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RESEARCH ARTICLE

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# SH2B1 promotes epithelial-mesenchymal transition through the IRS1/β-catenin signaling axis in lung adenocarcinoma

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Lung adenocarcinoma (LADC), the most prevalent type of human lung cancer, is characterized by many molecular abnormalities. SH2B1, a member of the SH2-domain containing family, have recently been shown to act as tumor activators in multiple cancers, including LADC. However, the mechanisms underlying SH2B1 overexpression are not completely understood. Here, we reported that SH2B1 expression levels were significantly upregulated and positively associated with EMT markers and poor patient survival in LADC specimens. Modulation of SH2B1 levels had distinct effects on cell proliferation, cell cycle, migration, invasion, and morphology in A549 and H1299 cells in vitro and in vivo. At the molecular level, overexpression of SH2B1 resulted in the upregulation of the EMT markers, especially induced β-catenin accumulation and activated β-catenin signaling to promote LADC cell proliferation and metastasis, while silencing SH2B1 had the opposite effect. Furthermore, ectopic expression of SH2B1 in H1299 cells increased IRS1 expression level. Reduced expression of IRS1 considerably inhibited H1299 cell proliferation, migration, and invasion which were driven by SH2B1 overexpression. Collectively, these results provide unequivocal evidence to establish that SH2B1-IRS1-β-catenin axis is required for promoting EMT, and might prove to be a promising strategy for restraining tumor progression in LADC patients.

**KEYWORDS**
β-catenin signaling, EMT, IRS1, lung adenocarcinoma, SH2B1

**Abbreviations:** EMT, epithelial-mesenchymal transition; IRS1, insulin receptor substrates 1; LADC, lung adenocarcinoma; SH2B1, Src homology 2 (SH2) B adaptor protein 1; TNM, tumor-nodule-metastasis.

Shaoqiang Wang and Yuanda Cheng contributed equally to this work.

## 1 | INTRODUCTION

Lung cancer occupies a peculiar place in the public mind and contributes substantially to the global cancer burden and healthcare costs both in the United States and in China.[1–3] Owing to fail the initial therapy and lack of effective treatment for advanced lung cancer, even if decades of extensive studies, the prognosis remains poor with approximately 15-18% of 5-year relative survival rates.[1,4] A multistage GWAS (genome-wide association studies) on lung cancer suggested that lung tumors attributed to differing carcinogens may have developed from individual genetic risk markers.[4] And most notably, lung adenocarcinoma (LADC), the most frequent histological type of lung cancer, has exploited and elevated the clinical application of effective molecular targeted therapies.[5,6] The effectiveness of precision medicine against LADCs urgently requires peculiar molecular markers and novel therapeutic strategies from vast changes in gene regulation.

The SH2B1 (Src homology 2 [SH2] B adaptor protein 1) gene maps to the chromosomal region 16p11.2, which microdeletion is frequently associated with developmental delay, congenital anomalies and obesity.[7] SH2B1 is a member of the SH2B adaptor proteins family, mainly characterized by an SRC homology 2 (SH2), a pleckstrin homology domain (PH), and phenylalanine zipper dimerization domain (DD).[8] And so SH2B1 performs classical adaptor functions to recruit specific proteins, but, it also has a unique ability to enhance cytokine receptor-associated tyrosine kinases (eg, JAK kinase) and several receptor tyrosine kinase activity,[9] including the receptors for insulin,[10] insulin-like growth factor (IGF-1),[11] fibroblast growth factor (FGF),[12] glial cell line-derived neurotrophic factor (GDNF),[13] platelet-derived growth factor (PDGF),[14] brain-derived neurotrophic factor (BDNF),[15] and nerve growth factor (NGF).[16] Cells often employ SH2B1 to connect signal proteins to achieve accurate and appropriate cellular responses from external environmental stimuli, that is termed as signal transduction and signal enhancement processes.[9] Both in central nervous system and in peripheral tissues, SH2B1 is widely expressed and systemic changes in SH2B1 expression, dominantly deletion mutation, could have profound effects to result in energy imbalance, obesity, severe leptin resistance, and type 2 diabetes in mice and humans.[17] Clinical importance for the field of SH2B1 research is not only focused on endocrine disease,[18,19] but also concerned with some solid tumors, including lung cancer,[20,21] esophageal cancer,[22] and thyroid carcinomas,[23] which detected somatic gain-of-function mutations. In addition, emerging several lines of basic research in cultured cells show that the function of SH2B1 is involved in actin cytoskeletal reorganization,[24,25] focal adhesion assembly and disassembly,[26] filopodium formation,[27,28] and mitogenic response,[29] suggesting that SH2B1 could regulate cells motility,[24,30] proliferation and differentiation[17,29] by enhancing Rac,[30] RET,[23] mTOR,[31] and STATs[29] signals, which are generally established mediators in tumorigenesis and EMT program in tumors.[32–35]

The epithelial to mesenchymal transition (EMT), as a spectrum of intermediate states between the epithelial and mesenchymal phenotypes, plays crucial roles in epithelial-derived neoplasia and tumor invasiveness and metastasis[36,37] by modifying adhesion molecules proteins to trigger cancer cells dissociation to adopt a migratory and invasive behavior. A key inducer of EMT is the canonical Wnt signal through β-catenin dependent, which implies its significance in maintaining an epithelial cell phenotype, proper cell-cell junctions, cell differentiation, and proliferation in a subset of cancer.[37,38] Intriguingly, insulin receptor substrates-1 (IRS1), have been identified as a major player in the regulation of Wnt-mediated EMT.[39] We previously determined that SH2B1 directly bound, via its PH and SH2 domain, to IRS1 to activate PI3-kinase pathway.[40] However, the roles of SH2B1 in LADC are poorly understood. In this study, SH2B1 is substantially overexpressed in clinical LADC and significantly correlated with poor patient prognosis. We first demonstrate that SH2B1 promoted cell migration and induction of EMT in response to Wnt stimulation, whereas depletion of IRS1 impaired the SH2B1-mediated cell migration and EMT phenotype of LADC both in vitro and in vivo. Our findings uncover that a novel SH2B1-IRS1-β-catenin axis is required for promoting EMT, and might prove to be a promising strategy for restraining tumor progression in LADC patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethical statement

Written informed consent was obtained from 159 participants before the study. The study protocol of using clinical specimens for research was approved by the Medical Research Ethics of Xiangya Hospital, Central South University (CSU) (#201403216). All mice experiments were approved by the Animal Ethics Committee and conducted by the official recommendations for the Care and Use Laboratory Animals of Xiangya Hospital, CSU (#201403217).

### 2.2 | Tissue samples and clinicopathological characteristics

From January 2010 to December 2011, a total of 159 pairs of LADC and matched ANLTs (adjacent non-tumor lung tissues) were gathered from patients who had surgical lung resection performed at the Department of Thoracic Surgery, Xiangya Hospital, CSU. we collected cancer samples from the edge of human lung adenocarcinomas.[36] All samples were evaluated by a board-certified pathologist. Clinicopathological clinical TNM stages were determined according to the criteria of the 8$^{th}$ lung cancer TNM classification. The clinicopathological characteristics of the 159 samples were summarized in Table 2. All the freshly collected LADC tissues and matched ANLTs were frozen and stored in liquid nitrogen until required.

### 2.3 | Follow-up study

The follow-up data were regularly collected through telephone or visiting by our research team. Patients undergoing lung resection were recommended to carry out blood routine test, CEA, ultrasonography, and chest low-dose computed tomography (CT) every 3 months in

postoperative first two years and twice a year after that. The study was followed up for 5 years until December 2016. For patients suspected recurrence, imaging examinations including high-resolution contrast enhanced CT and/or magnetic resonance imaging (MRI), bone scan and bronchoscopy were performed to validate recurrence lesions. In this study, the 5-year overall survival period (OS) was defined as the interval between the surgical resection date and the end date of follow-up or the date of death due to recurrence of LADC and complications associated with LADC. The 5-year disease-free survival period (DFS) was the interval between the surgical resection date and the follow-up deadline or the diagnosis of recurrence and metastasis of lung cancer by imaging examination. This study defined mortality or loss of follow-up as data deletion due to non-lung cancer related causes.

## 2.4 | Cell lines and cell culture

The LADC cell lines H1299, A549, PC-9, SPC-A-1, H1975, H2009, and LTEP-A-2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). A normal human bronchial epithelial cell line (HBE) was purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and then incubated at 37°C in a humidified 5% $CO_2$ atmosphere.

## 2.5 | RNA extraction and qRT-PCR analyses

Total RNA was extracted from LADC tissue specimens and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. The expression level was quantified using SYBR Green Assay Kit (Takara, Shiga, Japan). Real-time PCR was performed in triplicate using the Applied Biosystems ViiA 7 Sequence Detection system (Life Technologies, Grand Island, NY). GAPDH was used to normalize mRNA and calculate the relative expression of each transcript. The following pairs of primers were synthesized as: SH2B1 (F): 5′-GACAACCACAGCCCTGGAGAT-3′, (R) 5′-AGACACC-CAGGCCTTCACCAT-3′; GAPDH (F): 5′ACCCACTCCTCCACCTTT-GACG-3′, (R): 5′-TCTCTTCCTCTTGTGCTCTTG-3′.

## 2.6 | Immunohistochemistry

IHC analysis was performed on the 159 paraffin-embedded LADC tissue sections, using the following primary antibodies, anti-SH2B1 (1:100, ab196575, Abcam), anti-E-cadherin (1:500, ab40772, Abcam), anti-N-cadherin (1:200, ab76011, Abcam), anti-Vimentin (1:200, ab92547, Abcam), anti-β-catenin (1:500, ab32572, Abcam) antibodies (Abcam, Cambridge, MA, USA). The IHC staining scores were determined by combining the intensity of staining and the proportions of positively stained cells. The standard of staining intensity was graded: 1, no staining; 2, weak staining (light yellow); 3, moderate staining (yellow-brown); and 4, strong staining (brown). The positive cell proportions were scored according to the following standard: 0, <5% positive cell; 1, 6-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; 4, 76-100% positive cells. We evaluated protein expression using staining index (SI),[41] which was calculated in the proportion of positive cells and the staining intensity score as possible total scores of 0, 2, 3, 4, 6, 8, 9, 12, and 16. Samples with SI < 8 were defined as low (negative) expression and SI ≥ 8 as high (positive) expression. The degree of SI was reviewed and scored separately by two independent pathologists.

## 2.7 | Xenografted tumor model and H&E staining

Male BALB/c-nu mice (age of 4-5weeks, 16-18g) were purchased from the Hunan Slack King of Laboratory Animal Co., Ltd (Changsha, China). Mice were randomly divided into groups ($n = 5$ per group) before injection. The invasion effect of SH2B1 on LADC progression was examined in a lung colonization model.[42] Two groups were injected with shSH2B1 transduced A549 cells or control A549 cells ($1 \times 10^6$ cells in 0.1 ml phosphate-buffered saline) via lateral tail veins. Another two groups were intravenously injected with stable SH2B1-over-expressing or control H1299 cells. Mice were killed 60 days after injection, and lungs were collected to performed to H&E staining.

## 2.8 | Western blotting

Western blotting was performed using the primary antibodies, anti-SH2B1 (1:1000, ab196575, Abcam, Cambridge, MA), anti-E-cadherin (1:3000, ab40772, Abcam), anti-N-cadherin (1:1000, ab76011, Abcam), anti-Vimentin (1:2000, ab92547,Abcam), anti-β-catenin (1:5000, ab32572, Abcam), anti-TCF-4 (1:25000, ab76151,Abcam), anti-CyclinD1 (1:10000, ab134175, Abcam), anti-C-Myc (1:1000, ab32072, Abcam), anti-IRS1 (1:1000, ab40777), anti-DVL-2 (1:500, ab137528), antibodies (Abcam), and anti-GAPDH antibody (1:3000. D110016, Sangon Biotech, Shanghai, China). Following the Western blotting assay, the membranes were stripped, and the band intensities were relative to GAPDH. The quantification of protein bands was performed using ImageJ software.

## 2.9 | Immunofluorescent staining

Cells seeded on a coverslip were fixed in 4% paraformaldehyde for 15 min, permeabilized with 5% Triton X–100 for 20 min and then blocked with 5% bovine serum albumin for 30 min. Primary antibodies were incubated overnight against SH2B1 (1:100, ab196575, Abcam), β-catenin (1:100, ab22656, Abcam). The next day, PBST washed coverslips three times and added fluorescence-conjugated secondary antibodies of 1:1000 (Alexa Fluor® 555 Donkey Anti-Rabbit IgG, A31572, Invitrogen) for 1 h at 37°C. DAPI (4,6-diamidino-2-phenylindole, hoechst33342, C1026, Beyotime Biotechnology, Shanghai, China) were used for nuclear staining. Images were captured by fluorescent microscopy.

## 2.10 | In vitro migration and invasion assay

For wound healing assay, monolayer cells were grown in six-well plates without creating any scratch-wound until confluency. Cells

migration was observed and measured the width of a wound by microscopy 24 h later.

For transwell assays using polycarbonate transwell filters containing 8 μm pores (#3422, Corning coster, Cambridge, MA), cells ($1 \times 10^4$/100 μl) were plated in serum-free medium on the top chambers that was either uncoated, for the migration assay, or coated with Matrigel (BD Biosciences, Bedford, MA) for the invasion assay. The bottom chamber added 600 μl medium containing 10% fetal bovine serum. After 48h of incubation, the inserts were washed with PBS, and migrated cells were fixed, stained and then counted from six random fields. Experiments were performed in triplicate.

### 2.11 | Statistical data analysis

All statistical analyses in this study were carried out using SPSS 22.0 statistical software. Values were shown as mean ± SD, and 2-tailed paired Student's *t*-test was used for comparing the difference between groups unless otherwise stated. The chi-square test was used to analyze the protein expression levels and clinicopathologic parameters. Spearman's rank correlation determined the association between SH2B1 protein and EMT-related markers. The log-rank test was used to compared the patient survival curves. $P < 0.05$ was considered significant.

## 3 | RESULTS

### 3.1 | Increased SH2B1 expression correlates with LADC progression

Given the limited understanding of the clinical significance and biological role of SH2B1 in lung adenocarcinoma (LADC), we examined the expression of the SH2B1 protein using immunohistochemical (IHC) staining in 159 human lung adenocarcinomas and paired adjacent normal lung samples obtained from Xiangya Hospital Thoracic Tissue Bank. IHC staining showed that SH2B1 was strongly expressed in LADC tissues, especially in high clinical stage LADC samples, but was markedly repressed in adjacent normal tissues (ANLT) (Figure 1A, Table 1), as well as the results of SH2B1 mRNA by a quantitative real-time PCR (qRT-PCR) assay. LADC had a twofold increased mean SH2B1 expression comparing with adjacent normal lung tissues (Figure 1B). Moreover, statistical analysis demonstrated that SH2B1 protein levels were positively correlated with clinical stage ($P = 0.012$)

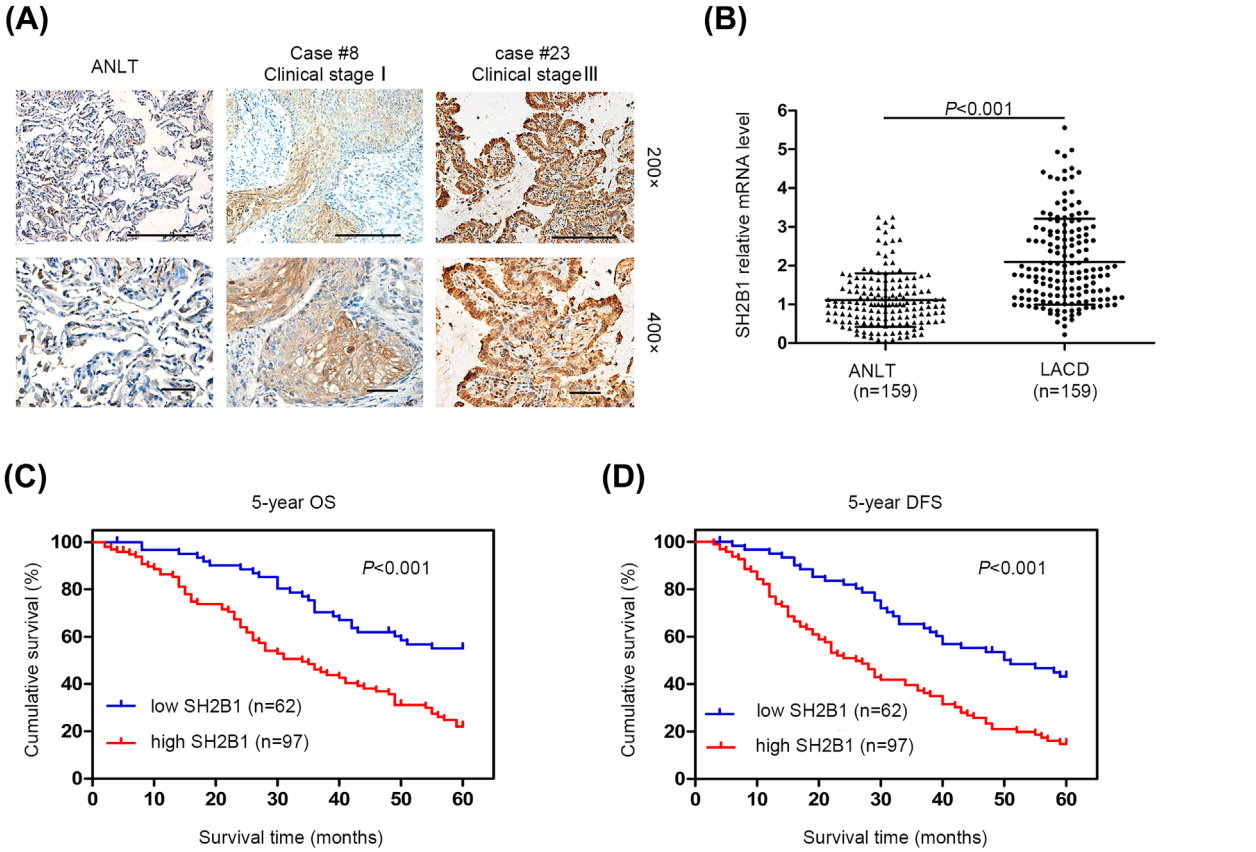


**FIGURE 1** Increased SH2B1 expression correlates with LADC progression. A, IHC staining indicating that SH2B1 protein expression is up-regulated in human LADC tumor tissues compared with adjacent normal lung tissues (ANLTs). Representative images from samples of LADC and ANLT were shown. Scale bars, 100 μm. B, RT-qPCR analysis of SH2B1 mRNA expression in 159 pairs of LADC and ANLT. The SH2B1 expression was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). C, Kaplan-Meier analysis of 5-year overall survival or (D) disease-free survival curves for LADC patients with low verse high expression of SH2B1 ($N = 159$; $P < 0.001$)

**TABLE 1** Expression of SH2B1, N-cadherin, E-cadherin, Vimentin, and β-catenin in 159 cases of lung adenocarcinomas and adjacent lung tissues

| Proteins | Adenocarcinomas tissues | Adjacent lung tissues | *P*-value |
|---|---|---|---|
| SH2B1 | | | |
| Positive | 98 | 37 | <0.001 |
| Negative | 61 | 122 | |
| N-cadherin | | | |
| Positive | 131 | 51 | <0.001 |
| Negative | 28 | 108 | |
| E-cadherin | | | |
| Positive | 87 | 136 | <0.001 |
| Negative | 72 | 23 | |
| Vimentin | | | |
| Positive | 109 | 44 | <0.001 |
| Negative | 50 | 115 | |
| β-catenin | | | |
| Positive | 123 | 40 | <0.001 |
| Negative | 36 | 119 | |

SH2B1, Src homology 2 B adaptor protein 1.

and tumor-nodule-metastasis (TNM) classification (T: $P = 0.017$; N: $P = 0.023$) in LADC patients (Table 2).

Given the higher levels of SH2B1 in LADC patients, we investigated whether SH2B1 correlated with patient survival. The 159 patients were divided into two groups using the IHC staining index (SI = 8) of SH2B1 as the cut-off value (negative: SI < 8; positive: SI ≥ 8). A threshold of 5-year follow-up revealed that patients with higher SH2B1 protein level had significantly shorter overall survival ($P < 0.001$, Figure 1C) and poor disease-free survival ($P < 0.001$, Figure 1D) compared with that of LADC patients with lower SH2B1 protein expression.

## 3.2 | Increased SH2B1 expression level is associated with EMT-related proteins in human LADC

The epithelial-mesenchymal transition (EMT) have been identified as crucial drivers of tumor progression and contributed to metastatic colonization.[36] In the last decade, more studies have still been focused on and further fueled the interest in EMT in the cancer field, especially the clinical significance of partial EMT.[36] Given the wide range of SH2B1 roles in cell motility,[24,30] we firstly raised the clinical question whether there existed associated relationship among the expression levels of SH2B1 and EMT-related proteins, such as E-cadherin, N-cadherin, Vimentin, and β-catenin.[43] To validate the connection in a larger cohort of LADC patients, we performed immunohistochemistry (ICH) staining in 159 paired LADC tumors and adjacent normal lung tissues obtained from Xiangya Hospital Thoracic Tissue Bank. Representative pictures were depicted in Figure 2A. N-cadherin (82.40% vs 32.08%), Vimentin (68.55% vs 27.67%), and β-catenin (80.50% vs 25.16%) were significantly higher in LADC tissues than in adjacent normal lungs ($P < 0.001$, respectively); E-cadherin (55.97% vs 85.53%), in contrast, showed decreased expression in LADCs compared with their counterparts (Table 1, $P < 0.001$). Additional tumor tissues from LADC patients were further analyzed to compare SH2B1, E-cadherin, N-cadherin, vimentin, and β-catenin with clinical features (Table 2). There were close relationships between SH2B1 or EMT-related proteins and LADC clinical stages. As shown in Table 2, compared with stages I and II, advanced LADC (stages III and IV) showed a significantly decreased E-cadherin (28.85% vs 70.09%, $P = 0.000$) and increased SH2B1 (54.21% vs 75.00%, $P = 0.012$), N-cadherin (77.57% vs 92.31%, $P = 0.022$), Vimentin (61.68% vs 82.68%, $P = 0.007$), and β-catenin (76.64% vs 86.54%, $P = 0.028$) expression. Furthermore, the expression levels of SH2B1 were negatively associated with E-cadherin expression but positively correlated with that of N-cadherin, Vimentin, and β-catenin expression in LADC cancer tissues (Figure 2B).

## 3.3 | SH2B1 alters EMT markers and induces the EMT phenotypes of LADC cells

A genetic EMT signature was common to various cancers.[44] Given that the wide range of EMT status in our clinical LADC specimens, the maintenance or loss of epithelial and mesenchymal markers was a good indicator of a potential EMT in LADC cell lineage analysis. We evaluated the effect of SH2B1 to promote EMT. We first examined SH2B1 expression using RT-qPCR and immunoblotting in a panel of LADC cell lines. As shown in Supplementary Figure S1A, approximately 71.43% (5 of 7) of the cell lines had significantly increased SH2B1 expression compared with that of an immortalized human lung epithelial cell (HBE). We observed considerably different SH2B1 expression among different LADC cell lines, ranging from hardly detectable in H1299 cells to quite more abundant in A549 cells (Supplement Figure S1A). The two LADC (A549 and H1299) cell lines were valuable tools to investigate the biological function of SH2B1 in LADC cells. Therefore, lentivirus-mediated knockdown of endogenous SH2B1 (in A549) and upregulation of SH2B1 (in H1299) were performed to evaluate the cellular outcomes using several assays. Efficient SH2B1 knockdown and overexpression were verified by RT-qPCR and immunoblotting (Supplementary Figures S1B and S1C).

Interestingly, when altering SH2B1 expression, we found cellular morphology changes in culturing LADC cells. As such, SH2B1 knockdown A549 cells exhibited a more epithelial-like phenotype compared with control A549 cells (Figure 3A); whereas H1299 cells overexpressing SH2B1 displayed a more fibroblast-like mesenchymal shape relative to control cells (Figure 3B), suggesting that SH2B1 was involved in EMT of LADC cell lines. In supporting of this finding, we quantitated the protein expression levels of EMT-specific markers in post-transfection SH2B1-shRNA A549 cells or SH2B1 overexpression H1299 cells. As expected, SH2B1-shRNA A549 cells robustly upregulated the epithelial marker E-cadherin and downregulated mesenchymal markers N-cadherin, Vimentin, and β-catenin (Figure 3C). Moreover, ectopic overexpression of SH2B1 in H1299

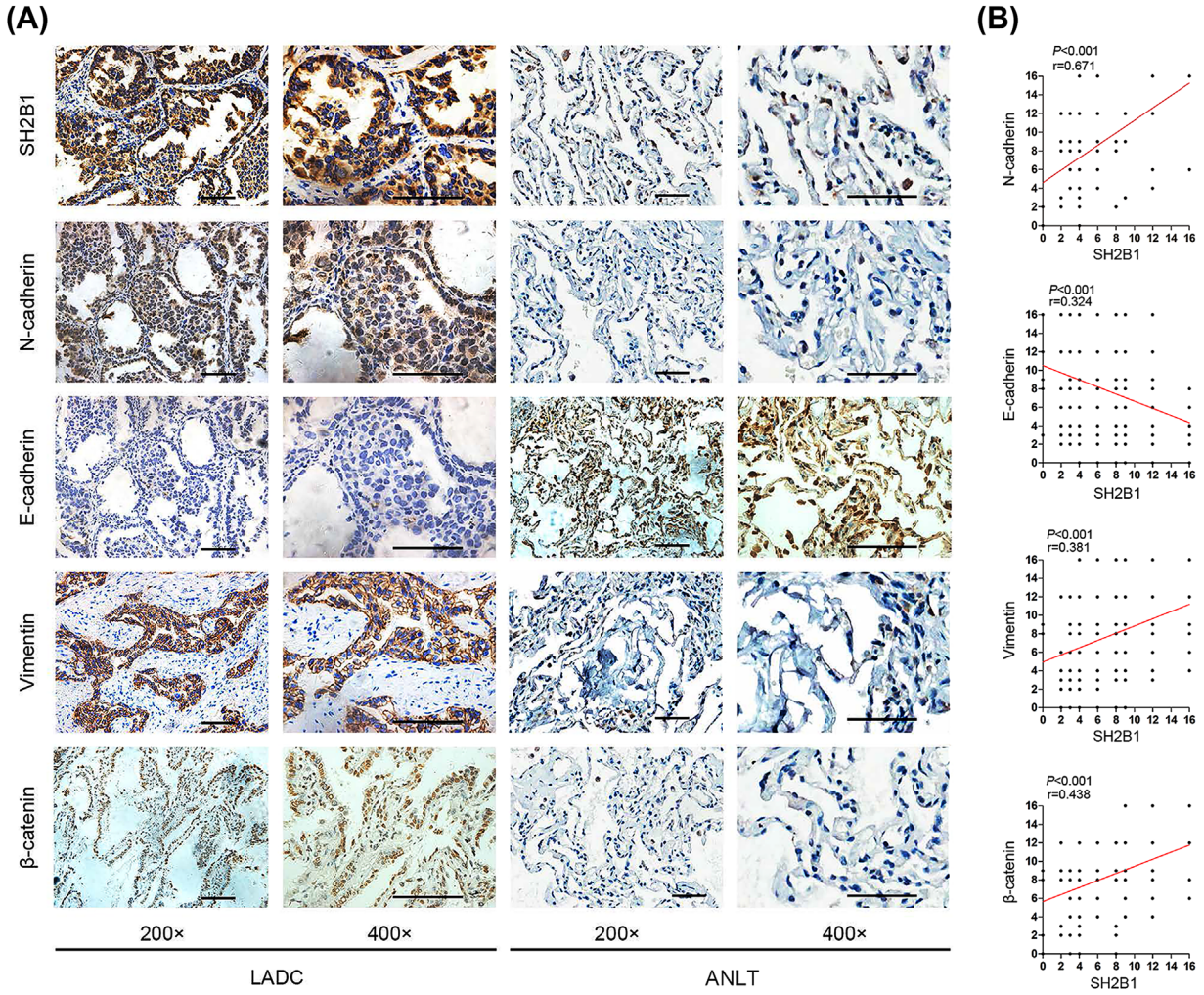


**FIGURE 2** Increased SH2B1 expression level is associated with EMT-related proteins in human LADC. A, Representative expression levels of SH2B1, N-cadherin, E-cadherin, Vimentin, and β-catenin in LADC and ANLT by IHC staining. Bars, 100 μm. B, Correlation between SH2B1 and EMT markers levels with liner regression and Pearson's significance. SH2B1 has a significant positive correlation with N-cadherin, Vimentin, and β-catenin expression and negative correlation with E-cadherin level ($P < 0.001$) by IHC staining

cells also affected the expression of EMT markers, resulting in E-cadherin downregulation and mesenchymal markers upregulation (Figure 3C).

We further studied whether SH2B1 had an active role in the promotion of LADC cells migration and invasiveness. To test this, using transwell migration and invasion assays, we observed SH2B1-knockdown significantly held back the cells migration and suppressed invasion (Figure 3D) of A549 cells. In line with this, elevated migration and invasion was seen in H1299-SH2B1 cells (Figure 3E). An in vivo metastasis assay further substantiated the invasive function of SH2B1 on LADC cells to form metastatic tumor nodules in the lungs. SH2B1 gene transfected LADC cells, A549 cells (shSH2B1 and shCtrl) and H1299 cells (SH2B1 and vector), were injected into nude mice via the tail vein, respectively. At 60 days after injection, mice were killed by cervical dislocation and lungs were collected for microscopic histological analysis. As shown in Figures 3E and S2A, compared with control A549-shCtrl cells, the macroscopic surface lung nodules and microscopic lung metastatic lesions were fewer in number and smaller in mean size of shSH2B1-A549 mice. Additionally, compared with lungs from control H1299 (H1299-vector) mice, we observed an increased surface lung nodules and microscopic lung adenocarcinoma both in number and size (Figure 3F). Together, our data demonstrated elevated SH2B1 expression promoted malignant lung tumor development.

## 3.4 | SH2B1 promotes EMT in LADC cells through the wnt/β-catenin pathway

Given that β-catenin expression was related to TNM stages of LADC specimens and positively correlated with the expression of SH2B1 in clinical and in vitro experiments, we hypothesized that SH2B1 would cooperate with β-catenin, activating Wnt pathway, to initiate EMT of LADC cells. We tested the effect of SH2B1 on the expression of TCF-4, Cyclin-D, and c-Myc,[45] well-characterized downstream targets of Wnt/β-catenin signaling. Consistent with the effect of SH2B1 on β-catenin expression, TCF-4, Cyclin-D, and c-Myc

**TABLE 2** Correlation between SH2B1, N-cadherin, E-cadherin, Vimentin, and β-catenin immunostaining and clinicopathologic features in 159 cases of LADC tissues

| | | SH2B1 | | | N-cadherin | | | E-cadherin | | | Vimentin | | | β-catenin | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Parameters** | **N** | **+** | **−** | ***P*-value** | **+** | **−** | ***P*-value** | **+** | **−** | ***P*-value** | **+** | **−** | ***P*-value** | **+** | **−** | ***P*-value** |
| Age (years) | | | | | | | | | | | | | | | | |
| <60 | 96 | 56 | 40 | 0.291 | 80 | 16 | 0.700 | 54 | 42 | 0.900 | 67 | 29 | 0.678 | 74 | 22 | 0.918 |
| ≥60 | 63 | 42 | 21 | | 51 | 12 | | 33 | 30 | | 42 | 21 | | 49 | 14 | |
| Gender | | | | | | | | | | | | | | | | |
| Male | 120 | 75 | 45 | 0.525 | 102 | 18 | 0.130 | 66 | 54 | 0.632 | 84 | 36 | 0.491 | 94 | 26 | 0.606 |
| Female | 39 | 23 | 16 | | 29 | 10 | | 21 | 18 | | 25 | 14 | | 29 | 10 | |
| Smoking history | | | | | | | | | | | | | | | | |
| Yes | 102 | 61 | 41 | 0.525 | 87 | 15 | 0.198 | 58 | 44 | 0.467 | 72 | 30 | 0.460 | 79 | 23 | 0.970 |
| No | 57 | 37 | 20 | | 44 | 13 | | 29 | 28 | | 37 | 20 | | 44 | 13 | |
| Differentiation | | | | | | | | | | | | | | | | |
| Well/moderate | 87 | 41 | 46 | <0.001 | 66 | 21 | 0.018 | 55 | 32 | 0.018 | 53 | 34 | 0.023 | 66 | 21 | 0.620 |
| Poor/undifferentiated | 72 | 57 | 15 | | 65 | 7 | | 32 | 40 | | 56 | 16 | | 57 | 15 | |
| Tumor size (cm) | | | | | | | | | | | | | | | | |
| ≤3 cm (T1) | 66 | 33 | 33 | 0.011 | 47 | 19 | 0.002 | 45 | 21 | 0.004 | 41 | 25 | 0.141 | 44 | 22 | 0.007 |
| >3 cm (T2-T4) | 93 | 65 | 28 | | 84 | 9 | | 42 | 51 | | 68 | 25 | | 79 | 14 | |
| Lymph node metastases (*N*) | | | | | | | | | | | | | | | | |
| No (N0) | 50 | 37 | 13 | 0.030 | 43 | 7 | 0.418 | 34 | 15 | 0.015 | 34 | 16 | 0.919 | 31 | 19 | 0.002 |
| Yes (N1-N3) | 109 | 61 | 48 | | 88 | 21 | | 53 | 56 | | 75 | 34 | | 92 | 17 | |
| Clinical stage | | | | | | | | | | | | | | | | |
| I-II | 107 | 59 | 48 | 0.016 | 83 | 24 | 0.022 | 75 | 32 | <0.001 | 66 | 41 | 0.007 | 82 | 25 | 0.028 |
| III-IV | 52 | 39 | 13 | | 48 | 4 | | 12 | 40 | | 43 | 9 | | 46 | 6 | |

SH2B1, Src homology 2 B adaptor protein 1.

expression were positively regulated by SH2B1 (Figure 4A). More interestingly, previous studies have demonstrated that tumor cells with nuclear accumulation of β-catenin, Wnt signaling pathway initiated this process,[46] appear to have undergone EMT.[43,47] Consistently, immunofluorescence assay revealed that the enhancement of SH2B1 expression in H1299 cells resulted in both an increase in cytoplasmic β-catenin and nuclear translocation of β-catenin (Figure 4B). Based on the result that SH2B1 promoted the nuclear accumulation of β-catenin, we further examined the effect of SH2B1 on Wnt/β-catenin pathway by using Wnt signaling specific inhibitor ICG001 to control the Wnt/β-catenin signaling in LADC cells. We observed that the protein changes in Wnt/β-catenin targets (TCF-4, Cyclin-D1, and c-Myc) induced by SH2B1 over-expression reversed by ICG001 (Figure 4C). Similar results were observed in the changes of EMT markers (E-cadherin, N-cadherin, and β-catenin). The results demonstrated that ICG001 promoted the expression of E-cadherin, but reduced N-cadherin and β-catenin in the presence of SH2B1. Importantly, LADC cells migration and invasion were increased in SH2B1 over-expression cells, while ICG001 impaired these effects (Figures 4D and 4E). Taken together, these results suggested SH2B1 promoted the EMT process mediated by Wnt/β-catenin signaling.

### 3.5 | SH2B1 regulates wnt/β-catenin signaling through IRS1

SH2B1 has been reported to recruit and directly bind to insulin receptor substrates (IRS-1, -2, -3 and -4), particularly IRS1 in HEK293 cells.[9] The physical SH2B1-IRS1 interaction could inhibit tyrosine dephosphorylation of IRS1 and promote IRS1 proteins to active the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway,[40,48] which has been emerging as a central feature of EMT.[49] Most importantly, it has been identified that IRS1 was a significant active player in the regulation of EMT[50] in response to Wnt/β-catenin signals by which interacted with and stabilized DVL2, a hub of Wnt signaling, via suppressing its autophagic degradation.[39,51] In the same clinical cohort study, IHC analysis revealed that IRS1 expression positively correlated with SH2B1 expression ($P < 0.001$, Figure 5A). In LADC cells (Figure 5B), a physical interaction between SH2B1 and IRS1 was detected by co-immunoprecipitation assays. To further tackled whether SH2B1 regulated Wnt/β-catenin signaling pathway through IRS1, we silenced endogenous IRS1 by a siRNA. The Western blotting assay showed that depletion of IRS1 decreased the expression of DVL2 and further weaken the effect of SH2B1 on Wnt/β-catenin signals, that is, Wnt/β-catenin targets (TCF-4, Cyclin-D1, and c-Myc)

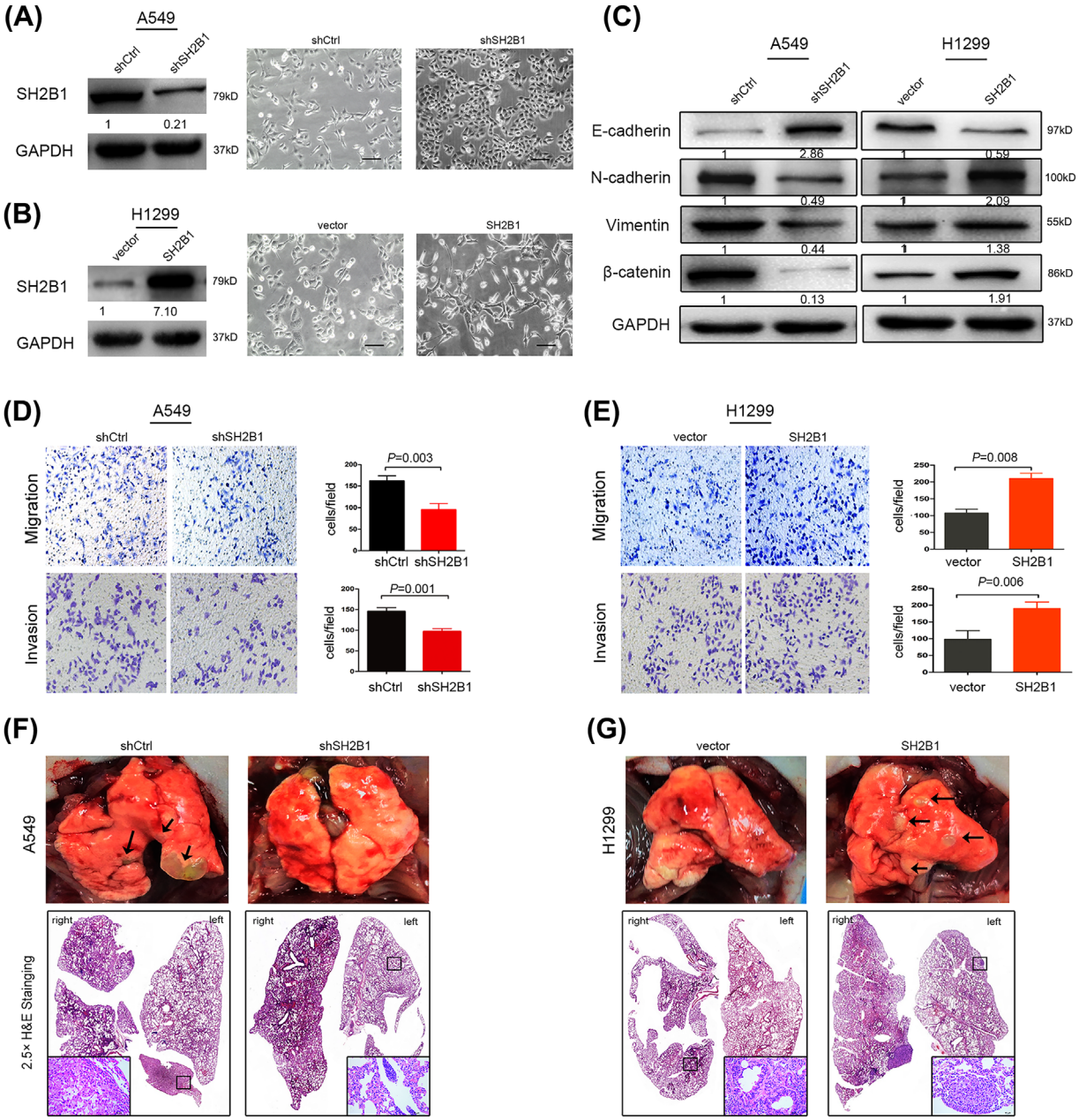


**FIGURE 3** SH2B1 alters EMT markers and induces the EMT phenotypes of LADC cells. A, Representative phase contrast images of SH2B1-konckdown A549 cells are shown a more polygonal epithelial-like shape compared with control cells. Scale bars, 100 μm. B, Overexpression of SH2B1 induces a more mesenchymal-like phenotype of H1299 cells. Scale bars, 100 μm. C, Western blotting shows that SH2B1-knockdown increases E-cadherin protein and decreases protein levels of N-cadherin, Vimentin, and β-catenin in A549 cells. SH2B1-overexpression suppresses E-cadherin protein and elevates protein levels of N-cadherin, Vimentin, and β-catenin in H1299 cells. Experiments were repeated three times, and representative pictures are shown for (C). D, SH2B1-knockdown suppresses the transwell migration (upper) and invasion (lower) of A549 cells. E, SH2B1-overexpression elevates the transwell migration (upper) and invasion (lower) of H1299 cells. Representative micrographs and quantification of the number of migrated and invasive cells are shown. Results are presented as mean ±SD. F, SH2B1-knockdown inhibits A549 cells metastasis in vivo ($n = 5$). G, SH2B1-overexpression promotes H1299 cells metastasis in vivo ($n = 5$). Representative photographs of gross lungs (upper), with arrows pointing to lung surface tumor nodules, and H&E-stained section of lung (lower), with original magnification: 2.5×, are shown. Micrograph indicates the magnified morphology of tumor tissues

and N-cadherin decreased, while E-cadherin increased. (Figure 5C). Importantly, depletion of IRS1 blocked the effect of SH2B1 induced cell migration and invasion (Figures 5D and 5E). These data suggested that depletion of IRS1 inhibited the Wnt/β-catenin signaling induced by SH2B1.

## 4 | DISCUSSION

Advanced LADC patients who miss the optimal opportunity of surgery have limited therapeutic options in the clinic at present. Hence, urgent priority is to accelerate progress toward investigating biological basis

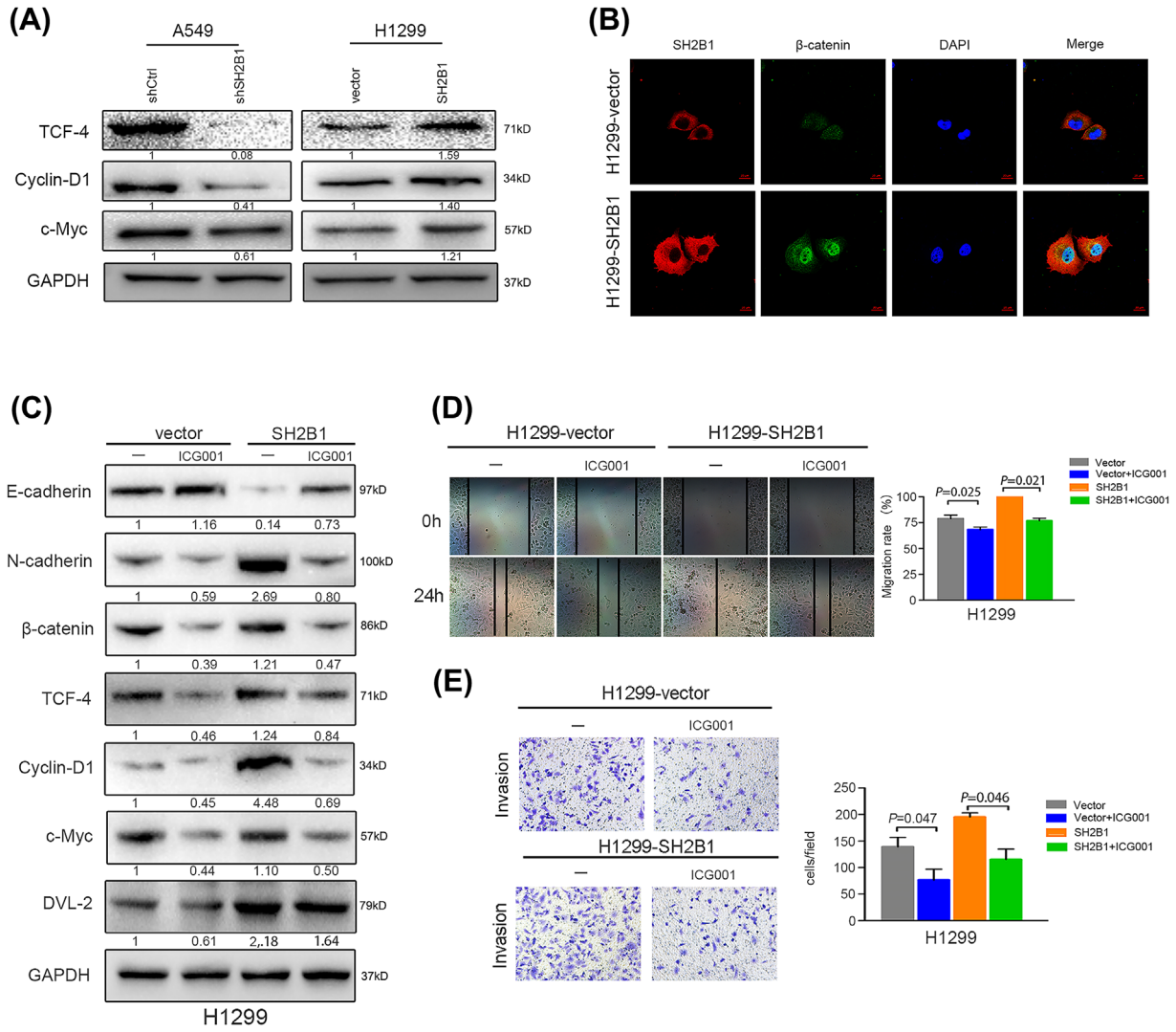


**FIGURE 4** SH2B1 promotes EMT in LADC cells through the Wnt/β-catenin pathway. A, The protein levels of Wnt/β-catenin pathway targets (TCF-4, c-Myc, Cyclin-D1) were assayed by Western blotting. GAPDH is used as an internal control. B, Immunofluorescence assay demonstrates that β-catenin nuclear translocation is markedly elevated in SH2B1-overexpression H1299 cells compared with control cells. SH2B1 protein is stained in red color, β-catenin protein is stained in green color. Nuclei were counterstained with DAPI (blue). B, The effect of SH2B1 on EMT and Wnt/β-catenin signaling. The protein levels of EMT markers (E-cadherin, N-cadherin, Vimentin, and β-catenin) and Wnt/β-catenin pathway targets (TCF-4, c-Myc, Cyclin-D1, and DVL-2) in different panels of H1299, which were subjected or not subjected to treatment: 20 μM/l ICG001 for 48 h, were assayed by Western blotting. GAPDH is used as an internal control. Relative quantifications of protein band intensity in different panels compared with the control are indicated. D, Scratch assays show statistically significant cell migration in H1299 cells with the different treatment (20 μM/l ICG001 or not) at 48 h after cell seeding (upper). Graphical presentation of the percentage of wound healing is measured with the following formula: $(W_0 - W_{48})/W_0\%$. (W, the width of wound) (lower). D, Transwell invasion assays are performed in H1299 cells with different treatment at 48 h after seeding (upper). Graphical presentation of the number of invasive cells is shown (lower)

and identifying novel targets for LADC prevention and therapy.[52] In this study, we firstly identified SH2B1 to be highly expressed in LADC patients and functionally required for EMT phenotype by positively regulating Wnt/β-catenin signaling. Intriguingly, we also found that SH2B1 could activate IRS1 to promote Wnt/β-catenin pathway. Therefore, our findings revealed that SH2B1 was verified to play a critical role in LADC progression.

During epithelial-derived carcinomas progression, EMT and intermediate states, highly dynamic and plastic manners, endow cells with migratory and invasive properties by modifying the adhesion molecules.[36] As such, E-cadherin is the most commonly used marker for the epithelial trait, while N-cadherin, vimentin, and β-catenin are for mesenchymal to confirm EMT.[43] However, even though EMT processes have been documented in many cancer cell models in vitro, considerably more important are the convincing evidence of EMT in cancer progression by direct imaging in vivo[53] and its relevance in human cancer tissues.[49] In this study, we collected cancer samples from edge of human lung adenocarcinomas because morphological

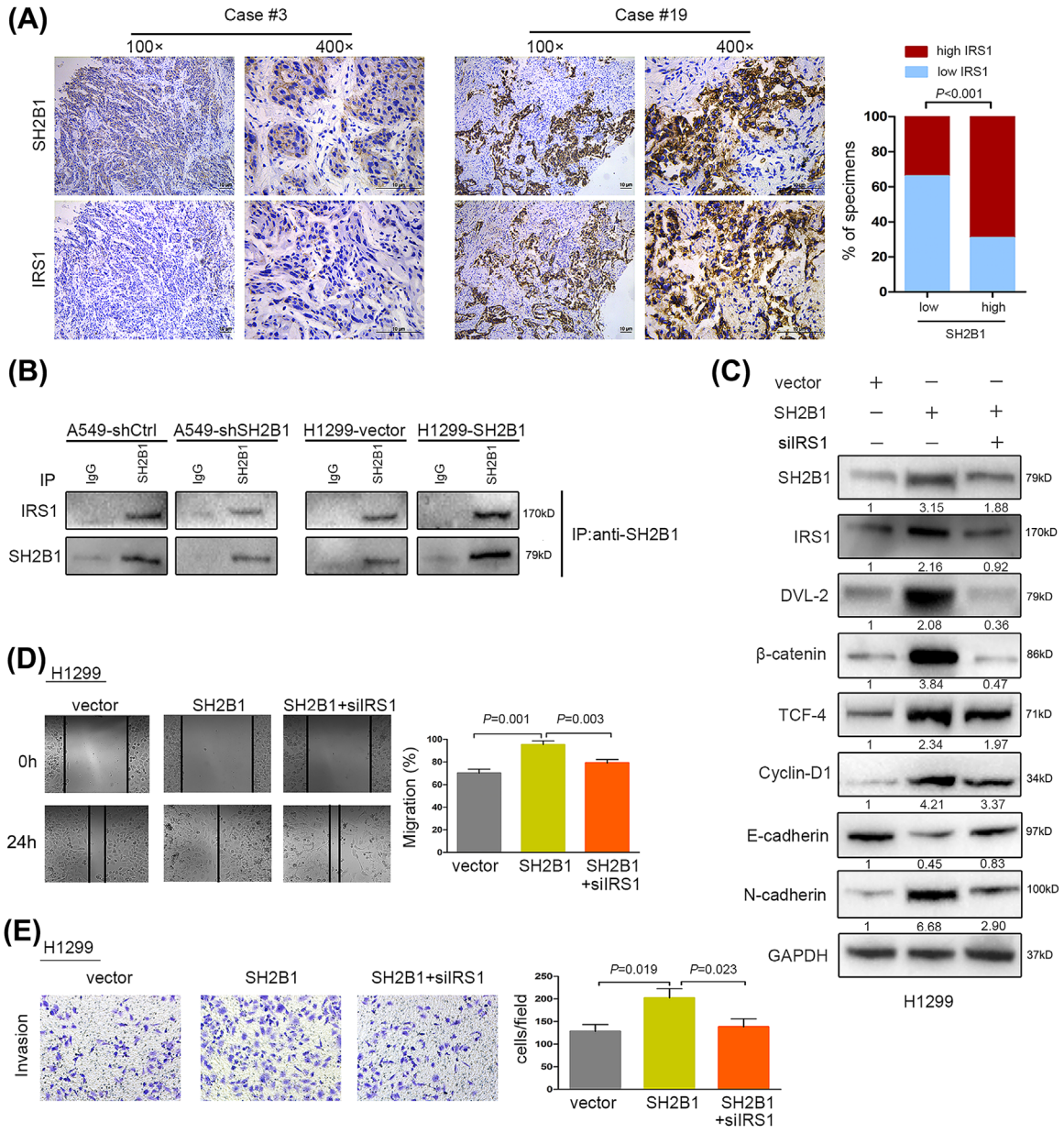


**FIGURE 5** SH2B1 regulates Wnt/β-catenin signaling through IRS1. A, IHC staining was indicating that SH2B1 levels were significantly associated with the expression of IRS1 in 159 primary human LADC specimens. B, Cell lysates from A549 and H1299 cells were collected and subjected to immunoprecipitation for SH2B1 and Western blotting for IRS1 and SH2B1. C, Western blotting analysis of indicated proteins in SH2B1-overexpressing and control H1299 cells transfected with a siRNA against IRS1. The changes of proteins level are quantified by ImageJ software. GAPDH is used as an internal control. Scratch assay (D) and Transwell invasion assay (E) show that depletion of IRS1 impairs the effect of SH2B1 on cell migration and invasion. Graphical presentation of the percentage of wound healing is measured with the following formula: $(W_0 - W_{48})/W_0\%$. (W, the width of wound). Representative micrographs and quantification of the number of invasive cells are shown for (D). Results are presented as mean ± SD

evidence of EMT[54] and some focal events were mainly executed at tumor edges.[36] Our results demonstrated that EMT markers changes were all correlated with clinical stages of LADC patients, suggesting that our findings were in agreement with recent studies highlighting the importance of EMT status in conferring tumor progression in cancer.[44]

Given the existence of EMT gradients in conjunction with LADC intrinsic molecules, such as the EGFR mutation[55] or ALK fusion transcript status,[56] we have yet to determine how far SH2B1 should affect EMT in LADC cells because of clinical evidence paid to the clear-cut relationship of SH2B1 and EMT markers in 159 LADC specimen. It has been reported that SH2B1 was a critical enhancer of proto-oncogene RET by interacting with proto-RET, RET/PTC, and *MEN2A*- and *MEN2B*-associated oncoproteins to mediate RET-induced neoplastic transformation.[23] Our study demonstrated that SH2B1 promoted metastatic properties of LADC cells, compatible with recent

data, indicating that SH2B1 implicated in cell motility,[24,30,57] which resulted in phenotypic cell transformation and potentiated cell survival. These findings contributed to our understanding of SH2B1 in carcinogenesis. Expectedly, the elevated expression of SH2B1 deteriorated LADC patient survival rates and was closely linked to active EMT status in both in cultured cell lines and in vivo tumors. Furthermore, in the presence of cell lineage analyses, the EMT program was observed in migrating cells with mesenchymal morphology and more filopodia structures.[58] Interestingly, SH2B1 promoted filopodium formation and increased the number of filopodia.[28] We observed that SH2B1 overexpression promoted, whereas SH2B1 knockdown impeded, cell morphological changes from a cobblestone-like to spindle shape accompanying with the alteration of EMT markers. Collectively, these in vitro and in vivo data indicate that SH2B1 was a better oncogene and promoted EMT.

Wnt/β-catenin alterations are prominent in human lung cancer and substantially impact tumorigenesis, prognosis, and resistance to therapy.[45] Wnt signals have been reported to regulate EMT program and cell proliferation.[59] As noted previously, β-catenin was higher expressed in 80.50% of LADC tumor tissues. On the other hand, western blotting and immunofluorescence analyses showed that SH2B1 elevated cytoplasmic accumulation of β-catenin and promoted β-catenin translocated into the nucleus and further regulated target genes expression, including TCF-4, cyclinD1, and c-Myc. The results supported the notions that SH2B1 overexpression could active Wnt signaling through β-catenin dependent. We also provided evidence for using an inhibitor or activator of Wnt/β-catenin signaling to reverse the response in which SH2B1 overexpression or knockdown mediated EMT phenotype. This result reinforced the concept that SH2B1 potentially promoted EMT via Wnt/β-catenin signaling in LADC cells.

To our knowledge, IRS1 is a signaling protein that interfaces with many pathways activated in cancer, including the phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase (MEK/ERK), and Janus kinase/transducers and activator of transcription (JAK/STAT) signaling pathways.[60] Consistent with this, some miRNAs mediate proliferation and metastasis of cancer cells through targeting IRS1.[61,62] Recently, Geng et al[51] reported that IRS1 could promote Wnt-mediated EMT and cell proliferation by interacting with and stabilizing Dvl2. Besides, our previous study in the SH2B1 knockout mice indicated that SH2B1 could interact with IRS1 and lead to intracellular cascades transduction, such as the activation of the Erk1/2 and PI 3-kinase pathways.[40,63] Constitutive activation of IRS1 is found in some solid human tumors.[64,65] In this study, we provide evidence that depletion of IRS1 inhibited SH2B1-overexpression-induced cell invasion and metastasis and uncovered that IRS1 has the positive effect on SH2B1-mediated Wnt signals activation. On the other hand, apart from Wnt/β-catenin signaling, SH2B1/IRS1 also active other pathways, including Erk1/2 and PI3-kinase. Therefore, these results provide an impetus to investigate a possible cross-talk between enhanced SH2B1-induced signal transduction pathways regarding the role of SH2B1 in EMT.

In conclusion, our findings reveal that SH2B1 has a major role in LADC progression and that SH2B1 is a critical activator of Wnt/β-catenin signaling. Understanding the precise function of SH2B1 in the pathogenesis of LADC and activation of the SH2B1-IRS1-β-catenin axis will extend our knowledge of the biological basis of LADC progression and may also offer new insight into a new therapeutic target, enhancing the clinical benefits of LADC patients.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## AUTHORS CONTRIBUTION

S-QW and YDC contributed equally to the manuscript. YG and C-JD provided clinical samples and Y-YZ and Z-ZP analyzed the data; S-QW and Y-DC carried out most of the experimental work, mainly the cell and animal experiments. Z-WH and W-LZ conducted the immunohistochemistry analysis and provided pathology evaluation. S-QW wrote the manuscript. R-MC and C-FZ contributed to manuscript revision. C-FZ, C-JD, and Y-DC designed the project and supervised all experiments.

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## SUPPORTING INFORMATION

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