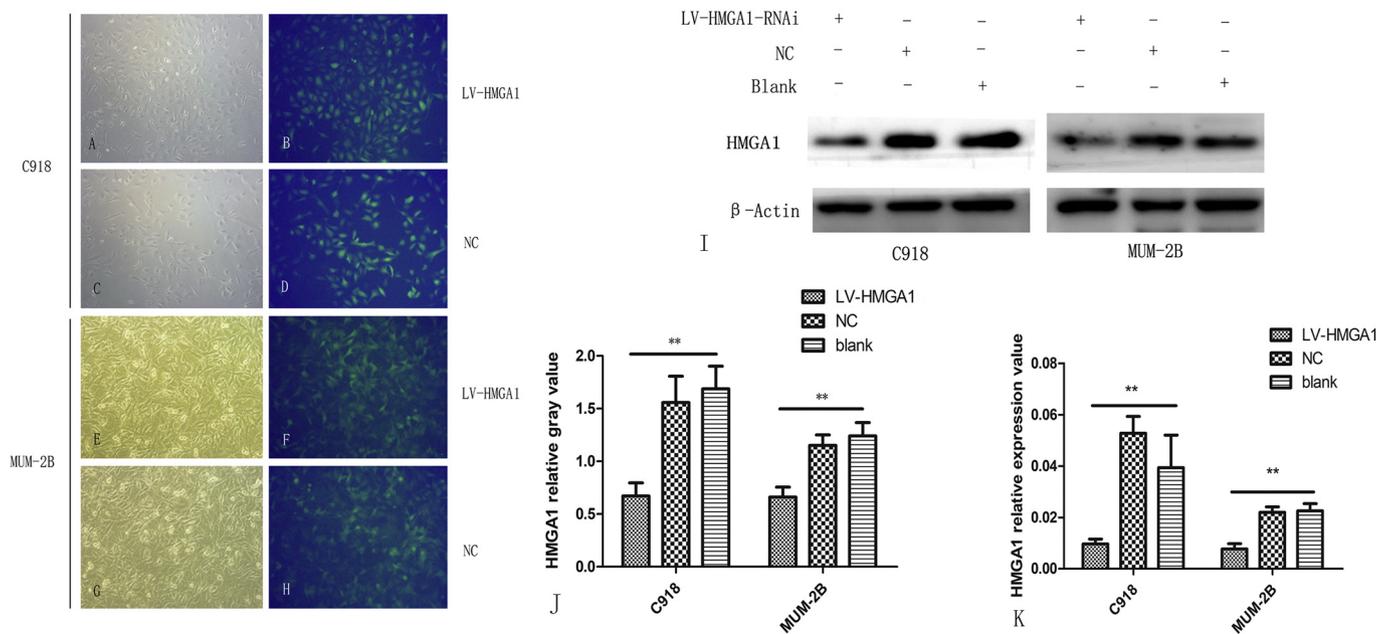


Fig. 1. Observation of lentivirus packaging and infection efficiency in C918 and MUM-2B. Transfection of LV-HMGA1 and negative control into the two UM cell lines for 48 h under fluorescence microscopy. A, C. white light fields of C918. B, D. Transfection efficiency to 90% of LV-HMGA1 and NC in C918 under fluorescent. E, G. white light fields of MUM-2B. F, H. Transfection efficiency of MUM-2B has reached to nearly 80% in LV-HMGA1 and NC. I-J. The protein expression of HMGA1 was significantly suppressed in LV-HMGA1 shown by western blot comparing to the control groups. Control groups were transfected with negative control vector (NC group) or without any handling (blank group). β -Actin was used as a loading control ($P < 0.001$, one-way ANOVA). K. The mRNA expression of HMGA1 was significantly downregulated in LV-HMGA1 ($P < 0.001$, one-way ANOVA). mRNA expression was relative to β -actin. * $P < .05$, ** $P < .001$.

V-positive cells, which provide a strong evidence that miR-222 promote the proliferation of UM cells and suppress their death through apoptosis (Fig. 3A). The proliferation of C918 and MUM-2B cells was also detected using the Cell Counting Kit-8 (CCK-8) assay. The CCK8 assays proved that miR-222 mimics could enhanced the proliferative capacities in UM cells (Fig. 3B), besides, proliferative capacities of C918

cells was suppressed by miR-222 inhibitor (all $P < .05$) (Fig. 3C). The migration of UM cells was evaluated using a wound-healing assay. Compared with the NC groups, the C918 and MUM-2B migration was increased when transfected with miR-222 mimics and attenuated with miR-222 inhibitor (all $P < .05$) (Fig. 3D-F). These data together suggested that miR-222 is able to promote the proliferation and migration

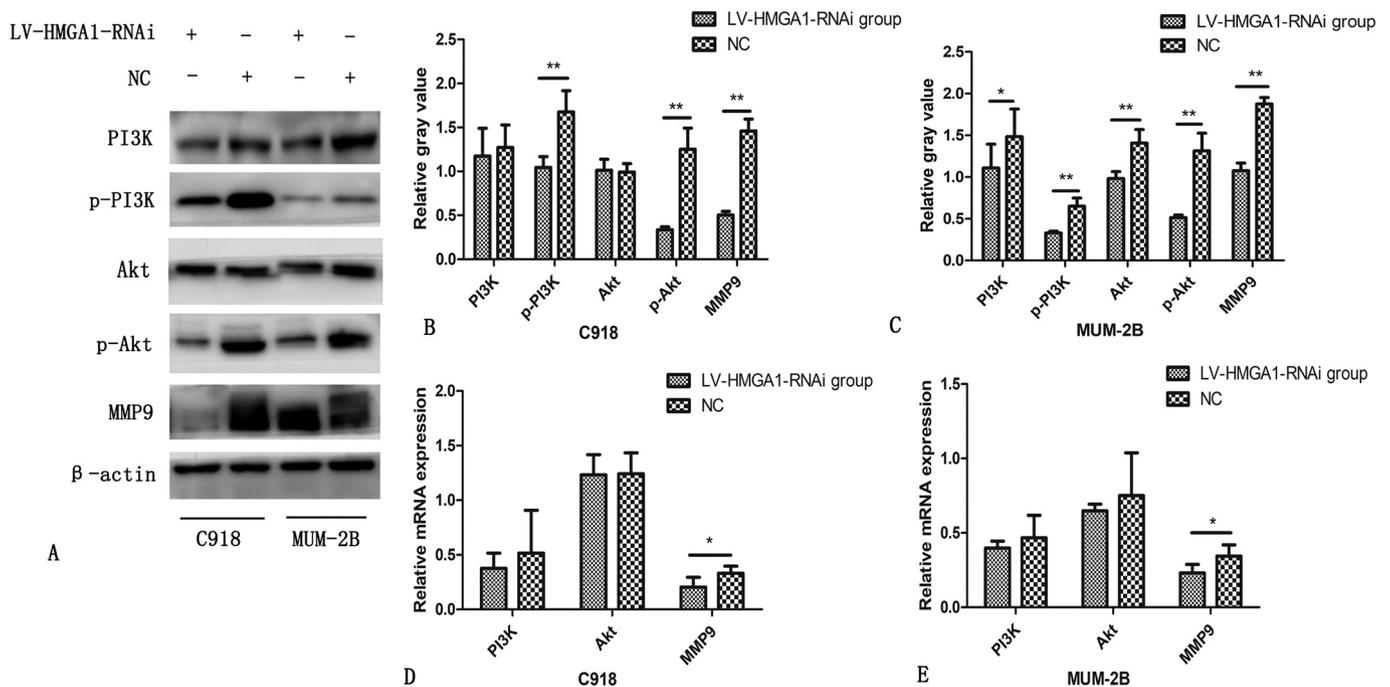


Fig. 2. Expression of PI3K/Akt/MMP-9 pathway in C918 and MUM-2B after lentivirus transfection. A-C. After 72 h of LV-HMGA1 and NC transfection in UM cells, the p-PI3K, p-Akt and MMP-9 protein expressions in these two UM cell lines were significantly decreased in LV-HMGA1 group shown by representative blots and relative gray values ($P < 0.001$, Student-*t*-test). D-E. Relative mRNA expressions of MMP-9 were significantly degraded in LV-HMGA1 group ($P < 0.05$, Student-*t*-test). β -Actin was used as a loading control for western blot analysis and RT-PCR. * $P < .05$, ** $P < .001$.

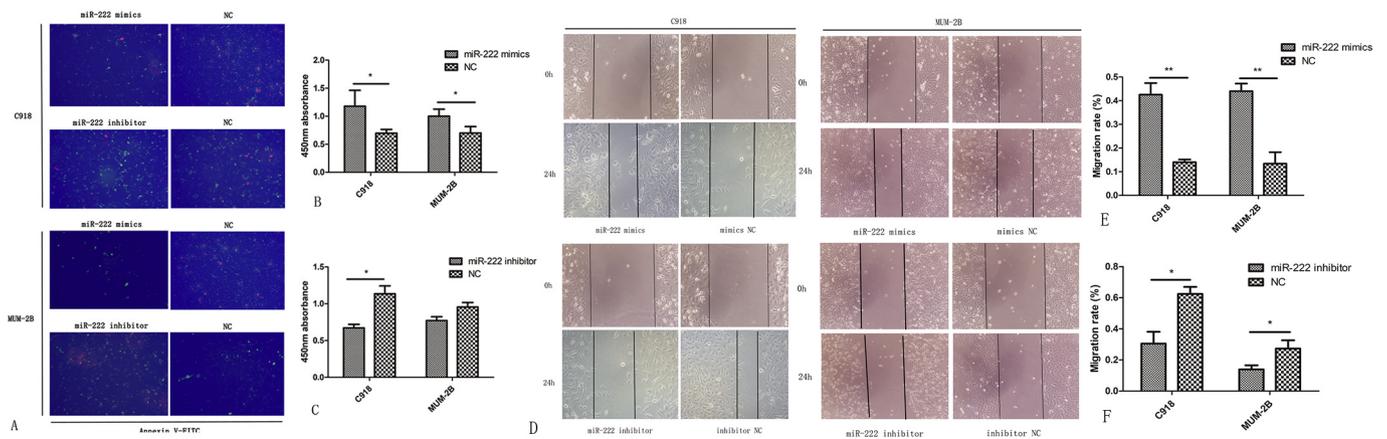


Fig. 3. The effect of miR-222 on UM cell apoptosis, proliferation and migration. After treating with miR-222 mimics and inhibitor, cells were stained with annexin V-FITC and propidium iodide and analyzed by fluorescence microscopy. A. C918 induced by miR-222 mimics showed less annexin V-positive and PI-positive cells comparing to the NC group, on the contrary, C918 cells interfered by miR-222 inhibitor showed much more annexin V-positive cells. Similar results were found in MUM-2B. (red: stained with Annexin V-FITC, green: stained with PI, mixture: stained with Annexin V-FITC and PI both). B–C. The proliferation of C918 and MUM-2B cells was measured by CCK-8 assay. The UM cells treated with miR-222 showed a higher 450 nm absorbance and miR-222 inhibitor-induced C918 represented a lower value of absorbance ($P < 0.05$, Student-*t*-test). The effect of miR-222 on UM cell migration. UM cell migration treated with LV-HMGA1 and NC was performed monitored by the wound healing assay. D-F. Migration rate of C918 and MUM-2B was enhanced with miR-222 mimics and attenuated with miR-222 inhibitor ($P < 0.05$, Student-*t*-test). These assays were analyzed and calculated using Image J. * $P < .05$, ** $P < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of UM cells in vitro.

Modulation of miR-222-3p enhances PI3K/Akt/MMP-9 pathway mediated by HMGA1 in UM cells.

MiRNA-222 has been reported in the progression of several malignancies, including cervical cancer and lung cancer [21,22,24]. Recently, HMGA1 overexpression has been proved to correlate with the increased expression of miR-222 and HMGA1 enhances its expression by directly binding with the proximal promoter of miR-222 in many tumors [21]. Further, we used the miRTarBase (<http://mirtarbase.mbc.ntu.edu.tw/php/index.php>) to reconfirm the relation between miR-222-3p and its putative binding sequence of HMGA1 (Fig. 4A). However, it was still unknown whether miR-222 participated in the

HMGA1-mediated proliferation and migration of UM cells.

Expression levels of PI3K/Akt/MMP-9 signaling pathway were checked by western blot in UM cells treated by miR-222 mimics and inhibitor. The results showed that p-PI3K, p-Akt and MMP-9 expression were upregulated in C918 and MUM-2B cells induced by miR-222 mimics (all $P < .05$). In contrast, the expression of p-PI3K, p-Akt and MMP-9 mitigated when transfecting miR-222 inhibitor in UM cells, compared with the NC group (all $P < .05$) (Fig. 4B-C). We didn't find the significant difference in pan-PI3K and pan-Akt, because the phosphorylated PI3K and Akt are the mainly functional proteins in this pathway. Thus, our data revealed that miR-222 exerted an oncogenic role in UM cells via PI3K/Akt/MMP-9, and HMGA1 is a positive

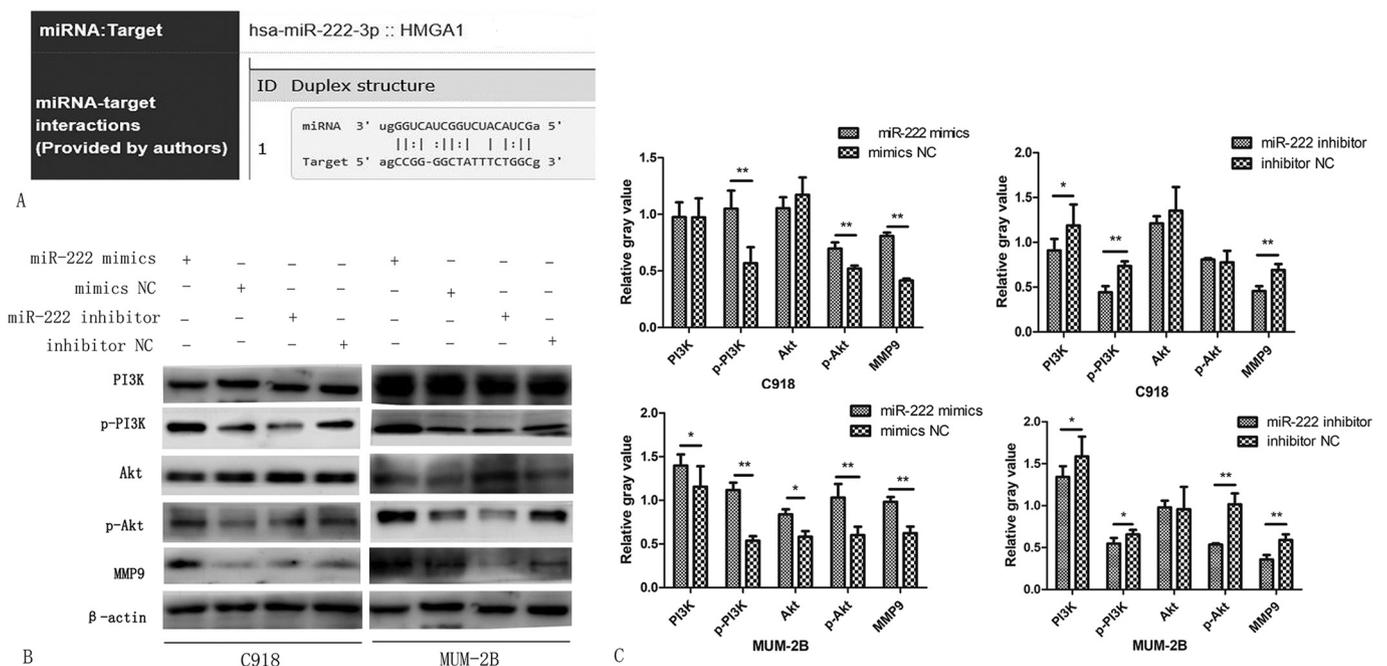


Fig. 4. A. miRTarBase provided the evidence of correlation between HMGA1 and miR-222. Representative blots (B) and relative gray values (C) of western blot showed that the miR-222 mimics increased the expression of p-PI3K, p-Akt and MMP-9 in C918 and MUM-2B ($P < 0.001$, Student-*t*-test) and miR-222 inhibitor reduced the p-PI3K, p-Akt and MMP-9 expression at 48 h ($P < 0.05$, Student-*t*-test). The protein expressions were relative to β -Actin. * $P < .05$, ** $P < .001$.

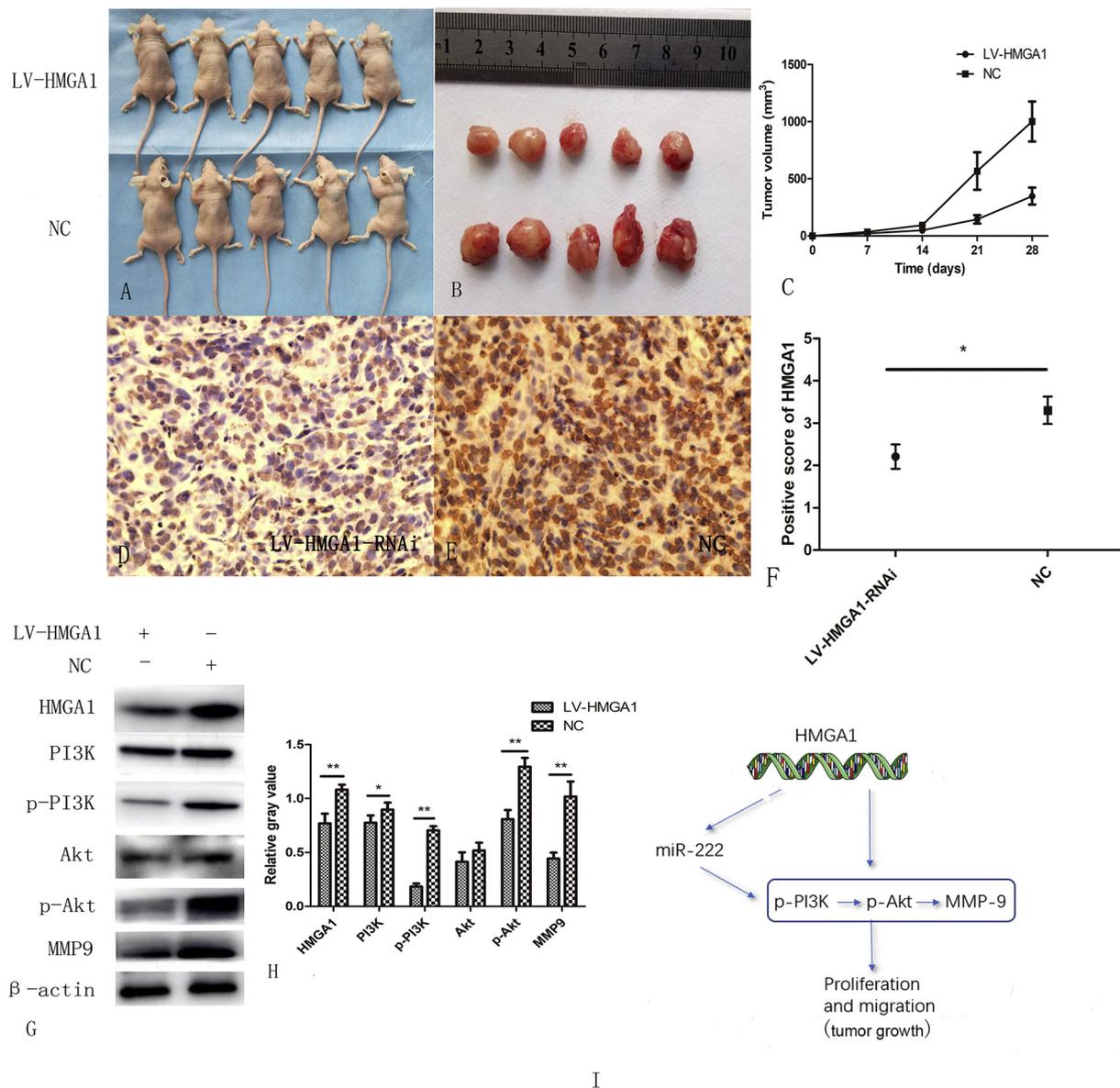


Fig. 5. The impact of HMGA1 on UM growth in xenograft mouse model. For the in vivo analyses, 5×10^6 C918 cells were injected subcutaneously into the posterior flank of nude mice. The mice were continuously observed for 28 days. A-C. The sizes of generated tumors in the two groups (LV-HMGA1 and NC) were calculated and compared in the right diagrams. Tumors in the LV-HMGA1 group have an average smaller size comparing to their NC group. Results were represented as mean \pm S.D. ($n = 5$). D-E. Representative immunohistochemical images showed the protein expression level of HMGA1 in the tumors from the C918 cells transfected with LV-HMGA1 is lower than NC group. The right bars (F) show the mean density scores of the HMGA1 protein staining by using IPP 6.0 ($P < 0.05$, Student-*t*-test). Protein was extracted from tumor tissue in xenograft mice induced by LV-HMGA1 and NC and then checked by western blot. Representative blots (G) and relative gray values (H) showed that HMGA1, PI3K, p-PI3K, p-Akt and MMP-9 expressions were suppressed in LV-HMGA1, comparing with the NC group ($P < 0.05$, Student-*t*-test). The protein expressions were relative to β -Actin. * $P < .05$, ** $P < .001$. I. Summary diagram of HMGA1 overexpression in UM progression through PI3K/Akt/MMP-9 pathway and mediated the oncogenic miR-222 function.

regulator of miR-222.

HMGA1 promotes the UM tumor progression in vivo.

To confirm the effects of HMGA1 on UM tumorigenesis in vivo, C918 cells treated by LV-HMGA1 and NC lentivirus were injected subcutaneously into the posterior flank of nude mice, which were killed after 28 days. Next, the sizes of the tumors were compared. As shown in Fig. 5A-C, the average tumor size was smaller in LV-HMGA1 groups than in the NC groups, and the individual growth velocity of tumor cells in the LV-HMGA1 group was clearly decelerated compared to that of their control group. These results suggest that HMGA1 facilitates UM tumor growth in vivo.

Additionally, we further detected HMGA1 protein expression levels in paraffin sections of tumor tissues from nude mice tumors using IHC

I

analysis. Similar results were obtained for HMGA1 expression in vivo, which showed lower expression in LV-HMGA1 groups than in the NC groups, supporting the conclusion that HMGA1 might participate in the tumorigenesis regulation of UM ($P < .05$) (Fig. 5D-F). Moreover, the tissue protein of tumor was extracted to analyze the PI3K/Akt/MMP-9 pathway expression using western blot. HMGA1, PI3K, p-PI3K, p-Akt and MMP-9 were dramatically attenuated in LV-HMGA1 group (Fig. 5G-H), which is in accordance with the influence of miR-222 in vitro.

4. Discussion

Burgeoning literatures have indicated that HMGA1 protein acts as

an oncogene in the tumorigenesis and progression of various cancers [34,35]. Our previous study demonstrated that HMGA1 overexpression is associated with worse prognosis using IHC in 89 tumor samples from UM patients [9], but the underlying mechanism of HMGA1 in the UM tumorigenesis is still poorly understood. In this study, we identified that HMGA1 could promote the progression of UM through PI3K/Akt/MMP-9 signaling pathway and positively regulated miR-222 in vitro and in vivo. There are several treatment measures on UM in the clinic currently, the main approaches include enucleation, proton beam radiotherapy and plaque radiotherapy. Despite the progress and availability of alternative therapeutic models, the five-year survival rates of UM patients are nearly unchanged [36–38]. Hence, there is an urgent need to identify new therapeutic targets that represent a molecular determinant of cellular progression. HMGA1 was described to function as a potent oncogene in different cell lines and transgenic mice [39,40]. The high expression level of HMGA1 protein was found to be linked to highly malignant phenotype of human cancers and a poor prognostic indicator in UM patients [9], we thus speculated that HMGA1 may serve as an effective and safe therapeutic molecular target.

In this study, we highlighted the prognostic and therapeutic value of HMGA1, which plays an important role through the PI3K/Akt/MMP-9 signaling pathway in UM. Our results showed p-PI3K, p-Akt and MMP-9 expression levels decreased in UM cell lines treated with LV-HMGA1. These data are similar to the previous study published by Janani Panneerselvam et al., where they reported the overexpression of HMGA1/miR222/AKT signaling axis in lung cancer cells [24]. PI3K/Akt signaling pathway is involved in tumor cell proliferation, survival and migration, which is positively mediated by HMGA1 [21–23]. Yunzhi Zhang et al. reported that HMGA1 activates Akt signaling through a feed-forward loop targeting PPP2R2A, HMGA1 might inhibit the transcription of PPP2R2A in lung cancer cells, which is independent of miR-222 function [21]. HMGA1-induced cellular invasiveness is in part due to PI3K/Akt dependent modulation of MMP-9 activity in pancreatic adenocarcinoma, which is consistent with our findings in UM [41]. Besides, it is well documented that HMGA1 protein phosphorylation reduces DNA-binding affinity and transcriptional activation, which may related to the mechanism involving the intracellular regulatory PI3K/Akt pathway [42,43]. Chiefari et al. demonstrated in their study that insulin is directly involved in the dynamic interaction of HMGA1 protein phosphorylation in vivo [42]. Hence, further study of molecular mechanism underlying HMGA1 protein phosphorylation and PI3K/Akt pathway is needed.

PI3K, a major downstream signal of growth factor receptor tyrosine kinases, catalyze the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the cell membrane, which involved in recruitment and activation of a wide range of cellular targets including Akt. In turn, Akt is fully activated through phosphorylation at threonine 308 and serine 473, the activation of Akt contributes to the regulation of cellular growth, cell survival and cell cycle progression [44,45]. Our observation that HMGA1 silencing suppressed p-PI3K and p-Akt protein expressions is significant, however, the total mRNA of PI3K and Akt in UM cells didn't show a significant difference. We deduced that phosphorylated PI3K and Akt proteins reflect the activity of PI3K/Akt signaling pathway, which explained that the p-PI3K and p-Akt proteins showed a dramatic inhibition in lentivirus-mediated HMGA1 silencing group compared to NC group.

Recently, a better understanding of biophysics implicates that MMPs are involved in nearly every stage of cancer metastasis. Numerous clinical trials show that MMP inhibitors would make effective therapeutic targets and focus on more specific inhibitors, rather than broad range inhibitors [46]. MMP-9 could degrade type IV collagen, which is the main structural component of the basement membrane. This biological process has a close relation with cancer cell migration [47]. Furthermore, MMP-9-mediated tumor angiogenesis provides a favorable microenvironment to tumor cell invasion by promoting gas exchange and supplying nutrients [48]. Several studies have

demonstrated that HMGA1 may involve in the up-regulation of some MMPs, in particular MMP-2, MMP-9 and MMP-11, which play a key role in the proliferation and progression of various human malignancies [49–51]. We found that HMGA1 inhibition could downregulate the MMP-9 expression via PI3K/Akt pathway, subsequently suppressing the proliferation and migration of UM cells in vivo and in vitro. Therefore, HMGA1, a regulator of the PI3K/Akt/MMP-9 pathway, could be a potential target for UM patients treatment.

MiRNA is small noncoding RNA that plays a critical role in basic biological and pathological processes, modulating target gene expression at the post-transcriptional level, mainly through binding to 3'-UTR of target messenger RNA [52,53]. Accumulating literatures show that miR-222 significantly overexpressed in many human malignancies, including pancreatic cancer, breast cancer, colorectal cancer and cervical cancer [54,55]. Our findings proved that miR-222 is able to promote the proliferation and migration of C918 and MUM-2B cells, indicating that silencing miR-222 might represent an intriguing approach for therapeutic studies. Moreover, miR-222 is involved in the activation of various pathways in cancer cells, including Akt signaling and TIMP3-MMP2/MMP-9 axis [22,24,56]. Consistent with these findings, we found that PI3K/Akt/MMP-9 pathway expression has been mediated by using miR-222-3p mimics and inhibitor in UM cells. Expressions of p-PI3K, p-Akt and MMP-9 increased in UM cells transfected by miR-222-3p mimic and declined in cells treated with miR-222-3p inhibitor, suggesting PI3K/Akt/MMP-9 pathway activity was regulated by miR-222 in UM.

Our study has demonstrated a prognostic and therapeutic value of HMGA1 and miR-222 in UM. The correlation between HMGA1 and miR-222 has been reported in some other tumors, including lung cancer and cervical cancer [21,22]. They indicated overexpressed HMGA1 is responsible for dysregulation of many important oncogenic genes or miRNAs, including miR-222. In line with our results, they reported the high expression of HMGA1 was significantly associated with increased levels of miR-222 in lung and cervical tumor specimens. In this regard, the combined treatment of pharmacological inhibitors of HMGA1 and functionally oncogenic miRNAs (such as miR-222) might acquire desired anti-tumorigenic effects. Further mechanism of oncogenic-related genes and miRNAs involved would lead to a better understanding of UM etiology and progression.

In conclusion, our novel work establishes a link between HMGA1 and PI3K/Akt/MMP-9 pathway, suggesting HMGA1 plays an important role in UM cell proliferation and migration. Moreover, current results indicated that oncogenic miR-222 could be positively mediated by HMGA1, which could be considered as diagnostic and therapeutic biomarkers for UM (Fig. 5I). Accordingly, we hope our findings merit further investigation of targeting HMGA1-related gene for the clinical treatment of UM.

Funding

This study was supported by National Natural Science foundation of China (31570789) and The Fundamental Research Funds of Shandong University (2017JC034). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

Conception and design: Yi Qu and Ying Cheng; Experimental operation: Ying Cheng, Tongjie Cheng and Yuqing Zhao; Manuscript writing: Ying Cheng; Final approval of the manuscript: all authors.

Declaration of Competing interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgements

The authors thank the grant support from National Natural Science foundation of China (31570789) and The Fundamental Research Funds of Shandong University (2017JC034).

References

- P.L. Triozzi, C. Eng, A.D. Singh, Targeted therapy for uveal melanoma, *Cancer Treat. Rev.* 34 (3) (2008) 247–258.
- M.J. Jager, M. Dogrusoz, S.E. Woodman, Uveal melanoma: identifying immunological and chemotherapeutic targets to treat metastases, *Asia-Pacific J. Ophthalmol.* (Philadelphia, Pa.) 6 (2) (2017) 179–185.
- K. Buder, A. Gesierich, G. Gelbrich, M. Goebeler, Systemic treatment of metastatic uveal melanoma: review of literature and future perspectives, *Cancer Med.* 2 (5) (2013) 674–686.
- E. Chiefari, S. Tanyolac, F. Paonessa, C.R. Pullinger, C. Capula, S. Iiritano, T. Mazza, M. Forlin, A. Fusco, V. Durlach, A. Durlach, M.J. Malloy, J.P. Kane, S.W. Heiner, M. Filocamo, D.P. Foti, I.D. Goldfine, A. Brunetti, Functional variants of the HMGAI1 gene and type 2 diabetes mellitus, *Jama* 305 (9) (2011) 903–912.
- M.D. Williams, X. Zhang, A.S. Belton, L. Xian, T. Huso, J.J. Park, W.F. Siems, D.R. Gang, L.M. Resar, R. Reeves, H.H. Hill Jr., HMGAI1 drives metabolic reprogramming of intestinal epithelium during hyperproliferation, polyposis, and colorectal carcinogenesis, *J. Proteome Res.* 14 (3) (2015) 1420–1431.
- J. Zhou, M. Xie, H. He, Y. Shi, B. Luo, G. Gong, J. Li, J. Wang, X. Wu, J. Wen, Increases urinary HMGAI1 in serous epithelial ovarian cancer patients, *Cancer Biomarkers* 15 (3) (2015) 325–331.
- I. Takeuchi, N. Takaha, T. Nakamura, F. Hongo, K. Mikami, K. Kamoi, K. Okihara, A. Kawachi, T. Miki, High mobility group protein AT-hook 1 (HMGAI1) is associated with the development of androgen independence in prostate cancer cells, *Prostate* 72 (10) (2012) 1124–1132.
- N. Sekimoto, A. Suzuki, Y. Suzuki, S. Sugano, Expression of miR26a exhibits a negative correlation with HMGAI1 and regulates cancer progression by targeting HMGAI1 in lung adenocarcinoma cells, *Mol. Med. Rep.* 15 (2) (2017) 534–542.
- Y. Qu, Y. Wang, J. Ma, Y. Zhang, N. Meng, H. Li, Y. Wang, W. Wei, Overexpression of high mobility group A1 protein in human uveal melanomas: implication for prognosis, *PLoS One* 8 (7) (2013) e68724.
- A. Esquela-Kerscher, F.J. Slack, Oncomirs - microRNAs with a role in cancer, *Nat. Rev. Cancer* 6 (4) (2006) 259–269.
- G.A. Calin, C.M. Croce, MicroRNA signatures in human cancers, *Nat. Rev. Cancer* 6 (11) (2006) 857–866.
- L. Falzone, L. Scola, A. Zanghi, A. Biondi, A. Di Cataldo, M. Libra, S. Candido, Integrated analysis of colorectal cancer microRNA datasets: identification of microRNAs associated with tumor development, *Aging (Albany NY)* 10 (5) (2018) 1000.
- S. Hafs, S. Candido, R. Maestro, L. Falzone, Z. Souza, B. Bonavida, D.A. Spandidos, M. Libra, Correlation between the overexpression of yin Yang 1 and the expression levels of miRNAs in Burkitt's lymphoma: a computational study, *Oncol. Lett.* 11 (2) (2016) 1021–1025.
- L. Falzone, G. Lupo, G.R.M.L. Rosa, S. Crimi, C.D. Anfuso, R. Salemi, E. Rapisarda, M. Libra, S. Candido, Identification of novel MicroRNAs and their diagnostic and prognostic significance in oral cancer, *Cancers* 11 (5) (2019) 610.
- L. Falzone, G.L. Romano, R. Salemi, C. Bucolo, B. Tomasello, G. Lupo, C.D. Anfuso, D.A. Spandidos, M. Libra, S. Candido, Prognostic significance of deregulated microRNAs in uveal melanomas, *Mol. Med. Rep.* 19 (4) (2019) 2599–2610.
- F. Felicetti, A. De Feo, C. Coscia, R. Puglisi, F. Pedini, L. Pasquini, M. Bellenghi, M.C. Errico, E. Pagani, A. Carè, Exosome-mediated transfer of miR-222 is sufficient to increase tumor malignancy in melanoma, *J. Transl. Med.* 14 (1) (2016) 56.
- K. Kang, J. Zhang, X. Zhang, Z. Chen, MicroRNA-326 inhibits melanoma progression by targeting KRAS and suppressing the AKT and ERK signalling pathways, *Oncol. Rep.* 39 (1) (2018) 401–410.
- S. Galardi, N. Mercatelli, E. Giorda, S. Massalini, G.V. Frangese, S.A. Ciarfè, M.G. Farace, miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1, *J. Biol. Chem.* 282 (32) (2007) 23716–23724.
- S. Wang, A.B. Aurora, B.A. Johnson, X. Qi, J. McAnally, J.A. Hill, J.A. Richardson, R. Bassel-Duby, E.N. Olson, The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis, *Dev. Cell* 15 (2) (2008) 261–271.
- E.M. Small, E.N. Olson, Pervasive roles of microRNAs in cardiovascular biology, *Nature* 469 (7330) (2011) 336.
- Y. Zhang, T. Ma, S. Yang, M. Xia, J. Xu, H. An, Y. Yang, S. Li, High-mobility group A1 proteins enhance the expression of the oncogenic miR-222 in lung cancer cells, *Mol. Cell. Biochem.* 357 (1–2) (2011) 363–371.
- F. Fu, T. Wang, Z. Wu, Y. Feng, W. Wang, S. Zhou, X. Ma, S. Wang, HMGAI1 exacerbates tumor growth through regulating the cell cycle and accelerates migration/invasion via targeting miR-221/222 in cervical cancer, *Cell Death Dis.* 9 (6) (2018) 594.
- J. Song, Y. Ouyang, J. Che, X. Li, Y. Zhao, K. Yang, X. Zhao, Y. Chen, C. Fan, W. Yuan, Potential value of miR-221/222 as diagnostic, prognostic, and therapeutic biomarkers for diseases, *Front. Immunol.* 8 (2017) 56.
- J. Panneerselvam, A. Srivastava, R. Muralidharan, Q. Wang, W. Zheng, L. Zhao, A. Chen, Y.D. Zhao, A. Munshi, R. Ramesh, IL-24 modulates the high mobility group (HMG) A1/miR222/AKT signaling in lung cancer cells, *Oncotarget* 7 (43) (2016) 70247–70263.
- K. Kessenbrock, V. Plaks, Z. Werb, Matrix metalloproteinases: regulators of the tumor microenvironment, *Cell* 141 (1) (2010) 52–67.
- C. Gialeli, A.D. Theocharis, N.K. Karamanos, Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting, *FEBS J.* 278 (1) (2011) 16–27.
- G. Tu, W. Xu, H. Huang, S. Li, Progress in the development of matrix metalloproteinase inhibitors, *Curr. Med. Chem.* 15 (14) (2008) 1388–1395.
- L. Falzone, R. Salemi, S. Travali, A. Scalis, J.A. McCubrey, S. Candido, M. Libra, MMP-9 overexpression is associated with intragenic hypermethylation of MMP9 gene in melanoma, *Aging (Albany NY)* 8 (5) (2016) 933.
- M. Labrie, Y. St-Pierre, Epigenetic regulation of mmp-9 gene expression, *Cell. Mol. Life Sci.* 70 (17) (2013) 3109–3124.
- A. Ucar, V. Vafaizadeh, H. Jarry, J. Fiedler, P.A. Klemmt, T. Thum, B. Groner, K. Chowdhury, miR-212 and miR-132 are required for epithelial stromal interactions necessary for mouse mammary gland development, *Nat. Genet.* 42 (12) (2010) 1101.
- P. Liu, M.J. Wilson, miR-520c and miR-373 upregulate MMP9 expression by targeting mTOR and SIRT1, and activate the Ras/Raf/MEK/Erk signaling pathway and NF-κB factor in human fibrosarcoma cells, *J. Cell. Physiol.* 227 (2) (2012) 867–876.
- E. Giovannetti, N. Funel, G.J. Peters, M. Del Chiaro, L.A. Erozceni, E. Vasilis, L.G. Leon, L.E. Pollina, A. Groen, A. Falcone, MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity, *Cancer Res.* 70 (11) (2010) 4528–4538.
- Y. Cheng, Y. Li, X. Huang, W. Wei, Y. Qu, Expression of EZH2 in uveal melanomas patients and associations with prognosis, *Oncotarget* 8 (44) (2017) 76423–76431.
- Y. Wang, L. Hu, Y. Zheng, L. Guo, HMGAI1 in cancer: cancer classification by location, *J. Cell. Mol. Med.* 23 (4) (2019) 2293–2302.
- T.F. Sumter, L. Xian, T. Huso, M. Koo, Y.T. Chang, T.N. Almasri, L. Chia, C. Inglis, D. Reid, L.M. Resar, The high mobility group A1 (HMGAI1) transcriptome in cancer and development, *Curr. Mol. Med.* 16 (4) (2016) 353–393.
- V.P. Papastefanou, V.M. Cohen, Uveal melanoma, *J. Skin Cancer* 2011 (2011) 573974.
- R.D. Carvajal, J.A. Sosman, J.F. Quevedo, M.M. Milhem, A.M. Joshua, R.R. Kuchadkar, G.P. Linette, T.F. Gajewski, J. Lutzky, D.H. Lawson, C.D. Lao, P.J. Flynn, M.R. Albertini, T. Sato, K. Lewis, A. Doyle, K. Ancell, K.S. Panageas, M. Bluth, C. Hedvat, J. Erinjeri, G. Ambrosini, B. Marr, D.H. Abramson, M.A. Dickson, J.D. Wolchok, P.B. Chapman, G.K. Schwartz, Effect of selumetinib vs chemotherapy on progression-free survival in uveal melanoma: a randomized clinical trial, *Jama* 311 (23) (2014) 2397–2405.
- K.M. Komatsubara, R.D. Carvajal, Adopting a new stance on immunotherapy for uveal melanoma, *The Lancet. Oncol.* 18 (6) (2017) 702–704.
- L.M. Resar, The high mobility group A1 gene: transforming inflammatory signals into cancer? *Cancer Res.* 70 (2) (2010) 436–439.
- A. Conte, S. Paladino, G. Bianco, D. Fasano, R. Gerlini, M. Tornincasa, M. Renna, A. Fusco, D. Tramontano, G.M. Pierantoni, High mobility group A1 protein modulates autophagy in cancer cells, *Cell Death Differ.* 24 (11) (2017) 1948–1962.
- S.-S. Liao, A. Jazag, E.E. Whang, HMGAI1 is a determinant of cellular invasiveness and in vivo metastatic potential in pancreatic adenocarcinoma, *Cancer Res.* 66 (24) (2006) 11613–11622.
- E. Chiefari, M.T. Nevelo, B. Arcidiacono, E. Maurizio, A. Nocera, S. Iiritano, R. Sgarra, K. Possidente, C. Palmieri, F. Paonessa, HMGAI1 is a novel downstream nuclear target of the insulin receptor signaling pathway, *Sci. Rep.* 2 (2012) 251.
- D.-Z. Wang, P. Ray, M. Boothby, Interleukin 4-inducible phosphorylation of HMG-1 (Y) is inhibited by rapamycin, *J. Biol. Chem.* 270 (39) (1995) 22924–22932.
- J. Luo, B.D. Manning, L.C. Cantley, Targeting the PI3K-Akt pathway in human cancer: rationale and promise, *Cancer Cell* 4 (4) (2003) 257–262.
- J.A.F. Vara, E. Casado, J. de Castro, P. Cejas, C. Belda-Iniesta, M. González-Barón, PI3K/Akt signalling pathway and cancer, *Cancer Treat. Rev.* 30 (2) (2004) 193–204.
- G.A. Conlon, G.I. Murray, Recent advances in understanding the roles of matrix metalloproteinases in tumour invasion and metastasis, *J. Pathol.* 247 (5) (2019) 629–640.
- H. Zheng, J.F. Liu, Studies on the relationship between PI3K/AKT signal pathway-mediated MMP-9 gene and lung cancer, *Eur. Rev. Med. Pharmacol. Sci.* 21 (4) (2017) 753–759.
- M. Stefanidakis, E. Koivunen, Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and cancer progression, *Blood* 108 (5) (2006) 1441–1450.
- M. Greco, B. Arcidiacono, E. Chiefari, T. Vitagliano, A.G. Ciriaco, F.S. Brunetti, G. Cuda, A. Brunetti, HMGAI1 and MMP-11 are overexpressed in human non-melanoma skin cancer, *Anticancer Res.* 38 (2) (2018) 771–778.
- N. Takaha, L.M.S. Resar, D. Vindivich, D.S. Coffey, High mobility group protein HMG(Y) enhances tumor cell growth, invasion, and matrix metalloproteinase-2 expression in prostate cancer cells, *Prostate* 60 (2) (2004) 160–167.
- S.S. Liao, A. Jazag, E.E. Whang, HMGAI1 is a determinant of cellular invasiveness and in vivo metastatic potential in pancreatic adenocarcinoma, *Cancer Res.* 66 (24) (2006) 11613–11622.
- J. Hausser, M. Zavolan, Identification and consequences of miRNA-target interactions—beyond repression of gene expression, *Nat. Rev. Genet.* 15 (9) (2014) 599–612.
- F.J. Slack, J.B. Weidhaas, MicroRNA in cancer prognosis, *N. Engl. J. Med.* 359 (25) (2008) 2720–2722.
- K. Gocze, K. Gombos, K. Juhasz, K. Kovacs, B. Kajtar, M. Benczik, P. Gocze, B. Patczai, I. Arany, I. Ember, Unique microRNA expression profiles in cervical cancer, *Anticancer Res.* 33 (6) (2013) 2561–2567.
- J. Song, Y. Ouyang, J. Che, X. Li, Y. Zhao, K. Yang, X. Zhao, Y. Chen, C. Fan, W. Yuan, Potential value of miR-221/222 as diagnostic, prognostic, and therapeutic biomarkers for diseases, *Front. Immunol.* 8 (2017) 56.
- J. Zhang, L. Han, Y. Ge, X. Zhou, A. Zhang, C. Zhang, Y. Zhong, Y. You, P. Pu, C. Kang, miR-221/222 promote malignant progression of glioma through activation of the Akt pathway, *Int. J. Oncol.* 36 (4) (2010) 913–920.