MDIG promotes cisplatin resistance of lung adenocarcinoma by regulating ABC transporter expression via activation of the WNT/β-catenin signaling pathway

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Abstract. Mineral dust-induced gene (MDIG) is a proto-oncogene associated with lung cancer that serves a key role in the biological processes of tumorigenesis. The aim of the present study was to determine whether MDIG is involved in cisplatin (DDP) resistance in lung adenocarcinoma, and to investigate the associated molecular mechanism. In the present study, MDIG-knockdown and MDIG-overexpressing A549 cells and DDP-resistant A549/DDP cells were initially constructed, and then the mRNA and protein expression levels of MDIG and ATP-binding cassette (ABC) transporters (ABCB1, ABCC1, ABCG2), and the expression levels of the major associated proteins in the WNT/β-catenin pathway were determined by reverse transcription-quantitative PCR and Western blotting experiments. The results revealed that the mRNA and protein expression levels of MDIG in A549/DDP cells were significantly higher compared with those in A549 cells, and that the protein expression levels of MDIG increased in a dose-dependent manner with increasing DDP concentrations. Overexpression of MDIG in A549 and A549/DDP cells led to an increase in the IC_{50} value, whereas silencing of MDIG led to a clear reduction in the IC_{50} value. The overexpression of MDIG in the A549 and A549/DDP cells markedly upregulated the mRNA and protein expression levels of ABCB1, ABCC1, ABCG2, WNT family member 5A, WNT family member 3A and active β-catenin, and these were markedly decreased following MDIG silencing. Taken together, these results demonstrated that the DDP resistance of lung adenocarcinoma may be associated with an upregulation of MDIG expression, and that the expression levels of MDIG are positively associated with the degree of DDP resistance. Furthermore, MDIG promoted the expression of ABC transporters in tumor cells by activating the WNT/β-catenin signaling pathway, which may, in turn, lead to DDP resistance in lung adenocarcinoma.

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Key words: mineral dust-induced gene, ATP-binding cassette transporter, lung cancer, WNT/β-catenin pathway, multidrug resistance

Introduction

Lung cancer is one of the most common malignancies, with high incidence and high mortality rates worldwide, and non-small cell lung cancer (NSCLC) accounts for ~80-85% of the total cases of lung cancer (1,2). In 2011 in the United States, the 5-year survival rate of lung cancer is <20%, which is mainly due to the development of drug resistance that occurs after 1 year of treatment (1,3,4). Cisplatin (DDP) is one of the most commonly used chemotherapeutic drugs for clinical NSCLC, although it is only 20-30% effective in patients with advanced non-surgical NSCLC (5). One of the reasons for this is the emergence of multidrug resistance (MDR) (5). The mechanism of MDR formation in tumor cells is complex, although it predominantly includes drug absorption reduction, an increase in metabolism, drug target alterations and damage sustained to the tumor cell apoptosis pathway. The most important mechanism of drug resistance is the high expression of the ATP-binding cassette (ABC) transporters in the efflux pump on the tumor cell membrane, particularly high expression of P-glycoprotein (P-gp; ABCB1 gene-encoded), MDR-associated protein 1 (MRP1; ABCC1 gene-encoded) and breast cancer resistance protein (BCRP; ABCG2 gene-encoded) (6,7). The chemotherapeutic drug binds to the transmembrane domain of the ABC transporter on the tumor cell membrane, resulting in the activation of the ATP-binding domain, which subsequently hydrolyzes ATP to ADP with the concomitant release of energy, altering the morphology of the ABC transporter under the action of Mg^{2+}; thus, it is transferred to the outside of the tumor cells prior to the cytotoxic action of the drug, which reduces the drug concentration in the tumor cells and causes drug resistance (8). Previous studies have revealed that the WNT/β-catenin pathway is an important signal transduction pathway regulating tumor cell DDP resistance (9,10). Following activation of the WNT signaling pathway, non-phosphorylated (activated) β-catenin is induced

to enter the nucleus, promoting the expression of downstream signaling molecules, including ABCB1, ABCC1 and ABCG2, thereby promoting the occurrence of DDP resistance in tumor cells (9,10).

Mineral dust-induced gene (MDIG) is a novel lung cancer-associated oncogene that was identified in the alveolar macrophages of coal miners in 2005, and was subsequently revealed to be the same gene as myc-induced nuclear antigen 53 (Mina53) and nuclear protein 52 (11-13). Previous studies have reported that MDIG is a downstream target gene of c-myc, serving an important regulatory role in the proliferation, growth, differentiation, invasion and migration, and genomic stability of tumor cells (11-16). However, whether MDIG is associated with the DDP resistance of lung cancer has, to the best of our knowledge, not yet been established.

In the present study, A549 (lung adenocarcinoma) and A549/DDP (lung adenocarcinoma resistant to DDP) cell lines were used, and the effects of MDIG on the expression levels of ABC transporters and the WNT/β-catenin signaling pathway in A549 and A549/DDP cells were investigated following MDIG silencing and MDIG overexpression to elucidate the role of MDIG in DDP resistance of lung adenocarcinoma and its molecular mechanism. The overall aim was to identify novel treatments for the effective targeted therapy of lung cancer.

Materials and methods

Cell lines and cell culture. The human lung adenocarcinoma cell line, A549, was purchased from the Type Culture Collection of the Chinese Academy of Sciences, and the lung adenocarcinoma cell line resistant to DDP, A549/DDP, was purchased from the BeNa Culture Collection. A549 cells were cultured in HyClone™ RPMI-1640 culture medium (HyClone; GE Healthcare Life Sciences) containing 10% HyClone™ FBS medium (GE Healthcare Life Sciences) in a humidified environment with 5% CO_2 in a cell incubator (Thermo Fisher Scientific, Inc.) at 37˚C. A549/DDP cells were cultured in RPMI-1640 medium containing DDP (final concentration, 1 μg/ml) (MedChemExpress) and 10% FBS to maintain their drug resistance with 5% CO_2 in a cell incubator at 37˚C.

Lentiviral transfection. MDIG-overexpression lentiviral vector (LV-MDIG; GenBank accession no. NM_032778) and control lentiviral vector (Vector), and MDIG-silenced lentiviral vector [LV-MDIG-RNA interference (RNAi) 1 sequence, 5'-GGGTGATTTGTTGTACTTT-3'; LV-MDIG-RNAi 2 sequence, 5'-AACGATTCAGTTTCACCAA-3'] and control lentiviral vector (LV-con sequence, 5'-TTCTCCGAACGT GTCACGT-3') were purchased from Shanghai GeneChem Co., Ltd. The medium used to dilute the virus was purchased from Gibco (Thermo Fisher Scientific, Inc.) and the reinforcing fluid was provided by Shanghai GeneChem Co., Ltd. The lentiviral vector carrying green fluorescent protein (GFP) gene was transferred to the target cell together with the aforementioned vectors. Aliquots (2.5 ml, $5x10^4$ cells/ml) of the target cells were inoculated into a T12.5 flask (Corning Inc.) the day prior to transfection, and cultured in a cell incubator at 37˚C in a humidified environment with 5% CO_2. When the cell confluence reached 30-50%, the cells were incubated with lentivirus according to the counterstaining index of the target cells (multiplicity of infection, A549-50 and A549/DDP-50, respectively). After 16 h, the medium was replaced with 5 ml fresh medium, and subsequently cells were cultured for a further 48 h. The cells were observed under an Axio Observer A1 inverted fluorescence microscope (Zeiss GmbH), and the transfection efficiency was expressed as a percentage of GFP-positive cells identified using the GFP fluorescence module (settings: Excitation, BP470/40; beam splitter, FT495; emission, BP525/50). The stably transfected cells were continuously exchanged, passaged and frozen, and subsequently used for further experiments. All experiments were performed after fourth-generation cells transfected with lentivirus.

Cytotoxicity assay. Cytotoxicity was determined using the Cell Counting kit-8 (CCK-8; MedChemExpress). The specific experimental procedures were performed according to the manufacturer's protocol. A549 and A549/DDP cells in the exponential growth phase, and the MDIG-silenced and overexpressing cells following transfection with the respective lentiviruses, were inoculated into 96-well plates (100 μl/well) at ~$5x10^3$ cells/well, and wells containing only RPMI-1640 medium were used as a blank control. Three replicate wells were used for each experimental group, with an incubation period of 24 h. Sterile saline (0.9%) was used to prepare and dilute DDP, as described previously (17). Subsequently, different concentrations of DDP (20, 40, 80, 100, 160, 200 and 320 μg/ml) (MedChemExpress) were sequentially added, and the cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 24 h. Subsequently, 10 μl/well CCK-8 solution was added, and the cells were incubated for a further 1-2 h at 37˚C. The optical density (OD) at 450 nm was measured using a microplate reader (Tecan Infinite M200 PRO; Tecan Group, Ltd.). The percentage cell viability was calculated as follows: Cell viability (%)=(the average OD value of the experimental group-average OD value of the blank control group)/(the average OD value of the negative control group-the average OD value of the blank control group) x100%. The concentration was plotted on the abscissa and the corresponding cell viability was plotted on the ordinate, then the curve was plotted and, finally, the IC_{50} value was calculated for DDP against the cells of each experimental group using GraphPad Prism version 7.0 software (GraphPad Software, Inc.). The IC_{50} value for DDP was defined as the value for which cell viability (%) was decreased to 50% by DDP. The resistance coefficient was determined by the ratio of the average IC_{50} value of A549/DDP cells to the average IC_{50} value of A549 cells. Each experiment was repeated at least three times.

RNA isolation, cDNA synthesis and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from each group of cells (including MDIG-knockdown and MDIG-overexpressing A549 cells, and DDP-resistant A549/DDP cells) was extracted using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.), and subsequently RNA quantification was performed on a NanoQuant plate™ (Tecan Group, Ltd.). The cDNA was reverse-transcribed and synthesized using the

Table I. Primer sequences of genes used in the present study.

Genes	Primer sequences
MDIG	Forward: 5'-GCAACGATTCAGTTTCACCAACC-3'
	Reverse: 5'-ATGTACACATTCGAGCCAACCAAG-3'
ABCB1	Forward: 5'-CACATTTGGCAAAGCTGGAGA-3'
	Reverse: 5'-CATCATTGGCGAGCCTGGTA-3'
ABCG2	Forward: 5'-TGCCCAGGACTCAATGCAAC-3'
	Reverse: 5'-TCGATGCCCTGCTTTACCAAATA-3'
ABCC1	Forward: 5'-GTGATGGCGATGAAGACCAAGA-3'
	Reverse: 5'-GCCAGCTCCCAGGCATAAAG-3'
Actin	Forward: 5'-CCTGGCACCCAGCCAAT-3'
	Reverse: 5'-GGGCCGGACTCGTCATAC-3'

MDIG, mineral dust-induced gene; ABCB1, ATP-binding cassette transporter B1; ABCC1, ATP-binding cassette transporter C1; ABCG2, ATP-binding cassette transporter G2.

PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol: 35˚C for 15 min, 85˚C for 5 sec and gradually decreased to 4˚C. Actin was used as the internal reference gene. The PCR primers used in this experiment were synthesized by Takara Biotechnology Co., Ltd., and the primer sequences are shown in Table I. RT-qPCR analysis was performed using SYBR Premix Ex Taq™ II (Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. The thermocycling conditions for the PCR reaction were as follows: Pre-denaturation at 95˚C for 30 sec, followed by 95˚C for 5 sec and 60˚C for 30 sec (40 cycles). The melting curve was established at 95˚C for 5 sec, 60˚C for 1 min, 95˚C for 15 sec, and the cooling process was 50˚C for 50 sec. The cycle threshold value (Cq) was determined using a LightCycler 480 machine (Roche Diagnostics), and, finally, the $2^{-\Delta\Delta Cq}$ method (18) was used to calculate the relative ratio of the gene of interest, expressed as a percentage relative to the normal control group.

Western blotting. The cells of each group were extracted with RIPA lysate (Beijing Suolaibao Technology Co., Ltd.) containing 10% PMSF and protease inhibitor cocktail (Roche Diagnostics GmbH) on ice. Subsequently, total protein concentration was determined using a Bicinchoninic Acid Protein Quantification kit (Thermo Fisher Scientific, Inc.). The protein samples (30 μg/lane) were separated using SDS-PAGE (8% gels; Bio-Rad Laboratories, Inc.), and subsequently transferred onto PVDF membranes (0.45 μm) for 1.5-2.5 h, followed by blocking at room temperature for 2 h with 5% non-fat dried milk. The membranes were washed 3 times with TBS with 0.1% Tween-20 (TBST) for 15 min each time, and then incubated with the primary antibodies of interest, as follows: Anti-Mina53 (cat. no. ab173573, 1:1,000 dilution, Abcam), anti-ABCB1 (cat. no. 13342, 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-ABCC1 (cat. no. 14685, 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-ABCG2 (cat. no. 42078, 1:1,000 dilution; Cell Signaling Technology, Inc.), non-phospho (active) β-catenin (cat. no. 8814, 1:1,000 dilution; Cell Signaling Technology, Inc.), anti-WNT family member 5A (WNT5A; cat. no. ab174963, 1:500 dilution; Abcam), anti-WNT family member 3A (WNT3A; cat. no. ab28472, 1:1,000 dilution; Abcam) and anti-GAPDH (cat. no. 5174, 1:1,000 dilution; Cell Signaling Technology, Inc.), overnight at 4˚C. Following washing of the membranes with TBST, they were incubated with horseradish peroxidase-conjugated secondary antibody [anti-rabbit immunoglobulin G (IgG); cat. no. 2306, 1:4,000 dilution; OriGene Technologies, Inc.] at room temperature for 2 h. Immunoreactive bands were detected with an ECL western blotting system (Clarity Western ECL Substrate; Bio-Rad Laboratories, Inc.). ImageJ version 1.8.0 software (National Institutes of Health) was used to analyze the gray value of each band, and to calculate the gray value percentage of each protein band compared with the internal reference (GAPDH). Each experiment was repeated at least three times.

Statistical analysis. All experiments were repeated at least three times. The data are expressed as the means ± SD. All data were tested for normality and homogeneity of variance: Comparisons among groups were performed using ANOVA, and the comparisons within groups were performed using the Bonferroni method (when the variance was uniform) and the Dunnett's T3 method (when the variance was not uniform). All statistical analyses were performed using GraphPad Prism version 7.0 software (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Construction of MDIG-knockdown and MDIG-overexpressing A549 cells. Inverted fluorescence microscopy was used to observe fourth-generation A549 cells transfected with lentivirus at bright-field x100 magnification, and subsequently, in the same field of view, the cells were analyzed for GFP fluorescence at GFP-field x100 and x400 magnification. All

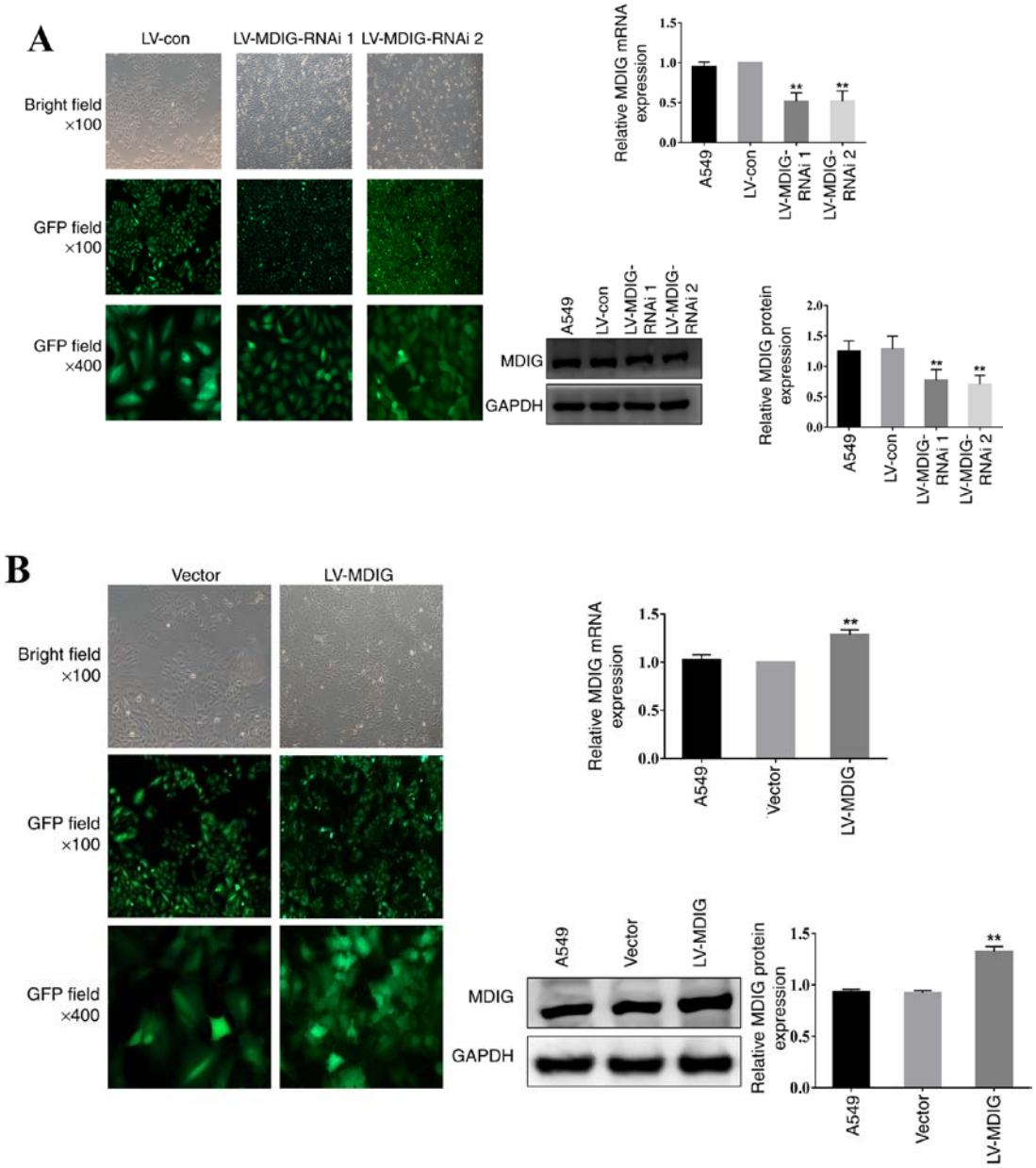

Figure 1. Knockdown and overexpression of MDIG in A549 cells. The transfection efficiency and cell morphology (bright field, x100 magnification; GFP field, x100 magnification; and GFP field, x400 magnification) were observed under an inverted fluorescence microscope, and the mRNA and protein expression levels of MDIG in each group were examined using reverse transcription-quantitative PCR and western blot analysis, respectively. (A) Stably transfected MDIG-silenced A549 cells. **P<0.01 vs. LV-con and A549 groups. (B) Stably transfected MDIG-overexpressing A549 cells. The details of the groups (LV-MDIG-RNAi 1, LV-MDIG, etc.) can be found in the Materials and methods section. **P<0.01 vs. vector and A549 groups. Con, control; GFP, green fluorescence protein; LV, lentiviral vector; MDIG, mineral dust-induced gene; RNAi, RNA interference.

cell groups exhibited a high cell viability state and high transfection efficiency (>90%; Fig. 1), and the efficiency of MDIG knockdown and overexpression was verified by RT-qPCR and western blot analysis. The results revealed that, compared with the normal A549 group and the LV-con group, the LV-MDIG-RNAi 1 and the LV-MDIG-RNAi 2 groups exhibited a marked reduction in the mRNA and protein expression levels of MDIG in the A549 cells (P<0.01; Fig. 1A). By contrast, compared with the A549 and vector groups, the LV-MDIG group was observed to have increased mRNA and protein expression levels of MDIG in the A549 cells (P<0.01; Fig. 1B). Taken together, these results indicated that the processes of lentiviral transfection for knockdown and overexpression were successful.

Construction of MDIG-knockdown and MDIG-overexpressing A549/DDP cells. As for A549 cells, fourth-generation A549/DDP cells transfected with lentivirus were observed by inverted fluorescence microscopy. All cells were shown to be in good condition, and the transfection efficiency was high (Fig. 2). The RT-qPCR and western blotting experiments demonstrated that the mRNA and protein expression levels of MDIG were lower in the LV-MDIG-RNAi 1 and LV-MDIG-RNAi 2 groups compared with the A549/DDP and

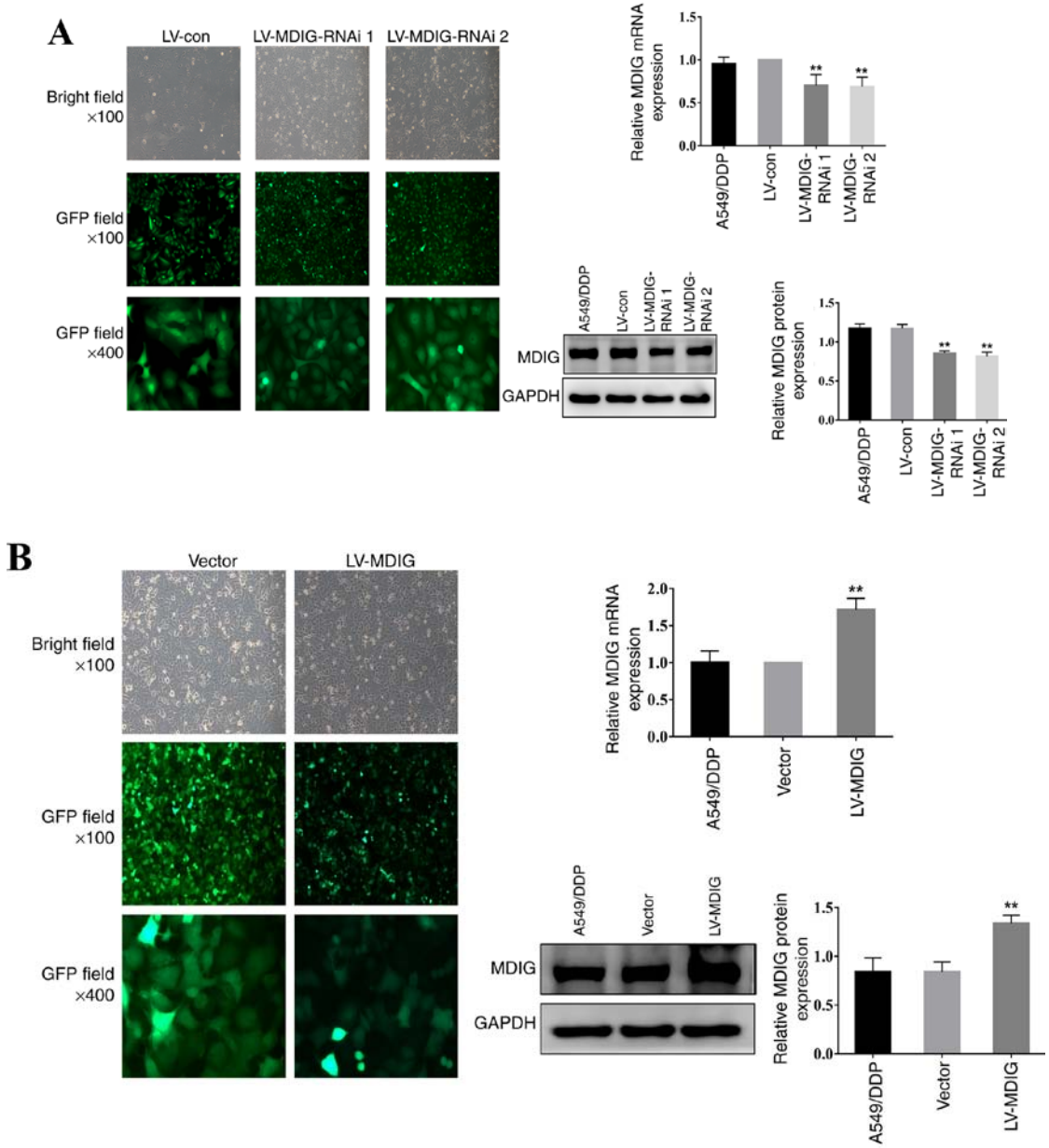

Figure 2. Knockdown and overexpression of MDIG in A549/DDP cells. The transfection efficiency and cell morphology (bright-field, x100 magnification; GFP field, x100 magnification; and GFP field, x400 magnification) were observed under an inverted fluorescence microscope, and the mRNA and protein expression levels of MDIG in each group were examined using reverse transcription-quantitative PCR and western blot analysis, respectively. (A) Stably transfected MDIG-silenced A549/DDP cells. **P<0.01 vs. LV-con and A549/DDP groups. (B) Stably transfected MDIG-overexpressing A549/DDP cells. **P<0.01 vs. vector and A549/DDP groups. The naming of the groups (LV-MDIG-RNAi 1, LV-MDIG, etc.) is detailed in the Materials and methods section. Con, control; DDP, cisplatin; GFP, green fluorescence protein; LV, lentiviral vector; MDIG, mineral dust-induced gene; RNAi, RNA interference.

the LV-con groups (P<0.01; Fig. 2A). Conversely, the mRNA and protein expression levels of MDIG were higher in the LV-MDIG group compared with those in the A549/DDP and vector groups (P<0.01; Fig. 2B). These results indicated that these stably transfected cell lines could be used in subsequent experiments.

Basic expression levels of MDIG in the A549 and A549/DDP cells, and the effect of DDP. In order to investigate the association between MDIG and platinum-based drug resistance in tumor cells, RT-qPCR and western blot analyses were used to detect the basic mRNA and protein expression levels of MDIG in the A549 and A549/DDP cells (Fig. 3). The results revealed that the mRNA and protein expression levels of MDIG in A549/DDP cells were significantly higher compared with those in A549 cells (P<0.01), which suggested that MDIG expression was associated with DDP resistance. To further determine the association between MDIG expression and DDP resistance, A549 and A549/DDP cells were treated with different concentrations of DDP (0, 40 and 80 μg/ml), and the protein expression levels of MDIG were revealed to increase in a dose-dependent manner with increasing DDP concentrations in the two cell lines (P<0.01; Fig. 4).

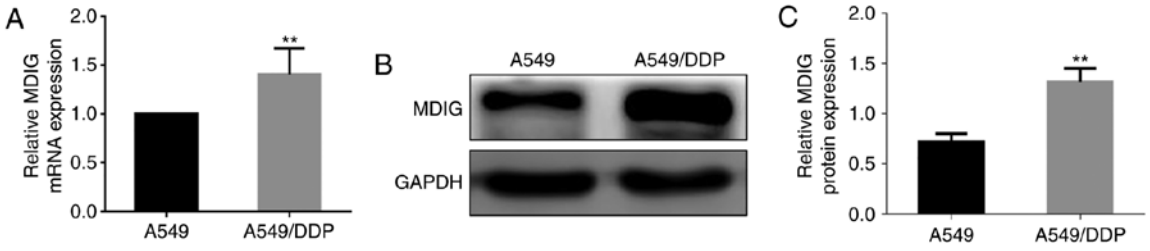

Figure 3. Basal mRNA and protein expression levels of MDIG in A549 and A549/DDP cells. (A) MDIG mRNA expression in the two cell lines was measured by reverse transcription-quantitative PCR analysis, and actin was used as the reference gene. (B) MDIG protein expression in the two cell lines was measured by western blot analysis, and GAPDH was used as a reference control. (C) Statistical analysis diagram of protein expression levels in A549 and A549/DDP cells. **P<0.01 vs. A549 group. DDP, cisplatin; MDIG, mineral dust-induced gene.

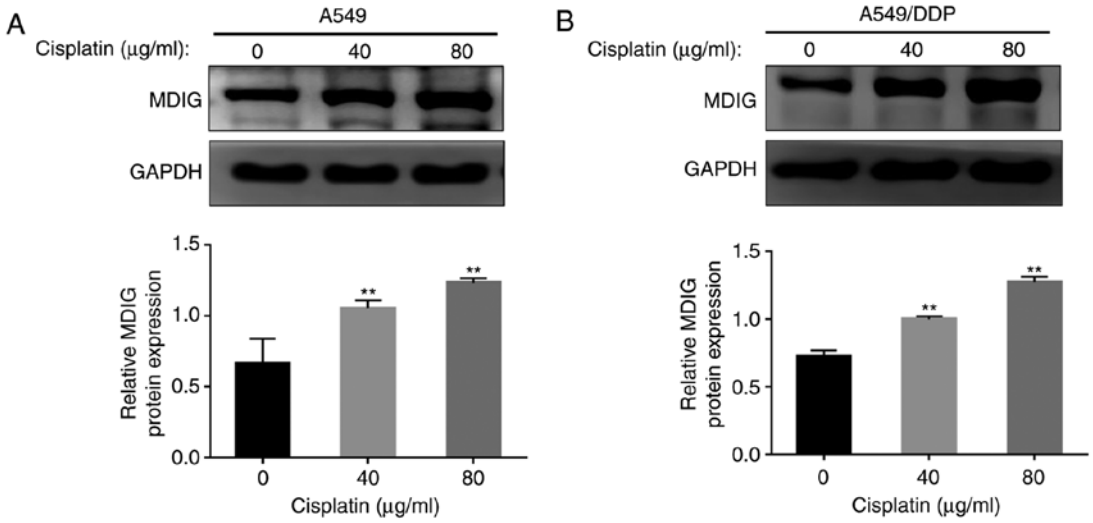

Figure 4. Effect of DDP on the protein expression levels of MDIG in A549 and A549/DDP cells. Protein expression levels were semi-quantified in (A) A549 and (B) A549/DDP cells. GAPDH was used as a reference control. **P<0.01 vs. A549 group. DDP, cisplatin; MDIG, mineral dust-induced gene.

*MDIG promotes resistance to DDP in A549 and A549/DDP cells. In order t*o further investigate the effect of MDIG on DDP resistance in lung adenocarcinoma cells, the IC_{50} values for DDP in A549 and A549/DDP cells were determined using a CCK-8 assay. The results demonstrated that the IC_{50} value for DDP in the A549/DDP cells was significantly higher compared with that in the A549 cells (93.79±8.70 vs. 29.91±1.43 μg/ml; P<0.01), and the resistance coefficient was 3.14 (Fig. 5A). Based on this, following MDIG overexpression (LV-MDIG) and MDIG knockdown (LV-MDIG-RNAi) in A549 and A549/DDP cells, the IC_{50} values for DDP of each group of cells was determined, revealing that, compared with the A549 and vector groups, the IC_{50} value of the A549 cells overexpressing MDIG was significantly increased (IC_{50} of LV-MDIG group, 176.90±13.40 μg/ml; IC_{50} of A549 group, 29.91±1.43 μg/ml; and IC_{50} value of vector group, 37.57±1.98 μg/ml; P<0.01; Fig. 5B), whereas the IC_{50} values of the A549 groups were significantly decreased following MDIG knockdown (IC_{50} of LV-MDIG-RNAi 1 group, 10.84±1.44 μg/ml; IC_{50} of LV-MDIG-RNAi 2 group, 8.471±1.74 μg/ml; IC_{50} of A549 group, 29.91±1.43 μg/ml; and IC_{50} of LV-con group, 26.67±1.059 μg/ml; P<0.01; Fig. 5C). Furthermore, in the MDIG-knockdown and MDIG-overexpressing A549/DDP cells, similar results were obtained, i.e., the IC_{50} values of the MDIG-overexpressing A549/DDP groups were significantly higher compared with those of the A549/DDP and vector groups (IC_{50} of LV-MDIG group, 262±25.51 μg/ml; IC_{50} of A549/DDP group, 93.79±8.70 μg/ml; and IC_{50} of vector group, 114.7±7.379 μg/ml; P<0.01; Fig. 5D). Conversely, following MDIG knockdown, the IC_{50} values were significantly lower (IC_{50} of LV-MDIG-RNAi 1 group, 31.05±1.73 μg/ml; IC_{50} of LV-MDIG-RNAi 2 group, 35.1±5.85 μg/ml; IC_{50} of A549/DDP group, 93.79±8.70 μg/ml; and IC_{50} of LV-con group, 104.8±21.13 μg/ml; P<0.01; Fig. 5E).

MDIG promotes ABC transporter expression in A549 and A549/DDP cells. Subsequently, in order to investigate the molecular mechanism of MDIG-promoted DDP resistance in lung adenocarcinoma cells, the expression levels of ABC transporters in A549 and A549/DDP cells were examined. As shown in Fig. 6, the basal mRNA and protein expression levels of ABCB1, ABCC1 and ABCG2 were significantly higher in A549/DDP cells compared with in A549 cells, indicating that efflux pump ABC transporters are involved in DDP resistance in lung adenocarcinoma cells. To further clarify whether MDIG affected the resistance of lung adenocarcinoma to DDP by regulating the expression levels of ABC transporters, MDIG was overexpressed (the LV-MDIG group) or silenced (the LV-MDIG RNAi 1 and LV-MDIG RNAi 2

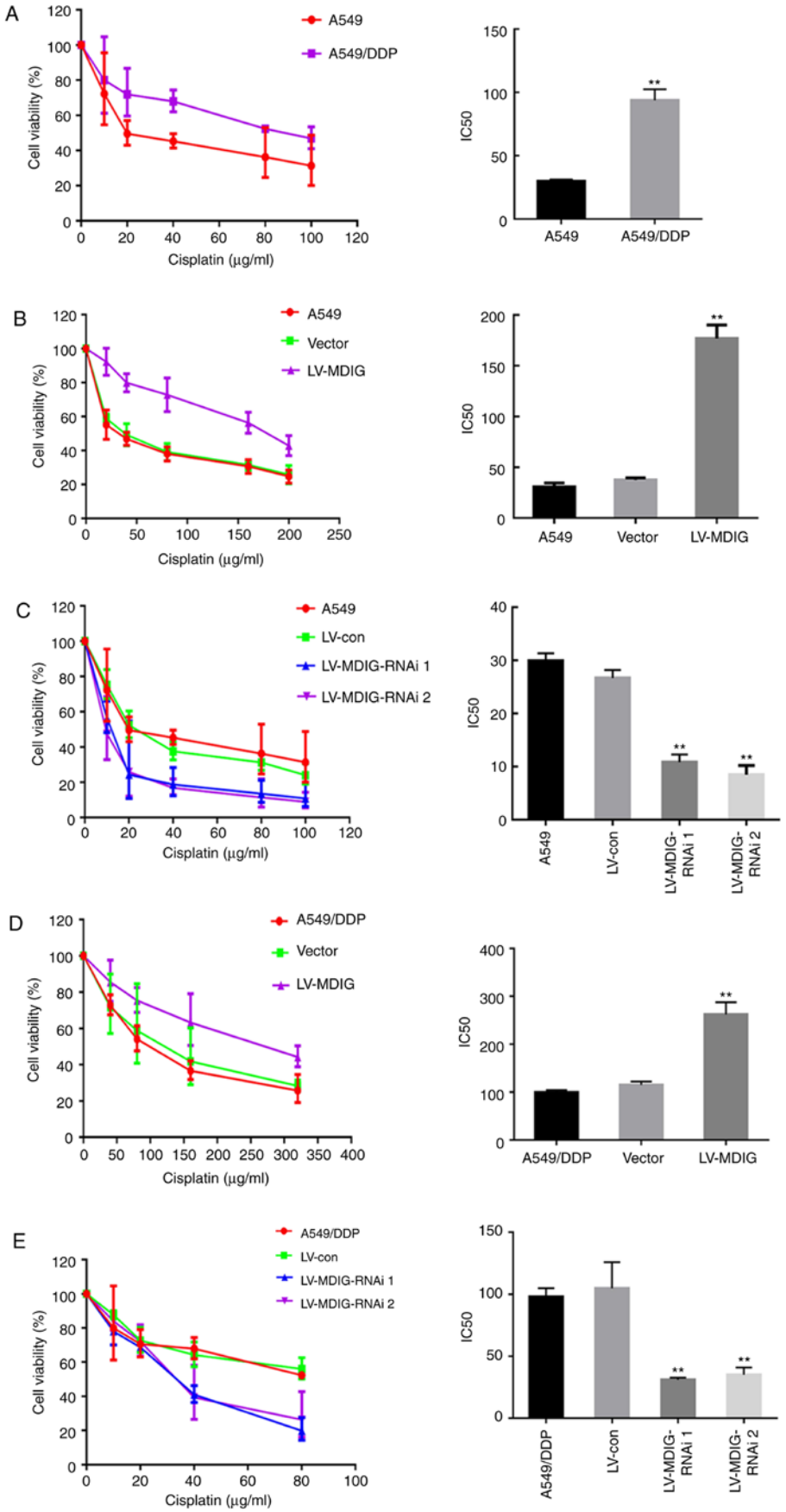

Figure 5. MDIG is associated with DDP resistance in A549 and A549/DDP cells. (A) A549 and A549/DDP cells were treated with different concentrations of DDP for 48 h, cell viability was determined by CCK-8 assay, and the IC_{50} values for DDP were calculated. $^{**}P<0.01$ vs. A549 group. A CCK-8 assay was used to determine the cell viability of (B) MDIG-overexpressing ($^{**}P<0.01$ vs. A549 group), and (C) MDIG-silenced A549 cells, and the IC_{50} values for DDP were calculated. $^{**}P<0.01$ vs. LV-con and A549 groups. A CCK-8 assay was used to determine the cell viability of (D) MDIG-overexpressing ($^{**}P<0.01$ vs. vector and A549/DDP groups) and (E) MDIG-silenced A549/DDP cells, and the IC_{50} values for DDP were calculated ($^{**}P<0.01$ vs. the LV-con and A549/DDP groups). Con, control; DDP, cisplatin; LV, lentiviral vector; MDIG, mineral dust-induced gene; RNAi, RNA interference.

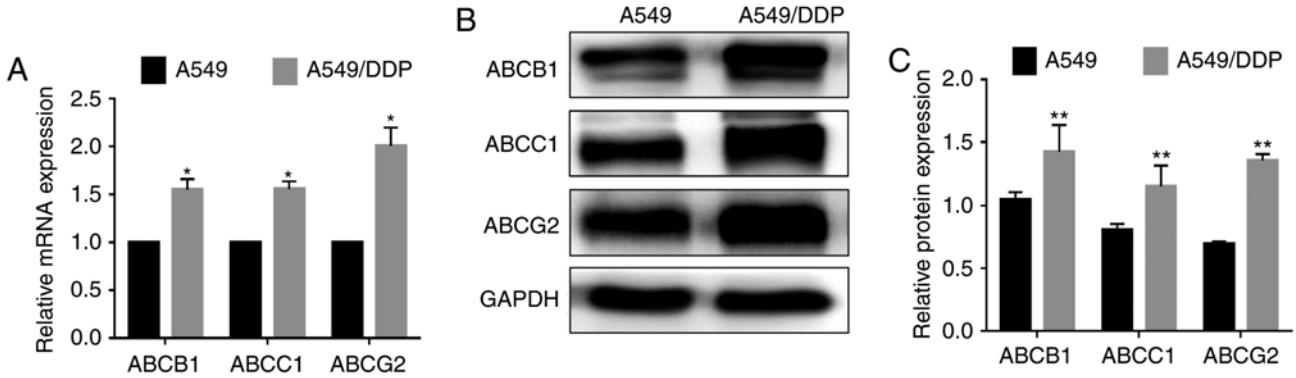

Figure 6. Basal expression levels of ABC transporter (ABCB1, ABCC1 and ABCG2) proteins. (A) mRNA and (B) protein expression levels in A549 and A549/DDP cells, (C) Statistical analysis of protein expression levels in A549 and A549/DDP cells. Actin was used as the reference control for reverse transcription-quantitative PCR analysis, whereas GAPDH was used as the reference control for western blot analysis. *P<0.05 and **P<0.01, vs. A549 group. ABC, ATP-binding cassette; ABCB1, ATP-binding cassette transporter B1; ABCC1, ATP-binding cassette transporter C1; ABCG2, ATP-binding cassette transporter G2; DDP, cisplatin.

groups) in A549 and A549/DDP cells, and the mRNA and protein expression levels of the ABC transporters in either of the cell types was determined by RT-qPCR and western blot analysis, respectively. The results revealed that the mRNA and protein expression levels of the efflux pump transporters, ABCB1, ABCC1 and ABCG2, in A549 and A549/DDP cells were significantly upregulated following MDIG overexpression compared with the normal and vector groups (P<0.05; Fig. 7). Conversely, following MDIG knockdown, the mRNA and protein expression levels of the ABC transporters were significantly lower compared with the normal and the LV-con groups (P<0.01; Fig. 8).

MDIG regulates the expression levels of ABC transporters by activating the WNT/β-catenin signaling pathway. A previous study demonstrated that WNT signaling is activated in A549/DDP cells, and that this is associated with DDP resistance (19). In addition, further studies have demonstrated that WN T leads to DDP resistance by regulating the expression levels of downstream signaling molecules; the ABC transporters (9,20). Therefore, in order to investigate whether MDIG also affected the expression levels of ABC transporters via the WNT signaling pathway, thereby promoting DDP resistance, the basal expression levels of WNT signaling pathway-associated proteins were examined in A549 and A549/DDP cells by western blot analysis. The results demonstrated that the expression levels of WNT5A, WNT3A and active β-catenin in the A549/DDP cells were significantly higher compared with those in the A549 cells (P<0.01; Fig. 9A). Based on this, the expression levels of WNT signaling pathway-associated proteins following overexpression or silencing of MDIG in the A549/DDP cells was examined. The results revealed that, the expression levels of WNT5A, WNT3A and active β-catenin in the MDIG-overexpression group were significantly higher compared with those in the normal A549/DDP and vector groups (P<0.01; Fig. 9B). Conversely, in the MDIG-silencing experiment, compared with the normal A549/DDP and the LV-con groups, the expression levels of WNT5A, WNT3A and active β-catenin in the MDIG-silencing groups were significantly lower (P<0.01; Fig. 9C).

Discussion

Lung cancer is the leading cause of cancer-associated mortality worldwide (1). NSCLC accounts for 80-85% of all cases of lung cancer (1,2). Out of all patients with NSCLC, ~70% are diagnosed with advanced metastasis. Chemotherapy is able to prolong the life of patients with advanced NSCLC, and to improve their quality of life. DDP is one of the most important first-line chemotherapy drugs for patients with advanced lung cancer. However, the development of lung cancer resistance to DDP leads to failure of chemotherapy, rendering the treatment of patients with advanced cancer less effective. According to previous studies, the overall 5-year survival rate of patients with stage IIIB/IV NSCLC is only 1-5% (21-23).

MDIG is a proto-oncogene associated with lung cancer, and is highly expressed in lung cancer tissues and the majority of lung cancer cell lines, but not in normal lung tissues (11). Previous studies have demonstrated that MDIG is highly expressed in a variety of tumor types in addition to lung cancer, including breast cancer, colon cancer, liver cancer, renal cell carcinoma and neuroblastoma (24-28). In addition, it exerts different biological effects in tumor progression, including regulation of tumor cell proliferation, invasion and migration, differentiation, and genomic stability (11-16). However, limited research has been conducted on whether MDIG is associated with tumor resistance. One study in this area was conducted by Huo *et al* (29), who reported in 2017 that MDIG is associated with the resistance of sorafenib in hepatoma cells *in vitro*. They demonstrated that MDIG knockdown increased the sensitivity of Huh7 (human hepatoma) cells and MHCC97-H (human high-metastatic-potential hepatoma) cells to sorafenib; however, the specific mechanism of drug resistance was not further explored, and the association between MDIG and DDP sensitivity in lung cancer and other tumor cells, to the best of our knowledge, has not yet been reported on.

In order to investigate the association between MDIG and platinum-like resistance in lung adenocarcinoma cells, in the present study, the mRNA and protein expression levels of MDIG in DDP-resistant lung adenocarcinoma cells (A549/DDP) were revealed to be significantly higher compared with those in normal lung adenocarcinoma cells (A549) according to

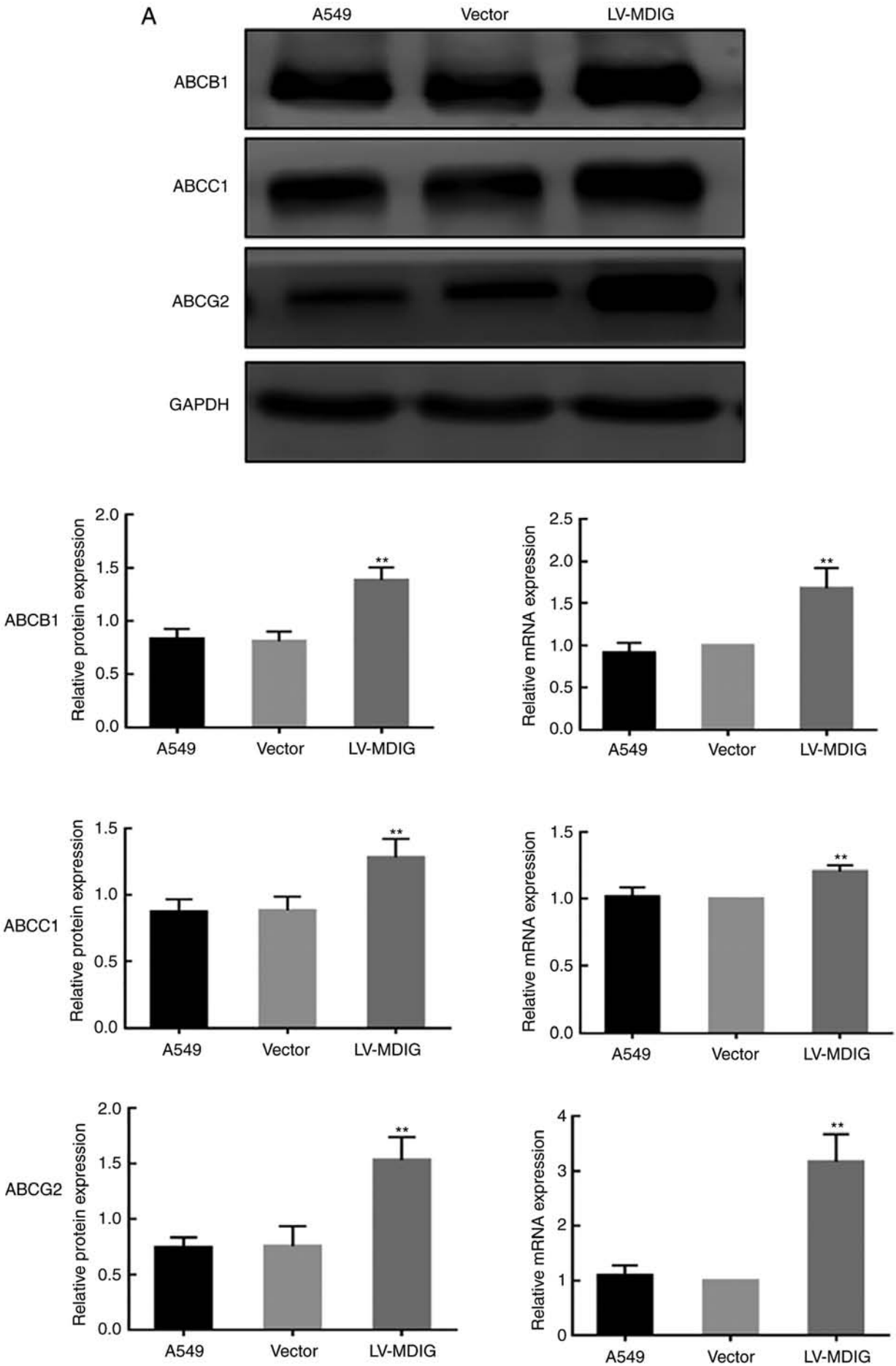

Figure 7. Alterations in the mRNA and protein expression levels of ABC transporters in (A) A549 cells following MDIG overexpression.

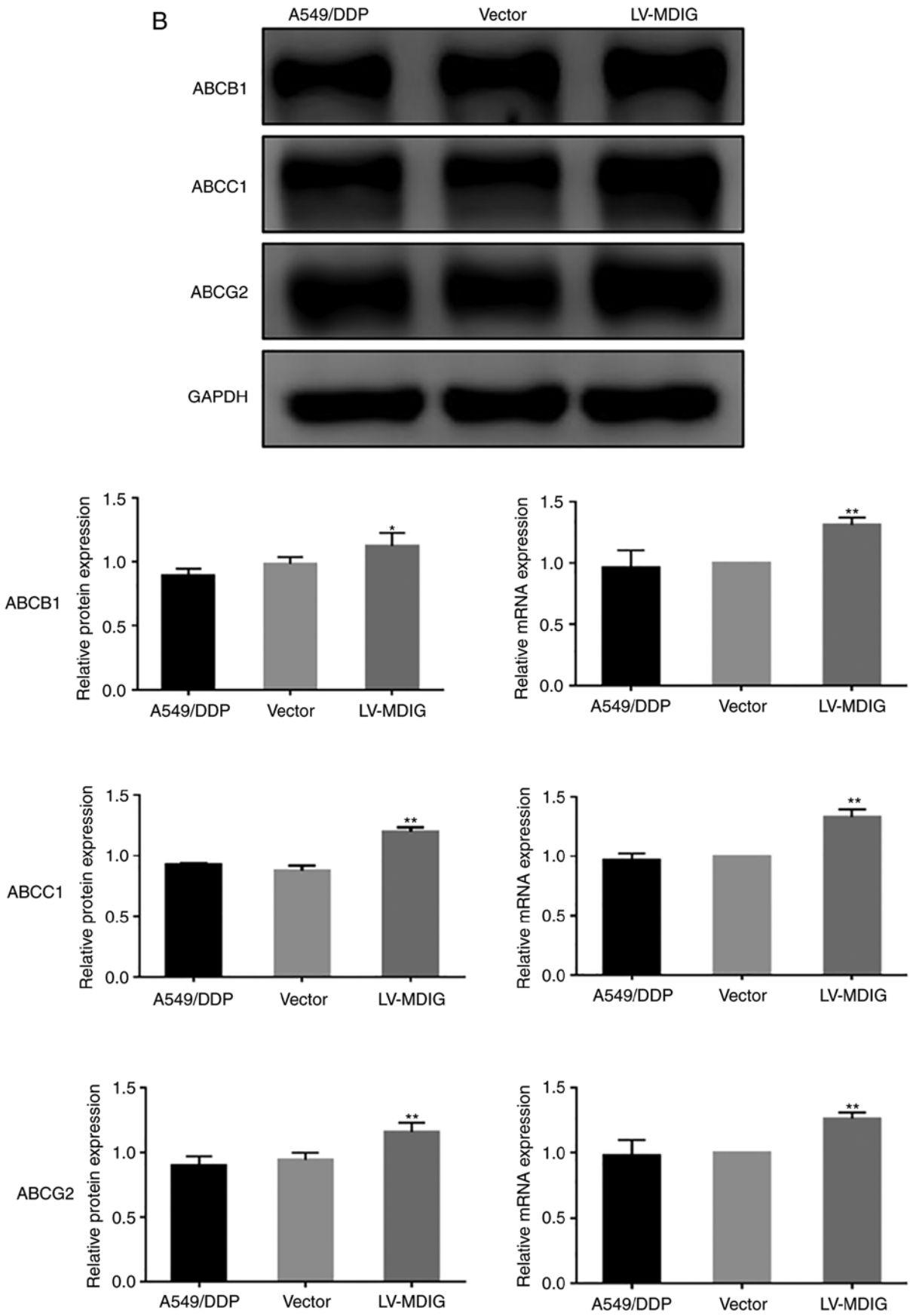

Figure 7. Continued. Alterations in the mRNA and protein expression levels of ABC transporters in (B) A549/DDP cells following MDIG overexpression. The LV-MDIG group was compared with the vector and normal groups. Actin was the reference control for reverse transcription-quantitative PCR analysis, whereas GAPDH was the reference control for the western blotting experiments. The naming of the groups (LV-MDIG-RNAi 1, LV-MDIG, etc.) is detailed in the Materials and methods section. *P<0.05, **P<0.01 vs. vector and A549 groups or vs. vector and A549/DDP groups. ABC, ATP binding cassette; ABCB1, ATP-binding cassette transporter B1; ABCC1, ATP-binding cassette transporter C1; ABCG2, ATP-binding cassette transporter G2; DDP, cisplatin; LV, lentiviral vector; MDIG, mineral dust-induced gene.

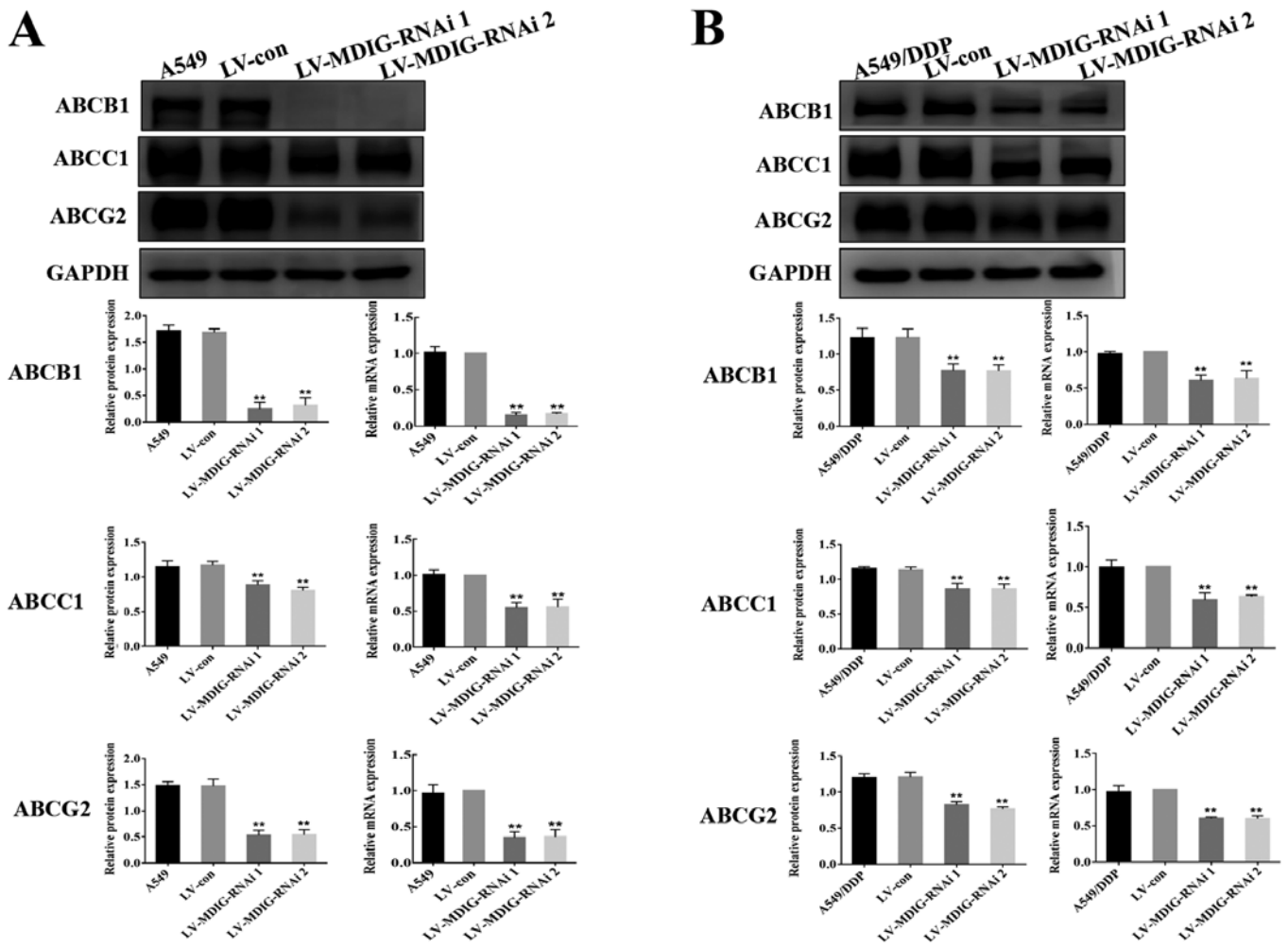

Figure 8. Alterations in the mRNA and protein expression levels of the ABC transporters. Expression levels of ABV transporter proteins in (A) A549 cells following MDIG knockdown. Expression levels of ABV transporter proteins in (B) A549/DDP cells following MDIG knockdown. $^{**}P<0.01$ vs. LV-con and A549 groups or vs. LV-con and A549/DDP groups. Actin was the reference control for reverse transcription-quantitative PCR analysis, whereas GAPDH was the reference control for the western blotting experiments. The naming of the groups (LV-MDIG-RNAi 1, LV-MDIG, etc.) is detailed in the Materials and methods section. ABC, ATP-binding cassette; ABCB1, ATP-binding cassette transporter B1; ABCC1, ATP-binding cassette transporter C1; ABCG2, ATP-binding cassette transporter G2; Con, control; DDP, cisplatin; LV, lentiviral vector; MDIG, mineral dust-induced gene; RNAi, RNA interference.

RT-qPCR and western blotting analyses. Additionally, the expression levels of MDIG in the two cell lines increased in a dose-dependent manner with increasing DDP concentration, which suggested that DDP resistance in lung adenocarcinoma may be associated with the upregulation of MDIG. The expression levels of MDIG were positively associated with the degree of DDP resistance.

To further investigate the association between MDIG and DDP resistance in lung cancer cells, the present study initially employed a CCK-8 assay to detect the IC_{50} value for DDP in A549 and A549/DDP cells. The results demonstrated that the IC_{50} value for DDP of the A549/DDP cells was significantly higher compared with that of the A549 cells, and the resistance coefficient was 3.14. Based on this, following MDIG overexpression (LV-MDIG group) or MDIG knockdown (LV-MDIG-RNAi groups) in A549 and A549/DDP cells, the IC_{50} values for DDP of each group were determined, revealing that, compared with the normal A549 and the vector groups, the IC_{50} values were significantly increased in the MDIG-overexpressing A549 groups, whereas the IC_{50} values were significantly decreased in the MDIG-silencing A549 groups, when compared with the normal A549 and the LV-con groups. Following overexpression and knockdown of MDIG in the A549/DDP cells, similar results to those described for the A549 cells were obtained. These results suggested that MDIG expression may promote the resistance of lung adenocarcinoma cells to DDP, and that the sensitivity to DDP can be restored following MDIG silencing.

The emergence of MDR is the most important cause of the failure of tumors to respond to chemotherapy drugs, and the ABC transporters in the efflux pump on the tumor cell membrane serve the most important role in this regard (6,7). The ABC transporter family is a group of transmembrane proteins, including seven subfamilies: ABCA-ABCG, of which the three subfamilies of ABCB, ABCC and ABCG are the ones that are mainly associated with tumor MDR. The most studied proteins are ABCB1 in the ABCB subfamily (also known as P-gp), ABCC1 in the ABCC subfamily (also known as MRP1) and ABCG2 in the ABCG subfamily (also known as BCRP). These proteins function predominantly by using the energy released by ATP hydrolysis to pump the intracellular drugs out of the cell, leading to a reduction in

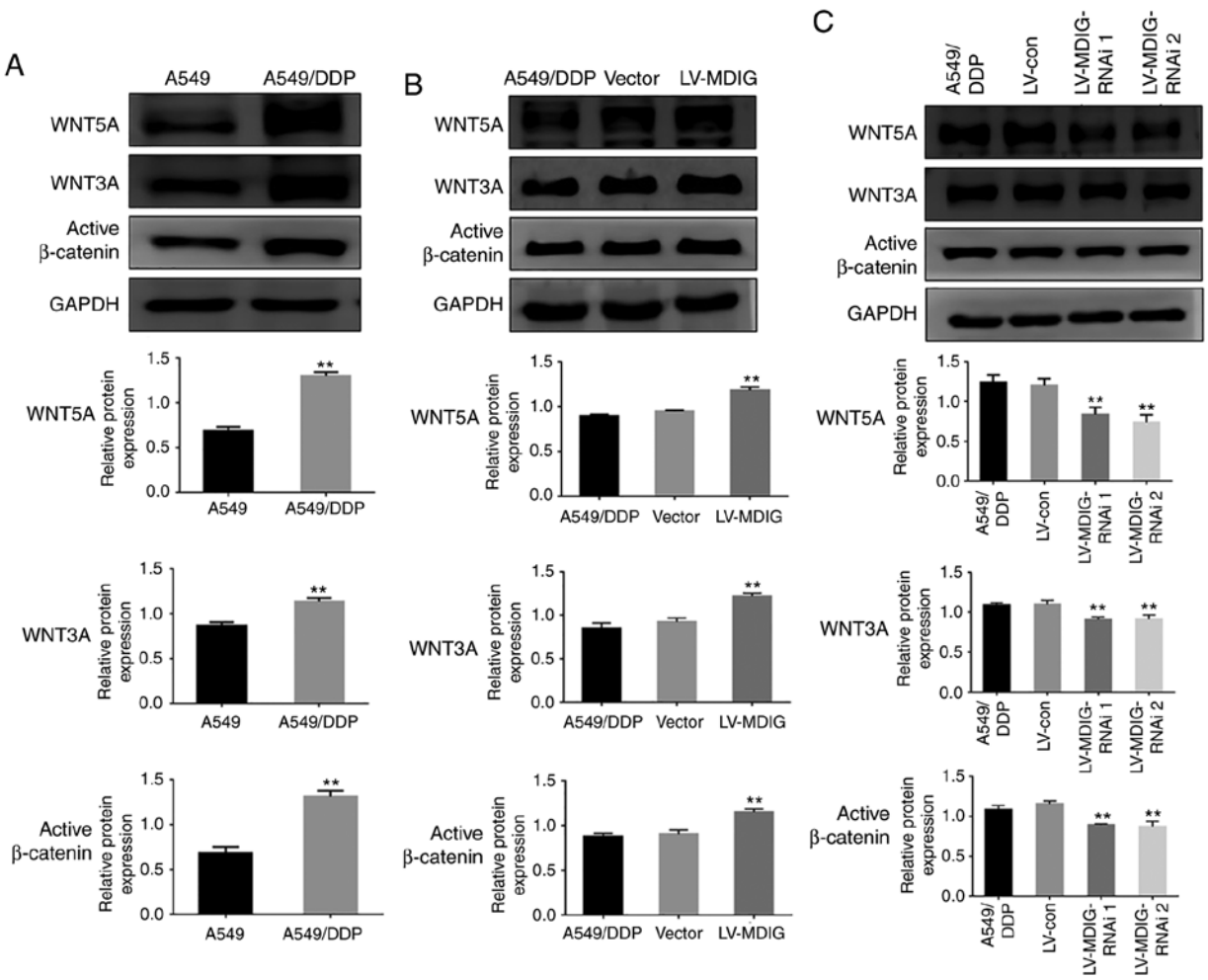

Figure 9. MDIG regulates ABC transporter expression by activating the WNT/β-catenin signaling pathway. (A) Basal expression of WNT signaling pathway-associated proteins in A549 and A549/DDP cells. **P<0.01 vs. A549 group. (B) MDIG overexpression promoted the expression of WNT/β-catenin signaling pathway-associated proteins in A549/DDP cells. **P<0.01 vs. A549 and vector groups. (C) MDIG knockdown reduces the expression of WNT/β-catenin signaling pathway-associated proteins in A549/DDP cells. **P<0.01 vs. A549 and LV-con groups. The naming of the groups (LV-MDIG-RNAi 1, LV-MDIG, etc.) is detailed in the Materials and methods section. GAPDH was used as a reference control. Con, control; DDP, cisplatin; LV, lentiviral vector; MDIG, mineral dust-induced gene; RNAi, RNA interference; WNT3A, WNT family member 3A; WNT5A, WNT family member 5A.

the intracellular drug concentration and consequent drug resistance (8). Robey *et al* (30) demonstrated that the use of specific targeting inhibitors of ABCB1 and ABCC1 is able to overcome the resistance of osteosarcoma to DDP. Similarly, Shi *et al* (31) reported that the epidermal growth factor tyrosine kinase inhibitor, AG1478, and erlotinib effectively reverse ABCG2-mediated MDR by directly inhibiting the drug efflux function of ABCG2 in ABCG2-overexpressing cells.

In order to investigate the molecular mechanism of MDIG-promoted DDP resistance in lung adenocarcinoma cells, the present study examined the basal expression levels of efflux pump ABC transporters in A549 and A549/DDP cells, and it was revealed that the mRNA and protein expression levels of ABCB1, ABCC1 and ABCG2 in A549/DDP cells were significantly higher compared with those of A549 cells. This was consistent with previous studies (32-34), indicating that efflux pump ABC transporters are involved in DDP resistance in lung adenocarcinoma cells. To further investigate whether MDIG affected the resistance of lung adenocarcinoma to DDP by regulating the expression levels of the ABC transporter family, MDIG was overexpressed and silenced in A549 and A549/DDP cells, and the mRNA and protein expression levels of the ABC transporters were subsequently determined by RT-qPCR and western blot analysis, respectively. The results revealed that the mRNA and protein expression levels of the extracellular pumping transporters, ABCB1, ABCC1 and ABCG2, were upregulated in the two cell lines compared with the normal and the control vector groups. On the other hand, following MDIG knockdown, the expression levels were significantly lower compared with the normal and LV-con groups. These findings indicated that MDIG regulated the expression levels of ABC transporters, which may cause DDP resistance in lung adenocarcinoma cells by promoting the expression levels of the efflux pump ABC transporters.

Previous studies have reported that DDP resistance of tumor cells is associated with activation of the WNT/β-catenin signaling pathway (9,10). For example, Luo *et al* (19) reported that the sensitivity of DDP-resistant lung cancer cells was enhanced upon downregulating WNT/β-catenin signaling and inhibiting BCRP and MRP4 expression, and Huang *et al* (35) demonstrated that abnormal activation of the WNT/β-catenin

signaling pathway in ovarian cancer cells promoted DDP resistance, and inhibition of WNT signaling effectively reversed DDP chemoresistance in SKOV3/DDP cells. Furthermore, Li *et al* (36) reported that the long non-coding RNA HOXA distal transcript antisense RNA induced resistance to DDP in osteosarcoma cells by activating the WNT/β-catenin pathway, a process that could be reversed by the addition of WNT signaling pathway inhibitors. Previous studies have also demonstrated that ABC transporters are the downstream signaling molecules of the WNT/β-catenin signaling pathway (9,20). In addition, WNT leads to DDP resistance by regulating the expression levels of the downstream signaling molecule ABC transporter (9,20). In order to investigate whether MDIG also affected ABC transporter expression via the WNT signaling pathway and promoted DDP resistance in lung adenocarcinoma cells, the present study examined the basal expression levels of WNT signaling pathway-associated proteins in A549 and A549/DDP cells by western blot analysis. The results demonstrated that the expression levels of WNT5A, WNT3A and active β-catenin in A549/DDP cells were significantly higher compared with those in A549 cells, indicating that the WNT/β-catenin signaling pathway was activated in DDP-resistant lung adenocarcinoma cells, a finding consistent with a previous study (19). On this basis, in the present study, MDIG in A549/DDP cells was respectively overexpressed or silenced, revealing that the MDIG-overexpression group was associated with significantly increased expression levels of WNT5A, WNT3A and active β-catenin. Conversely, the MDIG-silenced group was associated with significantly reduced expression levels of WNT5A, WNT3A and active β-catenin. These results suggested that MDIG promoted the expression of the downstream signaling molecules, the ABC transporters (ABCB1, ABCC1 and ABCG2), by activating the WNT/β-catenin signaling pathway, which may in turn lead to DDP resistance in lung adenocarcinoma cells.

In conclusion, in the present study, A549 cells and DDP-resistant A549 cells (A549/DDP cells) were used as target cells. Through silencing and overexpression of MDIG, it was demonstrated that: i) DDP resistance in lung adenocarcinoma cells may be associated with the upregulation of MDIG; ii) the expression level of MDIG was positively associated with the degree of DDP resistance; and iii) MDIG promoted the expression of downstream signaling efflux ABC transporters (ABCB1, ABCC1 and ABCG2) by activating the WNT/β-catenin signaling pathway, which may lead to DDP resistance in lung adenocarcinoma cells. Although a lack of experiments investigating ABC transporter inhibitors was a limitation of the present study, the present study has provided a putative novel target for effective DDP-based targeted therapy in the future.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

QW, FG, HZ, YC, JD, XZ, DS and HZ contributed to the experimental design. QW participated in the writing of manuscript and data interpretation. QW and FG conducted the transfection experiment. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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