**Objective:** Aplasia Ras homolog member I (ARHI) is associated with human ovarian cancer (HOC) growth and proliferation; however, the mechanisms are unclear. The purpose of this study was to investigate ARHI effects in HOC SKOV3 cells.

**Methods:** We transfected SKOV3 cells with Pires2-EGFP-ARHI and measured growth inhibition rates, cell cycle distribution, apoptosis rates, and expression of P-STAT3 (phosphorylated signal transduction and activators of transcription 3) and P-ERK (phosphorylated extracellular signal regulated protein kinase).

**Results:** Our data showed significant inhibition of growth, significantly increased S-phase arrest and apoptosis rates, and reduction of P-STAT3 and P-ERK1/2 expression levels.

**Conclusions:** We propose the mechanism may involve ARHI-induced phosphorylation of ERK1/2 and STAT3 protein kinases, thereby blocking proliferation signaling pathways, to induce HOC SKOV3 apoptosis.

**Key Words:** Ovarian neoplasms, ARHI, SKOV3, STAT3, Autophagy

**Abbreviations:** ARHI-aplasia Ras homolog member I, ERK-extracellular signal regulated protein kinase, GAPDH-glyceraldehyde phosphate dehydrogenase, GFP-green fluorescence protein, IR-inhibitory rate, OD-optical density, P-STAT3-phosphorylated STAT3, STAT-signal transduction and activators of transcription

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expression products and HOC, and the potential of altering expression of these genes and gene products for treating patients with HOC.

The ARHI gene (aplasia Ras homolog member I, also known as DIRAS3 or NOEY2) was first discovered by The University of Texas M. D. Anderson Cancer Center, United States. It is a member of the Ras superfamily and encodes a small GTP-binding protein. ARHI is highly expressed in normal human tissues, including mammary glands and ovaries, and heart, liver, pancreas, and brain. Conversely, it is reported to be down-regulated in tumor tissues including breast, HOC, and prostate cancers.1–3 The possible mechanisms underlying abnormal expression of ARHI in tumors may involve abnormal methylation, loss of heterozygosity, and low expression levels of acetylated histones.4–7 Depletion of histone deacetylases 1, 3, and 11 not only significantly increased the ARHI promoter activity of the transfected reporter but also activated the transcription of the endogenous ARHI gene.8 Some studies by Yu et al9 and Lu et al10 revealed ARHI is down-regulated by transcriptional mechanisms that involve E2F1 and E2F4, as well as by the loss of RNA binding proteins that decrease the half-life of ARHI mRNA. Mutation of the putative E2F binding site in the ARHI promoter reversed this inhibitory effect and significantly increased ARHI promoter activity.

Studies have shown that ARHI inhibits cell growth in HOC and that loss of ARHI expression contributes to the formation of HOC.11 When ARHI re-expression was promoted using demethylation factors and histone deacetylase inhibitors in different tissues, tumor growth rates were reduced, and apoptosis increased.12 Furthermore, several studies have found that continuous expression of ARHI in HOC epithelia was associated with expression of cyclin-dependent kinase inhibitor p21 (WAF1/CIP1)1 and extension of tumor-free survival time. Bao et al13 directly injected the ARHI adenovirus vector into human breast tumor in nude mice and found that the tumor volume was significantly reduced.

Although these reports demonstrated the possibility of tumor treatment by targeting the ARHI gene, the mechanism remains unclear. To address this, we did some research; according to our preliminary experiment, we found 3 ovarian cancer cell lines show low expression of ARHI in 9 ovarian cancer cell lines including HOSE, ES2, SKOV3, A2780, 3AO, OVCAR3, HO8910, HO8910PM, and CAOV3. They are SKOV3, OVCAR3, and CAOV3. We reconstructed the PIRES2-EGFP-ARHI plasmid and transfected it into HOC SKOV3 cells with low ARHI gene expression levels. We then investigated the effects and molecular mechanisms of ARHI on cell proliferation in HOC SKOV3 cells and changes to the signal transduction pathways of ARHI protein products. This study has provided an experimental basis for new approaches in the development of HOC therapies.

**MATERIALS AND METHODS**

**Reagents**

Fetal bovine serum was purchased from Hyclone (Logan, Utah), Lipofectamine 2000 from Invitrogen (Carlsbad, CA), Tripure Isolation Reagent from Roche Molecular Biochemicals (Mannheim, Germany), GAPDH (glyceraldehyde phosphate dehydrogenase) antibody from Abmart (Shanghai, China), and phospho-Stat3 (Tyr705) (D3A7) XP rabbit monoclonal antibody. Phospho-p44/42 mitogen-activated protein kinase (MAPK) (ERK1/2) (Thr202/Tyr204) (92G2) rabbit monoclonal antibody were all purchased from Cell Signaling Technology (Beverly, MA). Primer synthesis was performed by Sangon Biological Engineering Technology & Service Co (Shanghai, China). The cell cycle detection kit, cell lysis solution, and cell counting kit 8 (CCK-8) were from Beyotime (Shanghai, China). DNA marker, T4 DNA ligase, and Taq enzyme were all from Takara (San Diego, CA), DNA rapid purification kit was from Omega Bio-Tek (Norcross, GA).

**Construction of the Eukaryotic Expression Vector**

The ARHI gene sequence was obtained from GenBank (EMBL). The primers were designed based on the coding regions of the ARHI gene as follows: sense, 5’-CGGAATTC ATGGGTAACGCCAGCTT-3’; antisense, 5’-CGCCGATCTCC ACATGATTAGCAGTTT-3’ (the underlined GAATTC and CGCGGATCC sequences are the EcoRI and BamHI recognition sites, respectively). The amplified fragment was 690 base pairs. Polymerase chain reaction (PCR) was performed using pcDNA3.0-ARHI as the template (kindly donated by Dr D. B. Badgwell, at The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PCR products were separated by electrophoresis on a 1.5% agarose gel, followed by ethidium bromide staining. The resulting PCR fragments and plasmid were digested with BamHI and EcoRI. The fragments of interest were recovered from the agarose gel, purified, and ligated by T4 DNA ligase to express the PIRES2-EGFP-ARHI plasmid. The ligation mixtures were used for transformation of E. coli cells. The positive recombinant products were selected on LB agar plates using 100 µg/mL ampicillin and confirmed by PCR and DNA sequencing.

**Cell Culture and Transfection**

SKOV3 cells were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China) and were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin 100 U/mL, and streptomycin 100 U/mL at 37°C in a humidified 5% CO2 atmosphere. The cells were seeded in 6-well plates at a density of 34 × 104 cells per well in 2 mL of antibiotic-free medium and incubated for 1 day before transfection at 90% to 95% confluence. Transfection was performed by first diluting 2 µL plasmid and 5 µL Lipofectamine 2000 per well in serum-free medium to a final volume of 250 µL, gently mixing, and incubating at room temperature for 5 min. This was followed by further gentle mixing and incubation at room temperature for 20 minutes. The cells were washed with serum-free medium before the diluted plasmid/Lipofectamine complex was added to the 6-well plates for 4 to 6 hours, after which the complex was replaced with normal cell culture medium. The
cells were visualized 24 hours after transfection under a Nikon Eclipse Fluorescence microscope (Nikon, Japan).

**In Vitro Cell Growth Assay**

The SKOV3 cells were divided into the following 4 groups: cells transfected with PIRES2-EGFP-ARHI plasmid (treated group), cells transfected with PIRES2-EGFP plasmid (positive control group), cells without transfection (negative control group), and a blank control without cells. Cells were passaged at the logarithmic growth phase, trypsinized, and adjusted to 5 × 10^5 cells/mL. The cells were then seeded in 96-well plates in 100 μL 10% fetal bovine serum and cultured for 24, 48, 72, 96, and 120 hours at 37°C in a humidified 5% CO₂ atmosphere. Each experimental group included 6 wells, and all experiments were repeated in triplicate. After incubation for 4 hours under the same condition, 10 μL CCK-8 reagent was added to each well. The optical density value of each well was measured using a microculture plate reader at a wavelength of 450 nm. The growth inhibitory rate (IR) was calculated using the following formula:

\[
IR = \left( \frac{OD_{cell\ control\ well}}{OD_{experimental\ well}} \right) \times 100\%.
\]

**Apoptosis Assay**

The SKOV3 cells were divided into the following 3 groups: PIRES2-EGFP-ARHi-transfected group (treated), PIRES2-EGFP-transfected group (positive control), and cells without transfection (negative control). Each experimental group included 4 wells. The cells were harvested after 48 and 72 hours, and cell apoptosis was detected using Cell Cycle and Apoptosis Analysis Kits (Haimen, China), following the manufacturer’s instructions.

**Western Blotting**

The SKOV3 cells were grouped as described above. Aliquots of cell lysates containing 50 μg of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with TBST (Tris-buffered saline and Tween 20) buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skimmed milk; incubated with rabbit polyclonal antibodies to phospho-Stat3, p44/42 MAPK (ERK1/2 [extracellular signal regulated protein kinase 1/2]), or phospho-p44/42 MAPK (ERK1/2) at 4°C overnight; and followed by the addition of horseradish peroxidase–linked anti-rabbit immunoglobulin G. The bands were visualized by electrochemiluminescence, and the intensity of each Western blot band was analyzed using Quantity One software (BioRad).

**Statistical Analysis**

SPSS version 13.0 software was used for statistical analyses. Comparisons of growth IRs and apoptotic rates were performed by χ² test, and protein relative expression levels were compared by Student t test. P < 0.05 was considered statistically significant.

**RESULTS**

**Confirmation of PIRES2-EGFP-ARHI Plasmid Transfection**

The sequencing results confirmed that the ARHI gene was successfully inserted into PIRES2-EGFP. The nucleotide sequence is as follows:

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ATGGGTAACGCCAGCTTTGGCTCCAAGGAACAGAAGCTGCTTGAGGGTGCTGAGTACCTGCCGACCATTGAAAATACCTACCTGAGCACTACCCGCAGACAGAAGATGTCGACGCAACCGCGCTCTGCAGCGCCACGTTATAGCCCGGGGCCACGCCTTCGTCCTGGTCTACTCAGTCACCAAGAAACACTTGAAAGACAGTGCAAGGGGAGGTGGCCCTGAATGGTGCCACCTGTGC
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**FIGURE 1.** Green fluorescence protein expression 48 hours after transfection: (A) PIRES2-EGFP-ARHISKOV3 treated group; (B) PIRES2-EGFP-SKOV3 plasmid control group; (C) untransfected SKOV3 cells negative control group. The high level of GFP in the treated group compared with the control groups confirms successful transfection.
ARHI Protein Expression Levels in SKOV3 Cells

The micrographs (Figs. 1A-C) showed a high level of green fluorescence protein (GFP) in PIRES2-EGFP-ARHI-SKOV3 cells (Fig. 1A) compared with a low level in PIRES2-EGFP-SKOV3 cells (Fig. 1B); no green fluorescence was observed in untransfected SKOV3 cells (Fig. 1C). The high level of GFP in the treated group compared with the control groups confirms successful transfection.

Analysis of Growth Inhibitory Rates in SKOV3 Cells by ARHI

After the cells had been cultured for 24, 48, 72, 96, and 120 hours, the growth IRs were 64.69%, 70.17%, 67.01%, 66.87%, and 67.70%, respectively, in the test group, and were 27.20%, 31.10%, 29.80%, 30.73%, and 31.28%, respectively, in the plasmid control group (Fig. 2). This showed that the growth IR was significantly increased in the test group compared with the plasmid control group (P < 0.01).

Influence of ARHI on the Cell Cycle Phase Distribution and Apoptosis Rates

The proportions of S-phase cells and apoptosis rates in SKOV3 cells were determined by flow cytometry. After 48 hours of culture, the mean proportions of S-phase SKOV3 cells in the test, plasmid control, and negative control groups were 64.18%, 38.43%, and 15.15%, respectively, and the mean apoptosis rates were 47.97%, 26.53%, and 9.33%, respectively. After 72 hours of culture, the mean proportions of S-phase SKOV3 cells in the test, plasmid control, and negative control groups were 43.29%, 10.37%, and 10.89%, respectively, and the apoptosis rates were 51.34%, 24.70%, and 4.39%, respectively (Table 1). Data show that the proportions of S-phase cells, and thereby S-phase arrest, were clearly higher in the PIRES2-EGFP-ARHI-SKOV3 cell group compared with the control group at both 48 and 72 hours; the result at 72 hours was much more significant in the test group. In addition, the apoptosis rate was significantly increased in the test group compared with the control group (P < 0.01).

Analysis of Protein Expression in SKOV3 Cells by Western Blotting

The Western blot images for P-STAT3 (phosphorylated signal transduction and activators of transcription 3) and P-ERK protein expression for the control and treated groups: SKOV3, PIRES2-EGFP-SKOV3, and PIRES2-EGFP-ARHI-SKOV3, are shown in Figure 3. Compared with GAPDH reference protein, the relative expression levels of P-STAT3 were 1.1473 ± 0.0002, 1.0913 ± 0.0021, and 0.7424 ± 0.0006, respectively, and the relative expression levels of P-ERK were 1.2260 ± 0.0011, 1.1289 ± 0.0018, and 0.5866 ± 0.0013, respectively. In comparison to the 2 control groups, the expression levels of P-STAT3 and P-ERK proteins were significantly lower in the PIRES2-EGFP-ARHI-SKOV3 group (P < 0.05).

DISCUSSION

Cell cycle progression is regulated by multiple control points at different phases of the cell cycle; the 3 principal ones being G1/S, G2/M, and at metaphase/anaphase transmission during mitosis. Failure of these control points can lead to abnormal growth or apoptosis. The G1/S check point is the most critical for control of cell proliferation via intracellular and extracellular signals related to transportation and integration of molecules into the nucleus.14

By reconstructing the PIRES2-EGFP-ARHI plasmid and successfully transfecting it into HOC SKOV3 cells with low ARHI expression levels, as shown by high levels of green fluorescence from the plasmid observed by fluorescent

| TABLE 1. Influence of ARHI on the cell cycle phase distribution and apoptosis rate |
|---------------------|--------|-------------|--------|--|
|                   | n     | G0/G1, %    | S, %   | G2/M, % |
| 48 h              |       |             |        |       |
| SKOV3 group       | 4     | 63.326      | 15.150 | 21.520 | 9.333 |
| PIRES2-EGFP- SKOV3 group | 4 | 49.707      | 38.425 | 11.868 | 26.526 |
| PIRES2-EGFP-ARHI-SKOV3 group | 4 | 29.570      | 64.182 | 6.262  | 47.971 |
| 72 h              |       |             |        |       |
| SKOV3 group       | 4     | 70.511      | 10.888 | 18.597 | 4.387 |
| PIRES2-EGFP- SKOV3 group | 4 | 66.494      | 11.565 | 15.411 | 20.551 |
| PIRES2-EGFP-ARHI-SKOV3 group | 4 | 43.174      | 43.286 | 12.041 | 51.340 |
Changes in the expressions levels of P-ERK1/2 and P-STAT3 in the different SKOV3 groups: lane 1, protein expression in the untransfected SKOV3 control group; lane 2, protein expression 48 hours after PIRE52-EGFP was transfected into SKOV3 cells; lane 3, protein expression 48 hours after PIRE52-EGFP-ARHI was transfected into SKOV3 cells. The results show that ARHI significantly reduces P-ERK1/2 and P-STAT3 expression levels.

As studied by Lu et al., the ARHI gene main-

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