

IMPDH2 promotes cell proliferation and epithelial-mesenchymal transition of non-small cell lung cancer by activating the Wnt/ β -catenin signaling pathway

HAO XU, HONGDA MA, LIFEN ZHA, QIAN LI, GUANGHUI YANG, HUIMING PAN,
XIANGPING FEI, XINGXIANG XU, CHEN XING and LADI ZHANG

Department of Respiratory, The People's Hospital of Danyang, Affiliated Danyang Hospital of Nantong University,
Danyang, Jiangsu 212300, P.R. China

Received August 16, 2019; Accepted April 27, 2020

DOI: 10.3892/ol.2020.12082

Abstract. Inosine 5'-monophosphate dehydrogenase type II (IMPDH2) is an important enzyme involved in the biosynthesis of guanine nucleotides. Therefore, the present study aimed to investigate the potential and molecular mechanism of IMPDH2 in non-small cell lung cancer (NSCLC). Reverse transcription-quantitative PCR and immunohistochemistry were used to detect IMPDH2 expression levels in NSCLC tissues and cells. A Cell Counting Kit-8 assay, colony formation assay, flow cytometry, wound healing, Transwell assay, western blotting and immunofluorescence analyses were utilized to identify the effects of upregulated IMPDH2 levels on NSCLC cells. The expression levels of IMPDH2 have been discovered to be upregulated in several types of human cancer; however, the biological and clinical value of IMPDH2 in NSCLC remains unclear. The results of the present study revealed that the expression levels of IMPDH2 were significantly upregulated in NSCLC tissues. Furthermore, the genetic knockdown of IMPDH2 significantly hindered the proliferation, apoptosis, invasion, migration and epithelial-mesenchymal transition of NSCLC cells, whereas the overexpression of IMPDH2 achieved the opposite results. In addition, the results of the present study demonstrated that the inhibition of IMPDH2 inhibited the Wnt/ β -catenin signaling pathway by decreasing the expression levels of Wnt3a and β -catenin, while increasing the expression levels of phosphorylated glycogen synthase kinase-3 β in NSCLC cells. These findings of the present study indicated that IMPDH2 may promote NSCLC progression

by activating the Wnt/ β -catenin signaling pathway, which suggested that IMPDH2 may be a novel therapeutic target for patients with NSCLC.

Introduction

Lung cancer is divided into two types: Small-cell lung cancer and NSCLC (1). As the predominant form of lung cancer, NSCLC is the leading contributor to lung cancer-associated deaths, accounting for ~83% of all lung cancer cases (2). As NSCLC usually presents as a locally advanced disease or with distant metastasis, the majority of NSCLC cases are diagnosed at stages III or IV (3). Therefore, there is an urgent requirement to thoroughly investigate the underlying molecular mechanisms of NSCLC to develop accurate and efficient therapeutic methods for managing NSCLC.

Inosine 5'-monophosphate dehydrogenase (IMPDH) is a rate-limiting enzyme, which catalyzes the nicotinamide adenine dinucleotide⁺-dependent oxidation of inosine monophosphate to xanthosine monophosphate during the *de novo* biosynthesis of guanine nucleotides (4). In fact, IMPDH serves a crucial role in DNA synthesis (5). Human IMPDH exists in two ubiquitously expressed isoforms: IMPDH type I (IMPDH1) and IMPDH type II (IMPDH2), which serve different roles despite the 84% similarity in the amino acid sequence (6). For example, IMPDH1 is found constitutively expressed in normal human leukocytes and lymphocytes, whereas IMPDH2 is an inducible enzyme, which is generally found upregulated in tumor tissues and proliferating cells (7). Previous studies have revealed that IMPDH2 was involved in multiple types of malignancy, such as colorectal, prostate, kidney and bladder cancer (8-10). However, to the best of our knowledge, it remains unknown whether IMPDH2 may have an effect in NSCLC.

The aberrant expression levels of Wnt/ β -catenin signaling pathway components are involved in NSCLC invasion and metastasis (11). In addition, the pathway was identified to be critical for embryonic development and promoting rapid cell division and migration (12). However, in one previous study, the overactivation of the Wnt/ β -catenin signaling pathway was reported to promote the uncontrolled proliferation of cells,

Correspondence to: Dr Hao Xu, Department of Respiratory, The People's Hospital of Danyang, Affiliated Danyang Hospital of Nantong University, 2 Xinmin West Road, Danyang, Jiangsu 212300, P.R. China

E-mail: XHresp@163.com

Key words: inosine 5'-monophosphate dehydrogenase type II, non-small cell lung cancer, epithelial-mesenchymal transition, Wnt/ β -catenin

which promoted tumorigenesis (13). Thus, the present study aimed to investigate the relationship between IMPDH2 and NSCLC. The results indicated that IMPDH2 may promote NSCLC progression by activating the Wnt/ β -catenin signaling pathway, therefore IMPDH2 may represent a novel therapeutic target for patients with NSCLC.

Materials and methods

Patient studies. A total of 30 fresh primary NSCLC tissues and matched adjacent noncancerous lung tissues were collected from patients (including 18 males and 12 females 12 with age of 42 ± 13 years) at The People's Hospital of Danyang (Danyang, China) between February, 2018 and February, 2019. The tissues samples were obtained from patients who had not received chemoradiotherapy. The study protocol was approved by the Ethics Committee of The People's Hospital of Danyang (Danyang, China). All patient diagnose of NSCLC had been confirmed based on pathological assay, and none of the patients received any relative cancer treatment. Informed consent was written and provided by all patients.

Cell lines and cultures. The human lung adenocarcinoma epithelial cells A549, the normal primary human bronchial epithelium cell line BEAS-2B was purchased from the American Type Culture Collection. BEAS2B cells were cultured in bronchial epithelial cell growth medium (BEGM; Lonza Group, Ltd.). All cell lines were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific.) and 1% penicillin/streptomycin, and maintained at 37°C in an humidified atmosphere of 5% CO₂. Cells used for the experiments were harvested using 0.05% trypsin-EDTA upon reaching 80-90% confluence. All assays were performed in three separate wells.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the cultured cells and tissues using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a SuperScript Reverse Transcriptase kit (Thermo Fisher Scientific, Inc.) at 37°C for 15 min. Two-step qPCR was subsequently performed using SYBR Green (Takara Bio, Inc.) and a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). The thermocycling conditions using the following format: 40 cycles, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, elongation at 95°C for 10 sec and final extension at 65 to 95°C for 5 sec. The following primer sequences were used for the qPCR: IMPDH2 forward, 5'-GTT TCTGCGGTATCCCAATC-3' and reverse, 5'-CGAGCAAGT CCAGCCTAT-3'; β -actin forward, 5'-TCATCACCATTGGCA ATGAG-3' and reverse, 5'-CACTGTGTTG GCGTACAG GT-3. β -actin was used as the internal control for normalization. Three duplicated wells were set for each sample. The fold changes were calculated by means of relative quantification ($2^{-\Delta\Delta C_q}$ method) (14).

Western blotting. Total protein from cells were extracted using RIPA buffer (Cell Signaling Technology, Inc.) and the protein concentrations were determined using the BCA Protein Assay kit (Cell Signaling Technology, Inc.).

Proteins (1 mg) were loaded per lane and onto a 10% gel and separated by SDS-PAGE. The separated proteins were subsequently transferred onto a PVDF membrane and blocked with 5% skimmed milk at room temperature for 1 h. The membranes were incubated with the following primary antibodies: Anti-IMPDH2 (1:1,000; cat. no. ab131158), anti- β -actin (1:1,000; cat. no. ab8226), anti-E-cadherin (1:1,000; cat. no. ab1416), anti-N-cadherin (1:1,000; cat. no. ab18203), anti-vimentin (1:1,000; cat. no. ab92547), anti-p-GSK (1:1,000; cat. no. ab75814), anti-GSK (1:1,000; cat. no. ab40870), anti-Wnt (1:1,000; cat. no. ab219412) (all from Abcam), anti- β -catenin (1:1,000; cat. no. C2206; Sigma Aldrich; Merck KGaA), anti-Snail (1:1,000; cat. no. ab53519), anti-c-Myc (1:1,000; cat. no. ab32072) and anti-Twist (1:1,000; ab50581) (all from Abcam) overnight at 4°C. Following the primary antibody incubation, the membranes were incubated with secondary antibodies (anti-IgG; 1:20,000; cat. no. ab205718; Abcam) for 1 h at room temperature. Total protein was visualized using enhanced chemiluminescence reagent (ECL; SW2030, Beijing Solarbio Science & Technology Co., Ltd.) and the blots were analyzed using ImageJ version 1.48u software (National Institutes of Health).

Immunofluorescence assay. Cells (1×10^4) were fixed with 4% paraformaldehyde at room temperature for 15 min and then washed 3 times with PBS. The slides were subsequently blocked for 1 h at room temperature in 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) diluted in 0.5% Triton X-100 in PBS (blocking buffer). The slides were then incubated with the following primary antibodies in blocking buffer overnight at 4°C: Anti-E-cadherin, anti-N-cadherin and anti-vimentin (as aforementioned). Following the primary antibody incubation, the slides were incubated with a secondary DyLight488-conjugated anti-rabbit IgG antibody (1:2,000, D9542, Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. The slides were subsequently stained with DAPI at room temperature for 5 min (Sigma-Aldrich; Merck KGaA) and visualized using an Olympus FV1000 confocal laser-scanning microscope at 200x magnification (Olympus Corporation).

Immunohistochemistry. Immunohistochemical analysis was performed using a standard two-step method. Briefly, paraffin-embedded tissues (4 μ m) were deparaffinized in 0.5% xylene at 65°C for 2 h, rehydrated alcohol and blocked with 3% H₂O₂ for 10 min at 37°C. Deparaffinized sections were incubated in 3% hydrogen peroxide for 15 min at room temperature and boiled in citrate antigen retrieval solution (pH 6.5) for 20 min at 95°C for antigen retrieval. Subsequently, the slides were incubated with an anti-IMPDH2 primary antibody (1:1,000) at 4°C overnight and then incubated with IgG secondary peroxidase-conjugated antibody (1:20,000; cat. no. ab205718; Abcam) for 1 h at room temperature. After washing three times in PBS, the slides were stained with 3,3'-diaminobenzidine (DAB) at room temperature for 10 min and counterstained with Mayer's hematoxylin at room temperature for 3 min. Stained cells were visualized in three randomly selected fields using a light microscope (magnification, x200).

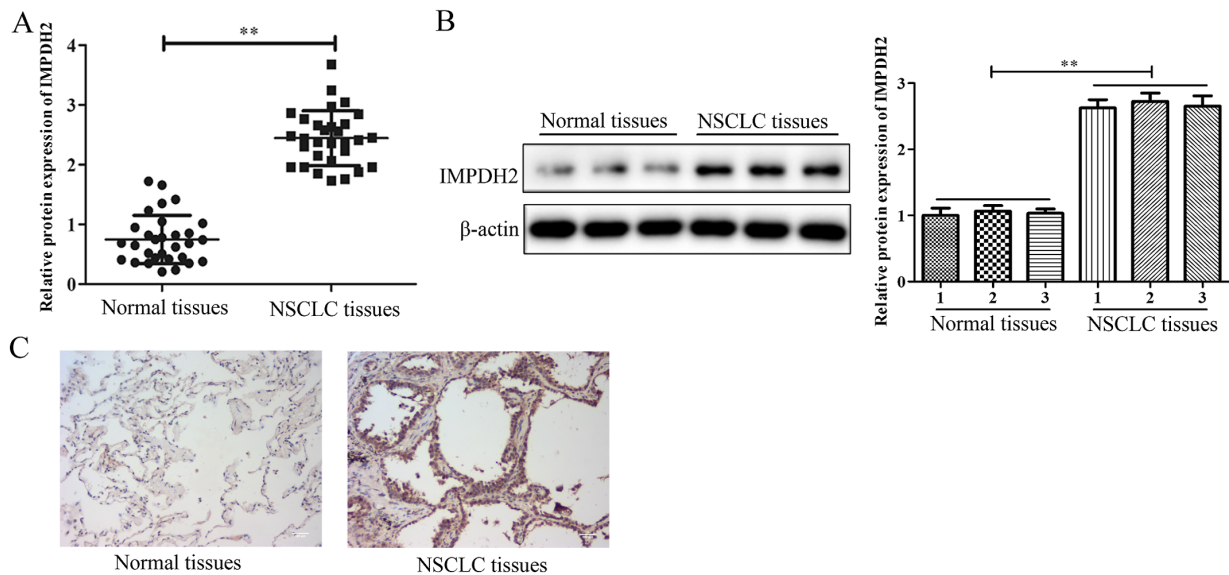


Figure 1. IMPDH2 expression levels are upregulated in NSCLC. Expression levels of IMPDH2 in 30 NSCLC tissues and normal tissues were analyzed using (A) reverse transcription-quantitative PCR, (B) western blotting and (C) immunohistochemistry Magnification, x200. **P<0.01, ns indicated no significance. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; NSCLC, non-small cell lung cancer.

Production of lentivirus for IMPDH2 overexpression and knockdown. The full-length open reading sequence of the human IMPDH2 gene (NM_000884.3) was PCR amplified from pcDNA3.1-IMPDH2 (pc-IMPDH2) and subcloned into the self-inactivating lentiviral vector pHIV-EGFP (both Addgene, Inc.). pcDNA3.1 empty vector was used as the control. Lentiviral preparations were produced by transient transfection of 293T cells (1×10^4) using pHIV-EGFP-IMPDH2, pRSV-Rev, pMDLg/pRRE and pMD2.G, which was purchased from Shanghai GenePharma Co., Ltd. at final concentration of 10 μ M for 48 h.

The corresponding oligo of short hairpin RNA (shRNA/sh) targeting IMPDH2 (sh-IMPDH2), β -catenin (sh- β -catenin) or scrambled sh-negative control (NC) was subcloned into the pLKO.1 vector (Addgene, Inc.). NC was empty plasmid and used as the negative control. Lentiviral preparations were generated as above, except for that the transfection cocktail was replaced with 6 μ g pLKO.1, 4.5 μ g psPAX2 and 1.5 μ g pMD2.G (Addgene, Inc.). The transfections were carried out for 48 h using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, Inc.).

Cell Counting Kit-8 (CCK-8) assay. A549 cells (1×10^4) were cultured in 96-well plates with DMEM medium at 37°C. Upon the cell confluence reaching 80%, the cells were cultured for 24, 48 and 72 h at 37°C, and 10 μ l CCK-8 solution (Gibco; Thermo Fisher Scientific, Inc.) was added to each well and incubated at 37°C for 2 h according to the manufacturer's protocol. The absorbance at 450 nm was measured using a microplate autoreader (Bio-Rad Laboratories, Inc.).

Colony formation assay. A total of 200 A549 cells/well were seeded into six-well plates and cultured at 37°C for 2 weeks. Following the incubation, the cells were fixed with 4% paraformaldehyde at 37°C for 30 min and stained with 1% crystal violet for 20 min at room temperature. The number

of colonies formed (>50 cells/colony) were counted manually. Stained cells were visualized using a light microscope (magnification, x40).

Flow cytometric analysis of apoptosis. A549 cells (1×10^4) were incubated for 72 h, collected and resuspended in PBS supplemented with 2% FBS, centrifuged at 12,000 \times g for 5 min at room temperature, and resuspend with PBS. The cells were subsequently stained with Annexin-V-FITC and propidium iodide at room temperature for 20 min using the Annexin V-FITC Apoptosis Detection kit (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Apoptotic cells were analyzed including early and late apoptosis using a flow cytometer (LSRFortessa X-20; Becton, Dickinson and Company). Apoptotic rate (%) = percentage of early + percentage of late apoptotic cells.

Wound healing assay. A549 cells were cultured in six-well plates until they reached 100% confluence. Before making the wound, the DMEM medium was replaced with fresh culture medium without FBS. Then, wounds were scratched into the cell monolayer using a 10- μ l pipette tip and culture for 48 h at room temperature. The wound closure was observed at 0 and 48 h using light microscope, after washing with PBS, and imaged to evaluate the migratory rate of cells in each well. The cell migratory ability was quantified by measuring the width of the advancing margins of cells in three randomly selected microscopic fields (magnification, x100) at the two time points.

Transwell migration assay. A total of 2×10^5 cells/well were plated in serum-free DMEM in the upper compartments of 8- μ m-pore Transwell plates. DMEM, supplemented with 10% FBS, was plated in the lower chambers. Following incubation at 37°C for 2 weeks, the migratory cells in the lower chamber were stained with 0.5% crystal violet for 15 min at room temperature. The invasion assay was performed as described

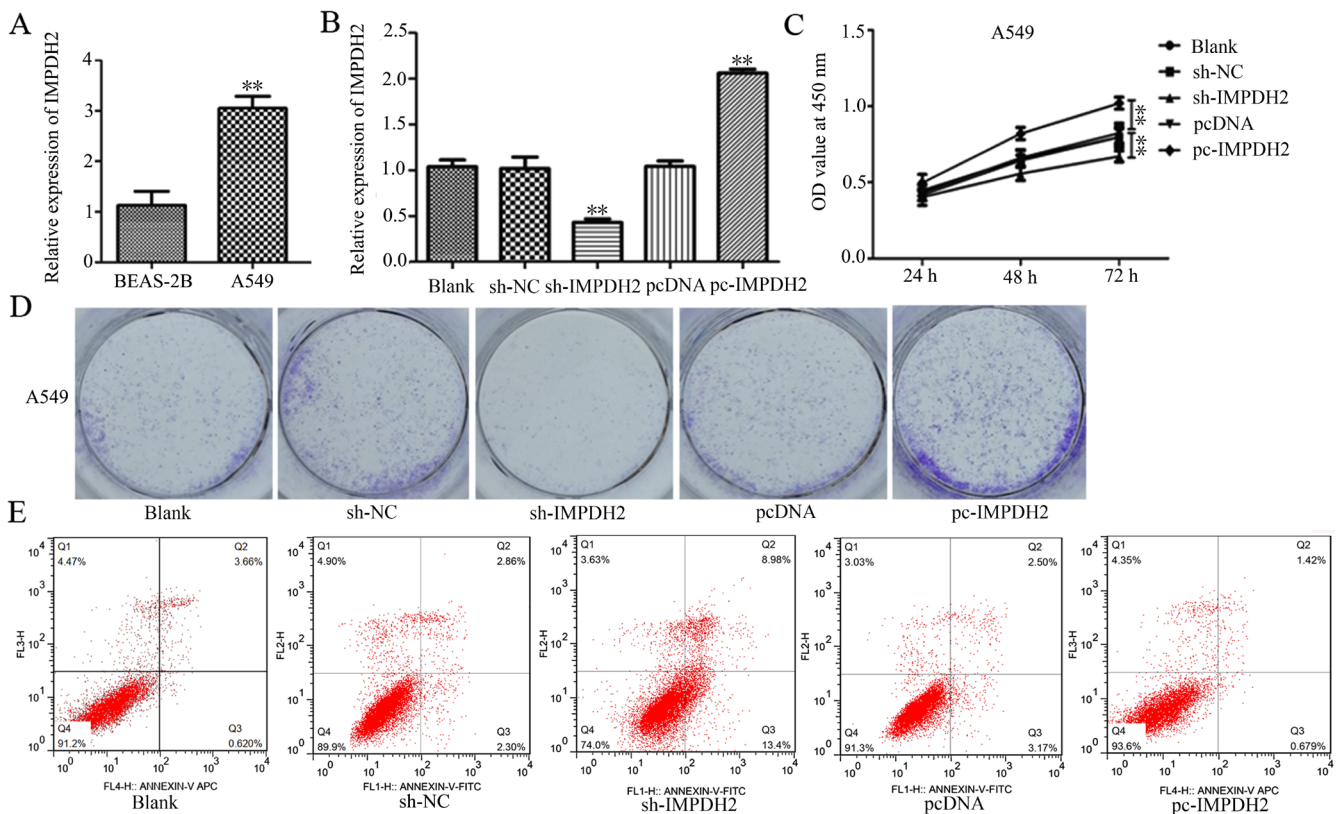


Figure 2. Overexpression and knockdown of IMPDH2 regulates the proliferation of NSCLC cells. (A) mRNA expression levels of IMPDH2 in NSCLC cell lines, A549 and BEAS-2B were analyzed using RT-qPCR. Data are presented as the mean \pm SD (n=3). **P<0.01 vs. BEAS-2B. (B) Transfection efficiency of the overexpression and knockdown of IMPDH2 was determined at the mRNA level in A549 cells using RT-qPCR. The data are presented as the mean \pm SD (n=3). (C) Cell Counting Kit-8 and (D) colony formation assays were used to determine the effect of the overexpression and downregulation of IMPDH2 on the proliferative rate of A549 cells. (E) Flow cytometry was used to analyze the effect of the overexpression and downregulation of IMPDH2 on the apoptotic rate of A549 cells. The data are presented as the mean \pm SD (n=3). **P<0.01 vs. sh-NC. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin RNA; NC, negative control; pc, overexpression vector; SD, standard deviation.

for the migration assay, except for the Transwell plates were pre-coated with Matrigel (50 μ g) at 37°C. Stained cells were visualized in three randomly selected fields using a light microscope (magnification, x200). The experiment was independently conducted three times.

Statistical analysis. All assays were conducted at least three times independently. Statistical analysis was performed using SPSS 21.0 software (IBM Corp.) and all experimental data are presented as the mean \pm SD. A paired Student's t-test was used to determine the significant differences between two groups, and one-way analysis of variance (ANOVA) followed Tukey's post hoc was used to test differences among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

IMPDH2 expression levels are upregulated in NSCLC. According to the RT-qPCR and western blotting analysis, the IMPDH2 expression levels were discovered to be significantly upregulated in NSCLC tissues compared with the adjacent normal tissues and there was no significance between 3 normal tissues and NSCLC tissues (Fig. 1A and B). In addition, the protein expression levels of IMPDH2 in NSCLC tissues were

investigated using immunohistochemistry; the results demonstrated that the NSCLC tissues exhibited markedly increased expression levels of IMPDH2 compared with the normal tissues (Fig. 1C). These data suggested that IMPDH2 may be upregulated in NSCLC.

IMPDH2 regulates the proliferation, invasion, migration and apoptosis of NSCLC cells. RT-qPCR analysis was performed to determine the expression levels of IMPDH2 in cell lines and revealed that A549 and SPC-A1 cells had significantly upregulated expression levels of IMPDH2 compared with the BEAS-2B cells, a normal primary human bronchial epithelium cell line (Fig. 2A).

To investigate the possible functional value of IMPDH2 in NSCLC progression, lentiviruses were constructed to over-express or knockdown IMPDH2. RT-qPCR analysis verified that the transfections of the overexpression and knockdown IMPDH2 lentiviruses into A549 cells were successful (Fig. 2B).

Results from the CCK-8 assays indicated that the cell proliferation rate was significantly attenuated in sh-IMPDH2 cells compared with the sh-NC cells, while enhanced in pc-IMPDH2 cells compared with the pcDNA cells (Fig. 2C). The colony formation assay, used to investigate cancer cell proliferation *in vitro*, displayed similar results; the number of

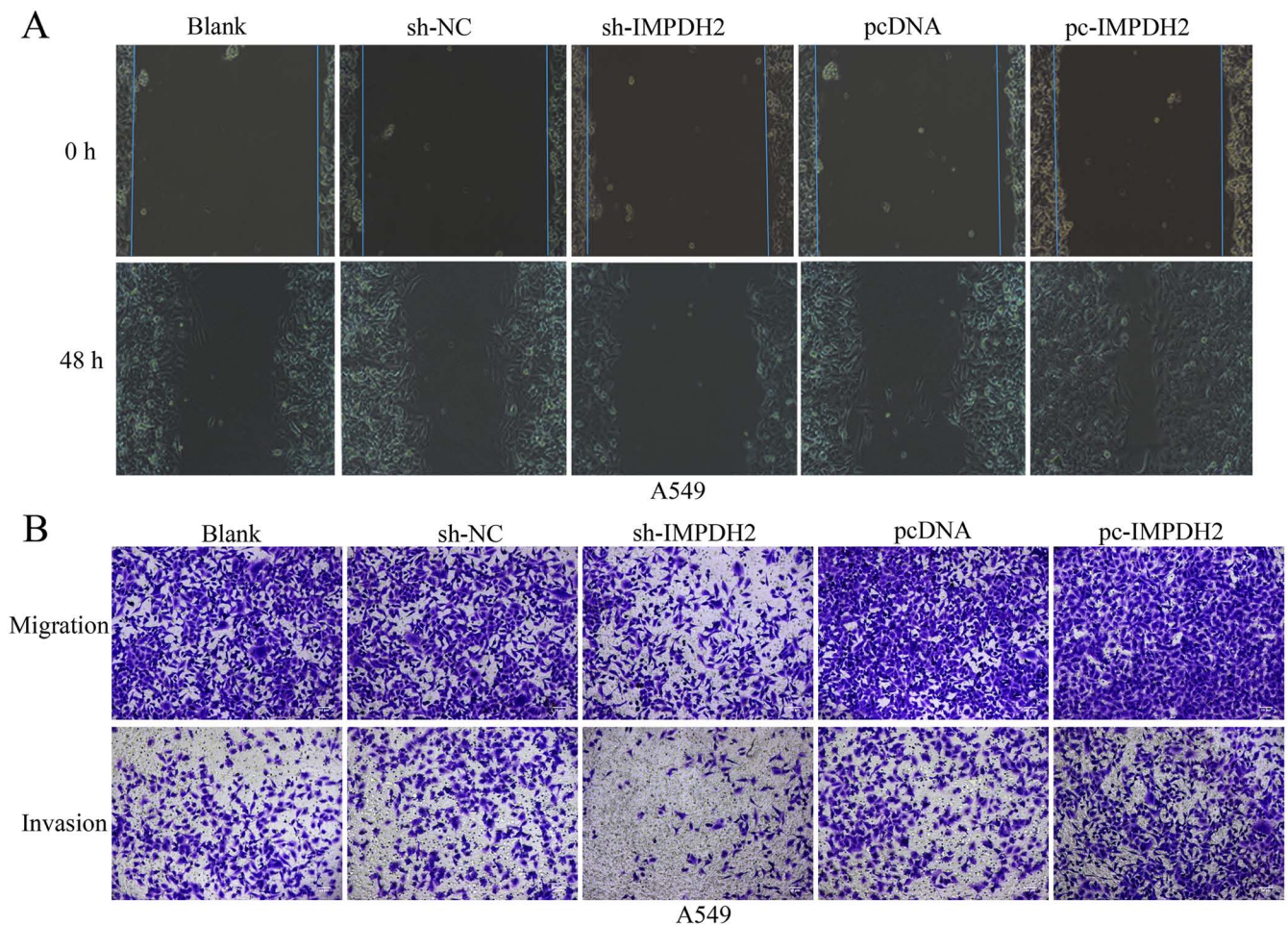


Figure 3. Overexpression and knockdown of IMPDH2 regulates the migration and invasion of non-small cell lung cancer cells. (A) Wound healing assays were used to determine that IMPDH2 regulated the migratory ability of A549 cells. Images were taken at 0 and 48 h. n=3. Magnification, x100. (B) Transwell assays were used to determine that IMPDH2 regulated the migration and invasion of A549 cells. The number of cells that migrated and invaded the extracellular matrix after 24 h was quantified in three randomly selected microscopic fields. Magnification, x200. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; sh, short hairpin RNA; NC, negative control; pc, overexpression vector.

colonies formed was markedly reduced following IMPDH2 knockdown and increased following the overexpression of IMPDH2 compared with their respective NCs (Fig. 2D). Flow cytometric analysis was subsequently used to determine whether IMPDH2 affected the apoptotic rate of A549 cells. The results indicated that the downregulation of IMPDH2 markedly increased the number of apoptotic cells compared with the sh-NC-transfected cells (Fig. 2E). Conversely, the upregulation of IMPDH2 markedly inhibited the rate of cell apoptosis compared with pcDNA3.1-transfected cells. Thus, these findings suggested that IMPDH2 may promote cell proliferation and inhibit apoptosis of NSCLC cells.

In addition to cell proliferation, one of hallmark characteristics of cancer is the ability to migrate and lead to metastasis (15). To investigate the effect of IMPDH2 on cell migration, a wound healing assay was performed. The cells transfected with pc-IMPDPH2 demonstrated a markedly increased migratory rate compared with the pcDNA3.1-transfected cells after 48 h incubation (Fig. 3A). Conversely, the knockdown of IMPDH2 inhibited the wound recovery, demonstrating a markedly decreased migratory rate compared with the sh-NC-transfected cells (Fig. 3A). Furthermore, the Transwell assays revealed that the

overexpression of IMPDH2 induced cell invasion compared with the pcDNA3.1-transfected cells, whereas the knockdown of IMPDH2 had the opposite effect compared with the sh-NC-transfected cells (Fig. 3B). These findings validated that knockdown of IMPDH2 had a suppressive effect on NSCLC cell migration and invasion.

IMPDH2 promotes the invasion and migration of NSCLC cells through epithelial-mesenchymal transition (EMT). IMPDH2 was previously reported to affect EMT in colon cancer cells (5), thus the present study investigated whether IMPDH2 may have the same influence on NSCLC cells. Following the overexpression and knockdown of IMPDH2 in A549 cells, the expression levels of the epithelial markers, E-cadherin, and the mesenchymal markers, vimentin and N-cadherin, were analyzed using western blotting. The expression levels of E-cadherin were significantly downregulated, whereas those of N-cadherin and vimentin were significantly upregulated in pc-IMPDPH2-transfected NSCLC cells compared with the blank control cells (Fig. 4A). An inverse trend was observed in the sh-IMPDPH2-transfected cells; the expression levels of E-cadherin were significantly upregulated and the expression levels of vimentin and N-cadherin were

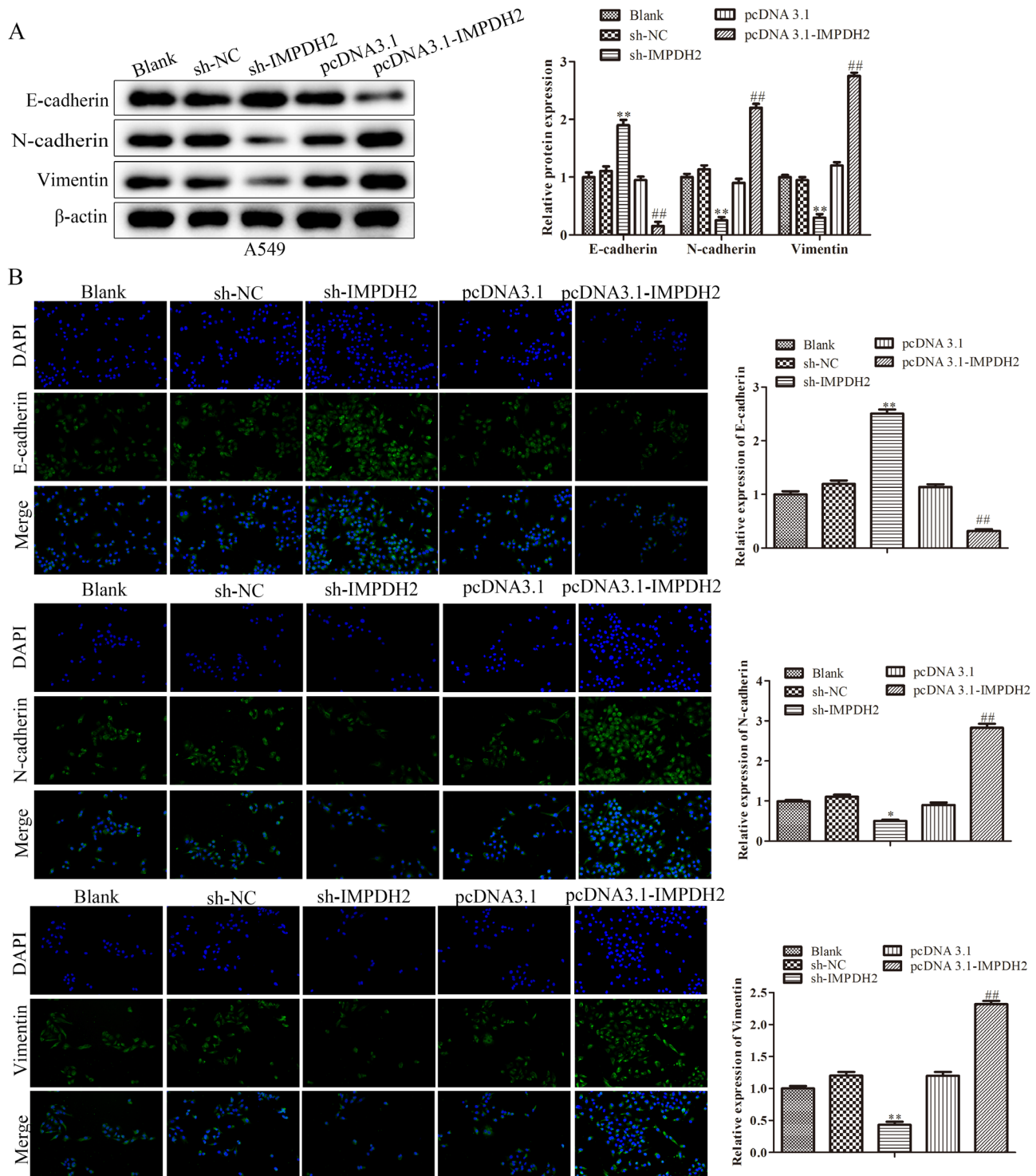


Figure 4. Effect of IMPDH2 on EMT in non-small cell lung cancer cells. (A) Western blotting was used to investigate the effect of IMPDH2 overexpression or downregulation on the expression levels of hallmark proteins of EMT, including E-cadherin, N-cadherin and vimentin, in A549 cells. (B) Immunofluorescence staining was used to investigate the expression levels of E-cadherin, N-cadherin and vimentin in IMPDH2 overexpressed and knockdown A549 cells. Data are shown as mean \pm SD. $n=3$. Magnification, $\times 200$. Scale bar, $100\ \mu\text{m}$. * $P<0.05$, ** $P<0.01$ vs. sh-NC, ## $P<0.01$ vs. pcDNA3.1. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; EMT, epithelial-mesenchymal transition; sh, short hairpin RNA; NC, negative control; pc, overexpression vector.

significantly downregulated compared with the sh-NC group. In contrast, The E-cadherin expression level was decreased and vimentin and N-cadherin expression levels were increased in pc-IMPDPH2 cells compared with their respective NCs. These findings were further confirmed using an immunofluorescence assay and the data are presented in Fig. 4B. With

downregulation of IMPDH2, the levels of N-cadherin and vimentin were significantly downregulated ($P<0.01$), while the levels of E-cadherin were markedly upregulated in A549 cells compared with the sh-NC group ($P<0.01$). These results suggested that IMPDH2 may induce the invasion and migration of NSCLC cells via EMT processes.

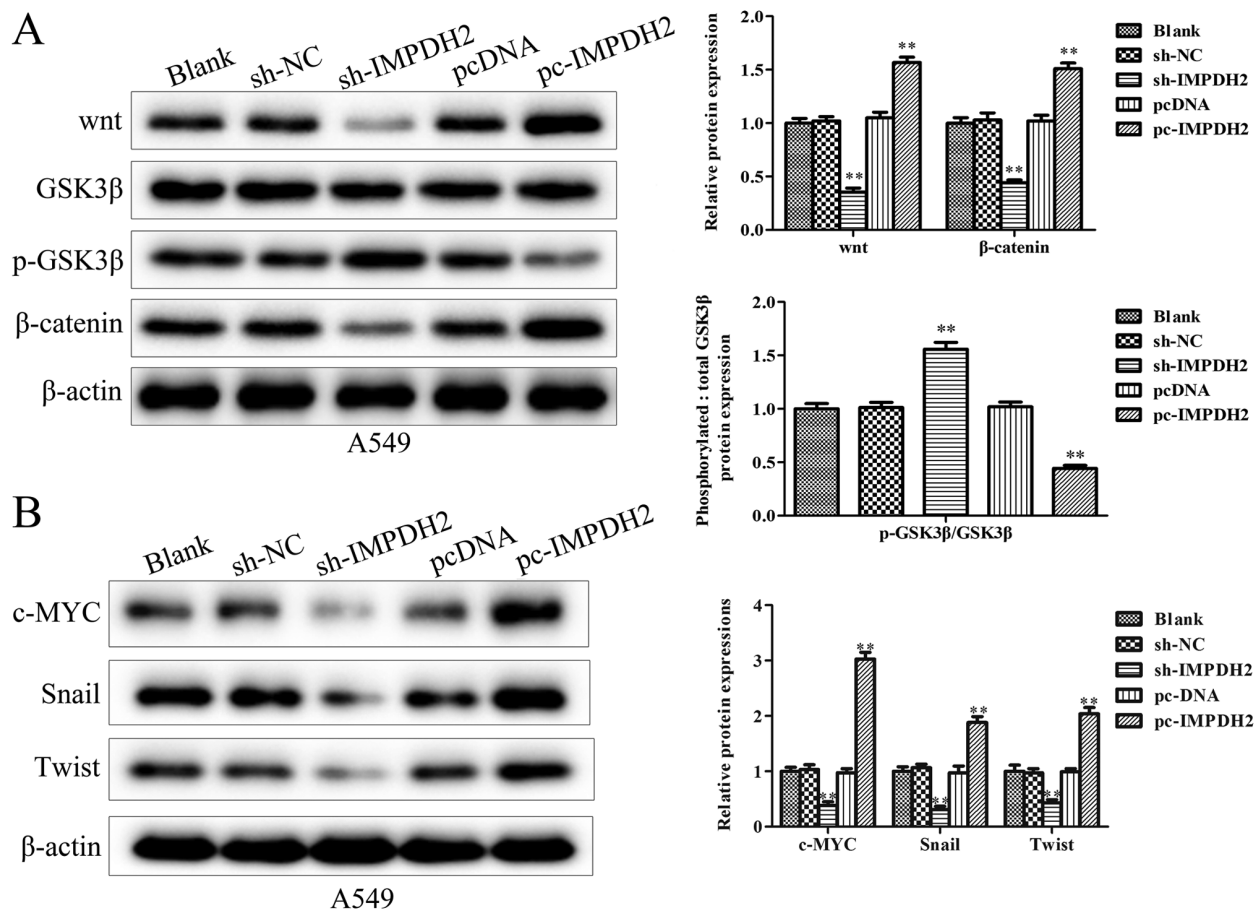


Figure 5. IMPDH2 promotes the Wnt/ β -catenin signaling pathway to regulate epithelial-mesenchymal transition in non-small cell lung cancer cells. Expression levels of (A) Wnt GSK3 β , p-GSK3 β and β -catenin and (B) c-Myc, Snail and Twist, in cells with overexpressed or knocked down IMPDH2 were analyzed using western blotting. The data are presented as the mean \pm SD. n=3. **P<0.01 vs. sh-NC. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; GSK3 β , glycogen synthase kinase 3 β ; p-, phosphorylated; sh, short hairpin RNA; NC, negative control; pc, overexpression vector.

IMPDH2 promotes NSCLC progression through the Wnt/ β -catenin signaling pathway. After determining the impact of IMPDH2 on NSCLC progression, the signaling pathways involved in this process were investigated. It was identified that the overexpression or knockdown of IMPDH2 affected the Wnt/ β -catenin signaling pathway. First, the western blotting analysis demonstrated that the overexpression of IMPDH2 significantly upregulated the expression levels of Wnt and β -catenin compared with the pcDNA3.1 group. In contrast, IMPDH2 overexpression significantly downregulated the expression levels of phosphorylated (p)-glycogen synthase kinase (GSK)3 β compared with the pcDNA3.1 group (Fig. 5A). Meanwhile, the knockdown of IMPDH2 expression levels presented the opposite trend; the expression levels of Wnt and β -catenin were significantly downregulated, while the expression levels of p-GSK3 β were upregulated in sh-IMPDH2-transfected cells compared with sh-NC group (Fig. 5A). Furthermore, the expression levels of downstream transcription factors involved in the Wnt/ β -catenin signaling pathway were investigated by western blotting. It was identified that the overexpression of IMPDH2 upregulated c-Myc, Snail and Twist expression levels, while silencing IMPDH2 expression reduced the expression levels of c-Myc, Snail and Twist compared with their respective NCs (Fig. 5B).

To further confirm that IMPDH2 influenced the Wnt/ β -catenin signaling pathway, shRNA targeting β -catenin was used. Western blotting was used to verify that the knockdown of β -catenin was successfully established in A549 cells. The result shown that sh β -catenin transfection markedly downregulated Wnt and β -catenin expression levels, while levels of p-GSK3 β were increased in A549 cell lines compared with sh-NC group (Fig. 6A). Subsequently, β -catenin shRNA was transfected into pc-IMPDH2+sh β -catenin-transfected cells and it was identified that the expression levels of c-Myc, Snail and Twist were downregulated compared with pc-IMPDH2+sh-NC group (Fig. 6B). These findings indicated that the effects of IMPDH2 in NSCLC cells may depend on β -catenin.

To determine whether the knockdown of β -catenin expression levels could suppress the function of IMPDH2, CCK-8 and Transwell assays were performed. Following the knockdown of β -catenin, the overexpression of IMPDH2 did not significantly promote cell proliferation to a further extent (Fig. 6C). In addition, cell migration and invasion were also determined, and the abilities in A549 cells were repressed in pc-IMPDH2+sh β -catenin group compared with pc-IMPDH2+sh-NC group (Fig. 6D). Notably, the expression levels of E-cadherin were increased, while N-cadherin and vimentin expression levels were decreased in

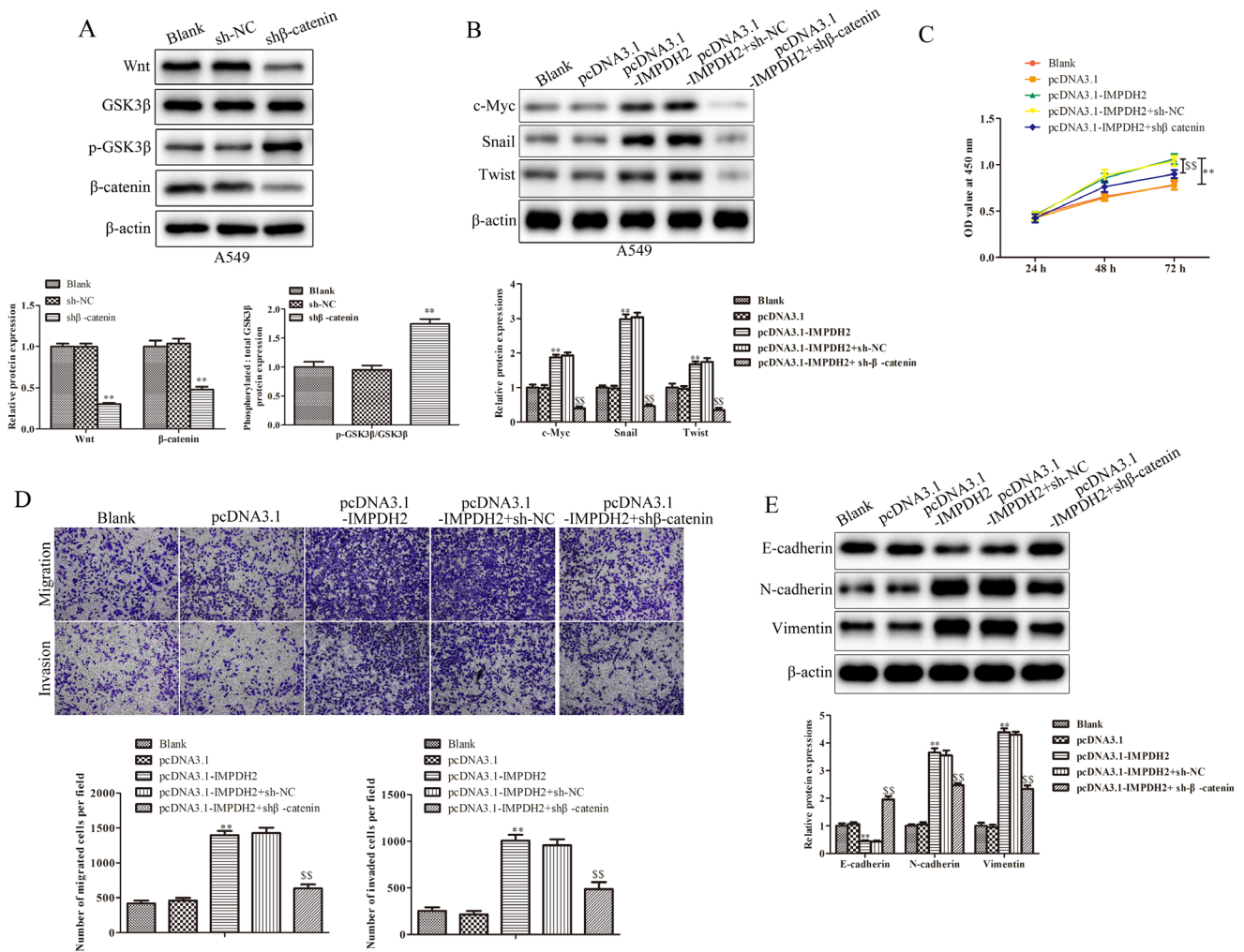


Figure 6. IMPDH2 promotes non-small cell lung cancer progression through the Wnt/ β -catenin signaling pathway. (A) The related protein expression levels of Wnt/ β -catenin signaling pathway were successfully inhibited in A549 cells using sh- β -catenin. (B) Protein expression levels of c-Myc, Snail and Twist were suppressed in pc-IMPDPH2 cells following β -catenin inhibition, as determined by western blotting. (C) Proliferative ability of cells was determined using a Cell Counting Kit-8 assay. (D) Migratory and invasive abilities were markedly reduced in pc-IMPDPH2 cells following β -catenin inhibition. (E) Expression levels of epithelial-mesenchymal transition-associated proteins, E-cadherin was upregulated, while N-cadherin and vimentin, were downregulated in pc-IMPDPH2-transfected cells following β -catenin knockdown. The data are presented as the mean \pm SD. $n=3$. Magnification, $\times 200$. ** $P<0.01$ vs. pcDNA3.1, $^{ss}P<0.01$ vs. pcDNA3.1-IMPDPH2+sh-NC. IMPDH2, inosine 5'-monophosphate dehydrogenase type II; GSK3 β , glycogen synthase kinase 3 β ; p-, phosphorylated; sh, short hairpin RNA; NC, negative control; pc, overexpression vector.

pc-IMPDPH2+sh β -catenin-treated cells when compared with pc-IMPDPH2+sh-NC group (Fig. 6E). In conclusion, these findings suggested that IMPDH2 may affect NSCLC progression through regulating the Wnt/ β -catenin signaling pathway.

Discussion

IMPDH serves as a rate-limiting enzyme in the synthesis of guanine nucleotides, thus it is an important regulator for DNA synthesis (16,17). IMPDH has been associated with cell proliferation and malignancy since 1975 (13). In particular, it has been previously reported that IMPDH2 was the predominant isoform in neoplastic and replicating cells (5). Accumulating evidence has also suggested that IMPDH2 may be closely implicated in different types of malignancy (18-21). In addition, it was revealed that IMPDH2 may be a useful biomarker for patient prognosis (20,21). In a previous study, IMPDH2 was also discovered to be a target of efficient immunosuppressive

agents (22) and an IMPDH inhibitor has been investigated and developed for tumor suppression (20,17,23-25). The present study demonstrated that the expression levels of IMPDH2 were upregulated in NSCLC tissues, which prompted the investigations into the potential role of IMPDH2 in NSCLC progression. CCK-8, colony formation, wound healing and Transwell assays were used in experiments involving the over-expression and knockdown of IMPDH2 in A549 cells, which revealed that IMPDH2 may positively affect cell proliferation, migration and invasion. Therefore, these findings suggested that IMPDH2 may serve oncogenic roles in NSCLC.

EMT is a hallmark of tumor invasion and metastasis, which can alter the adhesion of epithelial cancer cells, promoting them to invade and migrate to other distant sites (26-28). During EMT, the repression of genes encoding epithelial cell junction proteins is accompanied by the activation of genes promoting mesenchymal adhesion (29), and the 'Cadherin switch' refers to the process by which the downregulation of E-cadherin expression levels are

balanced by up-regulated N-cadherin expression levels (30,31). In the current study, the analysis of EMT-associated factors revealed that IMPDH2 suppressed E-cadherin expression levels, while up-regulating N-cadherin and vimentin expression levels. Therefore, IMPDH2 may induce EMT in NSCLC.

In addition, the present study identified the signaling pathway through which IMPDH2 exerted oncogenic functions. Western blotting analysis discovered that the expression levels of Wnt3a and β -catenin were significantly down-regulated in the sh-IMPDH2-transfected cells and up-regulated in the pc-IMPDH2-transfected cells, suggesting that Wnt/ β -catenin signaling may be involved in the IMPDH2 oncogenic activity. In addition, it was identified that the influence of IMPDH2 on tumor growth and EMT processes may be attributed to the activation of the Wnt/ β -catenin signaling pathway in NSCLC. Several studies have previously reported that the Wnt/ β -catenin signaling pathway participated in lung cancer, in addition to other types of cancer (32,33). Under normal conditions, GSK3 β , Axin, serine/threonine-protein phosphatase (PP2A), adenomatous polyposis coli protein and casein kinase I isoform α (CKK α) accumulate in the cytoplasm to form degradation complexes (34). Upon the degradation of complex β -catenin proteins, GSK3 β promotes the phosphorylation of β -catenin at Ser33 and Ser37, yielding ubiquitin-labeled β -catenin, which is subsequently degraded by proteasomes (35); this regulatory mechanism is useful for maintaining the stability of the Wnt/ β -catenin signaling pathway (36). However, upon the phosphorylation of GSK3 β , the kinase activity of GSK3 β is inactivated and can no longer promote the phosphorylation of β -catenin, leading to the accumulation and functioning of cytoplasmic β -catenin (37). The results of the present study suggested that β -catenin may be positively regulated by IMPDH2. β -catenin has been reported to reflect the changes of the Wnt/ β -catenin signaling pathway in cells (9). The expression levels of c-Myc, Snail and Twist, which are important transcriptional factors of the Wnt signaling pathway (10), were significantly upregulated following IMPDH2 overexpression. In addition, Snail and Twist are also crucial transcriptional factors in EMT and the Wnt/ β -catenin signaling pathway can regulate EMT via Snail and Twist (38,39). In addition, Mannava *et al* (40) discovered that IMPDH2 may be a direct target of c-Myc. Consistent with the findings of the present study, it can be inferred that IMPDH2 and c-Myc may influence each other. For instance, the current study suggested that IMPDH2 may promote NSCLC progression through Wnt/ β -catenin, which subsequently promoted the upregulation of c-Myc.

To identify the critical role of the Wnt/ β -catenin signaling pathway in NSCLC, the present study subsequently investigated the function of IMPDH2 during EMT after inhibiting β -catenin. The results revealed that the functions of IMPDH2 were suppressed following β -catenin inhibition; the cell proliferation rate and the EMT-associated phenotype were not affected by IMPDH2 overexpression following the inhibition of β -catenin.

In conclusion, the findings of the present study suggested that IMPDH2 may promote the cell proliferation and EMT of NSCLC by activating the Wnt/ β -catenin signaling pathway.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

Not applicable.

Authors' contributions

HX designed the experiments. HM, LZ, QL, CX and LZ performed the experiments. GY and HP contributed to data acquisition. XF and XX were responsible for data analysis. GY, HP, XF and XX were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of The People's Hospital of Danyang (approval no. JS-2018-04) and written, informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68: 394-424, 2018.
2. Liu R, Chen Y, Shou T, Hu J and Qing C: miRNA-99b-5p targets FZD8 to inhibit non-small cell lung cancer proliferation, migration and invasion. *Onco Targets Ther* 12: 2615-2621, 2019.
3. Miller KD, Nogueira L, Mariotto AB, Rowland JH, Yabroff KR, Alfano CM, Jemal A, Kramer JL and Siegel RL: Cancer treatment and survivorship statistics, 2019. *CA Cancer J Clin* 69: 363-385, 2019.
4. Zimmermann AG, Spychala J and Mitchell BS: Characterization of the human inosine-5'-monophosphate dehydrogenase type II gene. *J Biol Chem* 270: 6808-6814, 1995.
5. Dorn AR, Mountain LD, Ghoshal M, Hui RA, Klinedinst LK, Rugaber JE, Schamerloh AF and Salamone SJ: The use of inosine 5'-monophosphate dehydrogenase (IMPDH) in the development of a new liquid homogeneous enzyme immunoassay technology. *Clin Chem* 47: 1921-1922, 2001.
6. Carr SF, Papp E, Wu JC and Natsumeda Y: Characterization of human type I and type II IMP dehydrogenases. *J Biol Chem* 266: 27286-27290, 1993.
7. Colby TD, Vanderveen K, Strickler MD, Markham GD and Goldstein BM: Crystal structure of human type II inosine monophosphate dehydrogenase: Implications for ligand binding and drug design. *Proc Natl Acad Sci USA* 96: 3531-3536, 1999.
8. Duan S, Huang W, Liu X, Liu X, Chen N, Xu Q, Hu Y, Song W and Zhou J: IMPDH2 promotes colorectal cancer progression through activation of the PI3K/AKT/mTOR and PI3K/AKT/FOXO1 signaling pathways. *J Exp Clin Oncol* 37: 304, 2018.
9. Zhou L, Xia D, Zhu J, Chen Y, Chen G, Mo R, Zeng Y, Dai Q, He H, Liang Y, *et al*: Enhanced expression of IMPDH2 promotes metastasis and advanced tumor progression in patients with prostate cancer. *Clin Transl Oncol* 16: 906-913, 2014.
10. Zou J, Han Z, Zhou L, Cai C, Luo H, Huang Y, Liang Y, He H, Jiang F, Wang C and Zhong W: Elevated expression of IMPDH2 is associated with progression of kidney and bladder cancer. *Med Oncol* 32: 373, 2015.

11. Kahlert UD, Nikkhah G and Maciarczyk J: Epithelial-to-mesenchymal(-like) transition as a relevant molecular event in malignant gliomas. *Cancer Lett* 331: 131-138, 2013.
12. Zhang X, Ke X, Pu Q, Yuan Y, Yang W, Luo X, Jiang Q, Hu X, Gong YI and Tang K: MicroRNA-410 acts as oncogene in NSCLC through downregulating SLC34A2 via activating Wnt/ β -catenin pathway. *Oncotarget* 7: 14569-14585, 2016.
13. Clevers H: Wnt/ β -catenin signaling in development and disease. *Cell* 127: 469-480, 2006.
14. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) methods. *Methods* 25: 402-408, 2001.
15. Gao C, Yao H, Liu H, Feng Y and Yang Z: TM4SF1 is a potential target for anti-invasion and metastasis in ovarian cancer. *BMC Cancer* 19: 237, 2019.
16. Kozhevnikova EN, van der Knaap JA, Pindyrin AV, Ozgur Z, van Ijcken WF, Moshkin YM and Verrijzer CP: Metabolic enzyme IMPDH is also a transcription factor regulated by cellular state. *Mol Cell* 47: 133-139, 2012.
17. Bairagya HR, Mukhopadhyay BP and Bera AK: Role of salt bridge dynamics in inter domain recognition of human IMPDH isoforms: An insight to inhibitor topology for isoform-II. *J Biomol Struct Dyn* 29: 441-462, 2011.
18. Liu Y, Wang WM, Zou LY, Li LI, Feng LU, Pan MZ, Lv MY, Cao Y, Wang H, Kung HF, *et al*: Ubiquitin specific peptidase 5 mediates Histidine-rich protein Hpn induced cell apoptosis in hepatocellular carcinoma through P14-P53 signaling. *Proteomics* 17: 2017.
19. Jackson RC, Weber G and Morris HP: IMP dehydrogenase, an enzyme linked with proliferation and malignancy. *Nature* 256: 331-333, 1975.
20. Collart FR, Chubb CB, Mirkin BL and Huberman E: Increased inosine-5'-phosphate dehydrogenase gene expression in solid tumor tissues and tumor cell lines. *Cancer Res* 52: 5826-5828, 1992.
21. Nagai M, Natsumeda Y and Weber G: Proliferation-linked regulation of type II IMP dehydrogenase gene in human normal lymphocytes and HL-60 leukemic cells. *Cancer Res* 52: 258-261, 1992.
22. Zhao Y, Yang Y, Dai J, Xing D and Dong Y: IMPDH2 is highly expressed in breast cancer and predicts unfavourable prognosis. *Biomarkers* 1-9, Jun 30, 2018 (Online ahead of print).
23. Xu Y, Zheng Z, Gao Y, Duan S, Chen C, Rong J, Wang K, Yun M, Weng H, Ye S and Zhang J: High expression of IMPDH2 is associated with aggressive features and poor prognosis of primary nasopharyngeal carcinoma. *Sci Rep* 7: 745, 2017.
24. Zimmermann AG, Wright KL, Ting JP and Mitchell BS: Regulation of inosine-5'-monophosphate dehydrogenase type II gene expression in human T cells. Role for a novel 5' palindromic octamer sequence. *J Biol Chem* 272: 22913-22923, 1997.
25. Wu TY, Peng Y, Pellemounter LL, Moon I, Eckloff BW, Wieben ED, Yee VC and Weinshilboum RM: Pharmacogenetics of the mycophenolic acid targets inosine monophosphate dehydrogenases IMPDH1 and IMPDH2: Gene sequence variation and functional genomics. *Br J Pharmacol* 161: 1584-1598, 2010.
26. Ishitsuka K, Hideshima T, Hamasaki M, Raje N, Kumar S, Podar K, Gouill SL, Shiraishi N, Yasui H, Roccaro AM, *et al*: Novel inosine monophosphate dehydrogenase inhibitor VX-944 induces apoptosis in multiple myeloma cells primarily via caspase-independent AIF/Endo G pathway. *Oncogene* 24: 5888-5896, 2005.
27. Pua KH, Stiles DT, Sowa ME and Verdine GL: IMPDH2 is an intracellular target of the cyclophilin A and sanglifehrin A complex. *Cell Rep* 18: 432-442, 2017.
28. Guo Q, Huang Y, Xu J, Wang W, Gao J, Su Y, Gu Y and Yin X: Long non-coding RNA BANCRC contributes to cervical adenocarcinoma migration by affecting epithelial-mesenchymal transition. *Eur J Gynaecol Oncol* 40: 408-412, 2019.
29. Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. *Cell* 139: 871-890, 2009.
30. Gavert N and Ben-Ze'Ev A: Epithelial-mesenchymal transition and the invasive potential of tumors. *Trends Mol Med* 14: 199-209, 2008.
31. Lamouille S, Xu J and Derynck R: Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol* 15: 178-196, 2014.
32. Yilmaz M and Christofori G: EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* 28: 15-33, 2009.
33. Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y and Johnson KR: Cadherin switching. *J Cell Sci* 121 (Pt 6): 727-735, 2008.
34. Rao TP and Kühl M: An updated overview on Wnt signaling pathways: A prelude for more. *Circ Res* 106: 1798-1806, 2010.
35. Bienz M and Clevers H: Armadillo/ β -catenin signals in the nucleus-proof beyond a reasonable doubt. *Nat Cell Biol* 5: 179-182, 2003.
36. Li K, Pan W, Ma Y, Xu X, Gao Y, He Y, Wei L and Zhang J: A novel oncogene TRIM63 promotes cell proliferation and migration via activating Wnt/ β -catenin signaling pathway in breast cancer. *Pathol Res Pract* 215: 152573, 2019.
37. Segditsas S and Tomlinson I: Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* 25: 7531-7537, 2006.
38. Yang J, Chen J, He J, Li J, Shi J, Cho WC and Liu X: Wnt signaling as potential therapeutic target in lung cancer. *Expert Opin Ther Targets* 20: 999-1015, 2016.
39. Puisieux A, Brabletz T and Caramel J: Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* 16: 488-494, 2014.
40. Mannava S, Grachtchouk V, Wheeler LJ, Im M, Zhuang D, Slavina EG, Mathews CK, Shewach DS and Nikiforov MA: Direct role of nucleotide metabolism in C-MYC-dependent proliferation of melanoma cells. *Cell Cycle* 7: 2392-2400, 2008.