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Supplemental Information

Matrix Metalloproteinase 11 Is a Potential Therapeutic Target in Lung Adenocarcinoma

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Supplemental Materials and Methods

Microarray data mining and gene expression analysis

We analyzed microarray data sets containing lung adenocarcinoma patient samples from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). The DEGs were identified using four independent lung adenocarcinoma microarray datasets including GSE7670, GSE10072, GSE68465 and GSE43458. All datasets totally include 732 samples (607 primary tumor samples and 125 normal control samples). Among of them, data from 27 pairwise tumor samples and adjacent non-tumor samples in GSE7670 dataset were analyzed in this study, with excluding data derived from tissue mixtures and cell lines. GSE10072 includes 107 final expression values from 58 tumor tissues and 49 non-tumor tissues. Dataset of GSE68465 includes samples from 442 lung adenocarcinomas and 19 normal lung tissues. Above three datasets were generated from Affymetrix Human Genome U133A Array [HG-U133A]. GSE43458 dataset was measured using the Human Gene 1.0 ST platform [HuGene-1_0-st], consisting of 80 lung adenocarcinomas and 30 adjacent normal lung tissues. Annotations files for the probe arrays were simultaneously downloaded from GEO website. The RMA (robust multiarray average) algorithm was used to process the raw probe-level data (.CEL) via the Affy R package. The average expression value of multiple probes mapping to the same gene symbol was taken as real expression value of this gene. Expression values were exported after background correction and normalization.[1] To identify the differentially expressed genes (DEGs) between the tumor samples and non-tumor samples, the tool of the limma package in R was used.[2] TCGA lung adenocarcinoma gene expression data (counts based on IlluminaHiSeq) was downloaded from the GDC data portal (https://portal.gdc.cancer.gov/) and was processed by edger Bioconductor package.[3] T-test was conducted on the gene expression and the resulting P values were corrected using Benjamini-Hochberg (BH) procedure[4] for false positive control. Genes with adjusted P value < 0.05 and $|\log_2(\text{fold change})| > 1$ were selected out as differentially expressed genes in tumors.

Immunohistochemistry

The immunohistochemical staining for MMP11 was done according to standard protocol. Briefly, paraffin-embedded, formalin fixed tissue sections were deparaffinized in xylene solution at 70℃ for 2 hours, then rehydrated in 100% ethanol and 1 × PBS buffer. Antigenic epitope retrieval was performed in 0.1 mol/L citrate buffer (pH 6.0) at 95℃ for 20 min, subsequently blocked in 10% goat serum for 30 min. Slides were then incubated with primary antibodies in diluent solution at room temperature for 45 min, afterward washed with 1 × PBS buffer and incubated with secondary antibody before proceeding to DAB (3,3'-Diaminobenzidine) staining. Nuclei were counterstained with haematoxylin for 5 min, thus mounted in mounting medium. Slides were visualized under a bright-field microscope using Leica imaging software. Two independent observers evaluated staining for all samples. Staining intensities were classified in four classes (negative, weak, moderate and strong) and fractions of positive cells (0, 0-25%, 25–75%, 75-100%) for tumor cells and stromal cells were separately counted.

Western blot

Cells were washed with ice-cold PBS and lysated in Laemmli buffer containing proteinase inhibitor cocktail (Santa cruz). After centrifugation, supernatants were collected and protein concentrations were determined by BCA protein assay kit (Pierce). Equivalent cell lysates were separated on 10% SDS–polyacrylamide gel and proteins were then transferred to nitrocellulose membrane for blotting. Target proteins were detected with corresponding 1st and 2nd antibodies and visualized using WesternBright ECL HRP substrate (Advansta Inc.). β-actin was used as loading control.

Plasmids construction, Lentivirus packaging and infection

The sgRNAs were designed according to CRISPR Design webtool: https://zlab.bio/guide-design-resources. The oligoes designed for two different target

sequences were as follows, sgDNA1-F，CACCGACATCATGATCGACTTCGCC; sgDNA1-R ， AAACGGCGAAGTCGATCATGATGTC; sgDNA2-F ， CACCGTCGTGCTTTCTGGCGGGCGC; sgDNA2-R ， AAACGCGCCCGCCAGAAAGCACGAC. The oligoes were annealed and cloned into the lentiviral shuttle vector lenti-CRISPRv2 (addgene). The empty lenti-CRISPRv2 or vector containing target oligoes, together two helper plasmids psPAX2, pMD2.G, were co-transfected into the 293FT cells as previously described.[5] The medium was removed and changed to fresh medium 12–14 h after transfection. Medium containing the viral particles was harvested 48 h later and filtered through 0.45-μm filters. Virus containing medium with 8 μg/m Polybrene (Sigma) was added to A549 or PC9 cells for overnight infection, and the medium was replaced freshly in the following day. The infected cells were selected with puromycin for following assays.

Wound-healing assay

The wound-healing assay was used to evaluate the migration of cells in vitro. Briefly, A549 or PC9 cells with or without MMP11 were seeded in six-well plates at a density of 1×10^6 per well with complete medium. When reaching 100% confluency, cells were wounded by manual scratching with a 200-ml pipette tip. The wells were washed with PBS to remove cell debris and replaced with fresh DMEM. At indicated time points, images at specific wound sites were taken under microscope and the width of the wound was measured IPP6 image software.

Invasion assay

Ability of cell invasion was evaluated by the 24-well Transwell chamber (BD Biosciences). A 24-well unit with 8-mm polycarbonate nucleopore filters (Corning) was evenly coated with 100 μl of Matrigel Basement Membrane Matrix. Serum free medium containing 2×10^5 cells was placed in the upper compartment. DMEM medium supplied with 10% FBS was added to the lower compartment. After 48 hours incubation, cells that had not invaded were removed with a cotton swab. Cells that

had invaded to the lower surface of the membrane were fixed with 4% formaldehyde and stained with crystal violet and imaged under microscope. IPP6 software was used to calculate the area of the invaded cells.

ELISA

MMP11 levels in the serum of the patients were measured using an ELISA kit (Kete Biological Technology Co., Ltd, Jiangsu, China). The experiment was done according to instructional manual provided by the company. Briefly, serum samples and standard products were prepared and added to the bottom of the ELISA plate, then sealing the plate for incubation at 37℃ for 30 minutes. After incubation, removing the sealing membrane and discarding the reaction mixture, the plate was washed with cleaning solution for 5 times. Secondary antibody conjugated to horseradish peroxide was thereafter added to the wells and incubated for another 30 min at 37℃. Post washing, 50 μl color developing agent A and agent B were in turn added to the well, mixing well to coloring for 15 minutes, then stoping the reaction by adding 50 μl stop solution. The absorbance was read at 450 nm using a SpectraMax® Absorbance reader (Molecular Devices, USA).

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Supplementary Figure Legends

Fig. S1. Differential expression of MMP7, MMP9, MMP12 in LUAD datasets downloaded from TCGA database.

Fig. S2. Cell morphology undergoes alterations upon MMP11-depletion. The morphology of the MMP11-deficient A549 (A) or PC9 (B) cells changed to a cobblestone shape with loss of membrane ruffles/protrusions compared to control cells. Scale bar represents 50 μm.

Fig. S3. MMP11 deficiency leads to reduced p-AKT protein expression in PI3K signaling pathway. Total lysates of control M-KO-1 and M-KO-2 A549 cells were analyzed by western blots using antibodies against the indicated proteins.

Fig. S4. The body weight of xenografts at different time points was comparable. Nude mice were under subcutaneously injection of control (n = 8) or MMP11-depleted LUAD cells (n = 8) (A) or nude mice inoculated with LUAD cells were under treatment of saline (n = 8) or MMP11 antibody (Ab) (n = 8) (B).

Fig. S5. MMP11 (A) and Ki67 (B) expressions in xenograft tumors were assessed by immunohistochemistry. Scale bar represents 50 μm.

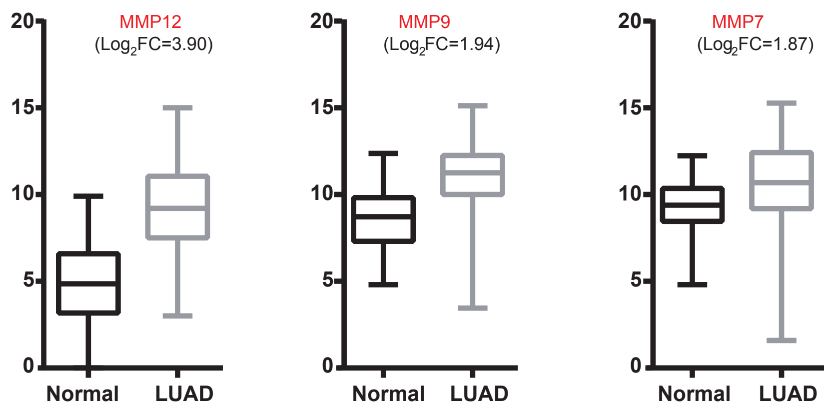


Figure S1

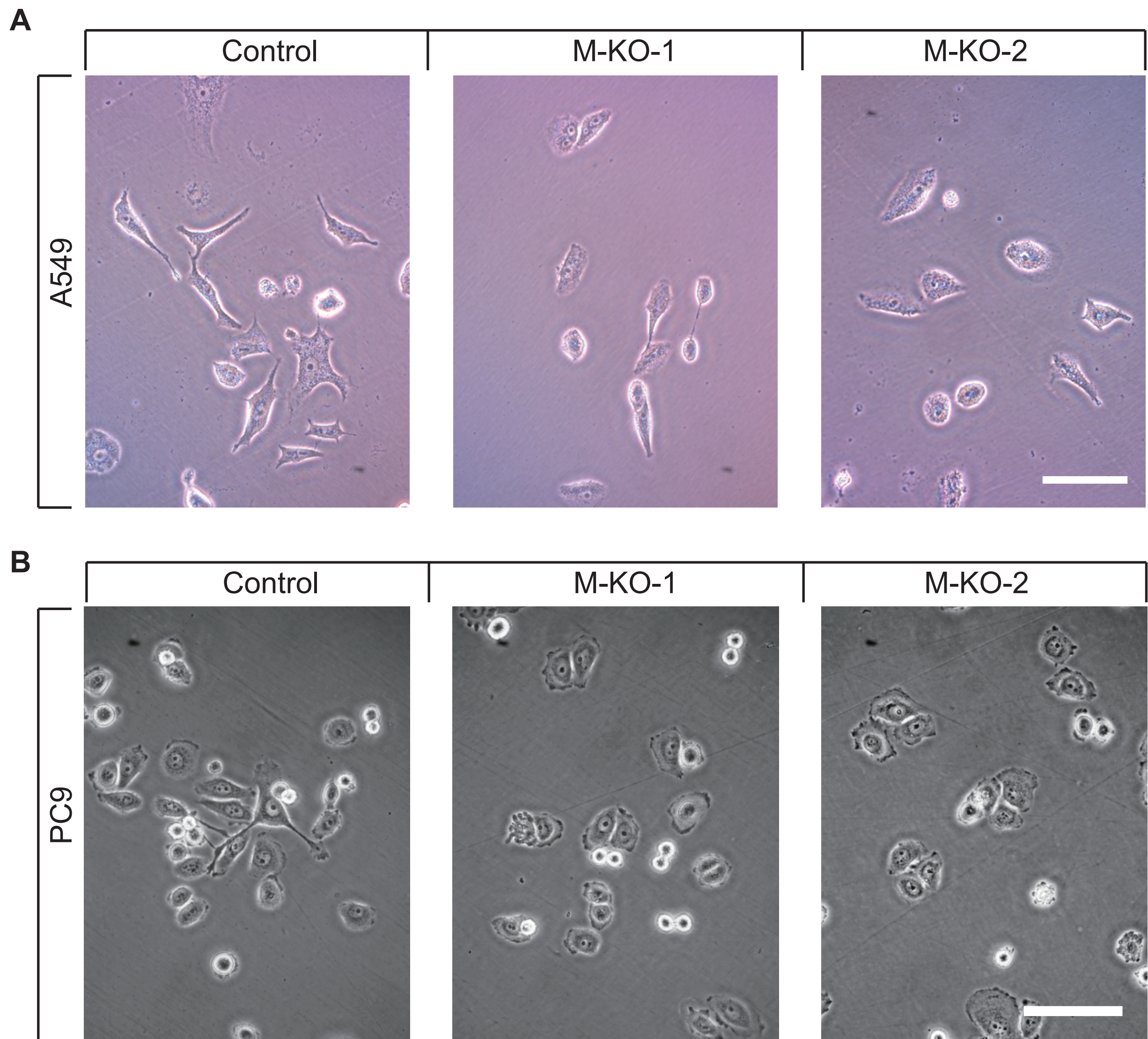


Figure S2

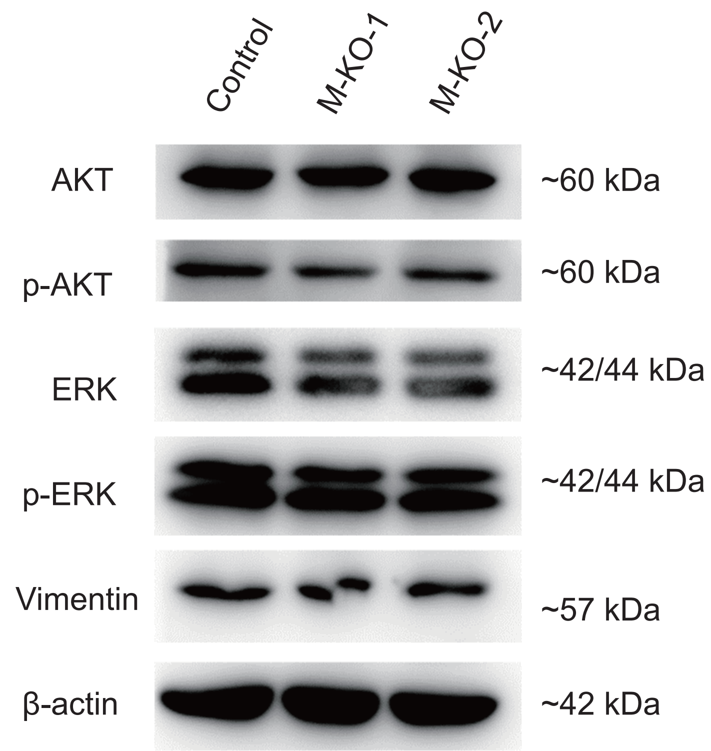
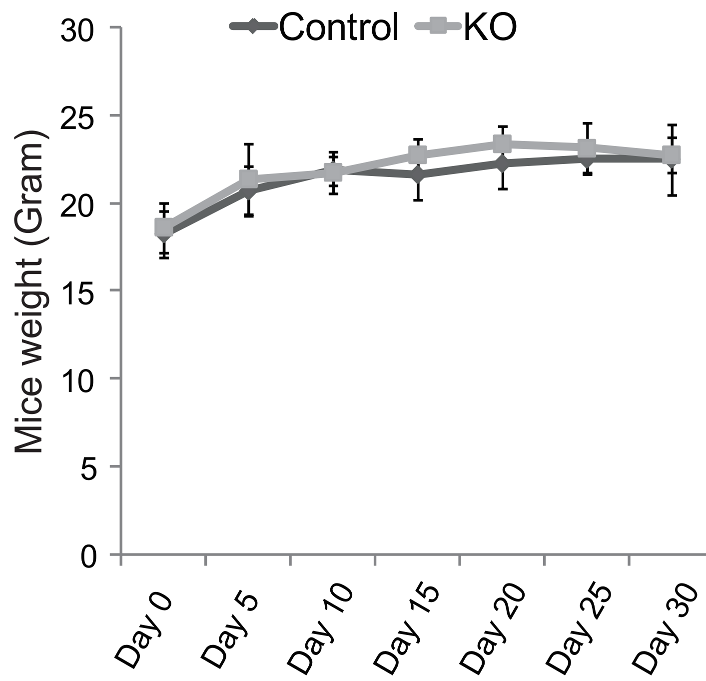


Figure S3

A



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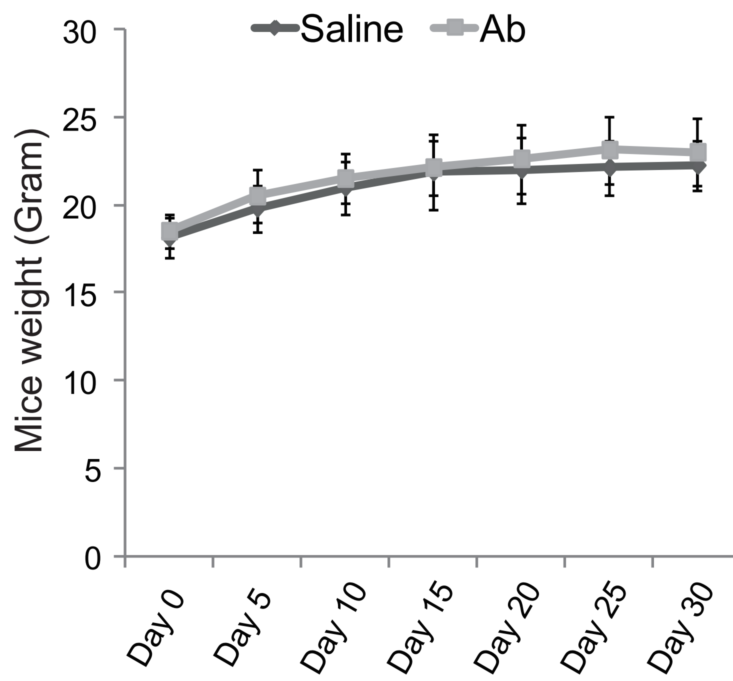

Figure S4

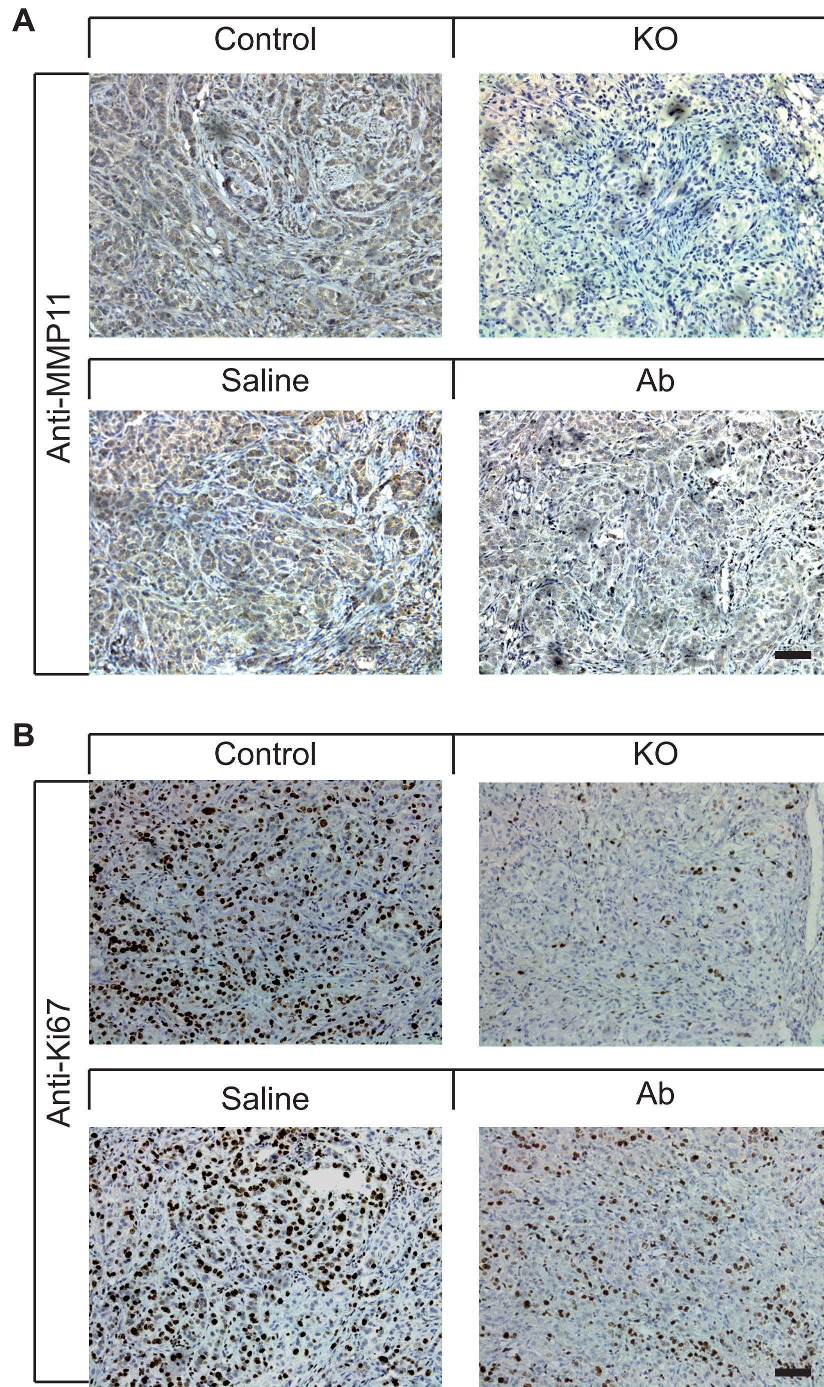

Figure S5