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RESEARCH ARTICLE

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The influence of the NRG1/ERBB4 signaling pathway on pulmonary artery endothelial cells

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Abstract

This study aimed to examine the influence of the Neuregulin-1 (NRG1)/ERBB4 signaling pathway on the function of human pulmonary artery endothelial cells (HPAECs) and investigate the underlying mechanisms. Enzyme-linked immunosorbent assay indicated that ERBB4 levels in the serum of patients with pulmonary embolism (PE) were significantly higher than those of healthy controls ($p < 0.05$). In cellular studies, thrombin stimulation for 6 h led to a significant decrease in cell viability and overexpression of ERBB4 compared to control ($p < 0.05$). In the NRG1 group, apoptosis of HPAECs was reduced ($p < 0.05$), accompanied by a decrease in ERBB4 expression and an increase in p-ERBB4, phosphorylated serine/threonine kinase proteins (Akt) (p-Akt), and p-phosphoinositide 3-kinase (PI3K) expression ($p < 0.05$). In the AG1478 group, there was a significant increase in HPAEC apoptosis and a significant decrease in p-ERBB4 and ERBB4 expression compared to the Con group ($p < 0.05$). In the AG1478 + NRG1 group, there was an increase in the apoptosis rate and a significant decrease in the expression of p-ERBB4, ERBB4, p-Akt, and phosphorylated PI3K compared to the NRG1 group ($p < 0.05$). In animal studies, the PE group showed an increase in the expression of ERBB4 and p-ERBB4 compared to the Con group ($p < 0.05$). NRG1 treatment led to a significant reduction in embolism severity with decreased ERBB4 expression and increased p-ERBB4 expression ($p < 0.05$). Gene set enrichment analysis identified five pathways that were significantly associated with high ERBB4 expression, including CHOLESTEROL HOMEOSTASIS, OXIDATIVE PHOSPHORYLATION, and FATTY ACID METABOLISM ($p < 0.05$). Therefore, NRG1 inhibits apoptosis of HPAECs, accompanied by a decrease in ERBB4 and an increase in p-ERBB4. NRG1 inhibition in HPAECs apoptosis can be partially reversed by inhibiting

Jin-Bo Huang, Qin Shen, and Zhi-Qi Wangs are contributed equally to this study.

ERBB4 expression with AG1478. ERBB4 has the potential to be a novel biological marker of PE.

KEYWORDS
chronic thromboembolic pulmonary hypertension, ERBB4, human pulmonary artery endothelial cells, Neuregulin-1

INTRODUCTION

Chronic thromboembolic pulmonary hypertension (CTEPH) is a rare and progressive disease characterized by persistent chronic thrombosis and mean pulmonary artery pressure >25 mmHg after 3 months of standard anticoagulation therapy, excluding other diseases.[1] However, its pathogenesis remains unclear. It is commonly attributed to pulmonary embolism (PE) initiated by deep venous thrombosis. Acute episodes of PE can lead to increased resistance in the pulmonary artery and mechanical damage to pulmonary artery endothelial cells (HPAECs), triggering inflammatory responses in vessels.[2] Inflammatory mediators contribute to sustained spasms in the pulmonary arterioles and increase pulmonary arterial pressure. This combined mechanical and inflammatory insult results in endothelial damage and dysfunction, as evidenced by abnormal cell proliferation and decreased susceptibility to apoptosis, leading to excessive growth of the pulmonary artery intima.[3] These changes are crucial in vascular remodeling and exacerbate pulmonary hypertension (PH), eventually progressing to CTEPH.[4] Recognized as the most severe long-term consequence of PE, CTEPH significantly affects morbidity and mortality. Without early diagnosis and intervention, it can progress to respiratory insufficiency and right-sided heart failure, resulting in mortality.[5] Therefore, understanding the dysfunctional behavior of HPAECs and their role in the transition from PE to CTEPH is of significant clinical importance, and research on the proliferative mechanisms of HPAECs is particularly critical.

The conventional approach to treat CTEPH includes surgical intervention, specifically pulmonary endarterectomy (PEA), which is the preferred treatment option with curative potential. However, approximately 40% of patients are not eligible for PEA due to various factors,[6] highlighting the importance of medical management. Medical therapy mainly involves anticoagulation and medications that improve right heart function. However, these are insufficient to address structural blockages in the small pulmonary arteries. The advent of novel targeted therapies has recently offered hope to nonsurgical candidates.[7] These new drugs target pathways such as nitric oxide (NO), endothelin (ET)-1 receptors, and prostaglandin (PGI),[8] and exert vasodilatory, antithrombotic, and antiproliferative effects. Studies have shown that targeted drug therapy can significantly improve hemodynamics, exercise capacity, and quality of life in patients with CTEPH,[9] highlighting the need to identify novel therapeutic targets.

The ERBB gene family encodes proteins belonging to the epidermal growth factor receptor (EGFR) subfamily, including ERBB1, ERBB2, ERBB3, and ERBB4, which are extensively expressed in human tissues.[10,11] These receptors are characterized by their single-pass transmembrane structure and comprise extracellular ligand-binding domains, transmembrane regions, intracellular tyrosine kinase domains, and C-terminal domains. Neuregulin-1 (NRG1), a member of the protein family with an epidermal growth factor-like motif,[12,13] influences neuronal differentiation, migration, survival, neurite outgrowth, and synaptic functions.[14,15] Among its receptors, ERBB4 is the only one specifically activated in response to NRG1 and functions as a tyrosine kinase upon ligand binding. This interaction leads to the phosphorylation of tyrosine residues, which facilitates interactions with various signaling molecules, such as growth factor receptors, the p85 subunit of phosphoinositide 3-kinase (PI3K), phospholipase Cγ2 (PLCγ2), and Src, thus activating downstream pathways. Furthermore, NRG1/ERBB4 signaling involves proteolytic cleavage that releases an active peptide fragment into the nucleus, influencing the transcription of genes related to inflammation, pulmonary fibrosis, endothelial proliferation, acetylcholine receptor subunit expression, and neural circuit stability.[16,17]

Studies have shown that H_2O_2 induces a significant increase in reactive oxygen species (ROS) in PC12 cells (expressing the ERBB4 receptor), and activation of PI3K or the use of NRG1 can prevent H_2O_2-induced ROS increase, block the ERBB4 receptor, or inhibit PI3K activity; NRG1 loses its protective effect against H_2O_2 damage.[18] Phosphorylation of the ERBB4 receptor in tyrosine has been suggested to inhibit ROS production through the PI3K signaling pathway. Other studies have

shown that blocking ERBB2 receptors with ERBB2 antibodies or siRNA can increase ROS content in cardiomyocytes.[19] Therefore, we hypothesized that the effect of ERBB4 receptor tyrosine phosphorylation on pulmonary vessels is related to the inhibition of ROS damage via PI3K/serine/threonine kinase proteins (Akt) signaling. Although NRG1/ERBB4 signaling is known to regulate the proliferation of cardiomyocytes and neuronal cells,[20] its effect on HPAECs warrants further investigation.

MATERIALS AND METHODS

Data collection

Serum samples were taken from 30 patients with PE and 30 healthy subjects at the Affiliated Hospital of Nantong University, Jiangsu Province, from 2020 to 2021. Samples were obtained before any treatment, in a fasting state in the morning, then left to stand at room temperature for 1 h, centrifuged at 3000 rpm for 20 min, and the supernatant was stored at −80°C. Diagnosis of PE was made according to the 2018 Chinese PE guidelines.

Reagents

Anti-ERBB4 antibody (ab219208, 1:1000)(Abcam), Anti-ERBB4 (phosphor Y1162) antibody (ab68478, 1:1000)(Abcam), Anti-pan-A kt (ab8805, 1:500)(Abcam), Anti-A kt (phospho T308) antibody (ab38449, 1:1000)(Abcam), PI3K antibody (ab86714, 1:1000)(Abcam), phosphorylated PI3K (p-PI3K) antibody (ab182651, 1:1000)(Abcam), polymerase chain reaction primers (Nantong Aoxiang Biology), fetal bovine serum, high-glucose Dulbecco's modified eagle medium DMEM culture medium (Gibco), lipofectamine 2000 transfection reagent (ThermoFisher), cell counting kit-8 (CCK-8, Biosharp Biology), and apoptosis assay kit (Nanjing Fumais Biology).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected in the morning after fasting, without prior medication, at 24°C for 1 h, centrifuged at 3000 rpm for 20 min, and the supernatant was transferred to clean EP tubes. After adding the biotin-conjugated antibody, the absorbance at 450 nm was measured using an ELISA reader. ERBB4 mass concentration was calculated from a standard curve using Microsoft Excel.

HPAECs culture and transfection

HPAECs, acquired from the Cell Bank of the Chinese Academy of Sciences, were cultured in a medium with 10% fetal bovine serum in an incubator at 37°C and 5% CO_2 until fully adherent (48−72 h). After reaching confluence, 2×10^4 cells were seeded in each well of 24-well plates and infected with the respective viruses. After 6 h of transfection, the medium was replaced.

CCK-8 proliferation assay

After harvesting, cells were seeded at a density of 2000 cells per well in 96-well plates to ensure an even distribution. Basic culture medium was added to the peripheral wells of the plates. The experiments included control, CD36 knockdown, and CD36 overexpression groups with multiple replicates in four 96-well plates. Cell viability was monitored by adding CCK-8 to each well and measuring optical density (OD) at 450 nm. The results were plotted with time on the x-axis and OD values on the y-axis to generate the growth curves.

Western blot (WB) analysis

Cellular proteins were extracted from collected cells, quantified, and subjected to WB using anti-β actin and anti-ERBB4 antibodies. Grayscale scanning was performed after exposure to determine the relative expression levels of the target proteins.

Flow cytometry for apoptosis

Approximately 100 μL of cell suspension was treated with 10 μL of 20 μg/mL propidium iodide and 5 μL Annexin V/AlexaFluor 647, mixed well, and incubated at room temperature for 15 min in the dark. The volume of phosphate-buffered saline added was adjusted according to cell density, and the samples were analyzed by flow cytometry.

Establishment of animal model

Mice weighing between 23 and 27 g were administered 20 U/kg thrombin intravenously in the internal jugular vein. Mice were assigned to sham, PE, and drug treatment groups. The sham group received only saline intravenously, all other procedures were identical,

while the NRG1 group was injected with 140 ng/kg of NRG1 intraperitoneally 24 h before the experiment. After 24 h, the mice were killed to harvest the left lung tissue for fixation in 4% paraformaldehyde, followed by paraffin embedding; the remaining tissues were stored at −80°C.

Hematoxylin and Eosin (H&E) staining

Lung and pulmonary artery samples were fixed in 10% formalin, embedded in paraffin, sectioned at 4 μm thickness, and stained with H&E for microscopic examination.

Immunohistochemistry (IH)

The samples were prepared in tissue microarrays for comparison with H&E-stained sections and processed by deparaffinization, rehydration, and antigen retrieval. Endogenous peroxidase activity was quenched using 3% H_2O_2. Blocking was carried out with goat serum, followed by incubation with monoclonal mouse anti-rabbit Vn antibody (1:100) at 4°C overnight. This was followed by secondary antibody application, DAB staining, hematoxylin counterstaining, dehydration, clearing, and mounting. The slides were then air-dried for observation.

Gene Expression Omnibus (GEO) data and gene set enrichment analysis (GSEA)

RNA sequencing data were sourced from the National Center for Biotechnology Information's GEO (NCBI-GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE130391. GSEA was executed using the "clusterProfiler" R package and gene sets from the Molecular Signatures Database obtained from the Broad Institute (http://software.broadinstitute.org/gsea/index.jsp).

Statistical analysis and graphical representation

In this study, intergroup comparisons were performed using the *t*-test, while multiple group comparisons with analysis of variance; the results were expressed as mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$. Statistical analyses and graphical representations were performed using GraphPad Prism version 7.00, Microsoft Excel, and Adobe Photoshop CS6.

RESULTS

ELISA analysis of ERBB4 expression differences between patients with PE and healthy controls

The ERBB4 concentration in the sera of 30 patients with PE and 30 healthy controls was determined using ELISA. Statistical analysis of serum ERBB4 concentrations between the two groups indicated that ERBB4 levels were significantly higher in patients with PE than in healthy controls ($p < 0.01$) (Figure 1).

Impact of thrombin on cellular behavior and overexpression of the ERBB4 receptor

After successful in vitro culture, HPAECs were stimulated with 5 U/ml thrombin at intervals of 0, 0.5, 1, 2, 6, and 24 h, and their viability was assessed using the CCK-8 assay. A significant reduction in cellular activity was observed 6 h after thrombin stimulation, which was statistically significant compared to the control group ($p < 0.01$) (Figure 2a). Consequently, for subsequent experiments, cells stimulated with thrombin for 6 h were designated as the experimental group. Following the ELISA findings, we evaluated ERBB4 expression in HPAECs 6 h after thrombin stimulation using WB analysis. Compared to the Con group, the experimental group exhibited a significant increase in ERBB4 expression after thrombin stimulation ($p < 0.05$) (Figure 2b,c).

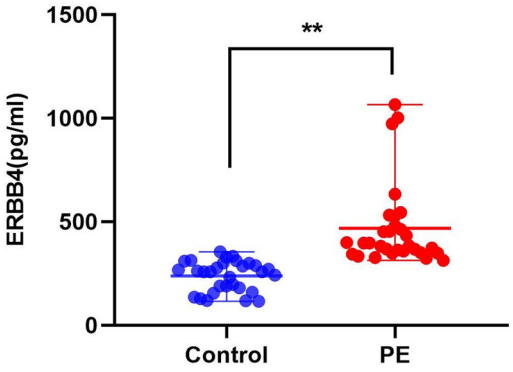

FIGURE 1 Levels of ERBB4 in the serum of healthy individuals versus patients with PE. **Indicates statistical significance compared to the control group, with $p < 0.01$. PE, pulmonary embolism.

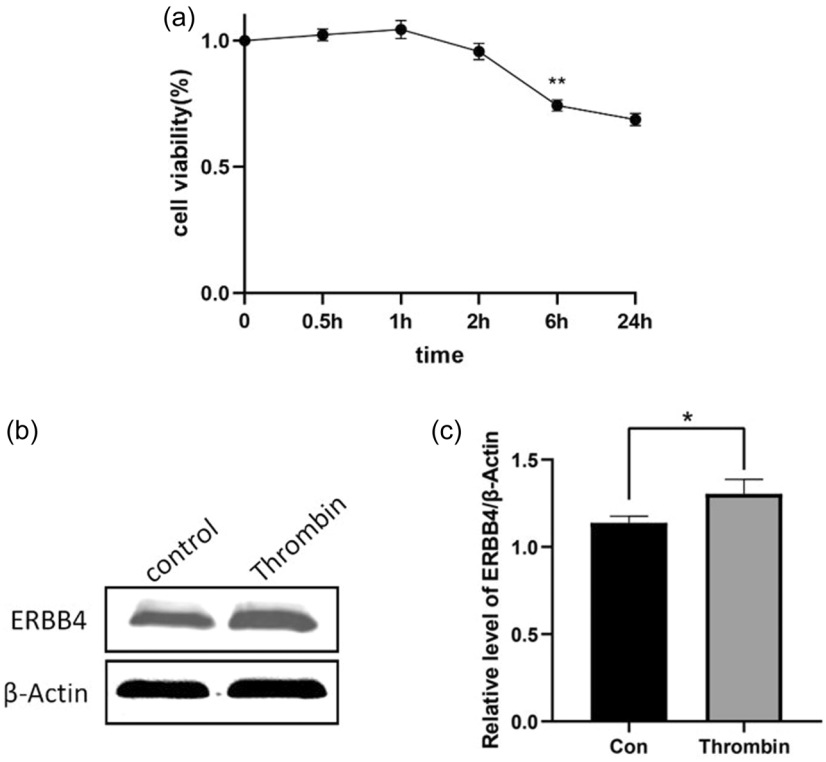

FIGURE 2 Thrombin influences the cellular behavior and expression of ERBB4. (a) The CCK-8 assay is used to measure the viability of HPAECs. Data are presented as mean ± standard deviation (SD), $n = 3$. (b) WB analysis is used to assess ERBB4 expression in HPAECs. (c) This figure provides a quantitative analysis of the results shown in Figure. Data are presented as mean ± SD, $n = 3$. (b). *Statistically significant difference compared to the Con group, with $p < 0.05$. CCK-8, cell counting kit-8; HPAEC, human pulmonary artery endothelial cell; WB, Western blot.

NRG1 induces ERBB4 phosphorylation to regulate the apoptosis of HPAECs, and AG1478 inhibits the NRG1-inhibited apoptosis of HPAECs induced by thrombin

For the study, four groups were designated as follows: the thrombin-HPAEC culture (treated with 5 U/mL thrombin for 6 h), thrombin-NRG1-HPAEC culture (cells treated with 5 U/mL thrombin and 25 ng/mL NRG1 for 6 h, referred to as NRG1 group), thrombin-AG1478-HPAEC culture (Tyrphostin AG1478, a selective inhibitor of EGFR tyrosine kinase) (pretreated with 10 uM AG1478 for 2 h before thrombin stimulation, referred to as AG1478 group), and thrombin-AG1478-NRG1-HPAEC (pretreated with AG1478 for 2 h before cotreatment with thrombin and NRG1, referred to as AG1478 + NRG1 group) groups, with the thrombin-HPAEC culture group acting as the Con group. Each group consisted of three replicate wells using the same cell culture methodology, as previously described. Flow cytometry analysis showed that the apoptosis rate in the NRG1 group was significantly lower than in the Con group and there was a significant increase in the apoptosis rate in the AG1478 group compared to the control group (Figure 3a,b), both statistically significant ($p < 0.05$). The WB analysis revealed an increase in the expression of phosphorylated ERBB4 (p-ERBB4) in the NRG1 group compared to the Con group and a reduction in p-ERBB4 expression in the AG1478 group compared to the Con group (Figure 3c,d) with a statistically significant difference ($p < 0.05$). These findings indicate that NRG1 enhances ERBB4 phosphorylation and regulates the apoptosis of HPAECs, and that AG1478 mitigates the activation of NRG1/ERBB4, thus increasing apoptosis in HPAECs.

The inhibitory effect of AG1478 on HPAECs mediated by the NRG1/ERBB4 pathway is likely associated with the downstream PI3K/Akt signaling pathway

WB analysis was used to assess the levels of PI3K, Akt, p-PI3K, and phosphorylated Akt (p-Akt) in the different groups. In particular, the phosphorylation levels of PI3K/Akt in the NRG1 group's HPAECs were significantly higher than those of the Con group, indicating a statistically significant increase ($p < 0.05$). In contrast, no significant changes were observed in the AG1478 and AG1478 + NRG1 groups compared to the Con group ($p > 0.05$). However, the phosphorylation levels of PI3K/Akt in the AG1478 + NRG1 group were significantly reduced compared to the NRG1 group, showing statistical significance ($p < 0.05$) (Figure 4a–c). These findings suggest that the NRG1/ERBB4 signaling pathway inhibits apoptosis by activating downstream PI3K/Akt phosphorylation. In contrast, AG1478 appeared to increase cell apoptosis by inhibiting the expression of

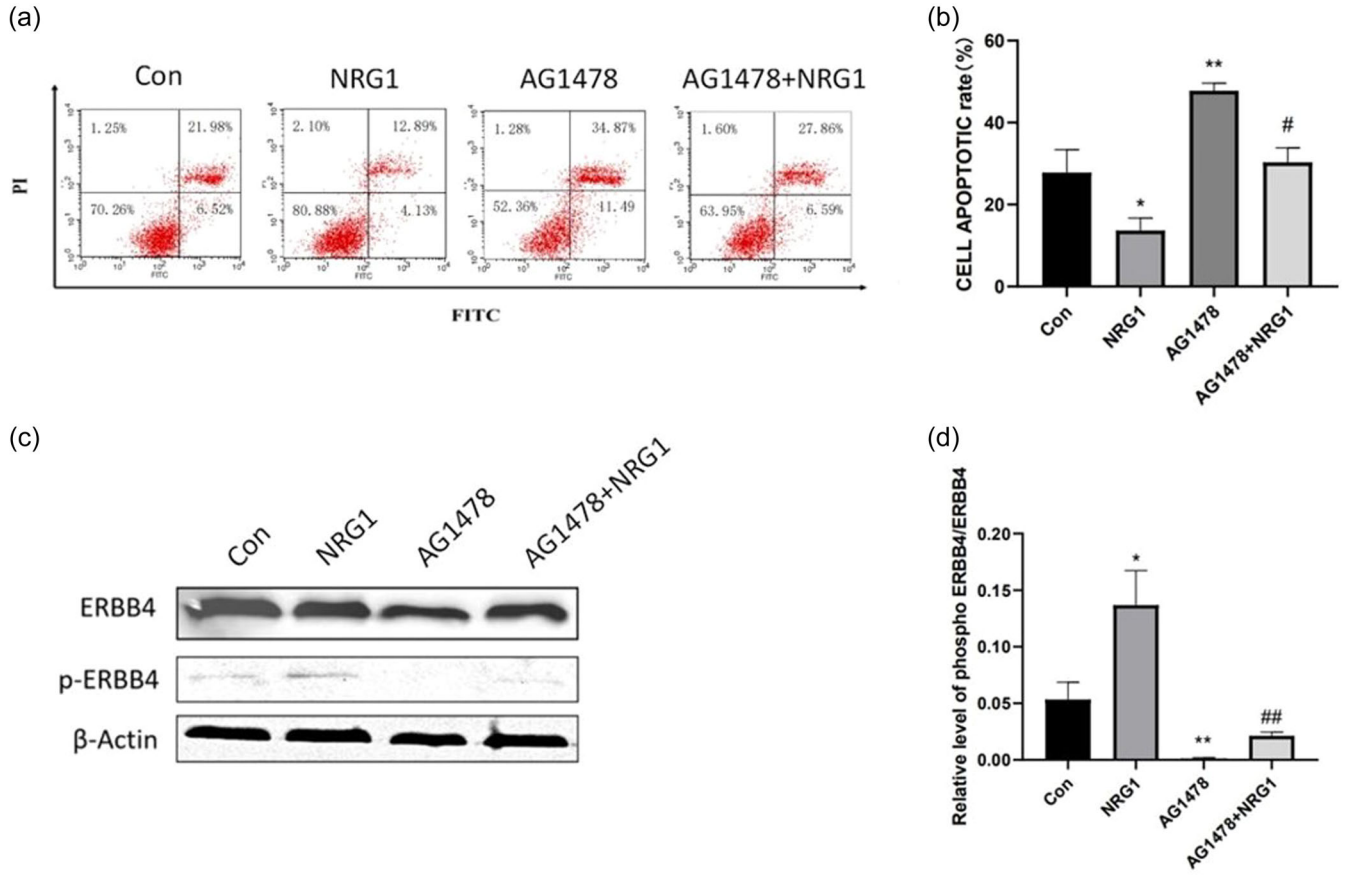

FIGURE 3 The effect of NRG1 and AG1478 on the function of HPAECs and expression of ERBB4. (a) Flow cytometric analysis is performed to determine the apoptosis rate. Data are presented as mean ± standard deviation (SD), $n = 3$. (b) A quantitative graph representing the overall apoptosis rates for each group, where *indicates $p < 0.05$ compared to the Con group. **Signifies $p < 0.01$ compared to the Con group, and #denotes $p < 0.05$ compared to the NRG1 group. (c) WB analysis is used to evaluate ERBB4 and p-ERBB4 expression between the groups. Data are presented as mean ± SD, $n = 3$. (d) This figure represents the quantitative analysis of the results of Figure (c), normalized against β-Actin protein expression, with *indicating $p < 0.05$ compared to the Con group, **representing $p < 0.01$ compared to the Con group, and ##signifying $p < 0.01$ compared to the NRG1 group. NRG1, Neuregulin-1; WB, Western blot.

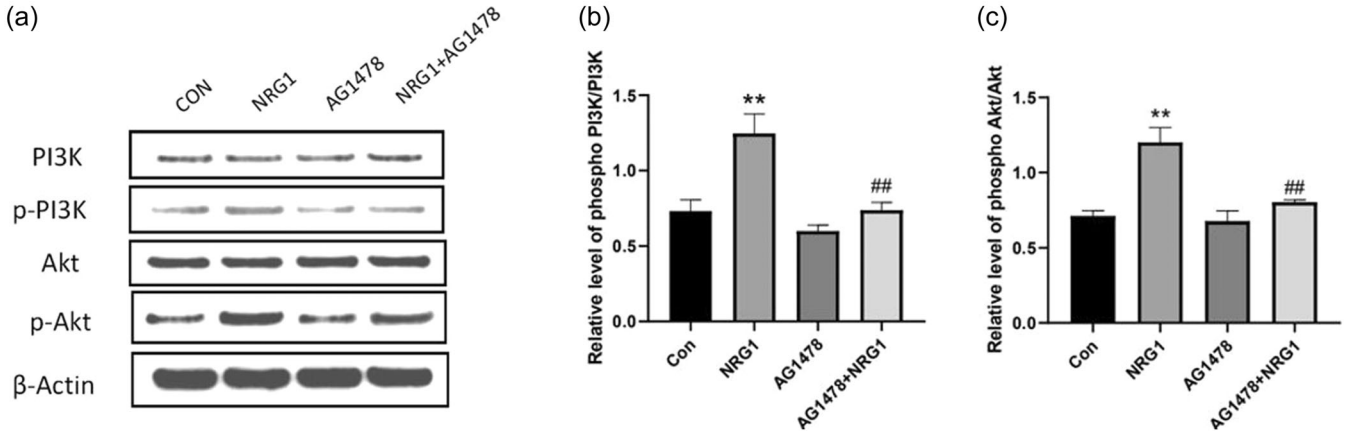

FIGURE 4 The effect of AG1478 on the function of HPAECs is associated with the PI3K/Akt signaling pathway. (a) WB is performed to measure the expression of PI3K, p-PI3K, Akt, and p-Akt in the groups. Data are presented as mean ± standard deviation (SD), $n = 3$. (b, c) These figures provide a quantitative analysis of the data presented in Figure (a), with normalization to β-Actin protein levels. Statistical significance is indicated as * for $p < 0.05$ and ** for $p < 0.01$ compared to the Con group, and ## for $p < 0.01$ compared to the NRG1 group. HPAEC, human pulmonary artery endothelial cell; NRG1, Neuregulin-1; p-Akt, phosphorylated Akt; WB, Western blot.

the ERBB4 receptor, subsequently reducing the activation of the downstream PI3K/Akt signaling pathway.

Impact of NRG1/ERBB4 pathway activation on a PE mouse model

Lung tissue sections from each mouse group were stained with H&E and four sections were randomly selected per group for thrombosis quantification. In the Con group, lung tissues from the PE and NRG1 groups showed extensive effusion within blood vessels, leading to complete blockage. In particular, the NRG1 group exhibited a significantly reduced number of thrombi compared to the PE group ($p < 0.05$) (Figures 5a,b,d). IH staining to assess ERBB4 expression revealed brown particulate positivity in the intima of small pulmonary arteries. Using Image-Pro Plus software (version 6.0) at 200× magnification standard, we identified consistent brown particles to standardize positive expression across all images, derived the integrated OD (IOD) and area metrics. Higher IOD values were correlated with a greater protein presence in the tissue, and the PE group showed significantly increased ERBB4 expression compared to the Con group ($p < 0.05$) (Figures 5c,e). WB analysis further confirmed that the expression of p-ERBB4 in the NRG1 group was significantly higher than in the PE group ($p < 0.05$) (Figures 5f,g). These in-vivo findings corroborated the in vitro data, suggesting that NRG1 facilitates the proliferative response in HPAECs by inducing ERBB4 phosphorylation.

Conducted GSEA on 18 patients with CTEPH from the GEO database

From the conducted experiments, we discovered a potential close association between high ERBB4 expression and PE. In the GEO database, 18 patients with CTEPH were classified according to ERBB4 expression levels into high ($n = 9$) and low ($n = 9$) expression groups. The GEO database, established by the National Center for Biotechnology Information (NCBI) in 2000, aggregates high-throughput gene expression data from global research entities. Following the GSEA of these data, with a significance threshold of corrected $p < 0.05$, five kyoto encyclopedia of genes and genomes (KEGG) pathways were enriched (Figure 6a). Among these, three pathways were associated with high ERBB4 expression: fatty acid metabolism, oxidative phosphorylation, and cholesterol homeostasis (Figure 6b).

DISCUSSION

CTEPH is the leading cause of severe PH and is characterized by intraluminal thrombosis and vascular remodeling, resulting in narrowing or complete occlusion of the pulmonary arteries. This pathology increases pulmonary vascular resistance, leading to PH and progressive right-sided heart failure. Although acute PE is widely considered the main initiator of CTEPH, pathologies in the small pulmonary arteries and in situ thrombus formation, which develop and worsen during the disease, are believed to be key contributors to hemodynamic changes and exacerbation of symptoms.[21] Pathological changes in the small pulmonary arteries are critical for PH, causing increased vascular resistance, reduced compliance, and marked proliferation of pulmonary artery smooth muscle and endothelial cells.

Endothelial cell hyperproliferation in PH shares several aberrant activation pathways with cancer cell proliferation.[22] ERBB4 has been implicated in tumor growth, proliferation, apoptosis, invasion, and metastasis, playing an important role, for example, in malignant peripheral nerve sheath tumors by activating crucial non-Ras-dependent signaling cascades such as STAT3, STAT5, and phospholipase-Cγ.[23] Ibrutinib effectively blocks ERBB4 activation in breast cancer cells, subsequently inhibiting the Akt and ERK pathways, causing cell cycle arrest and increased apoptosis, thus acting therapeutically against breast cancer.[24] MiR-193a-3p has been shown to inhibit tumor cell proliferation and invasion and promote apoptosis by downregulating ERBB4, demonstrating antitumor activity in xenograft mice.[25]

The influence of ERBB4 on cell proliferation extends beyond cancer cells. Upon binding to its ligand NRG1, ERBB4 undergoes structural changes, forming dimers with functionalities similar to those of ERBB1, ERBB2, and ERBB3. This phosphorylation activates downstream signaling crucial for neuronal development, migration, axonal guidance, and synaptic functions,[17,26] which are vital in organogenesis.[27] NRG1, a member of a family of growth factors, is known to stimulate neuronal growth and has been implicated in neurobiological research, including potential treatments for Alzheimer's disease, epilepsy, muscular atrophy, and neuritis. It also plays an important role in conditions such as schizophrenia and peripheral nerve damage.[28–30]

NRG1 has been shown to mitigate neuronal death induced by oxygen-glucose deprivation, an effect attenuated by pretreatment with the ERBB4 inhibitor AG1478.[31] Furthermore, studies have indicated a regulatory influence of the NRG1/ERBB4 pathway on cardiomyocytes, with NRG1 supplementation reducing apoptosis in ischemia-damaged cells.[32,33] The

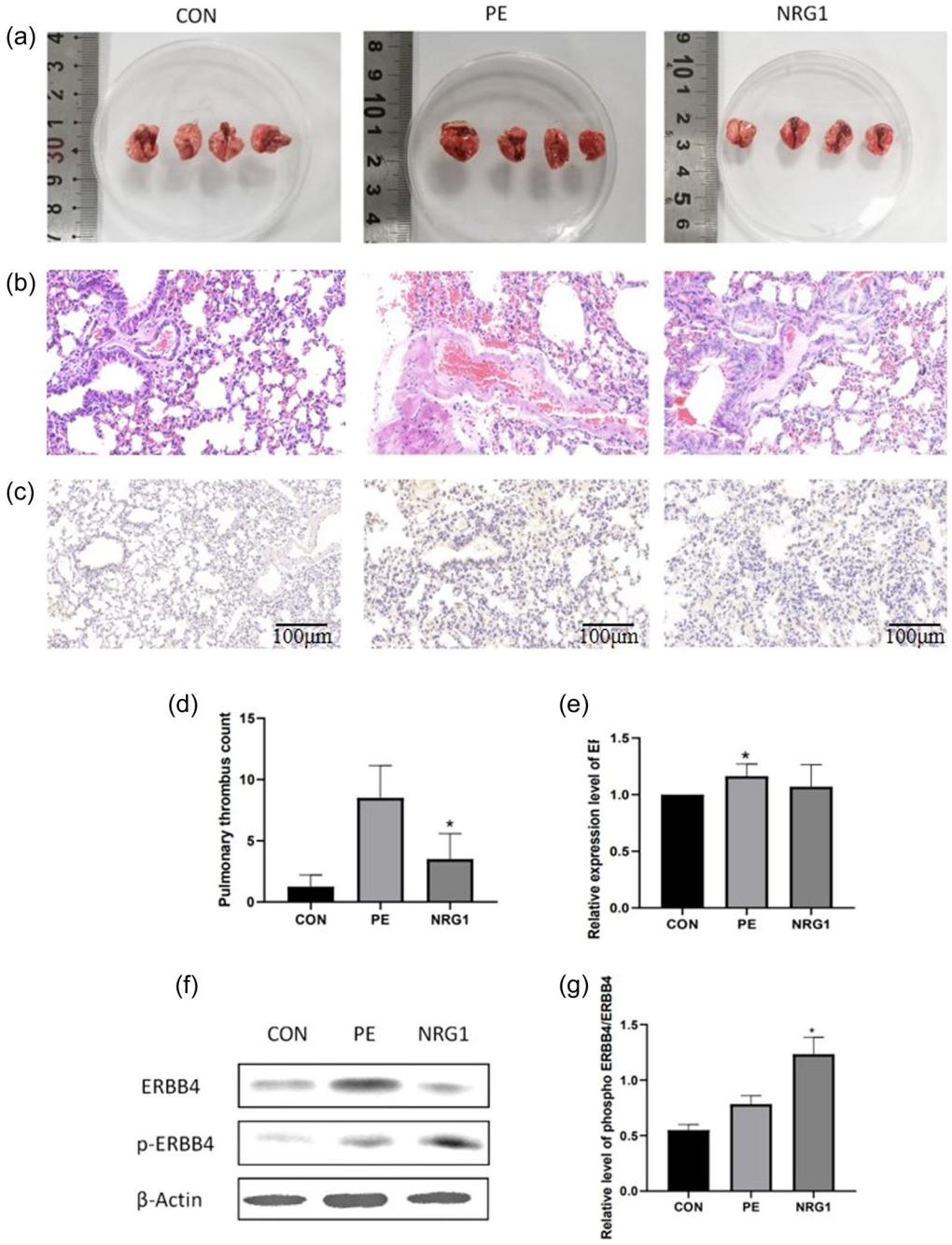

FIGURE 5 Impact of NRG1/ERBB4 on a PE mouse model. (a) The gross morphology of lung tissues from each group of mice is shown in this figure. Data are presented as mean ± standard deviation (SD), $n = 3$. (b) H&E staining of lung tissue sections of each group is illustrated. (c) ERBB4 IH staining is shown in lung tissues from each group. (d) Quantitative analysis of thrombus formation in lung tissue sections of each group is presented. (e) IOD for IH stained sections was calculated using Image-Pro Plus 6.0 software, providing a quantification of ERBB4 expression. (f) The expression of ERBB4 and p-ERBB4 in lung tissues of each group is assessed by WB. Data are presented as mean ± SD, n = 3. (g) A quantitative analysis of the WB results is shown in Figure (f). *indicates statistical significance compared to the Con group, with $p < 0.05$. H&E, Hematoxylin and Eosin; HPAEC, human pulmonary artery endothelial cell; IH, immunohistochemistry; IOD, integrated OD; NRG1, Neuregulin-1; SD, standard deviation; WB, Western blot.

inhibition of ERBB expression can lead to heart failure and dilated cardiomyopathy with ERBB tyrosine kinase inhibitors during early pregnancy in mice, causing ventricular hypertrophy and functional decline.[34] The absence of ERBB receptors in mouse embryos can cause lethal cardiac malformations.[35,36] Wang has been found to promote cardiomyocyte migration and angiogenesis and reduce apoptosis via the NRG1/ERBB4 pathway, which is mediated through the PI3K/Akt/mTOR pathway.[37]

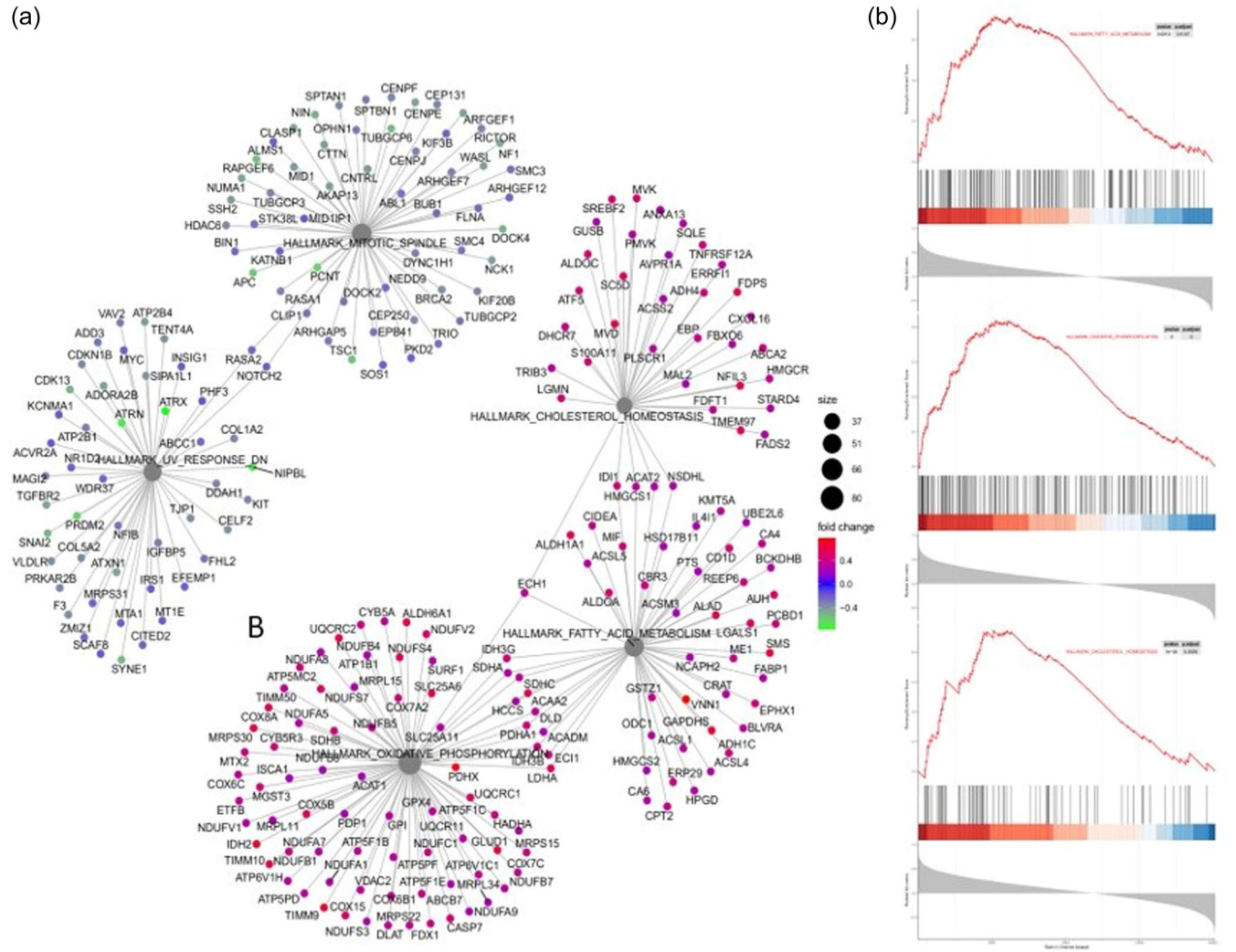

FIGURE 6 GSEA of 18 patients with CTEPH from the GEO database. (a) GSEA identified five pathways significantly associated with high ERBB4 expression, including CHOLESTEROL HOMEOSTASIS, OXIDATIVE PHOSPHORYLATION, and FATTY ACID METABOLISM ($p < 0.05$). (b) Three pathways are significantly associated with the high ERBB4 expression group: fatty acid metabolism, oxidative phosphorylation, and cholesterol homeostasis. CTEPH, chronic thromboembolic pulmonary hypertension; GEO, Gene Expression Omnibus; GSEA, gene set enrichment analysis.

To investigate differences in ERBB4 concentrations between patients with PE and healthy individuals, we collected serum samples from 30 patients with PE and 30 healthy controls for clinical analysis. ERBB4 expression was quantified using ELISA, revealing higher concentrations in patients with PE, which may indicate significant activation of ERBB4-related pathways. In vitro, the experiments involved stimulating HPAECs with thrombin to simulate endothelial conditions in PE, with WB analyses confirming an increase in ERBB4 expression, consistent with the ELISA findings.

Flow cytometry revealed a notable reduction in apoptosis rates in the NRG1-treated group, suggesting its potential role in HPAEC proliferation. Conversely, AG1478 treatment, an ERBB4 inhibitor, significantly increased apoptosis rates in the treated cells, negating the cytoprotective effects of NRG1, especially in the AG1478 + NRG1 group, indicating that AG1478 inhibits endothelial proliferation.

The WB analysis of these groups further elucidated the pathway dynamics, showing a decrease in p-ERBB4 levels post-AG1478 treatment, linking the regulatory effect on endothelial proliferation to the NRG1/ERBB4 pathway. Subsequent assessment of PI3K, p-PI3K, Akt, and p-Akt validated the activation of the downstream PI3K/Akt pathway by NRG1/ERBB4 signaling, which is essential to promote cell proliferation. Inhibition of AG1478 clearly reduced HPAEC proliferation.

These findings align with the known roles of the PI3K/Akt pathway in regulating essential cellular

functions and suggest that the NRG1-mediated protective effect on endothelial cells, which reduces thrombus formation, can be significant in PE management. However, unchecked cell proliferation can contribute to disease progression from PE to CTEPH, highlighting the dual role of ERBB4 regulation in this pathological spectrum. More research is imperative to delineate these mechanisms and optimize therapeutic interventions.

Given the elevated expression of ERBB4 in the disease groups studied, we sourced data from 18 patients with CTEPH from the GEO database and stratified them into high and low ERBB4 expression groups. GSEA carried out with the "clusterProfile" R package revealed that differentially expressed genes between these groups concentrated in KEGG pathways associated with high ERBB4 expression: fatty acid metabolism, oxidative phosphorylation, and cholesterol homeostasis. Oxidative phosphorylation is integral to cellular energy metabolism and involves sugars, proteins, and fats, with fatty acids and cholesterol as lipid entities. Dysregulated lipid metabolism can lead to hyperlipidemia, and significant lipid accumulation in the arterial walls can cause atherosclerosis and plaque formation. Plaque rupture triggers the coagulation cascade, expediting thrombus formation, and substantially increasing cardiovascular disease risk.[20] Although the link between high ERBB4 expression and related metabolic pathways warrants further investigation, it offers a direction for future research.

CONCLUSIONS

In conclusion, the NRG1/ERBB4 pathway likely modulates HPAEC function by affecting downstream PI3K/Akt phosphorylation. AG1478 partially mitigates this effect by inhibiting ERBB4. Furthermore, elevated expression of ERBB4 was significantly associated with PE, indicating its potential as a novel biomarker of this condition. These bioinformatic findings provide direction for future research.

AUTHOR CONTRIBUTIONS

Jin-Bo Huang, Qin Shen, Zhi-Qi Wang, and Jian-An Huang were study investigators and wrote and edited the manuscript. Song-Shi Ni, Fei Sun, and Yun Hua reviewed the data and edited the manuscript. All authors read and approved the final article.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors are accountable for all aspects of this work and ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was carried out in accordance with the Declaration of Helsinki (revised in 2013). This study was approved by the Ethics Board of the Affiliated Hospital of Nantong University (No. 2020-L088) and informed consent was obtained from all participants.

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