

MCM5 aggravates HDAC1 mediated malignant progression of lung cancer

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Research

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Abstract

Background

Lung cancer, as one of the most lethal malignant tumors, is closely related to epithelial mesenchymal transition (EMT).

Methods

Clinical specimens and TCGA database were used to analyze the expression of MCM and HDAC1 and their impact on the survival of lung cancer patients. Cell and animal experiments are used to verify the promotion of EMT of lung cancer cells by the expression of MCM5 and HDAC1.

Results

We found that MCM5 interacts with HDAC1 and promotes the EMT-mediated malignant progression of lung cancer. Lung adenocarcinoma patients with high expression of MCM5 and HDAC1 have poor survival time. Overexpression of MCM5 and HDAC1 in A549 and H1975 cells can promote metastasis and invasion in vitro and tumor growth and metastasis in vivo. Moreover, astragaloside IV can block the interaction of HDAC1 and MCM5, which can then inhibit the malignant progression of lung cancer in vivo and in vitro.

Conclusion

We described the interaction of MCM5 and HDAC1 to promote the EMT-dependent malignant progression in lung cancer. Astragaloside IV inhibited lung cancer progression by blocking the combination of MCM5 and HDAC1.

Background

Lung cancer is the most frequent cause of cancer-related deaths and causes more than one million deaths annually worldwide. Lung cancer is more frequently diagnosed at an advanced metastatic stage and survival rates are usually poor [1, 2]. During tumor development, the tumor microenvironment and external stimuli cause tumor cell stress responses and key gene transcriptional abnormalities [3–8]. Epigenetic regulation of abnormal gene expression leads to tumor malignant progression. Patients with lung cancer are usually accompanied by mutations of epidermal growth factor receptor (EGFR), P53 and KRAS[9–12]. It was also reported that mutations in ERBB2, another member of the EGFR family, are common in patients with lung adenocarcinoma[13].

The epithelial-to-mesenchymal transition (EMT) means that the epithelial cells elongate during the transition to a mesenchymal phenotype and acquire the characteristics of movement and invasion.[14]. This transformation of epithelial cells is involved in a series of biological processes, including wound healing, inflammation, fibrosis and cancer. In the pathogenesis of cancer, EMT promotes cancer progression by conferring highly invasive properties on tumor cells[15]. The expression and activity of highly conserved EMT-inducing factors, including Snail, Slug, Zeb1 and Zeb2, and Twist, can usually induce EMT transition in tumor cells[16]. Accumulated evidence indicates that the transcription complex contains EMT-inducing factors and histone-modifying enzymes play an important role in EMT-mediated malignant tumor progression.

MCM5, a mini chromosome maintenance protein, is closely related to the structure of chromatin[17]. HDAC1, a transcriptional repressor, is involved in a wide range of transcriptional regulatory behaviors and plays a crucial role in the development of tumors[18]. Our study found that MCM5 interacts with HDAC1. The co-expression of HDAC1 and MCM5 in lung cancer patients indicates a poor prognosis. In vitro and in vivo experiments also show that HDAC1 and MCM5 can promote the invasion and migration of EMT-dependent lung cancer cells. Furthermore, Astragaloside IV can block the interaction of HDAC1 and MCM5 to retard EMT progression in lung cancer. This study may provide a theoretical basis at the transcriptional level for drug development and the diagnosis and treatment of malignant tumors.

Material And Methods

Cell lines and plasmids

The Lung cancer cell lines A549 and H1975 were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences (Shanghai, China). H1975 cells were cultured in RPMI 1640 medium, and A549 cells were cultured in F12K medium. All culture media were supplemented with 10% fetal bovine serum (FBS) at 37°C in an incubator with 5% CO₂. MCM5-Flag, MCM5, and HDAC1 expression plasmids were purchased from Sino Biological (Beijing, China).

Clinical data analysis

The TCGA data were used to analyze the expression of MCM5 and HDAC1 and their effects on the survival of patients with NSCLC. A total of 61 primary NSCLC tissues were collected from patients. The use of these specimens in this study was approved by the Institutional Review Board and the Research Ethics Committee of Tianjin medical university cancer institute and hospital, and written consent was obtained from all participants. Twelve of these specimens were used to analyze the expression of MCM5 through Western blot analysis. All the patients' samples were divided in accordance with clinical stage and pathological grading. The expression levels of MCM5, HDAC1, and R-loop in these samples were analyzed.

Silver staining

Cells were transfected with flag-MCM5. After 48 h, the cellular extracts were collected and incubated with the anti-FLAG affinity gel (Sigma) for 12 h at 4°C. The eluents of the bed volume were collected and separated through 10% SDS–PAGE. Subsequently, silver staining was performed using the Fast Silver Stain Kit (Beyotime, China).

Immunoprecipitation and Western blot analysis

After transfection, the cell extracts were prepared through incubation in lysis buffer.

For Western blot analysis, the proteins were separated by 10% SDS–PAGE and transferred onto PVDF membranes. The membranes were blocked and incubated with the primary antibodies of MCM5 (ab75975, Abcam, UK), HDAC1 (ab7028, Abcam, UK), and GAPDH (ab8245, Abcam, UK) at 4°C. GAPDH (CAT: 5174, CST, USA) was used as the loading control. After 2 h, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody. Protein expression was visualized using an enhanced chemiluminescence substrate (Millipore). For immunoprecipitation, the cell extracts were centrifuged and incubated with specific primary antibodies at 4°C overnight with constant rotation. The extracts were incubated with Protein A agarose beads and washed thrice with lysate. After centrifugation and suspension with loading buffer, the denatured protein was boiled for 10 min, separated through 10% SDS–PAGE, transferred onto a PVDF membrane, and detected using a specific antibody.

Colony formation assay

Transfected cells were seeded in 6-well plates at a density of 500 cells per well. The medium was replaced every three days and cultured for 14 days. Then, the clones were fixed with 4% formaldehyde for 10 min and stained with crystal violet. The cell colonies with diameters exceeding 50 µm were counted.

Invasion assay

The Transwell upper chamber filters were coated with Matrigel (BD Biosciences). After transfection, the cells were placed in the upper chamber of the Transwell in serum-free media. The lower chamber media contained 10% FBS. These cells were incubated at 37°C for 24 h. The noninvasive cells in the top well were removed. Then, the membranes were stained with crystal violet and photographed using a microscope.

Immunohistochemistry

The paraffin-embedded tissue was cut into 4 µm thick sections. After antigen retrieval through heating in microwave oven and blocking with 3% H₂O₂ solution, the sections were incubated with primary antibodies at 4°C overnight. The immunohistochemistry staining scores were assessed by two pathologists. The staining score was assessed as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong).

Wound healing assay

The transfected cells were seeded in 24-well plates with 10% FBS culture for 24 h. Then, a linear wound was formed by scraping the cell layer with a 1 μ L pipette tip. After the suspended cells were removed with PBS, the culture was allowed to grow by adding a complete medium. Photographs were taken at 0 and 48 h under a microscope.

Scanning electron microscopy (SEM)

The cells were fixed, dehydrated in acetone/isoamyl acetate (1:1), and dried using a gradient concentration of acetonitrile. Gold-coated cells were photographed using a scanning electron microscope (JEOL 6000, Japan).

Molecular docking

The crystal structure of HIF-1 α was downloaded from the PDB database and used to perform molecular docking with Rg3 by using the Sybyl X1.1 software.

In vivo experiment

Five-week-old BALB/c mice were purchased from Charles River (Beijing,China), and 24 mice were randomly divided into four groups. Cells stably expressing GFP were transfected with MCM5 or HDAC1 expression plasmid. A total of 5×10^6 cells were inoculated into the lower part of the armpits of BALB/c nude mice. Tumor size and mouse survival were measured every three days. Tumor volume was calculated using the formula: Tumor volume = (length \times width²)/2. All animals were euthanized through the intravenous injection of barbiturate at a final concentration of 100 mg/kg. Then, the solid tumors were harvested from the mice by surgery, and the tumor volume was calculated. All tissues were fixed in 4% formalin and embedded in paraffin for H&E histological and immunohistochemical staining. All animal experiments were performed in accordance with relevant ethical standards, and the protocol was approved by the Tianjin Medical University Animal Ethics Committee.

Statistical analysis

All statistical analyses were performed using the GraphPad Prism V.6.0. Comparisons between two groups were performed using the Student's t-test, whereas comparisons among three or more groups were conducted using ANOVA with Dunnett's post-test. Differences were considered significant at $P < 0.05$ and labeled with *.

Results

MCM5 is highly expressed in lung cancer and associated with poor outcomes

In this study, the Human Protein Atlas revealed that MCM5 was highly expressed in lung cancer compared with that in normal tissues (Fig. 1A). Consistently, the database of The Cancer Genome Atlas (TCGA) showed that the MCM5 mRNA levels in human lung adenocarcinoma (LUAD) and lung squamous

cell carcinoma (LUSD) tissues were upregulated compared with that in normal tissues (Fig. 1B&1C). A high level of MCM5 expression was associated with reduced overall survival time in LUAD (Fig. 1D), whereas MCM5 expression was not significantly related to the survival of patients with LUSD. The protein levels of MCM5 in four pairs of fresh tumor tissues and their control tissues were determined to verify the expression of MCM5 in LUAD. Results were consistent with the TCGA results, which showed that the MCM5 expression in LUAD was higher than that in normal tissues (Fig. 1E). These results suggested that MCM5 was likely to be closely related to the malignant evolution of lung cancer rather than to tumor genesis.

MCM5 contributes to lung cancer progression in vitro

We detected the protein expression of MCM5 in 6 lung cancer cells (Fig. 2A). Next, we choose the A549 and H1975 cells with the lowest MCM5 expression to overexpress MCM5. (Fig. 2B). First, the changes in cell phenotype were observed through SEM. After overexpressing MCM5, the pseudopods in cells decreased, but more pseudopods. This finding suggested a reduction in cell adhesion (Fig. 2C). Next, clone formation, transwell, and wound healing assays were used to verify the function of MCM5 in lung cancer cell invasion and proliferation. The results of these assays indicated that the ectopic expression of MCM5 can promote lung cancer cell proliferation (Fig. 2D), invasion (Fig. 2E), and migration (Fig. 2F). These results suggested that MCM5 may be related to the proliferation and metastasis of lung cancer.

MCM5 interacts with HDAC1 and is correlated with poor survival in lung cancer

Given that MCM5 is widely involved in chromatin structure, functional specificity of MCM5 in lung cancer may be related to specific gene interactions. Therefore, after transfection with the MCM5 overexpression plasmid carrying the Flag tag, the pulldown experiment was performed using Flag magnetic beads. The HDAC5 interaction protein was found to have high HDAC1 enrichment (Fig. 3A). Then we used molecular docking to validate the binding of MCM5 and HDAC1 (Fig. 3B). The total proteins from lung cancer cells were extracted, and coimmunoprecipitation was performed to further confirm the interaction between MCM5 and HDAC1. The results of this assay demonstrated that MCM5 efficiently interacted with HDAC1 (Fig. 3C). According to the predicted binding site of MCM5 and HDAC1 by molecular docking, we expressed MCM5 fusion proteins with GST tags of different lengths. The interaction with HDAC1 protein showed that the full-length and 300-amino acid length MCM5 had strong binding to HDAC1, however, when the MCM5 protein was shortened to 200 amino acids length, the binding of MCM5 to HDAC1 was obviously weakened (Fig. 3D). Further analysis of the TCGA data showed that HDAC1 was highly expressed in lung cancer (Fig. 3E). In addition, the high expression of HDAC1 alone had a negligible effect on the prognosis of patients with lung cancer, whereas patients with both high expression of HDAC1 and MCM5 had short survival times (Fig. 3F).

MCM5/HDAC1 complex promotes the lung cancer cell invasion and migration

Overexpression of MCM5 or HDAC1 alone or together (Fig. 4A), and their effect on the ability of lung cancer cells invasion and migration were detected. Results showed that compared with the expression of

MCM5 and HDAC1 alone, the simultaneous expression of these two genes had a stronger promotion effect on the migration (Fig. 4B) and invasion (Figs. 4C) capabilities of lung cancer cells. Next, we knocked down one of the genes in cells overexpressing HDAC1 and MCM5 to test whether it can reverse the effect of the complex. SEM results showed that the cell morphology changes more obviously after knocking down HDAC1 (Fig. 4D). The transwell and wound healing experiments showed that Knockdown of HDAC1 has a more obvious reversal effect on the invasion and migration ability of H1975 cells (Figs. 4E & 4F). Finally, Western blot results showed that after knocking down HDAC1, the expression of E-cad was up-regulated, while the expression of Vimentin and MMPs was down-regulated (Fig. 4G).

MCM5/HDAC1 complex promotes tumor proliferation and lung cancer metastasis

The above in vitro experiments suggested that the co-expression of MCM5 and HDAC1 can promote the proliferation and invasion of lung cancer cells. H1975 cells stably expressing MCM5 and HDAC1 were inoculated into Balb/c nude mice to verify their effect in vivo. The subcutaneously inoculated tumors were removed, and results showed that the co-expression of MCM5 and HDAC1 can indeed promote tumor growth (Figs. 5A & 5B). The immunohistochemistry results for solid tumors showed that the expression of E-Cad was significantly inhibited in the co-expression of MCM5 and HDAC1 (Figs. 5C & 5D). In addition, the tail vein model showed that the co-expression of MCM5 and HDAC1 had a stronger effect on promoting tumor metastasis than other groups (Fig. 5E).

High level of MCM5 and HDAC1 causes poor prognosis in lung cancer patients

In order to verify the effect of the interaction between HDAC1 and MCM5 on the survival of patients with lung cancer, we used IHC to test the expression of 61 lung cancer or HDAC1 and MCM5. The results showed that the expression of MCM5 and HDAC1 was higher in high-grade tumors than in low-grade patients (Fig. 6A-C). While the expression of E-cad was lower in high-grade and metastatic lung cancer (Fig. 6D-E). We divided lung cancer patients into two groups based on the IHC results. According to the analysis of patient survival, it was found that patients with high expression of MCM5 and HDAC1 have shorter survival time (Fig. 6F).

Astragaloside IV blocks the interaction of HDAC1 and MCM5

The above results show that the interaction of HDAC1 and MCM5 can lead to the malignant progression of lung cancer, so we speculate that blocking the interaction of HDAC1 and MCM5 may achieve the effect of inhibiting this type of lung cancer. Through computer virtual screening, we found that Astragaloside IV can block the binding of HDAC1 and MCM5 (Fig. 7A). Next, we treated H1975 cells with Astragaloside IV, and then used Co-ip to detect the binding of MCM5 to HDAC1. As a result, it was found that the binding ability of MCM5 to HDAC1 became weaker after treatment with Astragaloside IV (Fig. 7B). This shows that Astragaloside IV can indeed block the interaction between MCM5 and HDAC1. Western blot experiments also confirmed that Astragaloside IV (50uM) treatment can restore E-cad expression (Fig. 7C). Next, we used wound healing and transwell to find that Astragaloside IV can inhibit the migration (Fig. 7D) and invasion ability (Fig. 7E) of H1975 cells. In addition, we also verified in vivo experiments

that Astragaloside IV can inhibit the growth of lung cancer tumors (Fig. 7F). High expression of E-cad was also found in solid tumors of animals treated with Astragaloside IV (Fig. 7G).

Discussion

Metastasis is a crucial determinant of cancer-related mortality[19]. In EMT related tumor metastasis, The decrease of E-cad and the increase of Vimentin weaken the adhesion of tumor cells and obtain stronger mobility[20]. The expression of E-cad is regulated by multiple transcription factors, including EMT-inducing factors, SNAIL, SLUG and TWIST, which can bind to the E-box of the promoter of E-cad to inhibit the transcription of E-cad[21–23]. HDAC1 has been reported to be recruited by ZEB1 and participate in the transcriptional regulation of E-cad[24]. This study found that after HDAC1 interacts with MCM5, the expression of E-cad is down-regulated, while the expression of Vimentin and MMP is up-regulated. This indicates that the MCM5/HDAC1 transcription complex is involved in the EMT process of lung cancer. Interestingly, we found that the R-loop level was up-regulated after overexpression of HDAC1 and MCM5, which may indicate that after recruitment of MCM5, HDAC1's regulation of EMT-related genes may be related to the higher-level RNA structure.

Epigenetic modifications, such as histone acetylation, are essential for regulating gene expression in cells. The pathological epigenetic modification of cancer cells will promote and maintain the occurrence and development of tumors[25]. Therefore, epigenetic manipulation is emerging as a new type of targeted therapy for cancer. Histone acetylases (HATs) and histone deacetylases (HDACs) regulate histone acetylation, thereby regulating gene expression[26]. HDAC1 is widely involved in the transcriptional regulation and plays a crucial role in the evolution of various tumors[27–29]. Here we also found the high expression of HDAC1 in lung adenocarcinoma. In addition, HDAC1 was found to interact with MCM5. Among patients with lung adenocarcinoma, the co-expression of HDAC1 and MCM5 had the worst survival. This seems to suggest the role of the interaction of HDAC1 and MCM5 in the progression of lung adenocarcinoma. Our in vitro and in vivo experiments also show that the co-overexpression of HDAC1 and MCM5 can promote the proliferation and metastasis of lung cancer cells. Of course, the HDAC1/MCM5 transcription complex may also contain other transcription factors or transcription cofactors, which we have not yet identified. In addition, after we used astragaloside IV to block the binding of HDAC1 and MCM5, it showed anti-tumor effects in cell and animal experiments. This illustrates the anti-tumor effect of astragaloside IV, and also confirms the role of HDAC1/MCM5 complex in the malignant progression of lung cancer. As astragaloside IV has been proven to have anti-inflammatory, enhance drug resistance and promote apoptosis [30–32]. Here we cannot rule out that astragaloside IV exhibits anti-tumor effects in addition to blocking the interaction of HDAC1 and MCM5 through other pathways.

Conclusion

Overall, we verified the interaction of HDAC1 and MCM5, which promoted the progression of lung adenocarcinoma. The high expression of HDAC1 and MCM5 in patients with lung adenocarcinoma

indicates a poor prognosis. In in vitro and in vivo experiments, we have found that the HDAC1/MCM5 complex promotes lung cancer proliferation and metastasis, and is closely related to EMT. In addition, we found that astragaloside IV can block the interaction of HDAC1 and MCM5 and inhibit the proliferation and metastasis of lung adenocarcinoma cells. This study may provide theoretical and experimental support for the development of anti-tumor small molecule drugs at the transcriptional level.

Abbreviations

MCM5

Minichromosome maintenance complex component 5

HDAC1

Histone deacetylase 1

EMT

Epithelial mesenchymal transition

NSCLC

Non-small cell lung cancer

EGFR

Epidermal growth factor receptor

FBS

Fetal Bovine Serum

Declarations

Ethics approval and consent to participate: Clinical specimens used in this study was approved by the Institutional Review Board and the Research Ethics Committee of Tianjin medical university cancer institute and hospital. All animal experiments were performed in accordance with relevant ethical standards, and the protocol was approved by the Tianjin Medical University Animal Ethics Committee.

Consent for publication: Not applicable

Availability of data and material: All data can be provided by contacting corresponding author.

Competing interests: All the authors claims that there is no conflict of interest.

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Author contributions: ZY was responsible for the experimental design and financial support, ZLL was responsible for article writing and cell experiments, LQ responsible for pathology experiment, ZWJ was responsible for data analysis and cell experiments, and SXJ responsible for animal experiments.

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Figures

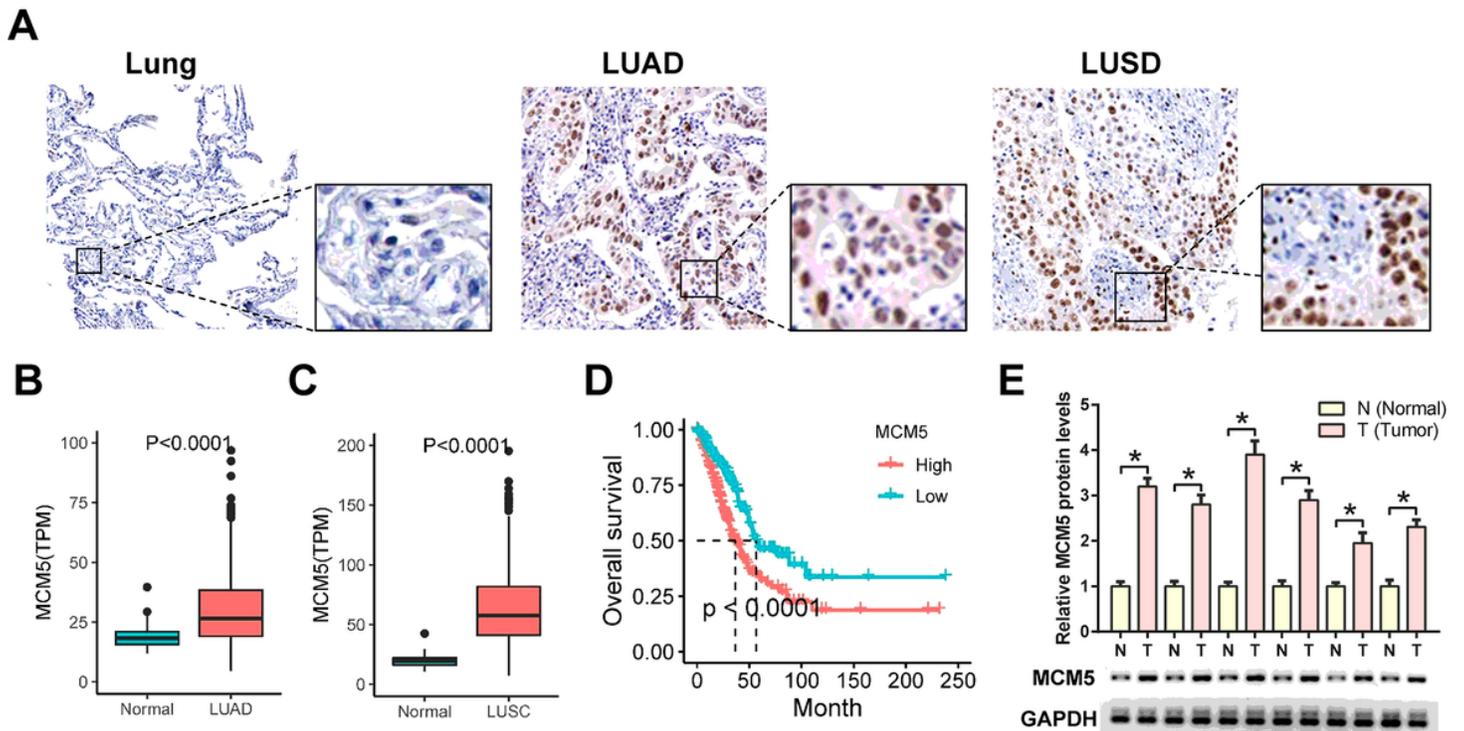


Figure 1

Figure 1

High levels of MCM5 in lung cancer A. IHC staining results for MCM5 in lung adenocarcinoma and lung squamous cell carcinoma from the Human Protein Atlas database. B. Expression of MCM5 in lung adenocarcinoma compared with that in normal lung tissue from the TCGA database. C. Expression of MCM5 in lung squamous cell carcinoma compared with that in normal lung tissue from the TCGA database. D. Effect of MCM5 expression on the overall survival of lung adenocarcinoma. E. Western blot detection of MCM5 protein expression in fresh lung adenocarcinoma and normal tissues. * $P < 0.05$

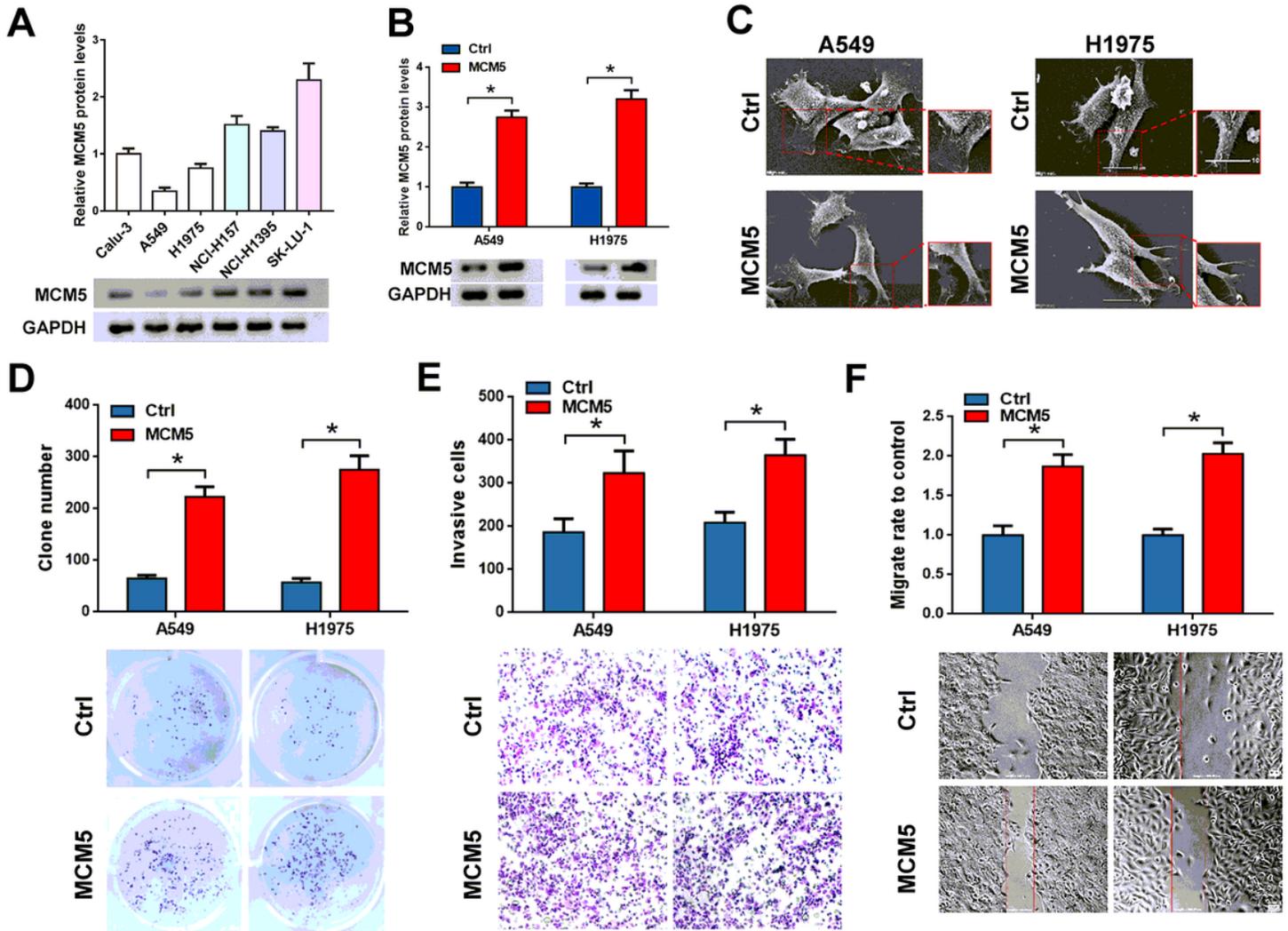


Figure 2

Figure 2

MCM5 promotes lung cancer cell proliferation and invasion A. Western blots was used to detect the expression of MCM5 in lung cancer cell lines. B. MCM5 protein level after overexpression of MCM5 in A549 and H1975 cells. C. Morphological changes in A549 and H1975 cells transfected with MCM5 observed with SEM. D. Colony formation of A549 and H1975 cells after MCM5 overexpression. E and F. Cell invasion and migration of A549 and H1975 cells after MCM5 transfection. *P < 0.05

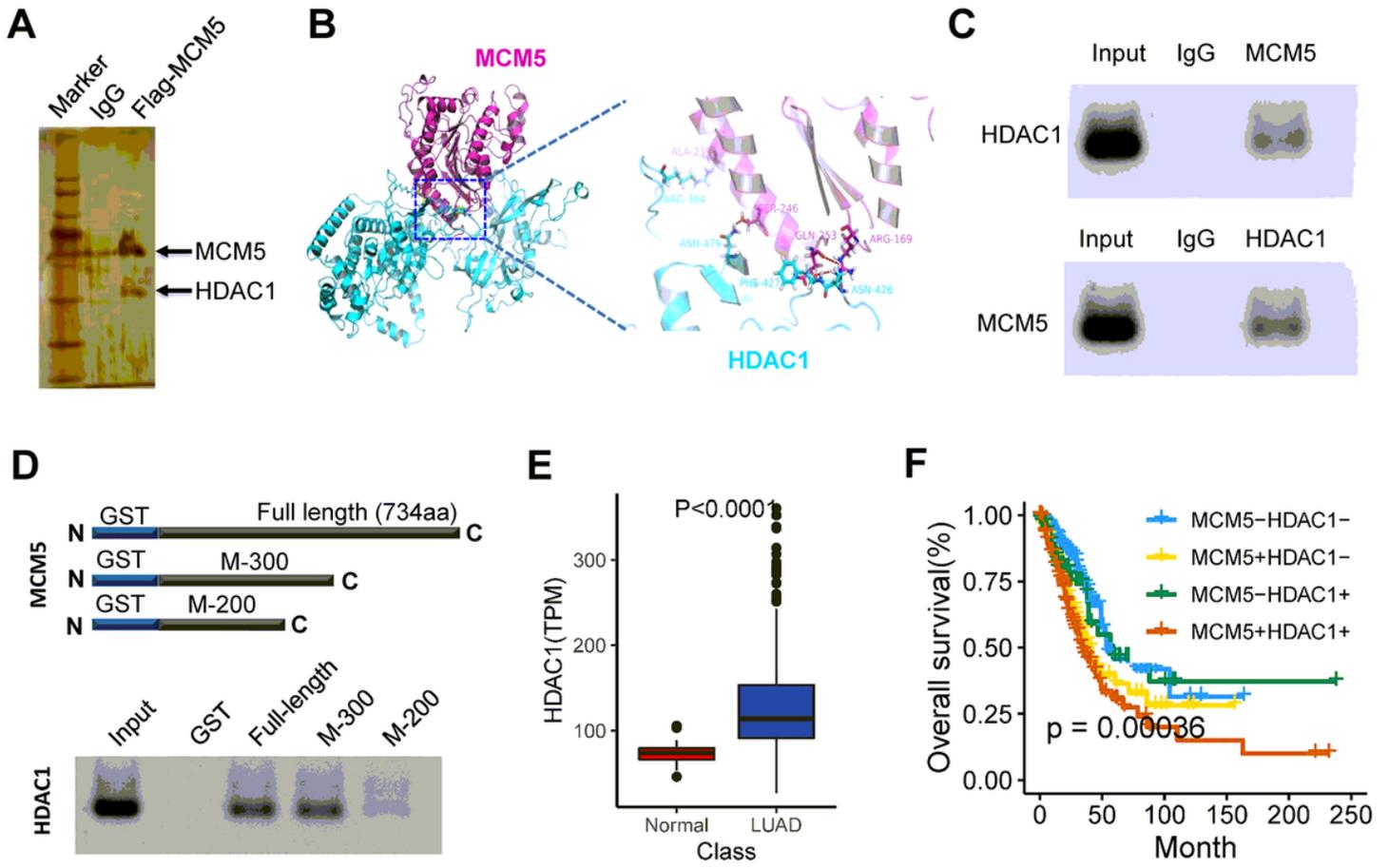


Figure 3

Figure 3

MCM5 interacts with HDAC1 and is correlated with poor survival in lung cancer. A. Silver staining identifies the interacting proteins of MCM5. B. Molecular docking between MCM5 and HDAC1. C. Co-IP of endogenous MCM5 and HDAC1 on A549 cells. D. In vitro interaction analysis of truncated MCM5 and HDAC1. E. HDAC1 expression level in lung cancer tumors and normal liver tissues based on the TCGA dataset. F. Survival analysis of TCGA samples for the relationship between the survival time and the MCM5 and HDAC1 expression levels of patients with lung cancer. Data are presented as mean \pm SD. *P < 0.05

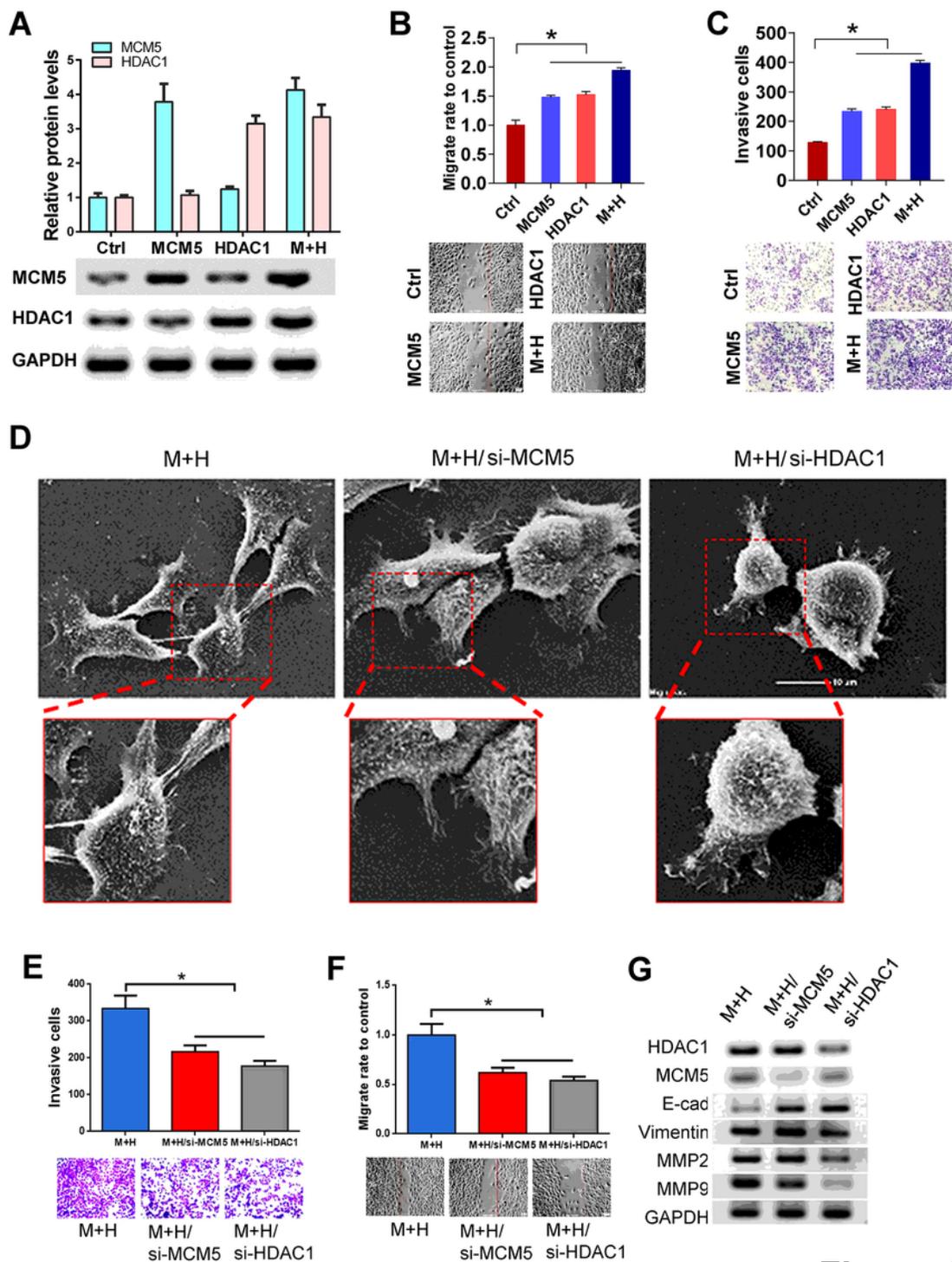


Figure 4

Figure 4

MCM5/HDAC1 complex promotes lung cancer migration and invasion A. Western blot analysis of HDAC1 and MCM5 expression. B and C. The invasion and migration of H1975 cells after HDAC1 and MCM5 expression. D. Morphological changes in MCM5/HDAC1 overexpression H1975 cells after transfected with siMCM5 or siHDAC1 observed with SEM. E and F. Results of the cell invasion and migration of

H1975 cells under different treatments. G. Western blots of H1975 cells of E-Cad, anti-Vimentin, anti-MMP2, and anti-MMP9 under different treatments. *P < 0.05

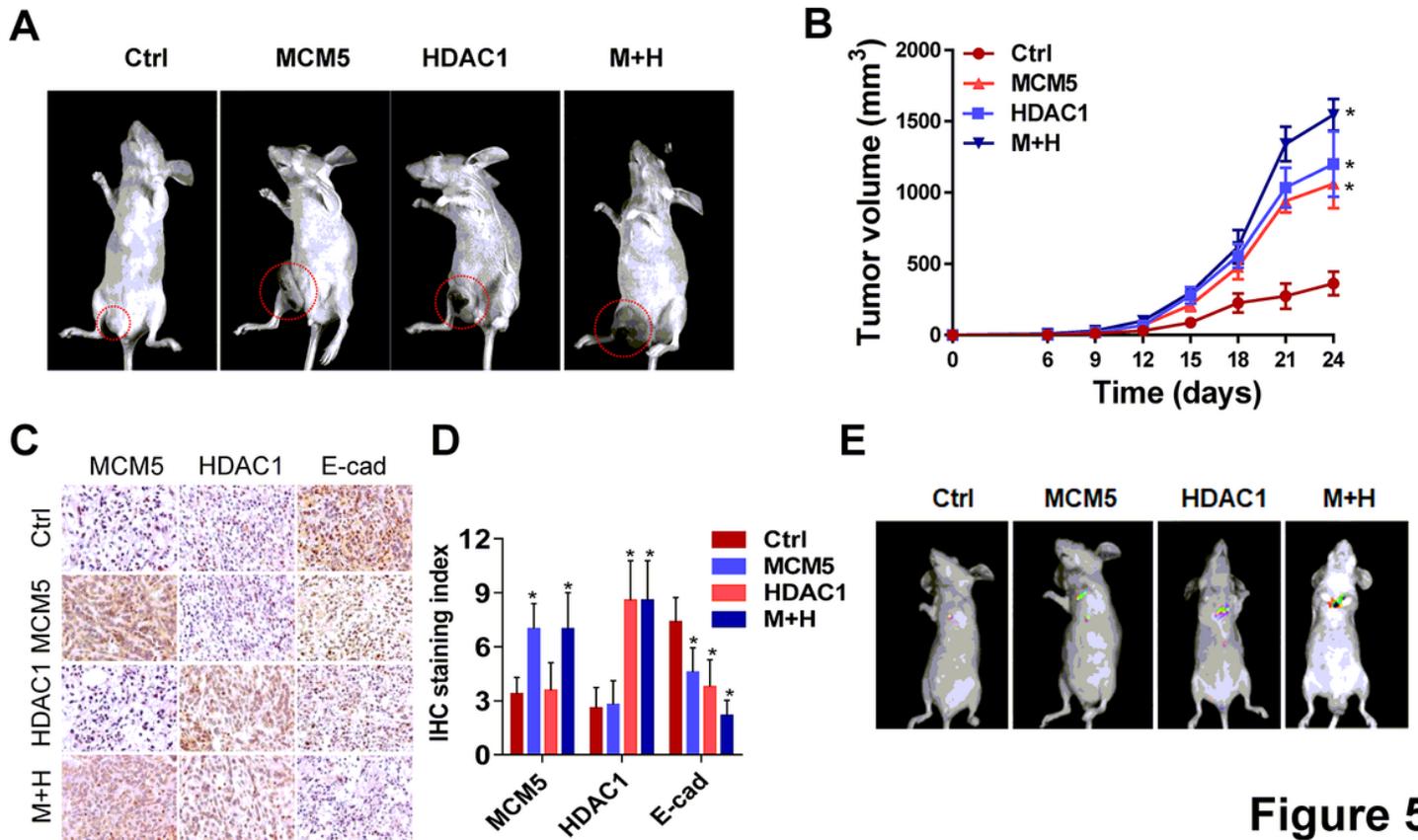


Figure 5

Figure 5

MCM5/HDAC1 complex promotes tumor proliferation and lung cancer metastasis A. Images of tumors under different treatments. B. Evaluation of tumor growth at different times. C and D. Detection of MCM5, HDAC1, and E-Cad expressions in tumors by immunohistochemistry. E. Representative images of in vivo bioluminescence imaging. *P < 0.05

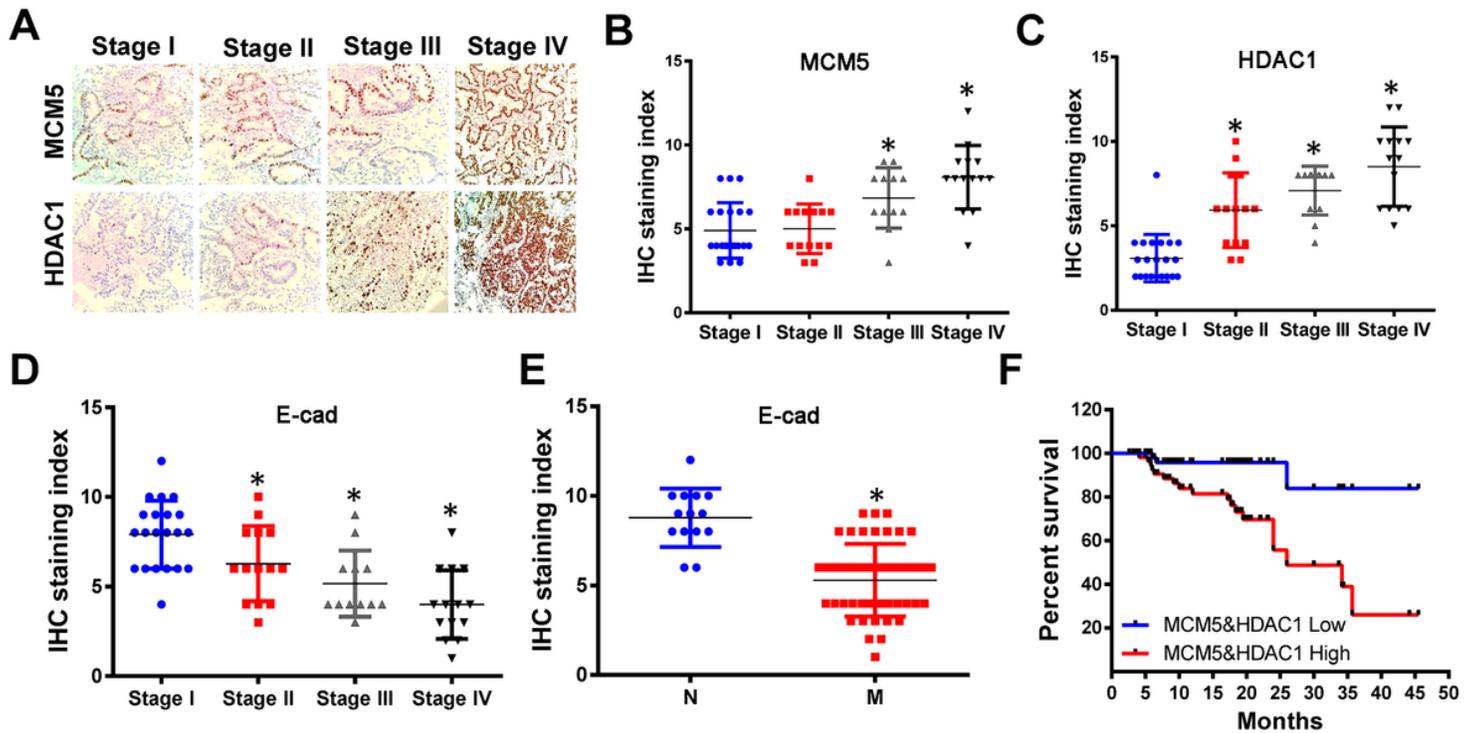


Figure 6

Figure 6

MCM5 / HDAC1 complex leads to poor prognosis in lung cancer patients A. IHC was performed to detect MCM5 and HDAC1 expression in lung cancer tissues with different clinical stages. B. MCM5 expression in lung cancer patients. C. HDAC1 expression in lung cancer patients. D. E-cad expression in lung cancer tissues with different clinical stages. E. The expression of E-cad in metastatic and non-metastatic lung cancer. F. Kaplan–Meier plots of the overall survival rate of patients with lung cancer with low (blue) or high (red) MCM5/HDAC1 levels. *P < 0.05

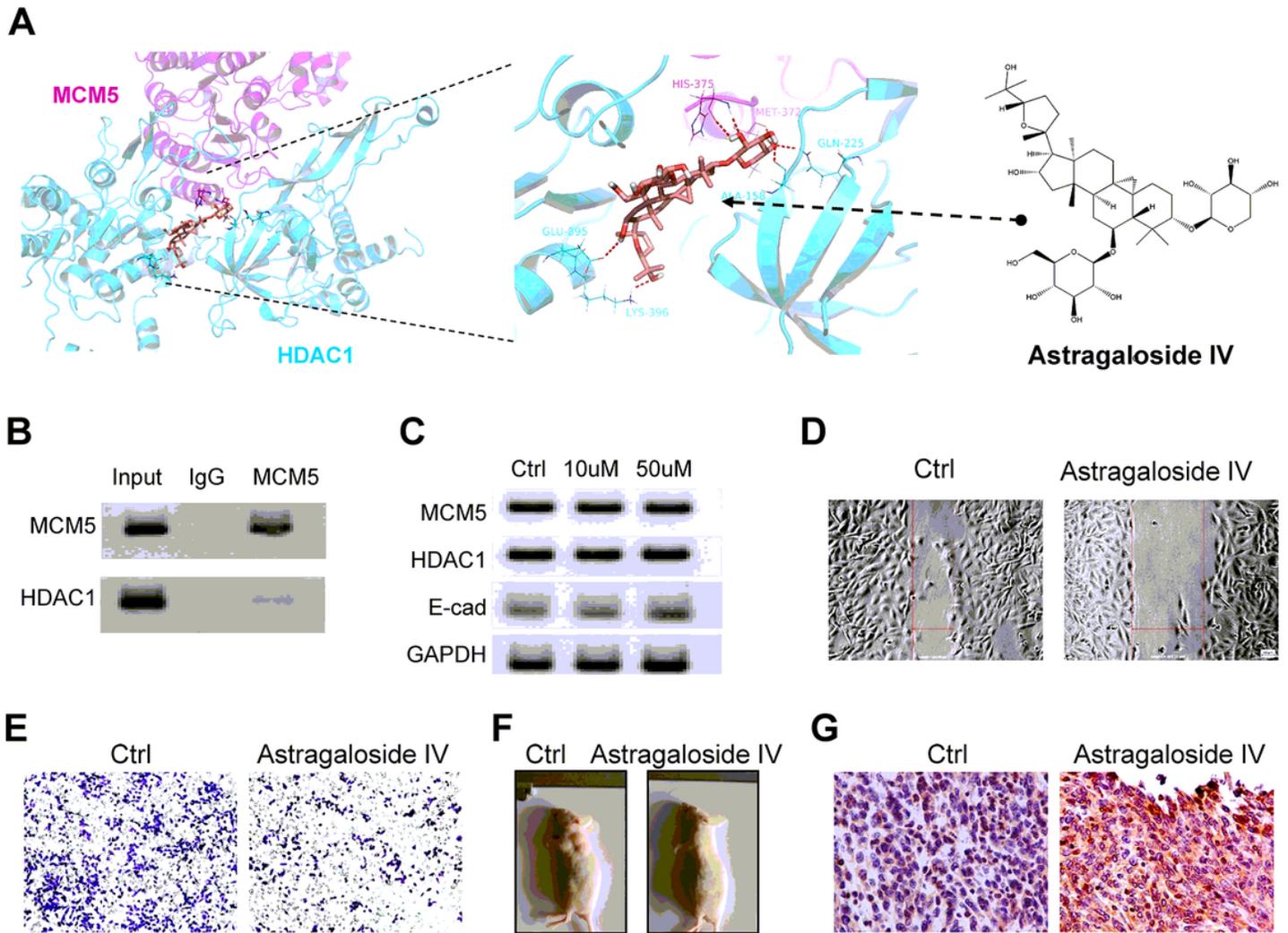


Figure 7

Figure 7

Astragaloside IV inhibits lung cancer malignant progression A. Molecular docking of MCM5/HDAC1 complex and Astragaloside IV. B. Co-ip analyzed the binding of MCM5 and HDAC1 after Astragaloside IV treatment. C. The expression of MCM5, HDAC1 and E-cad after Astragaloside IV treatment. D. Wound healing assay. E. Invasive ability analysis. F. Images of subcutaneous tumors. G. Detection of E-Cad in tumors by immunohistochemistry. *P < 0.05

Supplementary Files

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