

# The expression of *aplysia ras homolog 1* (*ARHI*) and its inhibitory effect on cell biological behavior in esophageal squamous cell carcinoma

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**Background:** *Aplysia ras homolog 1* (*ARHI*) is a Ras-related maternally imprinted tumor suppressor gene. Loss of *ARHI* expression contributes to the malignant progression of various tumors. However, reports on the clinical implications and functional role of *ARHI* expression in esophageal squamous cell carcinoma (ESCC) are limited. This study examined the role of *ARHI* in ESCC.

**Methods:** In total, 81 patients diagnosed with ESCC based on histopathological evaluations who were subjected to surgical resection were included in the study. *ARHI* expression was analyzed by immunohistochemistry and western blotting, examining the correlations between *ARHI* expression and patient clinicopathological features. The functional effects of *ARHI* overexpression were examined using a Cell Counting Kit-8 assay, flow cytometry, a Transwell assay, wound healing, and western blotting in the ECA109 cell line.

**Results:** *ARHI* was highly expressed in 27.5% (22/81) of ESCC specimens (adjacent non-cancerous tissues, 85.2%, 69/81;  $P < 0.05$ ). The *ARHI* expression level was significantly lower in patients with lymph node metastasis than in patients without ( $P < 0.05$ ). A Kaplan–Meier survival analysis showed that patients with low *ARHI* expression had shorter survival than patients with high expression ( $P < 0.05$ ), and a multivariate Cox analysis revealed that *ARHI* is an independent predictor of overall survival ( $P = 0.029$ ). Finally, overexpression of *ARHI* in ESCC cells indicates that *ARHI* suppresses proliferative capacity, invasive capacity, and cell cycle progression and may also suppress epithelial–mesenchymal transition and induce apoptosis and autophagy.

**Conclusion:** *ARHI* may be a prognostic biomarker and a potential therapeutic target in ESCC.

**Keywords:** *aplysia ras homolog 1*, esophageal squamous cell carcinoma, cell biological behavior

## Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressively malignant neoplasms, with a poor prognosis. Although considerable diagnostic and therapeutic progress has been made, substantial improvements in patient outcome have not been achieved.<sup>1</sup> The overall 5-year survival rate for ESCC is  $<40\%$ ,<sup>2</sup> mainly because it is usually diagnosed at a late stage, increasing instances with early metastasis to the lymph nodes and distant metastases.<sup>3–5</sup> Therefore, the identification of useful biomarkers for ESCC treatment is urgently needed.

*Aplysia ras homolog 1* (*ARHI*) is a recently identified maternally imprinted tumor-suppressor gene located on the human chromosome 1p31. It encodes a 26 kDa small GTP-binding protein and shares 54%–62% amino acid homology with Ras/Rap family

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members.<sup>6</sup> It is the first reported tumor-suppressor gene in the Ras superfamily. Recent research shows that *ARHI* is involved in breast, ovary, pancreas, liver, and lung carcinogenesis.<sup>7–13</sup> However, it is unclear whether it plays a role in ESCC.

One mechanism by which *ARHI* may mediate cancer progression is through the regulation of programmed cell death (PCD), which includes apoptosis and autophagic cell death. Apoptosis, also known as type I PCD, is a caspase-dependent process, and autophagy, type II PCD, is a type of non-apoptotic cell death. Autophagy is a physiological process in eukaryotic cells by which cytoplasm and other cellular components are targeted to lysosomes for degradation.<sup>14,15</sup> Autophagy has gradually become an important area in cancer research,<sup>16–18</sup> but data connecting cancer prognosis with autophagy are limited.

The aim of this study was to investigate the correlation between *ARHI* expression and patient clinicopathological characteristics. In addition, the effects of *ARHI* on invasion, proliferation, autophagy, apoptosis, and cell cycle progression were also investigated.

## Materials and methods

### Patients and tissue samples

A total of 81 ESCC tissue specimens were obtained from patients in the Department of Thoracic Surgery of Shengjing Hospital, the second affiliated hospital of the China Medical University, between 2007 and 2009. All patients underwent a resection with biopsy and diagnosis at the pathology department. The specimens consisted of 10% formalin-fixed, paraffin-embedded tissue sections; 4  $\mu$ m sections were cut for histopathological analysis. None of the patients received anti-cancer therapy or adjuvant treatment prior to the operation.

All the 81 cases were independently classified as ESCC by two experienced pathologists according to the World Health Organization classification. Patients were staged according to the International Union Against Cancer TNM classification of malignant tumors, seventh edition, 2009. All the 81 patients received follow-ups through telephone enquiry or questionnaires. The follow-up time ranged from 6 to 73 months (median, 29 months).

### Immunohistochemistry analysis

Immunohistochemical studies on *ARHI* were performed using 81 10% formalin-fixed, paraffin-embedded tissue sections (4  $\mu$ m thick) obtained from patients with ESCC. The expression of *ARHI* was detected using the DAB color system and the PV-9000 method. The primary antibody was anti-*ARHI* (working dilution 1:50; Santa Cruz Biotechnology Inc., Dallas, TX, USA). The DAB and PV-9000 kit were obtained from Zhongshan Chemical (Beijing, China). The tissue

sections were dewaxed in xylene and hydrated with graded alcohol and phosphate-buffered saline (PBS). Sections were then subjected to heat-induced epitope retrieval by boiling in 10 mM citrate buffer, pH 6.0, for 10 min. Sections were then incubated with primary antibodies overnight at 4°C, stained using the PV-9000 kit, and counterstained with hematoxylin following the manufacturer's instructions. For negative controls, the primary antibody was replaced with PBS.

Two experienced pathologists who were blinded to patient histopathological data independently scored immunohistochemically stained tissue sections. *ARHI*-positive staining was located in the cytoplasm. The evaluation was standardized based on the staining classification system of Rosen et al,<sup>19</sup> but it was modified slightly for simplification. Staining intensity was scored as follows: 0, no staining; 1, faint yellow or yellow; 2, tan. The extent of positive cells was classified as 0 ( $\leq 10\%$ ) or 1 ( $> 10\%$ ). The intensity and extent scores were multiplied to evaluate expression in each case. A score of 0 and 1 was classified as low expression and a score of 2 was classified as high expression.

### Western blot analysis

Total cell extracts were obtained, and the protein content was quantitatively analyzed using a bicinchoninic acid protein assay and separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). Samples were blocked in 5% bovine serum albumin for 2 h at room temperature and incubated with appropriate primary antibodies overnight at 4°C. The primary antibodies were as follows: 1) anti-*ARHI* (1:1,000; Santa Cruz Biotechnology Inc., Dallas, TX, USA), 2) anti-LC3 (1:1,000; Abcam, Cambridge, UK), 3) anti-GAPDH (1:5,000; Sigma-Aldrich Co., St Louis, MO, USA), 4) anti-bcl-2 (1:1,000; Santa Cruz Biotechnology), 5) anti-Mmp-2 (1:1,000; Santa Cruz Biotechnology), 6) anti-Mmp-9 (1:1,000; Santa Cruz Biotechnology), 7) anti-E-cadherin (1:1,000; Abcam), 8) anti-N-cadherin (1:1,000; Abcam). The membrane was washed in PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at room temperature for 1 h. The membrane was washed with PBST again, and the ECL kit was used for western blot detection. For whole images, ImageJ was used to estimate the band intensity. To normalize protein loading, monoclonal GAPDH antibody was used.

### Cell culture and transient transfection

The human ESCC cell line ECA109 (purchased from ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (GIBCO, Waltham, MA, USA; 1.5 g/L NaHCO<sub>3</sub>, 2.5 g/L glucose,

0.11 g/L sodium pyruvate), with 10% fetal calf serum, at 37°C and 5% CO<sub>2</sub>. PCMV-ARHI-AC-GFP and PCMV-AC-GFP were purchased from OriGene (Rockville, MD, USA). The *ARHI* plasmid PCMV-ARHI-AC-GFP was transfected into ECA109 cells; *ARHI* was overexpressed in vitro and verified by western blotting. The empty plasmid PCMV-AC-GFP (OriGene) was used as a negative control. *ARHI*-targeted small interfering RNA (siRNA) was purchased from GenePharma (Shanghai, China), the sequence of target was: CUGCUUGACAAGUGCAUAATT. Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used for transfection according to the manufacturer's instructions. After 48 h, the transfected cells were harvested and used for further experiments.

### Cell proliferation assays

Cell proliferation was examined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The ESCC cells were divided into an *ARHI* group, a control group, and an ECA109 group. Cells were plated in 96-well plates at a concentration of  $3 \times 10^3$  cells/well and incubated for 24 h. Each plate was supplemented with 90 µL of fresh complete RPMI-1640 medium. Then, 10 µL of CCK-8 was added to each well, and the plates were incubated for 3 h at room temperature. Absorbance was measured at wavelengths of 490 nm daily for 5 days.

### Cell invasion assay

Infected ECA109 cells ( $3 \times 10^4$ ) were transferred to the upper chambers of Transwell plates (Costar, Cambridge, MA, USA), and 200 µL of serum-free RPMI-1640 was added. The lower chamber was supplemented with 0.5 mL of RPMI-1640 with 10% fetal bovine serum as an inducer of cell migration. After incubation at 37°C for 48 h, cells in the upper chamber were removed using a sterile cotton tip. Invasive cells were fixed with 4% cold methanol for 15 min, rinsed with PBS, and stained with hematoxylin for 5 min. Cells were counted in five randomly selected areas for each well.

### Wound-healing assay

ESCC cells were divided into an *ARHI* group, a control group, and an ECA109 group. Infected cells were suspended by trypsin digestion containing RPMI-1640 with 10% fetal bovine serum and incubated at 37°C for 24 h. When the cells reached 90% confluence, wounds were made using a 20 µL pipette tip and washed three times with PBS. After replacing the medium with serum-free media, wounds were observed and photographed every 12 h under a light microscope.

### Apoptosis analysis using flow cytometry

For the apoptosis analysis, cells ( $1\text{--}500,000/\text{L}$  per well) were washed with PBS twice and single-cell suspensions were prepared by trypsin digestion. Cells were resuspended after adding 0.5 mL of Binding Buffer. Cells were stained with the Annexin V-Light 650/PI Apoptosis Detection Kit (Wanleibio, Shenyang, China) according to the manufacturer's instructions, and cell death was measured using a flow cytometer (BD).

### Statistical analysis

The data were analyzed using the SPSS 17.0 software package. The relationship between clinicopathological factors and the results of immunohistochemistry experiments was examined using  $\chi^2$  tests. Differences in protein expression among groups were assessed using paired *t*-tests. Kaplan–Meier survival curves and log-rank tests were used to describe patient survival. The multivariate Cox hazard model was used to identify independent prognostic factors for survival.  $P < 0.05$  was considered statistically significant.

### Ethics

This study was approved by the Research Ethics Committee of China Medical University. Written informed consent was obtained from all patients for this study.

### Results

#### Expression of *ARHI* is decreased in ESCC tissues

*ARHI* immunoreactivity was predominantly located in the cytoplasm (Figure 1), appearing as brownish-yellow granules. *ARHI* was expressed at a significantly lower rate (22/81, 27.16%) in ESCC tissues than in matched normal esophageal epithelia tissues (69/81, 85.19%,  $P < 0.01$ ).

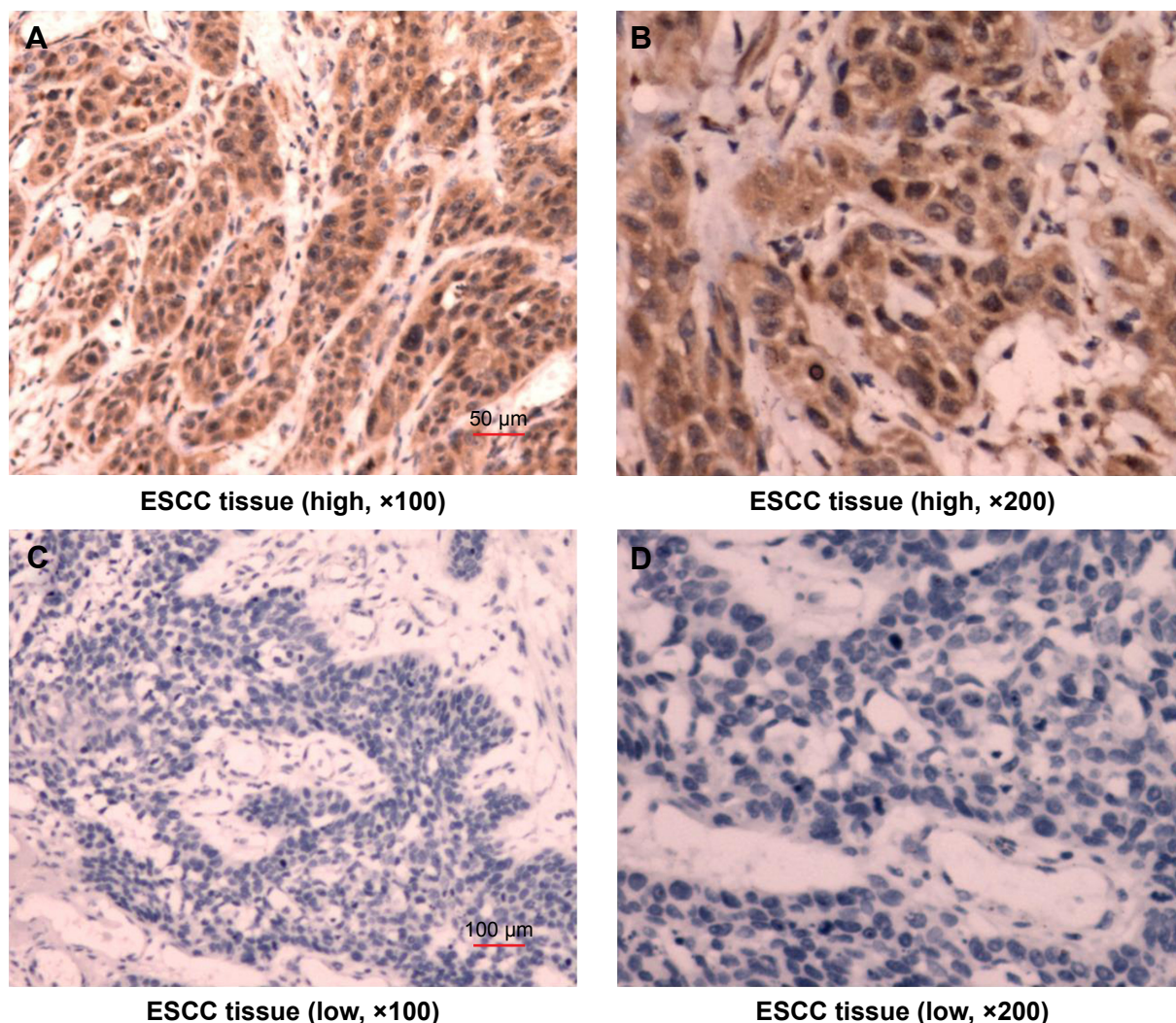
#### Low expression of *ARHI* in ESCC tissues is correlated with lymph node metastasis

The *ARHI* expression rate was significantly lower (11.11%) in ESCC patients with lymphatic metastasis than in those without lymphatic metastasis (40%,  $P < 0.05$ , Table 1). There was no significant correlation between *ARHI* expression and age, gender, TNM stage, T stage, M stage, or degree of differentiation in ESCC patients ( $P > 0.05$ , Table 1).

### Survival analysis

Patients with high *ARHI* expression had significantly higher 5-year survival rates than those with low levels of *ARHI* expression (log-rank test,  $P < 0.01$ ; Figure 2A).





**Figure 1** Immunostaining demonstrating the expression of *ARHI* in ESCC tissues.

**Notes:** (A) High expression of *ARHI* in ESCC,  $\times 100$ . (B) High expression of *ARHI* in ESCC,  $\times 200$ . (C) Low expression of *ARHI* in ESCC,  $\times 100$ . (D) Low expression of *ARHI* in ESCC,  $\times 200$ .

**Abbreviations:** *ARHI*, aplysia ras homolog I; ESCC, esophageal squamous cell carcinoma.

A univariate analysis demonstrated that N stage ( $P < 0.001$ ), TNM stage ( $P = 0.001$ ), and high *ARHI* expression ( $P = 0.005$ ) were significant prognostic factors (Table 2). A multivariate analysis using a forward stepwise Cox regression analysis showed that N stage ( $P < 0.001$ ) and high *ARHI* expression ( $P = 0.029$ ) were independent prognostic factors in ESCC patients (Table 2).

### Overexpression of *ARHI* inhibits the proliferation of ESCC cells

The ECA-109 cell line was transfected with the *ARHI* plasmid. After 48 h, the transfection efficiency was detected through western blot analysis. The *ARHI* cells had bands at 52 kDa, and the prompt endogenous *ARHI* protein expression quantity did not change, thus indicating that the *ARHI* gene was successfully transfected (Figure 2B). To evaluate

the impact of *ARHI* overexpression on cell proliferation, a CCK8 assay was performed. The proliferation of ECA109 cells following PCMV-*ARHI*-AC-GFP transfection was significantly inhibited compared with the control group and the blank group. The rates of cell proliferation inhibition for the ECA109 group, control group, and *ARHI* group were  $1.65 \pm 0.041$ ,  $1.613 \pm 0.083$ , and  $0.466 \pm 0.001$ , respectively, indicating that *ARHI* upregulation significantly suppressed the proliferation of ECA109 cells ( $P < 0.01$ , Figure 2C).

### Overexpression of *ARHI* inhibits the invasion and migration ability of ESCC cells

Transwell invasion assays were performed to evaluate the effect of *ARHI* on the invasive ability of ESCC cells. Following *ARHI* overexpression for 48 h, the average number of

**Table 1** Relationship between *ARHI* expression and clinical pathological features

Values	n	<i>ARHI</i>		$\chi^2$	P-value
		High	Low		
Gender				1.109	0.297
Female	4	2	2		
Male	77	20	57		
Age (years)				0.088	0.807
≤60	39	10	29		
>60	42	12	30		
Tumor invasion				0.657	0.426
T1–2	57	14	43		
T3–4	24	8	16		
Lymph nodes metastasis				8.437	0.004*
N0	45	18	27		
N1–3	36	4	32		
Tumor metastasis				0.541	0.472
M0	79	21	58		
M1	2	1	1		
TNM stage				3.126	0.086
I–II	46	16	30		
III–IV	35	6	29		
Differentiation				5.059	0.08
Good	26	11	15		
Moderate	45	10	35		
Poor	10	1	9		

Note: \*Statistical significance at  $P < 0.05$ .

Abbreviations: *ARHI*, alypsia ras homolog I; TNM, tumor node metastasis.

invading cells was  $257.8 \pm 12.13$ , significantly lower than that of the control group ( $415.4 \pm 20.48$ ) (Figure 3A and B). Wound assays indicated that the migration ability was also lower for ECA109 cells infected with *ARHI* than for the control group ( $P < 0.01$ , Figure 3C). We detected the expression of Mmp-2

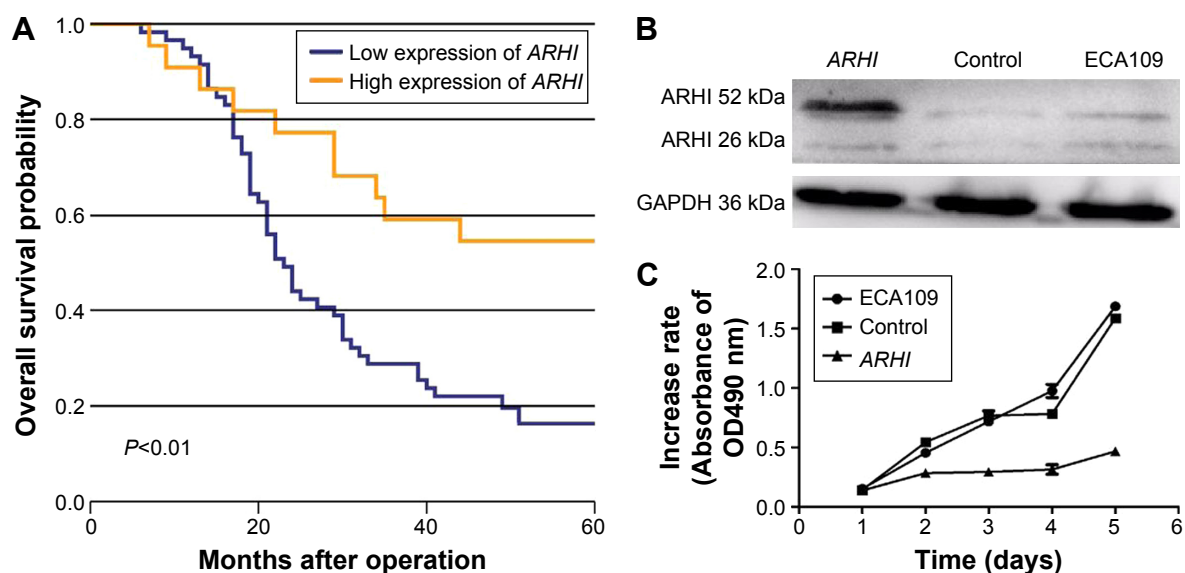
and Mmp-9 using western blotting. Compared with the control group, the *ARHI* plasmid-transfected group showed significantly lower levels of Mmp-2 and Mmp-9, two proteins commonly correlated with tumor progression and metastasis (Figure 4A). These results indicate that *ARHI* inhibits the invasion and migration capacities of ESCC cells.

### Inhibition of *ARHI* promotes the proliferation and invasion of ECA109 cells

In addition, siRNA was used to inhibit the level of *ARHI* in ECA109 cells. The results of cell proliferation assays and cell invasion assays showed that inhibition of *ARHI* can promote the proliferation and invasion of ECA109 cells (Figure S1). Thus, these results also confirmed the inhibitory effect of *ARHI* on ECA109 cell biological behavior.

### Overexpression of *ARHI* in ECA109 cells may induce apoptosis

Annexin V and light staining were performed to evaluate whether *ARHI* could induce ECA109 cell apoptosis. The proportion of apoptotic cells (Q2+Q4) was significantly higher in the *ARHI* group than the control and ECA109 groups ( $P < 0.01$ , Figure 4B and C). Furthermore, the expression of Bcl-2 was analyzed using western blotting. Compared with the control and ECA109 groups, the *ARHI* plasmid-transfected group showed lower levels of Bcl-2 expression (Figure 4A). In summary, *ARHI* induces apoptosis and inhibits cell cycle progression in ECA109 cell lines.



**Figure 2** (A) Kaplan–Meier analysis of the correlation between *ARHI* expression level and overall survival; (B) the relative expression levels of *ARHI* were determined by western blotting; (C) cell proliferation was assessed daily for 5 days using the Cell Counting Kit-8 assay in ESCC cells.

Abbreviations: *ARHI*, alypsia ras homolog I; ESCC, esophageal squamous cell carcinoma.

**Table 2** Univariate and multivariate analyses of survival in ESCC

Variable	n	Univariate		Multivariate	
		HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)					
≤60	39	I			
>60	42	1.473 (0.877–2.472)	0.143		
Sex					
Male	77	I			
Female	4	0.244 (0.034–1.769)	0.163		
T stage					
pT1–pT2	57	I			
pT3–pT4	24	0.981 (0.391–2.459)	0.967		
N stage					
pN0	45	I		I	
pN1–pN2	36	3.196 (1.871–5.460)	<0.001	2.803 (1.626–4.831)	<0.001*
M stage					
pM0	79	I			
pM1	2	3.101 (0.742– 12.971)	0.121		
TNM stage					
I–II	46	I			
III–IV	35	2.477 (1.464–4.192)	0.001*		
Differentiation					
Good	26	I			
Moderate– poor	55	1.475 (0.971–2.239)	0.068		
ARHI expression					
Low	59	I		I	
High	22	0.370 (0.186–0.738)	0.005*	0.458 (0.227–0.924)	0.029*

**Note:** \*Statistical significance at  $P < 0.05$ .

**Abbreviations:** ARHI, aplysia ras homolog 1; HR, hazard ratio; CI, confidence interval; TNM, tumor node metastasis.

## ARHI may be involved in cell autophagy and epithelial–mesenchymal transition (EMT) of ESCC cells

The expressions of LC3, E-cadherin, and N-cadherin were also detected through western blotting. There are two forms of LC3, LC3-I and LC3-II (an LC3-phospholipid conjugate). Many types of stressors upregulate LC3 expression and promote the binding of cytosolic LC3-I to phosphatidylethanolamine to form autophagosome-specific LC3-II. Therefore, LC3-II is considered a special marker of autophagy.<sup>20,21</sup> Compared to the control and ECA109 groups, the ARHI plasmid-transfected group showed significantly higher levels of LC3-II and E-cadherin and lower levels of N-cadherin expression (Figure 4A). Thus, ARHI may induce autophagy and inhibit EMT of ESCC cells.

## Discussion

The autophagy-related gene *ARHI* is a maternally imprinted tumor-suppressor gene that was discovered in 1999. It is the first reported tumor-suppressor gene in the Ras super family, sharing 54%–62% amino acid homology with Ras/Rap family members.<sup>6</sup> It is absent or downregulated in many cancers including breast, ovarian, pancreatic, liver, lung, and many others and is involved in tumor development.<sup>7–13</sup> These observations suggest an important role of *ARHI* in tumor development. However, to our knowledge, the role of *ARHI* in ESCC is not known.

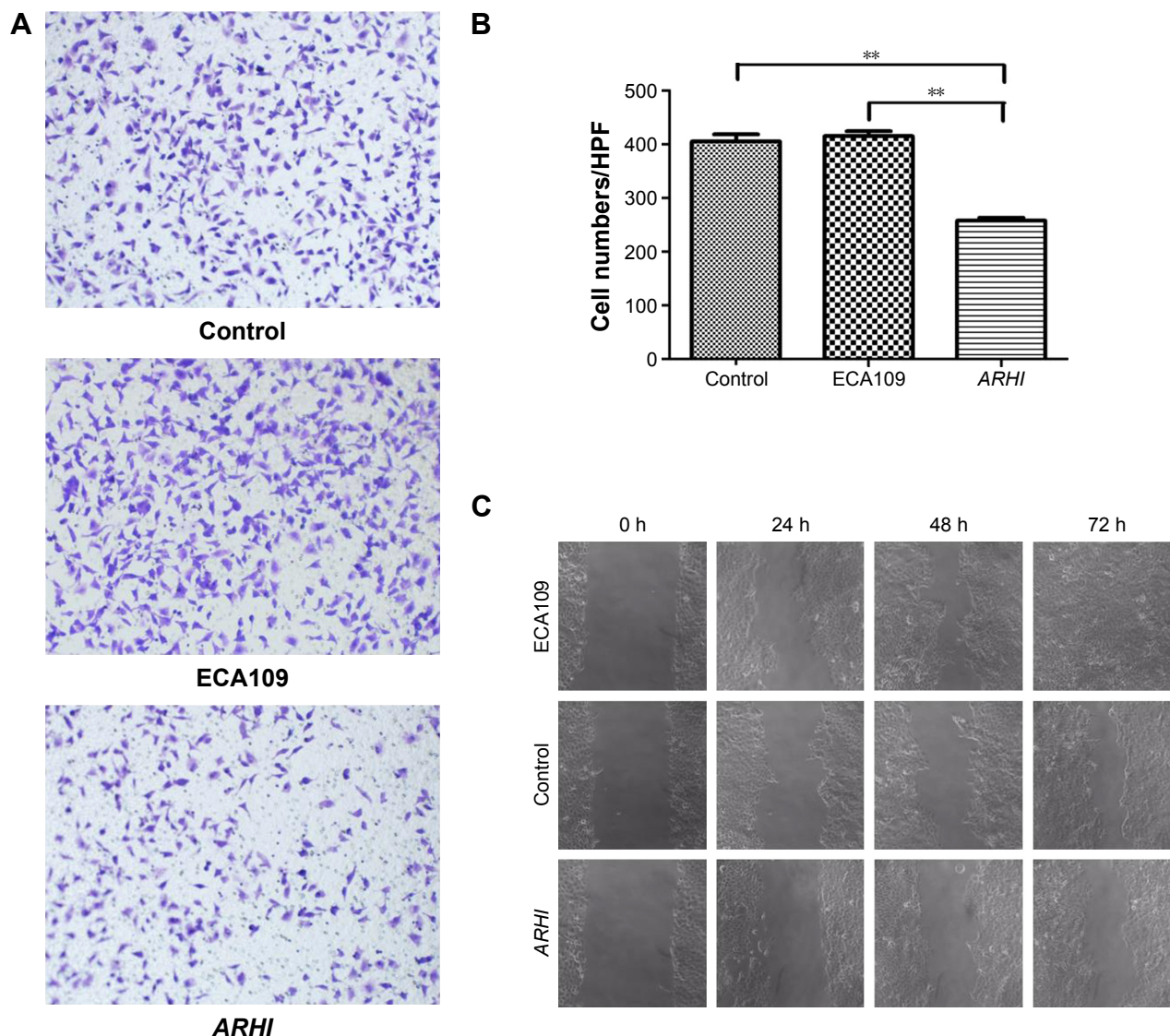
*ARHI* inhibits growth, reproductive development, proliferation, and migration and is involved in apoptosis, and autophagy.<sup>11,22–25</sup> *ARHI* may suppress pancreatic cancer by regulating the MAPK/ERK 1/2 pathway.<sup>26</sup> Yu et al<sup>27</sup> used in situ hybridization and immunohistochemical methods to examine *ARHI* expression in 64 cases of mammary ductal carcinoma and paired normal breast tissue samples and found that *ARHI* is absent or downregulated in breast cancer.

This study found that *ARHI* expression was absent or downregulated in ESCC tissue samples compared with normal tissues. Moreover, *ARHI* expression was associated with lymph node metastasis and poor prognosis. This finding suggests that *ARHI* is associated with ESCC progression and may act as a potential therapeutic target. Previous studies have revealed that low *ARHI* expression is associated with a shorter survival time for patients with pancreatic cancer.<sup>28</sup> Similarly, in this study, a multivariate Cox regression analysis indicated that low expression of *ARHI* is associated with poor prognosis in ESCC patients and is an independent prognostic factor and a potential therapeutic target.

Metastasis and invasion ability are basic biological characteristics of tumor cells and present major challenges for clinical treatment of esophageal cancer. A previous study suggests that *ARHI* regulates proliferation, invasion, and migration of ovarian carcinoma cells through the STAT3 and FAK/Rho pathways.<sup>29</sup> Accordingly, it was inferred that *ARHI* might also regulate these processes in ESCC cells.

Specifically, CCK8 assays, a Transwell invasion assay, wound assays, and flow cytometry were used to determine the influence of *ARHI* overexpression on proliferation, invasion, migration, and apoptosis in ECA109 cells. It was found that *ARHI* overexpression reduced the proliferation, invasion, and migration capacities of ECA109 cells. Previous studies have established that Mmp-2 and Mmp-9 are positively correlated with tumor progression, metastasis, and poor overall prognosis.<sup>30</sup> Consistent with this, significantly lower levels of Mmp-2 and Mmp-9 expression were observed in





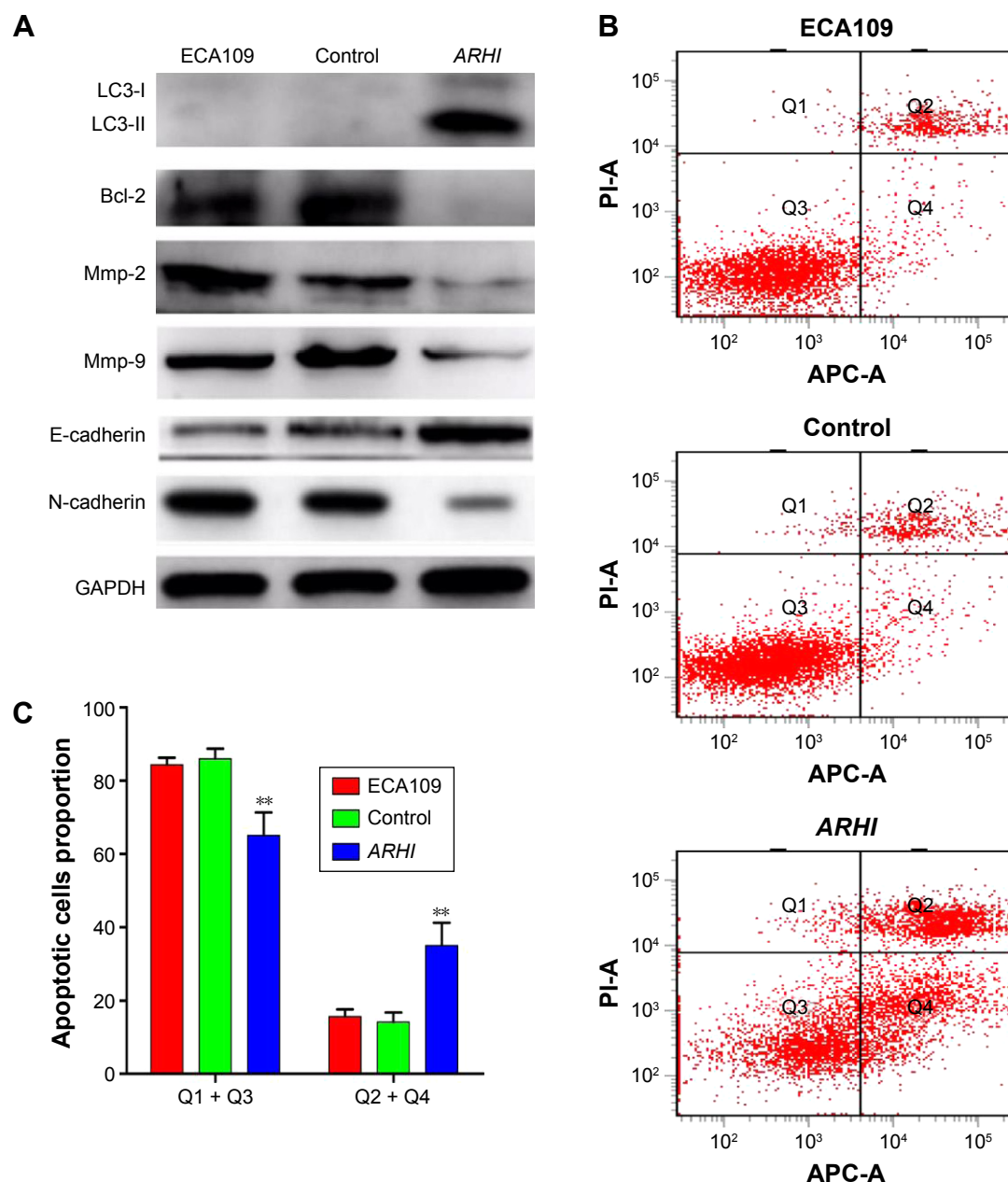
**Figure 3** (A) Transwell assays were used to assay the involvement of *ARHI* for invasion in ESCC cells, original magnification  $\times 200$ ; (B) the cell counts of the ECA109 group, control group, and *ARHI* group; (C) wound assays were monitored at 0, 24, 48, and 72 h in ESCC cells, original magnification  $\times 100$ .  $**P < 0.01$ .  
**Abbreviations:** *ARHI*, alypsia ras homolog I; ESCC, esophageal squamous cell carcinoma; HPF, high power field.

the *ARHI*-transfected group than the control group. Proteins in the BCL-2 family control the mitochondrial pathway of apoptosis.<sup>31</sup> The evasion of apoptosis is the main cause of therapeutic resistance.<sup>32</sup> Bao et al<sup>33</sup> found that *ARHI* mediates apoptosis through a caspase-independent calpain-dependent pathway. Based on Annexin V and light staining, it was found that apoptosis was significantly higher in the transfected group than the control group.

Bcl-2 was suggested to be an anti-apoptosis molecule that develops adverse effects on the modulation of cell death.<sup>34</sup> It has been reported that in HeLa cells, PI3K/AKT lies upstream of the Bcl-2 and can regulate its expression.<sup>35</sup> Li et al found that *ARHI* can trigger PI3K/AKT pathway to modulate the expression of Bcl-2, and overexpressed *ARHI*

could reduce the phosphorylation of AKT and increase the levels of PI3K inhibitor LY294002 to suppress the expression of Bcl-2 in epithelial ovarian cancer cells.<sup>36</sup> This study found that overexpression of *ARHI* could reduce the protein expression of Bcl-2 to induce apoptosis in ECA109 cells. Thus, it indicates that Bcl-2 may be one of the targets that lie downstream of *ARHI* pathways.

Abundant evidence supports the role of EMT in tumor progression, and E-cadherin and N-cadherin are important hallmarks of EMT.<sup>37</sup> In the current study, the results of western blotting showed that overexpressed *ARHI* could increase the expression of E-cadherin and decrease the expression of N-cadherin, indicating that overexpression of *ARHI* may inhibit EMT in ECA109 cells. On the other hand, Wu et al reported that



**Figure 4 (A)** The translational levels of invasive, autophagy, and EMT-related markers were determined by western blotting; **(B)** cells were stained with both Annexin V-APC and PI, and cells that were positive for Annexin V-APC were counted as apoptotic cells; **(C)** the proportion of apoptotic cells for ECA109 group, control group, and *ARHI* group, \*\* $P < 0.01$ . **Abbreviations:** *ARHI*, aplysia ras homolog I; EMT, epithelial–mesenchymal transition; APC, allophycocyanin; PI, propidium iodide.

Bcl-2 can regulate EMT by calcium signals in cancers.<sup>38</sup> Thus, *ARHI* may regulate the expression of cadherin and EMT by acting on Bcl-2. However, the exact mechanism between them remains unknown, and further studies are needed to confirm.

Abundant evidence supports the role of EMT in tumor progression. Lowered E-cadherin is a hallmark of EMT.<sup>34</sup> In the current study, E-cadherin expression was significantly higher in the *ARHI* plasmid-transfected group than the control group. Hence, *ARHI* may inhibit EMT in ESCC cells.

In summary, the results suggest that *ARHI* is absent or downregulated in ESCC tissues and is associated with lymph

node metastasis and prognosis. High *ARHI* expression is a good independent prognostic factor in ESCC patients. In the ECA-109 ESCC cell line, the overexpression of *ARHI* can suppress proliferative and invasive capacities and induce apoptosis and autophagy. This study provides the first evidence that *ARHI* may inhibit EMT in ESCC cells.

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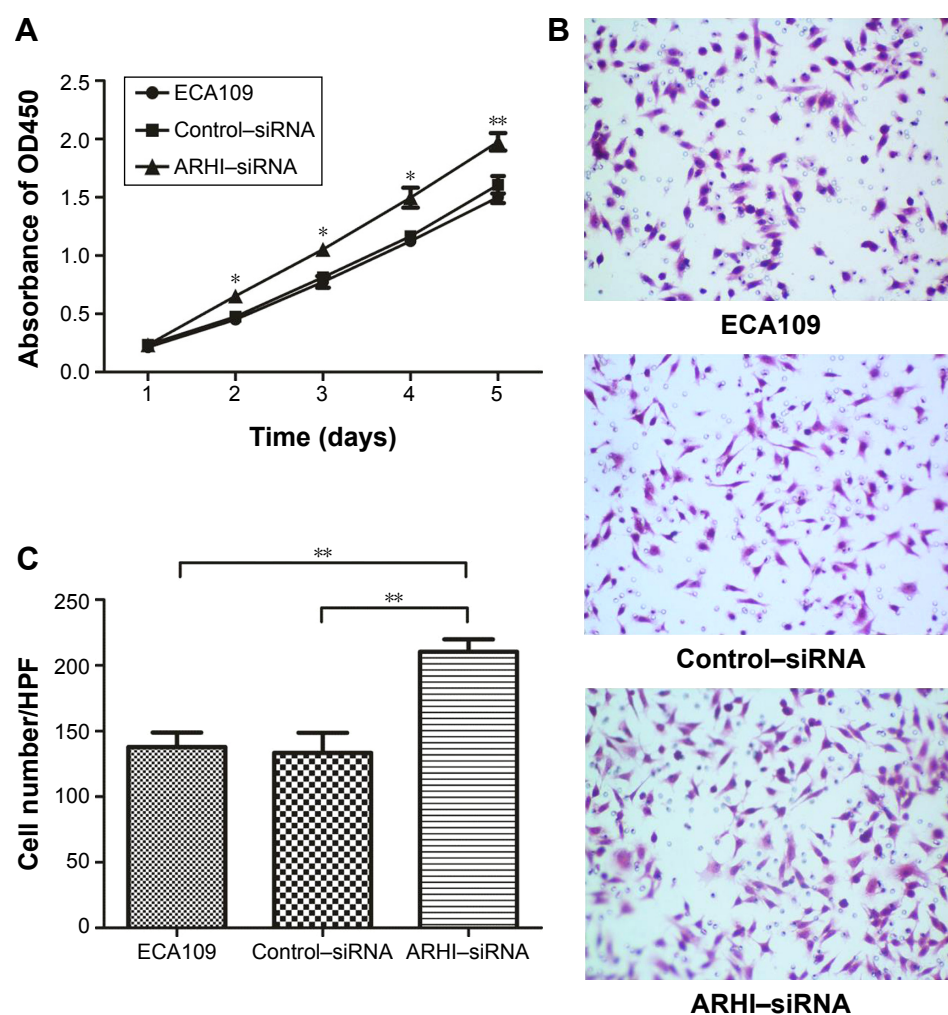
## Disclosure

The authors report no conflicts of interest in this work.

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# Supplementary material



**Figure S1** (A) Cell proliferation was assessed daily for 5 days using the Cell Counting Kit-8 assay in ECA109 group, Control-siRNA group and ARHI-siRNA group; (B) Transwell assays were used to examine the involvement of ARHI for invasion in ECA109 group, control-siRNA group, and ARHI-siRNA group (original magnification ×200); (C) the cell counts of the ECA109 group, control-siRNA group, and ARHI-siRNA group in transwell assays.

**Notes:** \*P < 0.05, \*\*P < 0.01.

**Abbreviations:** ARHI, aplysia ras homolog I; siRNA, small interfering RNA; HPF, high power field.

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