Downregulation of *HSPA2* inhibits proliferation via ERK1/2 pathway and endoplasmic reticular stress in lung adenocarcinoma

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Background: To explore the mechanisms of *HSPA2* downregulation in inhibiting the proliferation of lung adenocarcinoma.

Methods: We obtained 85 specimens of human lung adenocarcinoma and specimens of adjacent nontumor tissues from the First Affiliated Hospital, School of Medicine, Zhejiang University. We then analyzed the expression of *HSPA2* in these tissues and in lung adenocarcinoma and normal lung cell lines. Human lung adenocarcinoma cell lines were transfected with siRNA silencing *HSPA2* and subjected to colony forming, Thiazolyl blue tetrazolium bromide (MTT), propidium iodide flow cytometry, immunofluorescence assay and western blotting to explore the causes of the reduction in the proliferation of lung adenocarcinoma cells and the endoplasmic reticulum stress induced by *HSPA2* downregulation. Finally, we confirmed these mechanisms via rescue assay.

Results: Greater *HSPA2* expression was found in the lung adenocarcinoma specimens than in the specimens of adjacent nontumor tissues, and greater expression was found in lung adenocarcinoma cell lines than in normal cell lines. *HSPA2* knockdown via siRNA reduced proliferation and led to G1/S phase cell cycle arrest in the lung adenocarcinoma cell lines. G1/S phase cell cycle arrest triggered by *HSPA2* downregulation could be attributed, at least in part, to phosphorylation and activation of the Erk1/2 pathway and probably to activation of IRE1α/PERK-mediated endoplasmic reticulum stress.

Conclusions: *HSPA2* plays an important role in the origin and development of lung adenocarcinoma. It is thus deserving of further study as a promising clinical therapeutic target.

Keywords: Lung adenocarcinoma; *HSPA2*; G1/S cell cycle arrest; Erk1/2

Introduction

Malignant tumors are among the most horrible diseases of the 21st century, and lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death (1). Most lung cancer diagnoses (85%) are non-small cell lung cancer (NSCLC) (2), including lung adenocarcinoma. The 5-year survival estimates for NSCLC range from 73%

for stage IA disease to 13% for stage IV disease (3). The increasing morbidity rates of lung adenocarcinoma has led to a relative flood of research.

HSPA2 is a variant in the HSP70 family and it is an essential in spermiogenesis. In oncology research, *HSPA2* was first reported in renal cell carcinoma (4). Several studies have shown that *HSPA2* plays important roles in the cell growth and proliferation of hepatocellular carcinoma,

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nasopharyngeal carcinoma, esophageal squamous cell carcinoma, NSCLC, pancreatic ductal adenocarcinoma, colorectal carcinoma and breast carcinoma (5-10). Increased expression of *HSPA2* was significantly related to a shorter overall duration of survival in patients with stage I-II carcinoma (7).

However, unlike other members of the HSP70 family, the function of *HSPA2* in carcinogenesis and the development of lung cancer has rarely been reported. We thus explored the role of *HSPA2* in lung cancer cell proliferation to determine how the downregulation of *HSPA2* inhibits the proliferation of lung adenocarcinoma cells via multiple mechanisms.

Methods

Patients and tissue specimens

Eight-five clinical pathology specimens of lung adenocarcinoma tissue and matched adjacent nontumor tissue were collected from the Department of Thoracic Surgery, the First Affiliated Hospital, School of Medicine, Zhejiang University with the approval of Research Ethics Committee of the First Affiliated Hospital, College of medicine, Zhejiang University. The procedure was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All patients had given signed informed consent. The specimens of adjacent nontumor tissue for each patient were obtained more than 3 cm from carcinoma tissue.

Cell lines and cell culture

We used human lung adenocarcinoma cell lines (including A549, H1975, and H292), human bronchial epithelium, and human embryonic lung fibroblasts (MRC-5) for our experiments. All cell lines were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were cultured at 37 ℃ in a humidified atmosphere (Thermo Fisher Scientific) in the presence of 5% CO_2-95% air and were grown in RMPI-1640 supplemented with 10% Fetal Bovine Serum (FBS).

Real-time PCR

Total RNA was extracted from tissues by TRIzol reagent (Invitrogen Life Technologies) and used to synthesize cDNA (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed according to standard protocols.

Transfection of HSPA2 siRNA and overexpressing plasmid

The cells were plated in six-well plates and allowed to attach overnight. With the application of lipofectamin2000, HSPA2 siRNA sequenced and overexpressing plasmid was transfected into A549 and H1975 cell lines to knockdown or overexpress the *HSPA2* gene respectively.

Colony formation assay and MTT assay

After the cells being transfected as needed, colony formation assay was performed in six-well plates with cell culture for 14 days without disturbance. We washed the media in triplicate with phosphate-buffered saline (PBS) solution followed by 0.1% crystal violet for 15 minutes.

We seeded A549 and H1975 cells (being transfected as needed) into 96-well plates at 80% to 90% subconfluence. MTT was added to the medium for 3 hours of incubation after 24, 48, and 72 hours of incubation. The cell culture medium was then mixed with 150 μL of dimethyl sulfoxide for 10 minutes, and the optical absorbance was measured at 490 nm.

Immunofluorescence assay

We performed transfection in culture dishes and labeled ER with ER-Tracker™ Green dyes (Invitrogen Life Technologies) according to the instructions of the manufacturer. Washed the cell for three times with PBS for 5 minutes and visualized at room temperature using a Carl Zeiss microscope (Carl Zeiss AG, Jena, Germany). The exposure times between treatments were consistent and the brightness and contrast of the images were adjusted using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA) for presentation.

Cell cycle assay

We performed flow cytometry according to standard protocols of DNA staining solution (Multisciences Biotech Co., Ltd., China). After transfection, the cells were collected and washed with PBS. The cell pellets were obtained by centrifugation and the supernatant was discarded. Then added 1 mL of DNA staining solution incubated the cell samples for 30 minutes at room temperature in the dark for the flow cytometry.
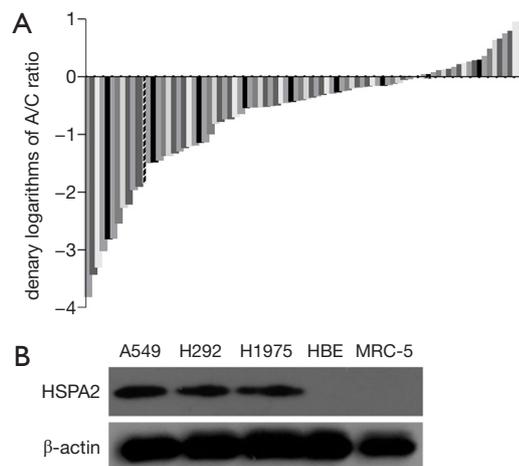
Figure 1 Expression of *HSPA2* was higher in tissue and cell lines of lung adenocarcinoma. (A) Expression level comparison of *HSPA2* in lung adenocarcinoma specimens and specimens of adjacent nontumor tissue. *HSPA2* expression was greater in the lung adenocarcinoma specimens than in those of adjacent nontumor tissue. The denary logarithms of the A/C ratio (C indicates the expression of *HSPA2* in cancer tissue, and A indicates the expression of *HSPA2* in the adjacent nontumor tissue, all with ΔCT value) are shown from low to high. Negatives showed greater expression in lung adenocarcinoma specimens than in those of adjacent nontumor tissues. (B) Expression level comparison of HSPA2 in lung adenocarcinoma and normal cell lines. Western blot result shows greater HSPA2 expression in lung cancer cell lines (A549, H1975, H292) than in normal lung cell lines (human bronchial epithelium, MRC-5).

Western blot

The cells were sonicated in the proper amount of radioimmunoprecipitation assay buffer, and 20 μg of protein was resolved on 12% polyacrylamide sodium dodecyl sulfate gels. The proteins were then transferred to polyvinylidene difluoride membranes, which were blocked with 3% bovine serum albumin in Tris-buffered saline-Tween 20. The membranes were incubated successively with the corresponding primary antibodies and secondary antibodies. Finally, the proteins were visualized with chemiluminescent horseradish peroxidase substrate.

Statistical analysis

All experiments were performed in triplicate, and the results were analyzed with GraphPad Prism 7.0. P values of less than 0.05 were considered to indicate statistical significance.

Results

HSPA2 showed greater expression in tissue and cell lines of lung adenocarcinoma than in adjacent nontumor tissue and normal cells

We applied qRT-PCR to measure tissue specimens from 85 patients. Every column represented one patient, and the A/C ratio indicated the ratio of PCR results between group A (adjacent nontumor tissues) and group C (lung adenocarcinoma tissues). The qRT-PCR results of demonstrated that HSPA2 showed greater expression in lung adenocarcinoma tissues than in the adjacent nontumor tissues (*Figure 1A*).

Western blotting showed that the lung adenocarcinoma cell lines had a significantly higher expression level of HSPA2 than the human bronchial epithelium and MRC-5 cell lines (*Figure 1B*).

Knockdown of HSPA2 reduced proliferation of adenocarcinoma cells

Selection of an effective siRNA sequence to knock down *HSPA2* gene expression

We found siRNA sequence no.1532 to be the most effective, so it was applied in the subsequent experiments (*Figure 2A*).

Knockdown of *HSPA2* reduced proliferation of A549 and H1975 cells

MTT assay and colony formation assay demonstrated that knockdown of *HSPA2* reduced the proliferation of A549 and H1975 cells (*Figure 2B,C*).

Knockdown of HSPA2 induced G1/S phase cell cycle arrest of A549 and H1975 cells

The results of flow cytometry show that G1 phase cell increased and G2 phase cell decreased in the *HSPA2* knockdown group of A549 and H1975 cell lines (*Figure 3A*). These results indicated that knockdown of *HSPA2* induced G1/S phase cell cycle arrest.

Cell cycle regulation proteins, including CDK4, cyclin D1, cyclin D3, and cyclin E1, showed reduced expression in the *HSPA2* knockdown group. G1/S phase checkpoint protein p-Rb(S795) and p-Rb(S807/811) were also downregulated, whereas Rb protein showed no significant difference between the two groups (*Figure 3B*).
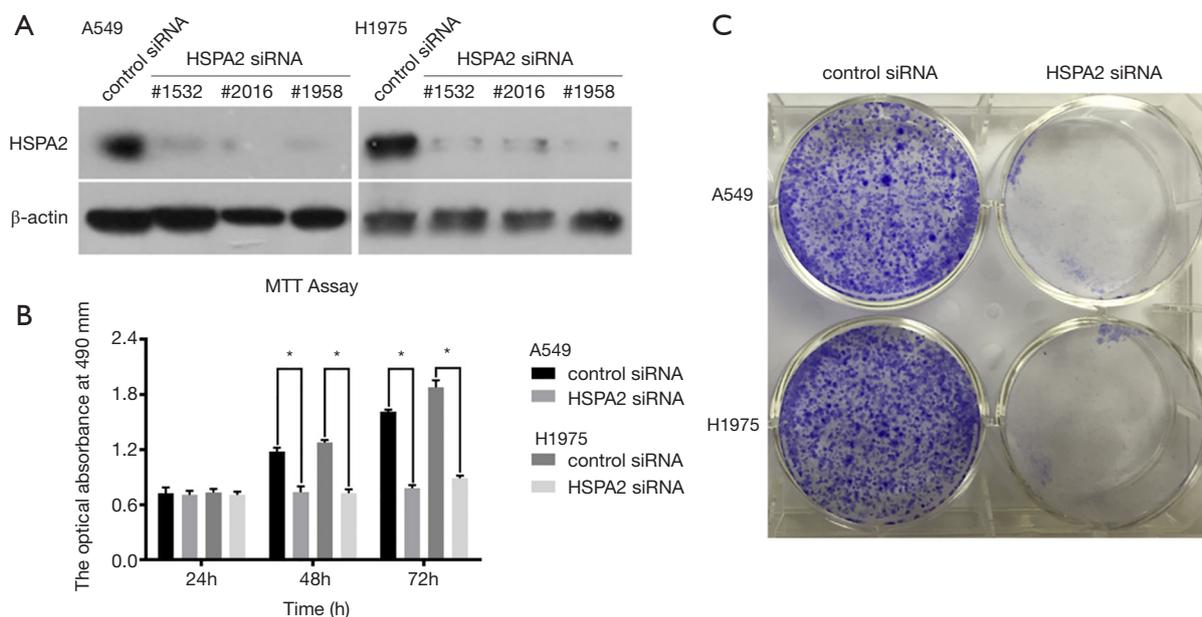
Figure 2 Knockdown of *HSPA2* reduced proliferation of adenocarcinoma cells. (A) Effects of HSPA2 siRNA on the expression of *HSPA2*. Western blot analysis demonstrates the effect of HSPA2 siRNA transfection on *HSPA2* expression in A549 and H1975 cells. (B) *HSPA2* knockdown reduced the proliferation of A549 and H1975 cells by MTT assay. The values obtained from the transfected and control cells represent the mean ± SD of three independent experiments. (C) *HSPA2* knockdown reduced the proliferation of A549 and H1975 cells by representative colony formation assay. *P<0.05.

Knockdown of HSPA2 activated Erk1/2 signaling transduction pathway and Erk1/2 inhibitor rescue experiment

We found an increased phosphorylation level of Erk1/2 protein in both A549 and H1975 cell lines. The total protein level, relative protein level of STAT3, and the Akt pathway remained stable (*Figure 4A*). We presumed that *HSPA2* could participate in the Erk1/2 cell signal transduction pathway. Activation of the Erk1/2 pathway induces cell cycle arrest in lung adenocarcinoma.

To confirm the former result, we rescued the G1/S phase cell cycle arrest by 24-hour treatment with Selumetinib (specific phosphorylation Erk1/2 inhibitor) after *HSPA2* knockdown in A549 and H1975. *Figure 4B* shows that Selumetinib was able to rescue cell cycle arrest of A549 and H1975 cells.

Knockdown of HSPA2 enhanced ER stress in A549 and H1975 cells

Immunofluorescence

We hypothesized that organelles may participate in the process of G1/S phase arrest induced by *HSPA2* knockdown.

We thus selected a specialized endoplasmic reticulum (ER) marker to stain the ER of A549 and H1975 transfected by HSPA2 siRNA or blank siRNA. We then observed them via laser confocal microscopy (LCM). Immunofluorescence demonstrated greater signal enhancement in the HSPA2 knockdown group than in the control group (*Figure 5A*), which indicates an increase in ER stress (11). These results confirmed our hypothesis.

Western blot

We focused the ER stress signaling pathway protein (ATF6, IRE1α, PERK) and Bip (modulin of IRE1α protein). Western blotting showed that an increase in the expression levels of IRE1α and PERK and a decrease in Bip (*Figure 5B*).

Rescue assay via HSPA2 knockdown and overexpression

We observed the expression levels of HSPA2, CDK4, cyclin D1, cyclin D3, cyclin E1, p-Rb(S795), p-Rb(S807/811), p-Erk1/2, IRE1α, PERK, and Bip in the previous experiments. *HSPA2* overexpressing plasmid was applied in A549 cell line. By comparing the changing protein levels in the control group (Con), the knockdown group (KD), the overexpression group (OE), and the rescue group (KD +
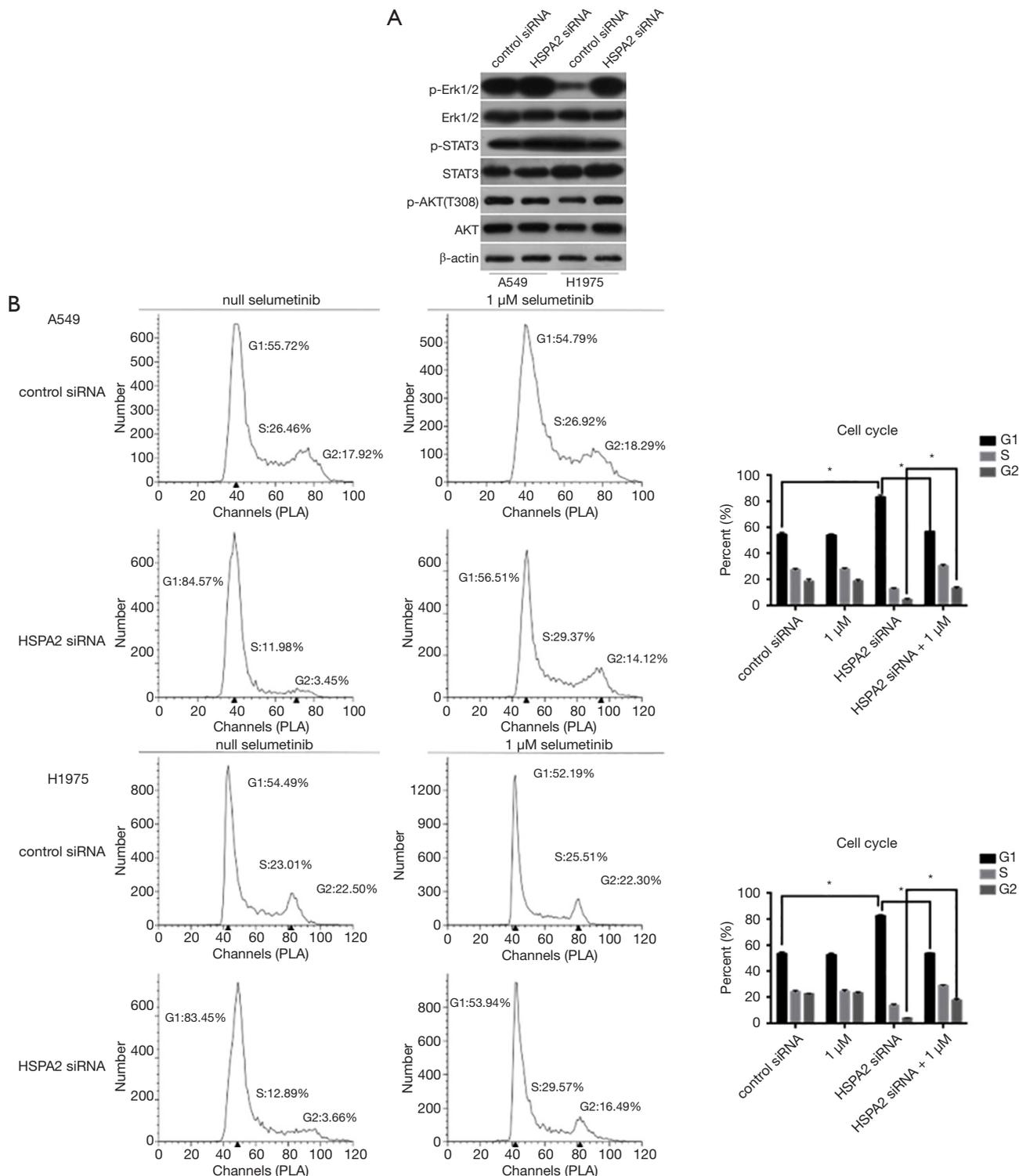
Figure 3 Knockdown of *HSPA2* induced G1/S phase cell cycle arrest. (A) *HSPA2* knockdown induced the G1/S phase cell cycle arrest of A549 and H1975 cells. Cell cycle profiles of A549 and H1975 cells transfected with HSPA2 siRNA and control cells were determined by flow cytometry and error bars. Cell count versus PI staining is shown (10,000 per treatment). (B) *HSPA2* knockdown induced the change of G1/S phase regulation and checkpoint proteins of A549 and H1975 cells. Western blot analysis of G1/S phase cell cycle regulation and checkpoint proteins with A549 and H1975 cell transfected with HSPA2 siRNA and control cells are shown. *, P<0.05.
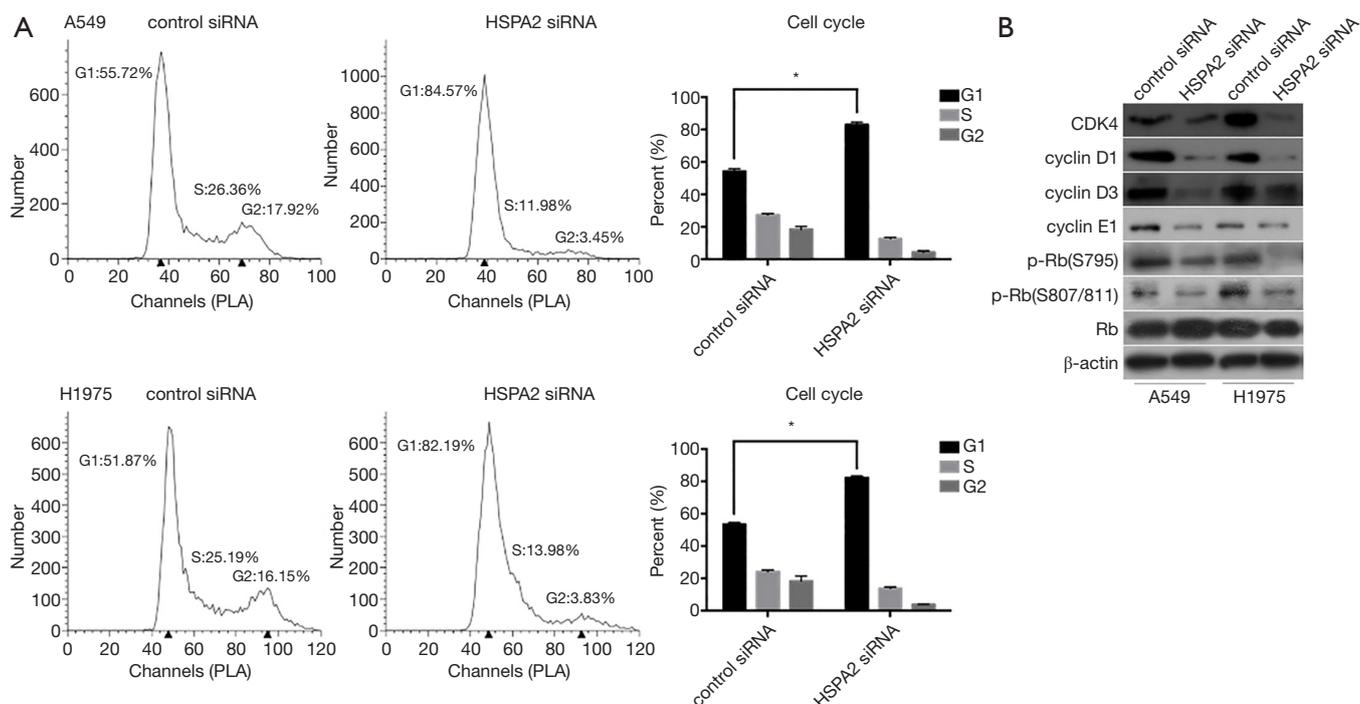
Figure 4 Knockdown of *HSPA2* activated Erk1/2 signaling transduction pathway. (A) Downregulation of *HSPA2* related with Erk1/2 signaling transduction pathway and c-Myc protein. Western blot analysis of proteins in pathways involved in cell cycle regulation with A549 and H1975 cells transfected with HSPA2 siRNA and control cells. (B) Verification of the finding that *HSPA2* knockdown triggers the activation of the Erk1/2 pathway. Cell cycle profiles of A549 and H1975 cells with or without *HSPA2* knockdown or Selumetinib (1 μM) and error bars were determined by flow cytometry. *, P<0.05.

OE), we confirmed the conclusion that downregulation of *HSPA2* inhibits proliferation via the ERK1/2 pathway and ER stress in lung adenocarcinoma (*Figure 6*).

Discussion

Ferrucio Ritossa proposed the existence of the heat shock response in the 1960s (12). It is now recognized that the heat shock response is widespread in most kinds of species and that cells surrounding the exchanging protein structure would show a heat shock response. A major characteristic of the heat shock response is that it encodes the gene signals of heat shock protein (HSPs) and then induces and amplifies the follow-up biological response. Heat shock response decreases the negative effect by means of environmental or endogenous molecular stress (13).

Molecular chaperone *HSPs* control protein folding to participate in the intracellular balance. Some *HSPs* serve as the molecular chaperones of other hyperspecialization proteins, such as signal transduction proteins and transcription factors.

Some *HSPs* show resistance to apoptosis and participate in some cellular immune responses (14). These findings have attracted scholars to study and induced lots of clinical applications.

The largest family of *HSPs* is the *HSPA (HSP70)* family, which belongs to the human protein group (15). *HSPA2* is a variant of this family. It was first found in testicular tissue and is highly expressed in pachytene stage spermatocytes in rodents. We therefore regard *HSPA2* as a specific expression protein of the testes (16).

HSPA2 protein is thought to be abnormally expressed in lung alveolar carcinoma (17). The construction of HSPA2 protein directly influences its regulated function in the progression of lung cancer (18). Biomarkers (7,19,20) and polymorphism (21) analysis have been the main achievements of *HSPA2* research for lung cancer, but studies of the mechanism are rare. In 2016, Chang *et al.* showed interaction between HSPA2 and JAG1, one of the ligands in the Notch pathway. The improved transcriptional level of JAG1 was related with HSPA2, and both participated in the
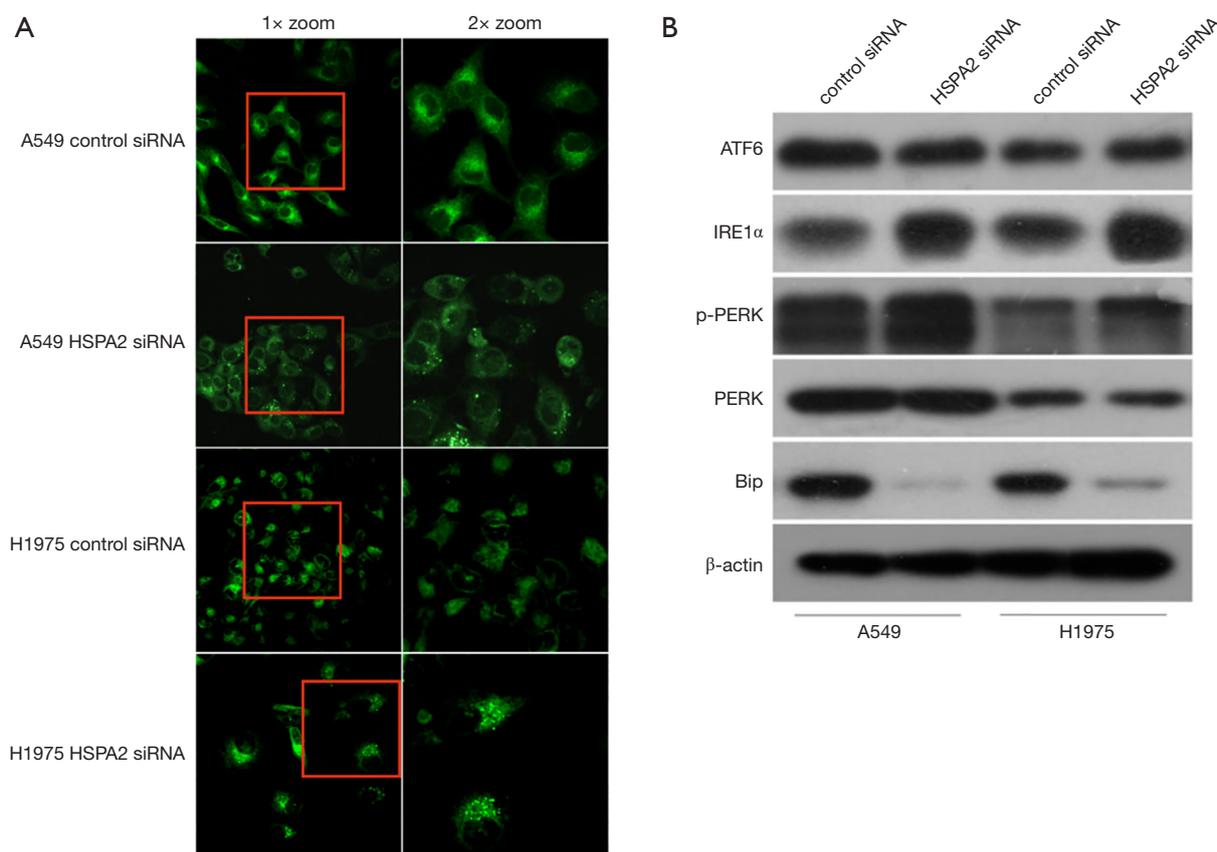
Figure 5 Knockdown of *HSPA2* enhanced ER stress. (A) *HSPA2* participates in the ER stress of A549 and H1975 cells. Representative ER-Tracker green dye assays of HSPA2 siRNA–transfected and control A549 and H1975 cells. Highlights of the cells represent the occurrence of ER stress. Left: 1×Zoom; Right: 2×Zoom. (B) Verification of the participation of *HSPA2* in the ER stress of A549 and H1975 cells. Western blot analysis of proteins in ER stress pathways with A549 and H1975 cells transfected with HSPA2 siRNA and control cells.
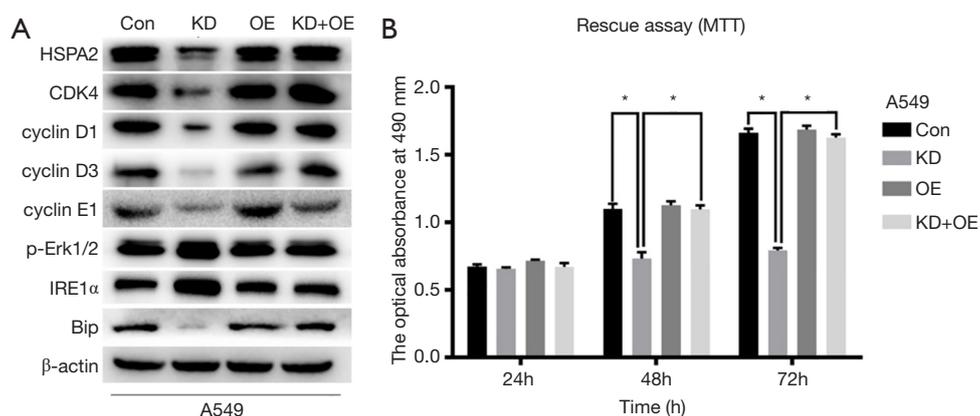
Figure 6 Rescue assay via HSPA2 knockdown and overexpression. (A) Rescue assay shows that *HSPA2* overexpression and plasmid transfection could rescue the downregulation of *HSPA (HSP70)*, *CDK4*, *cyclin D1*, *cyclin D3*, *cyclin E1* and *Bip*. Meanwhile, the overexpression of p-Erk1/2 and IRE1α were reversed. (B) *HSPA2* knockdown reduced the proliferation of A549 cells and *HSPA2* overexpression reversed the decreasing trend by MTT assay. The values obtained from the transfected and control cells represent the mean ± SD of three independent experiments. *, P<0.05.

process of malignant transformation of lung cancer (22). We have found no further studies of the mechanism of *HSPA2* function in lung adenocarcinoma.

In this study, we observed *HSPA2* expression in lung adenocarcinoma tissue and relative cell lines. Our results show greater *HSPA2* expression in the lung adenocarcinoma specimens than in the adjacent nontumor tissues, which was confirmed by western blot of the cell lines.

The proliferation of lung adenocarcinoma cell lines (A549 and H1975) decreased after *HSPA2* knockdown, which was induced by G1/S phase cell cycle arrest as the expression of relative protein in the G1/S phase cell cycle changed accordingly. After downregulation of *HSPA2*, western blotting showed increasing phosphorylation of Erk1/2. The extracellular signal–regulated kinase ERK1 and ERK2 (ERK1/2) cascade regulates a variety of cellular processes via phosphorylation of multiple target proteins. The outcomes of its activation range from stimulation of cell survival and proliferation to triggering of tumor suppressor responses such as cell differentiation, cell senescence, and apoptosis (23). The hyperphosphorylation of Erk1/2 protein could promote cell cycle arrest by regulating p16 (24), p21 (25), and p27 (26), which are common inhibitors of G1/S cyclin-dependent kinase. The function of increasing the phosphorylation level of Erk1/2 protein in cell cycle regulation was consistent with our results.

The ER is the major site of protein synthesis and transport in eukaryotic cells and can transport abnormal proteins to the cytoplasm. Those proteins were degraded by proteasome via ER-associated degradation. A disorder of ER-associated degradation would induce ER stress and trigger the unfolded protein response. IRE1α, PERK, and ATF6 protein represent three branches of the unfolded protein response (27). Studies of ER stress in the regulation of tumor cell cycle arrest have been reported (28,29). Meanwhile, the functions of *HSPs* in ER stress have been frequently studied (30-34). However, the relationship between *HSPA2* and ER stress remains unknown. Our study shows that upregulation level of IRE1α and PERK protein in the *HSPA2* knockdown group is comparable with that in the control group. It is worth noting that the Erk1/2 cascade have a reported relationship with ER stress. Erk1/2 has been intimately linked with the IRE1α and PERK stress pathway. Erk1/2 protein activation was inhibited after ER stress and IRE1α pathway activation, but the mechanism was unclear (35). A similar phenomenon has been observed in gastric cancer cells, in which activation of Erk1/2 inhibited Bip protein in the IRE1α pathway, after which

apoptosis was induced by ER stress (36).

The activation of Erk1/2 mediated phosphorylation of CHOP (downstream of the PERK pathway). This process could change the activity of its own transcriptional activation domain, subsequently changing its affinity with the binding protein and ultimately leading to changes in the gene expression profiles (37). In a study of HeLa cells, Erk1/2 also played an important role in the process of apoptosis induced by the high expression level of CHOP, and Erk1/2 also mediated the phosphorylation of CHOP (38). It remains uncertain which genes were dependent on CHOP phosphorylation and were responsible for alteration of the cellular phenotype associated with triggering the subsequent progression of cell apoptosis.

Therefore, when the downregulation of *HSPA2* induces ER stress, crosstalk may occur with the high phosphorylation level of Erk1/2. Erk1/2 activation leading to ER stress or ER stress happening before Erk1/2 protein phosphorylation require further study, and the relationship between ER stress and the cell cycle requires further discussion.

Conclusions

We observed greater expression of *HSPA2* in lung adenocarcinoma tumor tissues and cell lines than in the adjacent nontumor tissues and normal cell lines. It was confirmed that *HSPA2* gene silencing effectively inhibited the proliferation of lung adenocarcinoma cell lines. *HSPA2* gene silencing led to the G1/S phase arrest of A549 and H1975 cells. It was demonstrated that *HSPA2* caused G1/S phase arrest in A549 and H1975 cells via regulation of the Erk1/2 pathway and by triggering ER stress, thus inhibiting the proliferation of A549 and H1975 cells. We confirmed, for the first time, that *HSPA2* plays an important role in the occurrence and development of lung adenocarcinoma. It is thus worthy of further study as a promising clinical therapeutic target.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study has been approved by the ethics committee of the First Affiliated Hospital of Zhejiang University in China and written informed consent was obtained from eligible guardians. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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