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Mitotic Arrest-Deficient Protein 2B Overexpressed in Lung Cancer Promotes Proliferation, EMT, and Metastasis

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As the noncatalytic subunit of mammalian DNA polymerase, mitotic arrest-deficient protein 2B (MAD2B) has been reported to play a role in cell cycle regulation, DNA damage tolerance, gene expression, and carcinogenesis. Although its expression is known to be associated with poor prognosis in several types of human cancers, the significance of MAD2B expression in lung malignancies is still unclear. Our study showed that MAD2B expression significantly increased in lung cancer, especially in the metastatic tissues. We also found that knockdown of MAD2B inhibited the migration, invasion, and epithelial–mesenchymal transition of lung cancer cells in vitro and the metastasis in vivo, while overexpression of MAD2B had the opposite effect. Microarray and Western blotting data indicated that slug might be its downstream target since knockdown of MAD2B inhibited, while overexpression increased, the expression of slug. Moreover, the expression of MAD2B was found to be positively correlated with slug in lung cancer tissues as well. Collectively, these findings indicate an oncogenic role of MAD2B in lung cancer, and slug might be involved in the process.

Key words: MAD2B; Lung cancer; Epithelial–mesenchymal transition (EMT); Slug

INTRODUCTION

Lung cancer is by far the leading cause of cancer deaths among both men and women; about 1 out of 4 cancer deaths are from lung cancer[1]. Each year, more people die of lung cancer than of colon, breast, and prostate cancers combined[1]. Although there are significant advances in diagnosis and treatment, lung cancer still causes the most cancer-related deaths all over the world[2,3]. Therefore, there is a great need to develop novel treatments.

Mitotic arrest-deficient protein 2B (MAD2B) is the noncatalytic subunit of DNA polymerases that are involved in translesion DNA synthesis (TLS) polymerases to repair DNA damage during DNA replication[4,5]. When the replication fork is encountered at DNA damage sites during DNA replication, the replication fork stalls, which arrests DNA replication and causes genome instability and cell death; however, TLS polymerases help cells to bypass DNA damage sites and relieve this arrest[6,7]. Therefore, the TLS polymerases are necessary in maintaining genome integrity and tolerance to DNA damage in mammalian cells, and the lack of the TLS polymerases tends to show defects in DNA integrity and stability[8,9]. Together, MAD2B was reported to be associated with many processes including immunologic response, regulation and control of the mitotic cycle, and tumor cells' drug resistance. However, the function and mechanism that MAD2B plays in lung cancer are still unknown.

In this study, the roles of MAD2B were explored in lung cancer. The expression of MAD2B was found to be increased in most of the human lung cancer tissues examined compared with normal tissues. In the lung cancer cell line DMS53, our data showed that overexpression of MAD2B significantly enhanced the cell migration, invasion, and epithelial–mesenchymal transition (EMT). Further study revealed that overexpression of MAD2B led to increased slug. In contrast, knockdown of MAD2B in A549 cells inhibited migration, invasion, EMT, and slug levels. The expression of MAD2B was positively correlated with slug in lung cancer tissues. Together, these results suggest an important role of MAD2B in lung cancer cell migration, invasion, and EMT by regulating slug.

MATERIALS AND METHODS

Samples and Cell Lines

Human lung cancer tissue samples were provided by Linyi People's Hospital affiliated to Shandong Medical College. All investigations described in this study were

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carried out after obtaining informed consent and were in accordance with an institutional review board protocol approved by the Partners Human Research Committee at the Shandong Medical College. Human lung cancer cell lines BEAS-2B, DMS53, A549, H1650, and H1299 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Plasmid Construct

According to a previous report[10], a human complementary DNA (cDNA) of MAD2B was cloned. The full-length cDNAs were subcloned into the multiple cloning sites of the vector plasmid forming the vector-MAD2B expression plasmids. Short hairpin RNA (shRNA) targeting MAD2B (sense: 5 -TCGACGCGAATCTCTCT TTGGCAAGTTCAAGAGACTTGCCAAAGAGAG ATTCGTTTTTTGGAAT-3 ; antisense: 5 -CTAGATTC CAAAAAACGAATCTCTCTTTGGCAAGTCTCTTG AACTTGCCAAAGAGAGATTCGCG-3) was initially inserted into the *Sal*I and *Xba*I sites of the vector plasmid, forming the vector-shMAD2B plasmids.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

By using the TRIzol reagent (Invitrogen), total RNA was extracted from lung cancer tissues and cell lines. RT was performed by using the Thermoscript RT System (Invitrogen). PCR conditions were set as follows: 45 s at 94°C, 30 s at 55°C, and 1 min at 72°C for 28–30 cycles (for MAD2B) or 26 cycles [for glyceraldehyde 3-phosphate dehydrogenase (GAPDH)]. The following primers were used in the study: MAD2B, 5 -AGAGTTTCATCCCCAAAGACAA-3 (sense) and 5 -A GTTCAGGCAGTAGGCAAAGTC-3 (antisense); slug, 5 -TCATCTTTGGGGCGAGTGAG-3 (sense) and 5 -T GCAGCTGCTTATGTTTGGC-3 (antisense); GAPDH, 5 -TGCCTCCTGCACCACCAACT-3 (sense) and 5 -C CCGTTCAGCTCAGGGATGA-3 (antisense).

Western Blotting

Cells were quantified after washing and scraping in a lysis buffer. Twenty micrograms of protein was loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a 0.2-µm nitrocellulose membrane. Membranes were incubated with primary antibodies [MAD2B, 1:1,000 (BD); -actin, 1:5,000 (Sigma-Aldrich); E-cadherin, 1:500 (CST); -catenin, 1:2,000 (CST); N-cadherin, 1:500 (CST); vimentin,1:1,000 (BD); slug, 1:500 (CST)] overnight after blocking with 5% nonfat dry milk in Tris-buffered saline with 1% Tween 20 (TBS-T). The membrane was incubated for 1 h with the horseradish peroxidase (HRP)-conjugated secondary antibody after washing. Then membranes were washed three times with TBS-T, and the proteins were visualized with SuperSignal (Pierce, Bonn, Germany) enhanced chemiluminescence.

Establishment of Stable Expression and Knockdown Cell Lines

A549 cells were transfected with the vector vector-shMAD2B plasmid by using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). DMS53 cells were similarly transfected with the vector vector-MAD2B plasmid. Stable transfection was obtained after selection by puromycin (10 µg/ml; Invitrogen) for 2 weeks. Expressions of MAD2B mRNA and protein in stable cell lines were analyzed by RT-PCR and Western blot, respectively.

Cell Invasion and Motility Assay

Invasion of cells was measured in Matrigel-coated (BD, Franklin Lakes, NJ, USA) Transwell inserts (6.5 mm; Costar, Manassas, VA, USA) containing polycarbonate filters with 8-µm pores as detailed previously[11–13]. According to the manufacturer's recommendations, the inserts were coated with 50 µl of 1 mg/ml Matrigel matrix. Cells (2 10^5) were plated in the upper chamber with 200 µl of serum-free medium, and 600 µl of medium with 10% FBS was added to the lower well. The top cells were removed and the bottom cells were counted after 24 h of incubation. Cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet after migrating to the lower surface of the membrane. Five random fields were counted at 10 magnification for each membrane. The mean cell number was calculated from three independent experiments done in triplicate. Motility assays were similar to the Matrigel invasion assay except that the Transwell insert was not coated with Matrigel.

Microarray

RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA from the different cells according to the manufacturer's protocol, and RNA were stored in liquid N_2 at −80°C until further processing. The quantity and quality of the RNA were assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer, and only samples with an RNA integrity number (RIN) >7 were used for further analysis. For microarray hybridizations, 100 ng of total RNA was amplified and labeled using the MessageAmp Premier Kit (Ambion-ThermoFisher, Waltham, MA, USA). Equal amounts of labeled cRNA were hybridized to the Affymetrix Genome 2.0 microarray (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. Partek Genomics Suite 6.4 (Partek Inc., St. Louis,

MO, USA) was used to perform data analysis. Robust multichip analysis (RMA) normalization was done on the entire data set. Multiway analysis of variance (ANOVA) and fold change were performed to select target genes that were differentially expressed between the different comparisons. Top differentially expressed genes were selected with a *p* value cutoff of 0.05 based on ANOVA test and fold change cutoff of 2. Gene Ontology Enrichment analysis on the gene lists was performed with chi-square test and limited to functional groups with more than two genes. Hierarchical clustering was performed on differentially expressed genes based on average linkage with Pearson's dissimilarity. Additionally, the gene lists were analyzed using the GeneGo software for obtaining pathway maps, biological networks, and diseases relevant to the list.

In Vivo Metastasis

Male BALB/c nude mice aged 4 to 6 weeks were purchased from the Slac Jingda Laboratory Animal Co., Ltd. (Shanghai, P.R. China). All the animal studies were approved by the Animal Care and Welfare Committee of Shandong Medical College and conducted according to the guidelines of the National Animal Welfare Law of China. The cells at their exponential growth phase were harvested and washed twice in phosphate-buffered saline (PBS). For metastasis assay, cells were suspended in PBS at a concentration of 3 10^7 cells/ml. Cell suspension (0.1 ml) was injected into the tail veins of nude mice. All of the mice were sacrificed by CO_2 inhalation 60 days after inoculation.

Statistical Analysis

Data were described as the mean ± standard deviation (SD). Student's two-tailed *t*-test was used to compare the data between different groups. SPSS 11.0 (SPSS, Inc., Chicago, IL, USA) was used to analyze the statistical significance of the differences between mean values determined by a value of $p < 0.05$.

RESULTS

MAD2B Is Highly Expressed in Metastatic Lung Cancer Tissues and Cell Lines

To investigate whether MAD2B expression is correlated with the tumorigenesis of lung cancer, we examined

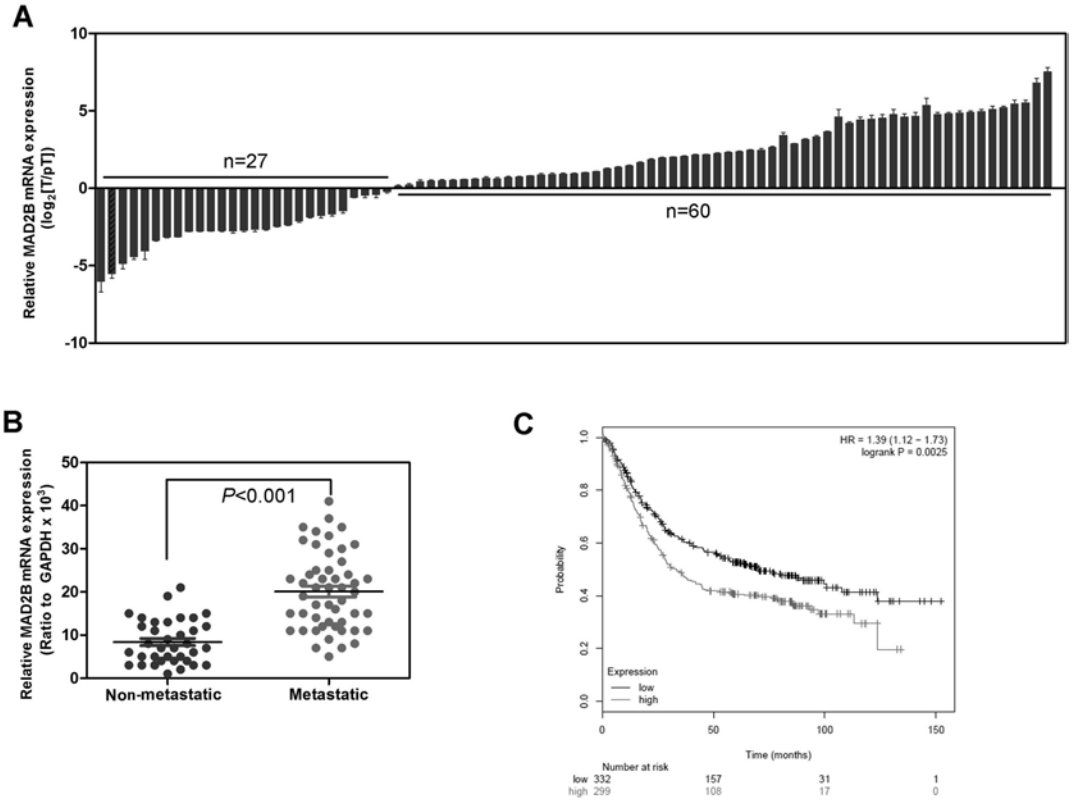

Figure 1. Mitotic arrest-deficient protein 2B (MAD2B) is highly expressed in human metastasis lung cancer tissues. (A) MAD2B mRNA expression was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 87 lung cancer tissues. (B) qRT-PCR results between nonmetastatic and metastatic lung cancer tissues were summarized by a statistical software. (C) Survival analysis of patients with lung cancers was based on the Kaplan–Meier survival analysis in The Cancer Genome Atlas (TCGA). A value of $p < 0.001$ is based on the Student's *t*-test. Error bars, standard deviation (SD).

MAD2B expression levels in 87 human lung cancer tissues (Fig. 1A). We found that the level of MAD2B was elevated in most of the metastatic lung cancer tissues compared with that in the nonmetastatic samples (Fig. 1B). A curve graph was drawn to describe the correlation of patients' survival time and MAD2B expression using The Cancer Genome Atlas (TCGA) database. We found that patients with higher MAD2B level in lung cancer tissues had a shorter life than those with lower MAD2B (Fig. 1C), suggesting a negative correlation between MAD2B and patient survival.

We then examined the MAD2B protein levels in a panel of four widely used human lung cancer cell lines in comparison to levels in the nonmalignant lung cell line BEAS-2B (Fig. 2A) by Western blotting. Similarly, MAD2B expression levels are consistently increased in lung cancer cell lines, especially in the invasive lung cancer cell lines (A549, H1650, and H1299) (Fig. 2B). We also found the same trend of mRNA expression in these cell lines (Fig. 2C).

Knockdown of MAD2B Inhibits Capacities of Migration and Invasion of Lung Cancer Cells In Vitro

To further study its role in lung cancer, we used retroviral vectors to establish a stable cell line with silenced MAD2B. The expression of MAD2B in the A549 cell line was examined by Western blotting (Fig. 3A) and quantitative RT-PCR (qRT-PCR) (Fig. 3B). We next examined the effects of deficient MAD2B on the migration and invasion of lung cancer cells. Transwell migration and Matrigel invasion assays were performed on the established A549 stable transfection to determine whether knockdown of MAD2B would have an effect. The results showed that A549-shMAD2B cell migration and invasive activities significantly decreased compared to those of the control cells (Fig. 3C and D). These results revealed that knockdown of MAD2B inhibited the migration and invasion abilities of lung cancer cells.

Knockdown of MAD2B Inhibits Metastasis of Lung Cancer Cells In Vivo

To further confirm whether MAD2B promotes metastasis in vivo, A549-shMAD2B and control cells were inoculated into the tail vein of BALB/C athymic mice. Sixty days later, we observed that fewer mice injected with the A549-shMAD2B cells had distant metastasis (Table 1). Moreover, less metastatic foci in the liver (Fig. 4A and B) were counted in each mouse injected with A549-shMAD2B cells.

Overexpression of MAD2B Promotes Capacities of Migration and Invasion in Lung Cancer Cells In Vitro

Since knockdown of MAD2B inhibits migration and invasion both in vitro and in vivo, we also want to know whether overexpression would have an opposite effect.

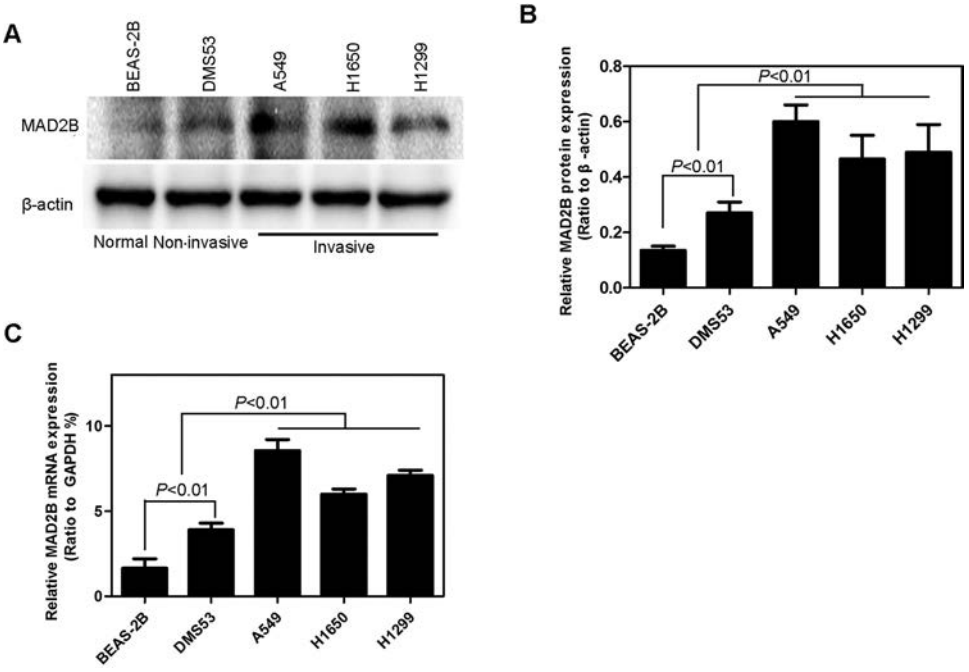

Figure 2. MAD2B is highly expressed in metastasis lung cancer cell lines. (A) MAD2B expression in lung cancer cell lines was analyzed by Western blotting. (B) Quantitative chart of MAD2B protein levels in liver cancer cell lines. (C) MAD2B expression in lung cancer cell lines was analyzed by qRT-PCR. A value of $p < 0.001$ is based on the Student's *t*-test. Error bars, SD.

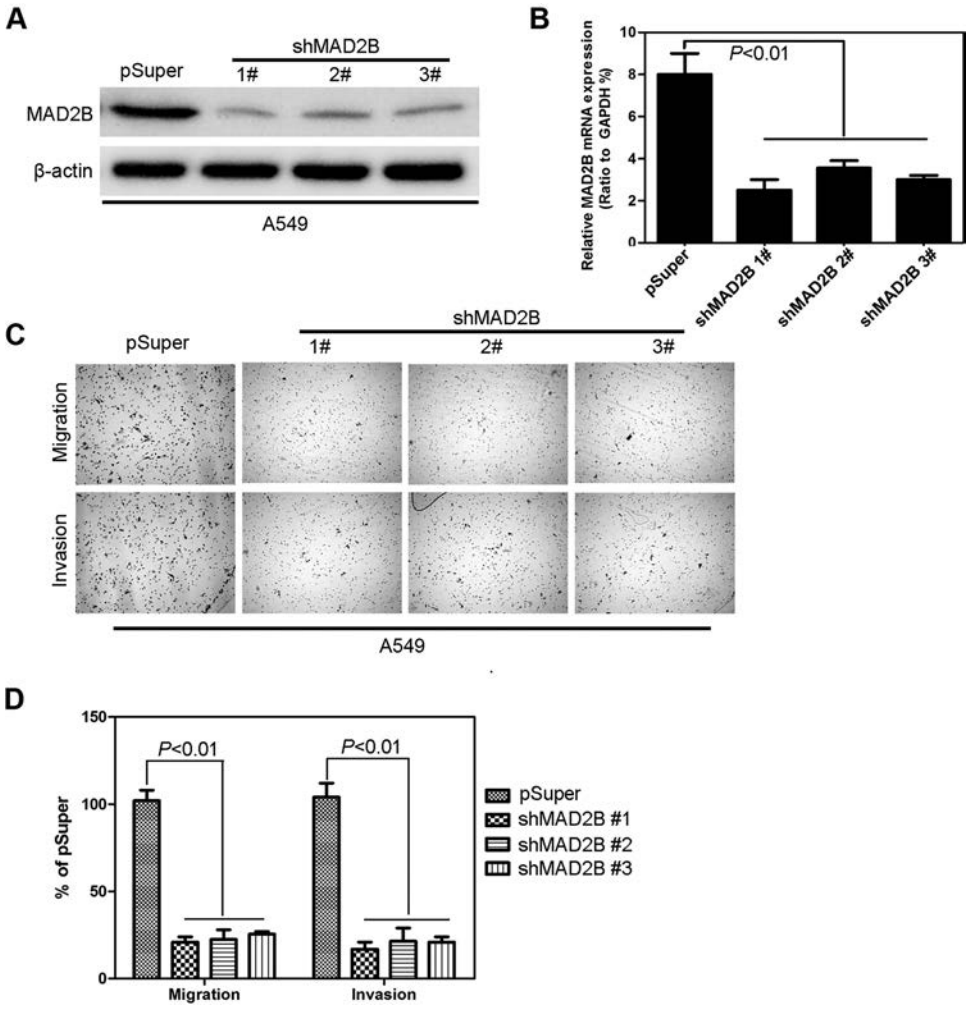

Figure 3. Knockdown of MAD2B inhibits capacities of migration and invasion of lung cancer cells in vitro. (A) MAD2B protein's low expression in the A549-shMAD2B cell lines was verified by Western blot assay compared with the control cells. (B) MAD2B mRNA's low expression in the A549-shMAD2B cell lines was verified by qRT-PCR assay compared with the control cells. (C) A549-shMAD2B and its control cells were subjected to Transwell migration and Matrigel invasion assays. (D) Quantification of migrated and invasion cells through the membrane is shown as proportions of their vector controls in a column diagram from (C). A value of $p < 0.001$ is based on the Student's *t*-test. Error bars, SD.

We used retroviral vectors to establish a lung cancer cell line stably overexpressing MAD2B. The expression levels of MAD2B in the DMS53 cell line were examined by Western blotting (Fig. 5A) and RT-PCR (Fig. 5B). We next examined the effects of overexpressed MAD2B on the migration and invasion of lung cancer cells. Transwell migration and Matrigel invasion assays were performed on the established DMS53 stable transfection to determine whether overexpression of MAD2B would influence migration or invasion. The results showed that DSM53-MAD2B cells' migration and invasive activities significantly increased compared to those of control cells (Fig. 5C and D). These results demonstrated that overexpression of MAD2B promotes capacities of migration and invasion of lung cancer cells.

Overexpression of MAD2B Promotes Capacities of Metastasis of Lung Cancer Cells In Vivo

To further confirm whether MAD2B promotes metastasis in vivo, DSM53-MAD2B and its control cells were inoculated into the tail vein of BALB/C athymic mice. Sixty days later, we observed that more mice injected with DSM53-MAD2B cells had distant metastasis (Table 2).

Table 1. Knockdown of MAD2B Significantly Decreased the Number of Mice With Distant Metastasis

A549	No. of Mice With Distant Metastasis
pSuper	8/10
shMAD2B #1	2/10

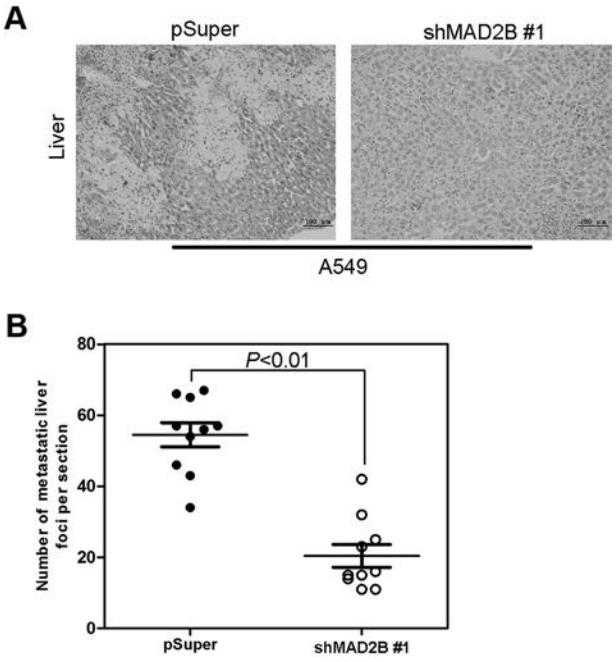

Figure 4. Knockdown of MAD2B inhibits metastasis capacity of lung cancer cells in vivo. (A, B) Fewer metastasis foci in the liver were counted in each mouse injected with lung cancer cells silencing MAD2B. A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

Table 2. Overexpression of MAD2B Significantly Increased the Number of Mice With Distant Metastasis

DSM53	No. of Mice With Distant Metastasis
pBabe	4/10
MAD2B	9/10

More metastatic foci in liver (Fig. 6A and B) were counted in each mouse injected with DSM53-MAD2B cells.

Knockdown of MAD2B Inhibits EMT

EMT progression between A549-shMAD2B and control cells was first detected by Western blotting. Knockdown of MAD2B led to elevated expression of E-cadherin and -catenin, and decreased expression of N-cadherin and vimentin compared with the control (Fig. 7A). The same results were found in the mRNA levels (Fig. 7B) in A549-shMAD2B and control cells.

Overexpression of MAD2B Promotes EMT

EMT progression was also detected by Western blotting and qRT-PCR between DMS53-MAD2B and control cells. Overexpression of MAD2B downregulated the expression of E-cadherin and -catenin but upregulated the expression of N-cadherin and vimentin compared

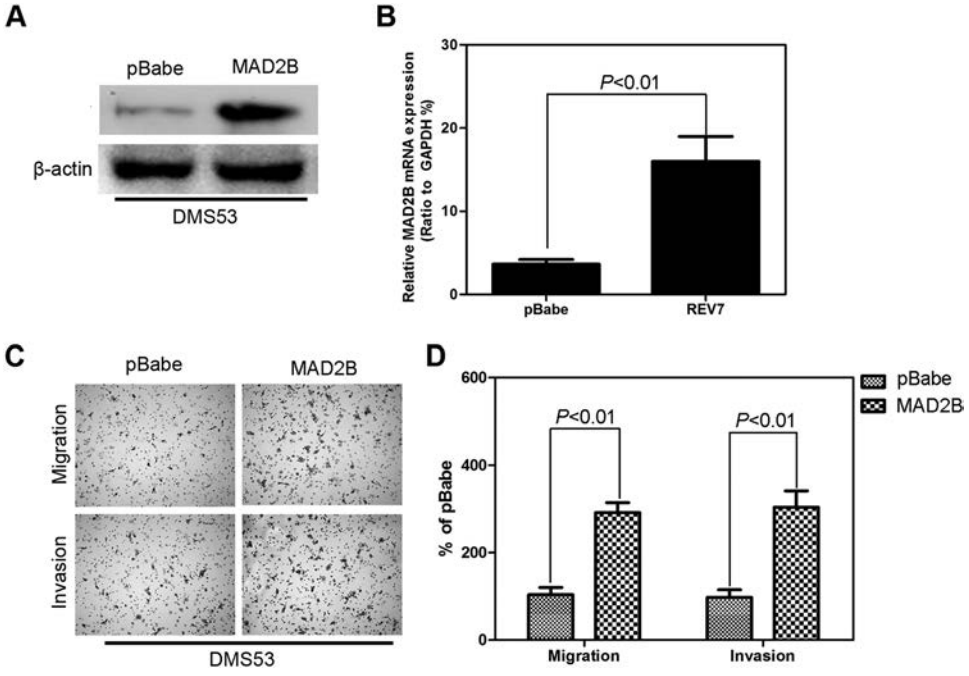

Figure 5. Overexpression of MAD2B promotes capacities of migration and invasion in lung cancer cells in vitro. (A). Western Blot analysis of MAD2B levels in the established cell lines. (B) qRT-PCR analysis of MAD2B levels in the established cell lines. (C) Lung cancer cells with high expression of MAD2B possessed stronger invasion abilities in Transwell and Matrigel assays. (D) Quantification of migrated and invasion cells through the membrane is shown as proportions of their vector controls in a column diagram from (C). A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

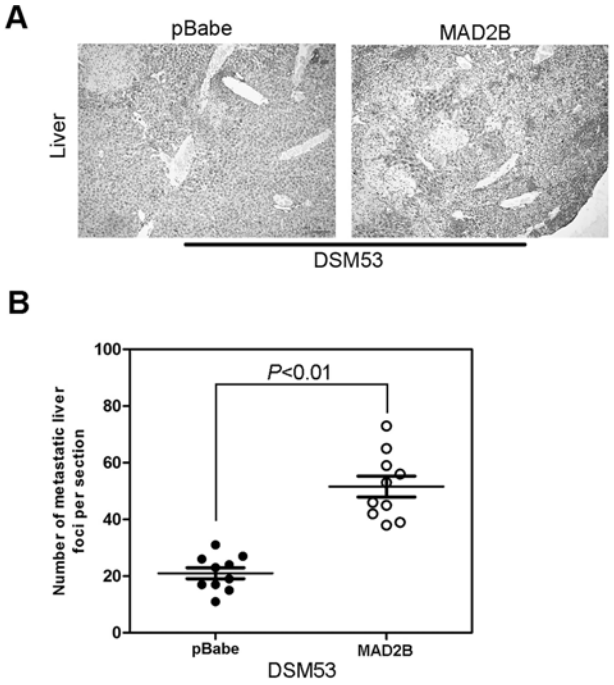

Figure 6. Overexpression of MAD2B promotes metastasis capacity of lung cancer cells in vivo. (A, B) More metastasis foci in the liver were counted in each mouse injected with lung cancer cell overexpressing MAD2B. A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

with the control (Fig. 8A). The same results were found in the mRNA levels (Fig. 8B) in DSM53-MAD2B and control cells.

MAD2B Can Regulate the Expression of Slug

MAD2B was demonstrated to be related with metastasis of lung cancer in the above findings. To better understand the mechanisms by which MAD2B engaged in metastasis, we performed gene expression profiling on A549-shMAD2B #1 and control cells. Microarray analysis identified a number of genes significantly differentially expressed after silencing MAD2B (Fig. 9A), and slug was the top related gene (Fig. 9B). To confirm the microarray data, we next examined the expression of slug in lung cancer cells stably overexpressing or silencing MAD2B. As shown in Figure 10A and B, decreased expression of slug was identified in lung cancer cells with deficient MAD2B by Western blotting (Fig. 10A) and qRT-PCR (Fig. 10B). In contrast, increased slug expression was found in lung cancer cells overexpressing MAD2B by Western blotting (Fig. 10C) and qRT-PCR (Fig. 10D). In addition, we also found that the expression of slug was positively correlated with MAD2B in the lung cancer tissues (Fig. 10E). Collectively, MAD2B may promote the metastasis of lung cancer through regulating slug.

DISCUSSION

In this study, we characterized the role of MAD2B in lung cancer. Compared to the nonmetastatic tissues, we detected increased levels of MAD2B expression in

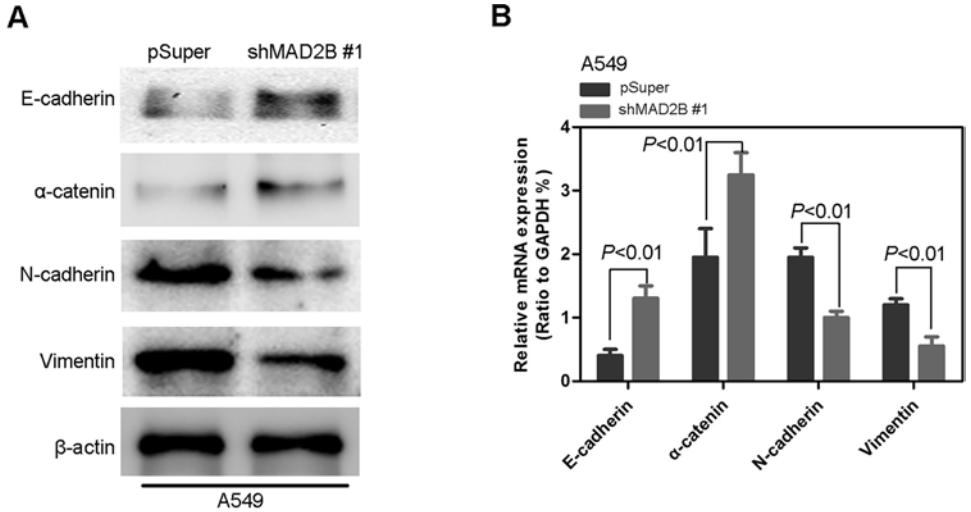

Figure 7. Knockdown of MAD2B inhibits epithelial–mesenchymal transition in lung cancer cells. (A) Western blot analysis showed that silencing MAD2B upregulated the epithelial cell markers (E-cadherin and -catenin) and downregulated the mesenchymal cell markers (N-cadherin and vimentin) in A549 cells. (B) qRT-PCR analysis showed that silencing MAD2B upregulated the epithelial cell markers (E-cadherin and -catenin) and downregulated the mesenchymal cell markers (N-cadherin and vimentin) in A549 cells. A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

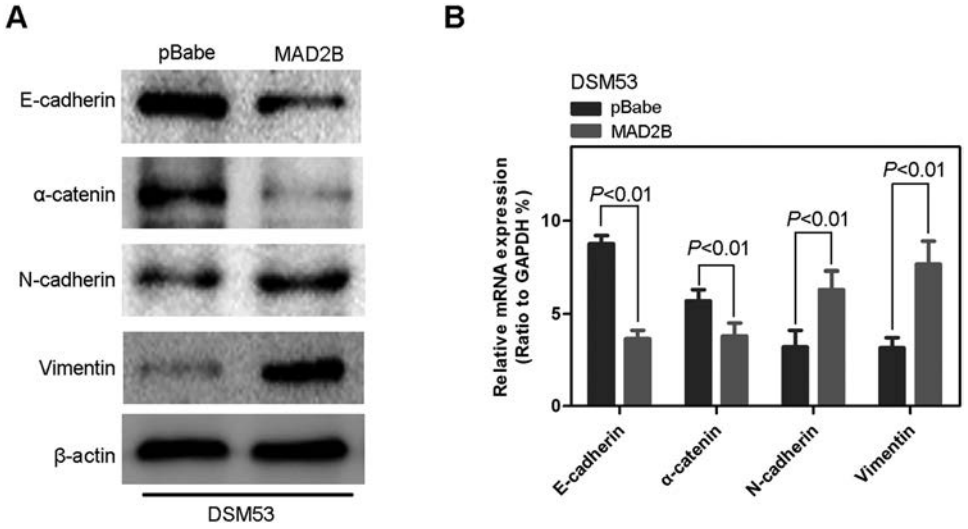

Figure 8. Overexpression of MAD2B promotes epithelial–mesenchymal transition. (A) Western blot analysis showed that overexpression of MAD2B downregulated the epithelial cell markers (E-cadherin and -catenin) and upregulated the mesenchymal cell markers (N-cadherin and vimentin) in DSM53 cells. (B) qRT-PCR analysis showed that overexpression of MAD2B downregulated the epithelial cell markers (E-cadherin and -catenin) and upregulated the mesenchymal cell markers (N-cadherin and vimentin) in DSM53 cells. A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

metastatic lung cancer tissues. We also found that knockdown of MAD2B inhibited cell migration and invasion as well as EMT progression. Similarly, overexpression of MAD2B in lung cancer cells was found to significantly promote migration, invasion, and EMT progression. It was found that MAD2B affects the expression of slug in lung cancer cells.

TLS is an important damage tolerance system that rescues cells from severe injuries caused by DNA damage[14]. It requires low-fidelity DNA polymerases, including

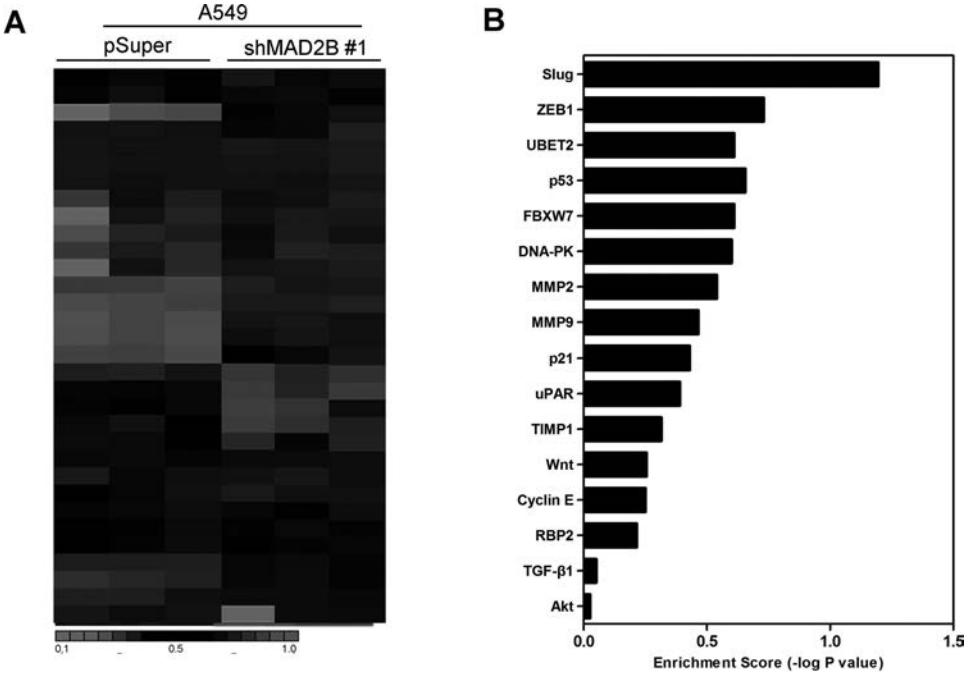

Figure 9. MAD2B regulates the expression level of slug. (A) Clustering of the genes differentially expressed after silencing of MAD2B in A549 cells. (B) The enrichment scores of differentially expressing genes in the MAD2B silencing cell line.

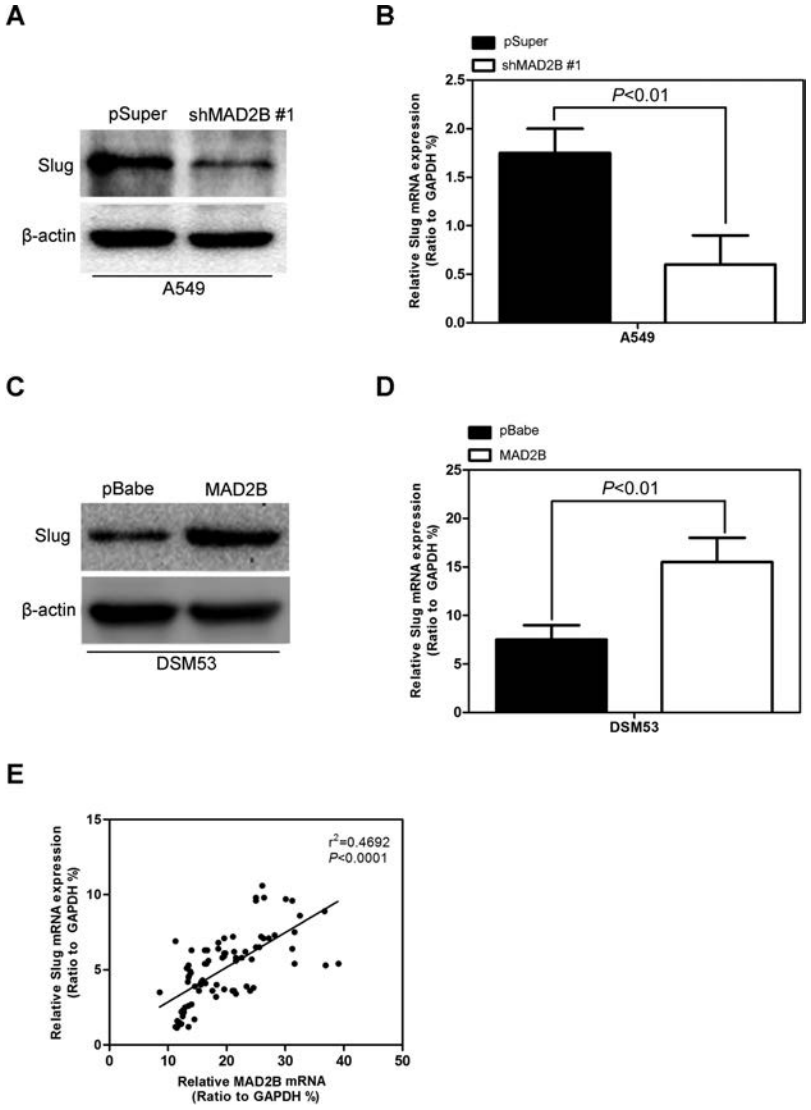

Figure 10. Slug expression was affected by MAD2B. (A) The slug expression levels in the MAD2B silencing cell line were assayed by Western blot. (B) The slug expression levels in the MAD2B silencing cell line were assayed by qRT-PCR. (C) The slug expression levels in the MAD2B overexpression cell line were assayed by Western blot. (D) The slug expression levels in an MAD2B overexpression cell line were assayed by qRT-PCR. (E) The expression of slug was positively correlated with MAD2B in the lung cancer tissues. A value of $p < 0.01$ is based on the Student's *t*-test. Error bars, SD.

DNA polymerase (Pol), which was originally found in *Saccharomyces cerevisiae*[15]. Pol involving subunits of Rev3 and MAD2B proteins plays an important role in eucaryons[16]. MAD2B was first identified as a gene that complements the reversionless phenotypes of yeast *S. cerevisiae* mutant strains[17].

Although the MAD2B gene was found to be associated with the immunologic response, regulation and control of mitotic cycle, and tumor cell drug resistance[16,18,19], the function MAD2B plays and its underlying mechanism in lung cancer are still not clear. We detected the protein expression of MAD2B in lung cancer and then investigated the relationships between MAD2B expression and the clinicopathologic characteristics of patients with lung cancer. Our results showed that MAD2B was highly expressed in lung cancer tissues with metastasis and in a majority of the high-grade lung cancer tissues examined.

Next, we found that overexpression of MAD2B promoted lung cancer cells' migration and invasion abilities well as EMT progression, whereas deficient MAD2B had the opposite effect. There were four proteins (E-cadherin, -catenin, N-cadherin, vimentin) involved in EMT progression examined as an index to recognize EMT[20].

EMT occurs gradually in the process of tumor development and is extremely complicated[21]. As a feature of aggressive tumors, EMT is characterized by reduced E-cadherin and -catenin and increased N-cadherin and vimentin expression[22]. E-cadherin plays an important role in sustaining the completeness and polarity of the structure and shape of epithelial cells. Downregulation of E-cadherin protein expression has a close relationship with the epithelial cell[23]. Cells in which EMT progression occurred usually showed upregulated expression of the proteins originating from mesenchymal cells, especially vimentin[24]. In contrast to E-cadherin, the upregulation of N-cadherin protein was found to be a positive sign of EMT[25]. -Catenin can modify cell adhesion activity , whose downregulation expression could lead to E-cadherin's deprivation of function, and the contact inhibition among epithelial cells immediately followed[25,26]. Thus, -catenin protein's downregulated expression is positively correlated with EMT progression.

To investigate the mechanism of MAD2B modifying the development of lung cancer, microarray assay was carried out. We identified slug as a potential mediator of MAD2B-induced phenomena. Slug has been proposed to play an important role in epithelial carcinogenesis, since it is known to be a potent negative regulator of epithelial cell growth. In cell lines with deficient MAD2B, the expression level of slug significantly decreased compared with the controls. Ectopic overexpression of MAD2B promoted the expression of slug. Therefore, our data suggest that MAD2B may regulate oncogenic activities through modulating the expression of slug in lung cancer cells.

In conclusion, we demonstrated that MAD2B is highly expressed in metastatic lung cancer tissues, and knockdown of MAD2B inhibited migration, invasion, and EMT in lung cancer cells. Therefore, these findings may shed light on potential targets in lung cancer prevention and therapy.

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