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ORIGINAL ARTICLE

P2X7 receptor promotes migration and invasion of non-small cell lung cancer A549 cells through the PI3K/Akt pathways

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Abstract

It has been demonstrated that the ATP-gated ion channel P2X7 receptor is involved in tumor progression and plays an important role in regulating tumor cell growth, invasion, migration and angiogenesis. However, P2X7 receptors have been relatively poorly studied in non-small cell lung cancer (NSCLC) cells. Therefore, the aim of this study was to investigate the effects of P2X7 receptor on A549 cells (NSCLC cell line) migration and invasion and to reveal the molecular mechanisms mediated by it. We detected the expression and function of P2X7 receptor in A549 cells. The effects and mechanisms of P2X7 receptor on A549 cells migration, invasion, and epithelial-mesenchymal transition were detected *in vitro* and *in vivo*. The results showed P2X7 receptor expressed by A549 cells had ion channel and macropore formation function. In addition, activation of P2X7 receptor by adenosine triphosphate (ATP) or 2′(3′)-O-(4-Benzoylbenzoyl)-adenosine-5'-triphosphate (BzATP) promoted Epithelial-mesenchymal transition (EMT), migration and invasion of A549 cells, which was attenuated by treatment of cells with P2X7 receptor antagonist A438079 and Oxidized ATP. Furthermore, activation of P2X7 receptor increased phosphorylated protein kinase B (p-Akt) levels, and the phosphatidylinositol-tris–phosphate kinase 3 (PI3K)/protein kinase B (Akt) inhibitor LY294002 blocked migration and invasion of A549 cells induced by ATP or BzATP. At the same time, *in vivo* results showed that P2X7 receptor could also promote EMT and PI3K/Akt expression in transplanted tumors. Our study indicated that P2X7 receptor promotes A549 cells migration and invasion through the PI3K/Akt signaling pathway, suggesting that P2X7 receptor may be a potential therapeutic target for NSCLC.

Keywords P2X7 receptor · Non-small cell lung cancer · Migration · Invasion · PI3K/Akt signaling pathway

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Introduction

In the world, lung cancer is one of the main causes of cancer-related death, which can be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) [1].

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Among them, NSCLC accounts for about 85% of all lung cancer cases, and the incidence is increasing, which is significantly higher than that of SCLC [2]. NSCLC is mostly a disease of the elderly, with a peak age of onset over 65 years and an annual incidence rate of up to 1.3 million cases [3, 4]. Because disease is often asymptomatic in the early stages, most patients with NSCLC are diagnosed in the late stages [5]. Currently, immunotherapy using immune checkpoint inhibitors has become the standard of care for the treatment of advanced NSCLC [6]. Tumor metastasis is the leading cause of death in approximately 90% of cancer patients. Among them, the main sites of NSCLC metastasis are brain, bone, liver, etc. [7]. Although NSCLC has made great progress in surgery, chemotherapy, immunotherapy and targeted therapy, its 5-year survival rate is still unsatisfactory [8–10]. The 5-year survival rate of patients with early stage and non-metastatic NSCLC is as high as 70%, while the 5-year overall survival rate of patients with metastatic NSCLC is less than 5% [11, 12]. Therefore, further studies on the molecular mechanisms and potential therapeutic targets of NSCLC progression are essential for the treatment of NSCLC.

Adenosine triphosphate (ATP) is not only a well-known intracellular energy source, but also an important extracellular signaling molecule [13]. Extracellular ATP levels within healthy tissues have been studied to be low, while in the tumor microenvironment, extracellular ATP levels (> 100 μM) are significantly increased due to inflammatory reactions, cell damage caused by hypoxia, and tissue destruction caused by tumor invasion [14]. Extracellular ATP exerts biological effects mainly by activating purinergic P2 receptors on the cell membrane [15]. P2 receptors are divided into two subfamilies that include ligand-gated P2X ion channel receptors and G protein-coupled P2Y receptors [16]. P2X7 receptor is a unique member of P2X receptor subfamily, which is expressed in a variety of cells and tissues *in vivo* and is involved in cell signal transduction, regulation of inflammation, and mediation of cell proliferation and apoptosis [17–20]. The P2X7 receptor gene is located on human chromosome 12q24.31 and consists of 595 amino acids [21]. The main function of P2X7 receptor is to form ion channels and macropores. When P2X7 receptor is stimulated by low concentrations of extracellular ATP, it leads to the opening of cation channels, allowing K^+ efflux as well as Ca^{2+} and Na^+ influx, however, prolonged activation of P2X7 receptor leads to the formation of irreversible pores, allowing macromolecules up to 900 Da to pass through, and ultimately to cell swelling, vacuolization, and cell death [22–25].

It has been found that P2X7 receptor is expressed in most tumors and is closely related to tumor migration, invasion and prognosis [26]. Although numerous studies have shown the involvement of P2X7 receptor in tumor progression, the role mediated after its activation remains controversial. It is well-known that P2X7 receptor was originally thought to be a cytotoxic receptor [27]. It has been found that P2X7 receptor activation by high levels of ATP exhibits direct cytotoxic effects on certain cancer cells. P2X7 receptor activation induces apoptosis in colorectal cancer cells and acute myeloid leukemia cells [28, 29]. In addition, P2X7 receptor activation by ATP induced cell death in the glioma cell line GL261, and ATP-induced cell death was blocked using P2X7 receptor antagonists [30]. However, recent studies have reported that activation of P2X7 receptor can promote tumor cells proliferation and growth [31, 32]. P2X7 receptor also has a significant growth-promoting effect *in vivo*, which is mainly manifested in accelerated cell proliferation, reduced apoptosis, and enhanced angiogenesis and release of vascular endothelial growth factor (VEGF) [33]. In addition, P2X7 receptor can also promote the migration and invasion of tumor cells [34, 35]. Studies have reported that autocrine ATP-P2X7 signaling can increase transforming growth factor-β1 (TGF-β1) -induced lung cancer cell migration and actin remodeling [36, 37], but the role of P2X7 receptor in lung cancer cell migration and invasion still needs to be further clarified, especially, the potential mechanism mediated by P2X7 receptor needs to be revealed. In addition, Schmid et al. [38] analyzed expression level of P2X7 receptor in NSCLC patients and showed that expression of P2X7 receptor is elevated in NSCLC patients. Also, expression of P2X7 receptor is higher in metastatic than non-metastatic tumors. Similarly, Zanini et al. [39] found that stage IV NSCLC patients showed an increase in P2X7 receptor expression in lymphocytes. Moreover, studies have shown that significant differences in clinical outcomes of NSCLC patients with highly-expressed P2X7 receptor, suggesting that P2X7 receptor may be a potential prognostic marker and therapeutic target [40].

Tumor metastasis is a major hurdle to cancer treatment. EMT is a critical process in cancer cell metastasis, which consists of a complex series of biological changes that result in cell losing its differentiated epithelial-like state and acquiring a mesenchymal-like phenotype [41]. During cancer progression, EMT promotes cancer progression by conferring mesenchymal phenotypes associated with highly aggressive tumor cells [42]. Changes in levels of epithelial and mesenchymal markers are commonly used to indicate the occurrence of EMT, which is characterized by the down-regulation of epithelial cell markers such as E-cadherin, while up-regulation of mesenchymal cell markers such as N-cadherin and vimentin [41].

Therefore, in this study, we investigated the effect of P2X7 receptor on NSCLC migration and invasion as well as the molecular mechanism by *in vitro* and *in vivo* experiments. The results showed that A549 cells expressed functional P2X7 receptors, and activated P2X7 receptors promoted EMT, migration and invasion of A549 cells, and promoted EMT and PI3K/Akt expression in transplanted

tumors. Alternatively, we further revealed the P2X7 receptor-mediated molecular mechanism, involving the PI3K/Akt signaling pathway.

Materials and methods

Reagents and antibodies

ATP, 2′(3′)-O-(4-Benzoylbenzoyl)-adenosine-5'-triphosphate (BzATP, P2X7 receptor agonist), Oxidized ATP (oxATP, P2X7 receptor antagonist), YO-PRO-1, PI3K/Akt inhibitor LY294002 were purchased from Sigma (St Louis, MO, USA). P2X7 antagonist A438079 was purchased from MCE (Shanghai, China). P2X7 antibody (NBP1-20180) was purchased from Novus Biologicals (Littleton, Colorado, USA), and antibodies of Akt, p-Akt, E-cadherin, N-cadherin, vimentin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish per oxidase (HRP) -labeled goat anti-mouse and goat anti-rabbit secondary antibodies were purchased from Proteintech (Wuhan, China). Fluo-4, AM was purchased from Beijing Solarbio Science & Technology (Beijing, China).

Human cell culture

The human NSCLC A549 cells were kindly provided by Stem Cell Bank, Chinese Academy of Scinences (Shanghai, China). The cells were maintained in Ham's F-12 K medium (Procell, Wuhan, China) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin (Servicebio, Wuhan, China) at 37 °C in a humidified atmosphere of 5% CO_2.

Immunohistochemistry

The collected tissue paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol, and subjected to antigen retrieval using 0.4% pepsin. Appropriate amounts of endogenous peroxidase blockers were added to the tissue sections, blocked with normal goat serum working solution for 10 min, and then incubated overnight at 4 °C with mouse monoclonal anti-E-cadherin antibody (1:200), anti-N-cadherin antibody (1:200), and anti-vimentin antibody (1:200). After washing with PBS, the sections were incubated with biotinylated secondary antibody for 30 min at room temperature, followed by incubation with horseradish enzyme-labeled streptavidin working solution for 20 min at room temperature. Immunostaining was visualized using diaminobenzidine (DAB) substrate and counterstained with hematoxylin. Microscopic examination was performed after dehydration and mounting.

Intracellular Ca^{2+} measurement

Changes in intracellular calcium concentration were detected using the calcium fluorescent probe Fluo-4, AM. A549 cells were seeded in 12-well plates, and some cells were preincubated with A438079 (100 μM) for 2 h at 37 °C and then washed three times with PBS. Added 4 μM Fluo-4, AM working solution and incubated at 37 °C for 20 min, followed by five volumes of Hanks balanced salt solution (HBSS) containing 1% fetal bovine serum and incubation was continued for another 40 min. Cells were washed three times with HEPES buffer saline, resuspended and transferred to a black 96-well plate. After treatment with or without ATP (3 mM) and BzATP (300 μM) [43], intracellular fluorescence intensity was detected using a multifunctional microplate reader (Tecan, Switzerland).

YO-PRO-1 uptake assay

P2X7 activation-induced pore formation was assessed using the DNA-binding fluorochrome YO-PRO-1 uptake assay. Cells were seeded in 12-well plates, and some cells were preincubated with A438079 (100 μM) for 2 h at 37 °C, followed by the addition of ATP (5 mM) or BzATP (500 μM) for an additional 1 h [44]. They were then incubated with 0.3 μM YO-PRO-1 for 15 min in the dark and washed three times with PBS. Cells were resuspended into a black 96-well plate and fluorescence intensity was detected using a multifunctional microplate reader. In addition, cells were imaged using fluorescence microscopy.

Cell migration assays

Cell migration assays were performed using wound healing assay and transwell chamber migration assay (Corning Costar, San Diego, CA, USA). For wound healing assay, A549 cells were seeded in 12-well plates and cultured to 80% ~ 90% confluence. Monolayer cells were scratched by a sterile pipette tip to create a linear wound area and then washed three times with PBS. Ham's F-12 K medium was then added and treated with ATP (1 mM), BzATP (200 μM), A438079 (100 μM) + ATP, A438079 + BzATP, oxATP (150 μM) + ATP, oxATP + BzATP for 24 h, or with ATP, BzATP, LY294002 (10 μM) + ATP, LY294002 (10 μM) + BzATP for 24 h [45, 46]. The wound healing was observed and images were captured in different treatment groups using an inverted microscope at 0 and 24 h. The wound width between cell scratches was calculated by image J software to evaluate cell mobility.

For transwell chamber migration assays, the upper compartment was seeded with A549 cells (2×10^4 cells) in basal culture, and the lower compartment was filled with medium with 10% FBS as a chemoattractant. They were treated with

ATP (1 mM), BzATP (200 μM), A438079 (100 μM) + ATP, A438079 + BzATP, oxATP (150 μM) + ATP, oxATP + BzATP for 24 h, or with ATP, BzATP, LY294002 (10 μM) + ATP, LY294002 (10 μM) + BzATP for 24 h. cells were fixed with methanol for 15 min at room temperature and stained with 0.1% crystal violet for 30 min. Cells not traversed on the membrane were removed and cells were counted in five random fields using an inverted microscope (100 × magnification).

Cell invasion assay

Cell invasion ability was analyzed using a transwell chamber invasion assay. This method was the same as the transwell migration assay described above, and the polycarbonate membrane of the upper insert was coated with Matrigel before use. After the cells were fixed and stained, the number of invasive cells was counted in five randomly selected fields under a microscope.

RNA extraction and Quantitative Real Time-PCR (qRT-PCR)

A549 cells were treated with or without ATP (1 mM), BzATP (200 μM), A438079 (100 μM) + ATP, A438079 + BzATP, oxATP (150 μM) + ATP, oxATP + BzATP. Total RNA was extracted from the A549 cells by the TRIzol reagent (TaKaRa) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using Evo M-MLV Reverse Transcription Kit (Accurate Biology, China). Subsequently, qRT-PCR reactions were performed using cDNA as a template using the TB Green™ Premix Ex Taq™ II kit (TaKaRa). Amplification was carried out for 40 cycles (pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing at 58 °C for 15 s, extension at 72 °C for 20 s). The relative expression of mRNA for each objective gene was calculated based on the $2^{-\Delta\Delta Ct}$ method using GAPDH as an internal control. Sequence of primers are presented in the Table 1.

Western blotting

A549 cells were treated with or without ATP (1 mM), BzATP (200 μM), A438079 (100 μM) + ATP, A438079 + BzATP, oxATP (150 μM) + ATP, oxATP + BzATP. Proteins were extracted from treated or untreated A549 cells. The protein concentration in each sample was determined using a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were separated with 10% SDS-PAGE gel and then bands were transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 2 h at room temperature and then incubated with primary antibodies against P2X7 receptor (1:300), Akt (1:200), p-Akt (1:200), E-cadherin (1:200), N-cadherin (1:200), vimentin (1:200), GAPDH (1:5000) at 4 °C overnight. After three washed with TBST, the membranes were incubated with HRP-labeled goat anti-mouse (1:5000) or goat anti-rabbit (1:7000) secondary antibody for 1 h at room temperature. Specific proteins were detected using the electrochemiluminescence (ECL) kit. Protein bands were quantified by densitometry analysis using Image J software.

Table 1 List and sequence of primers

Genes	Sequence
E-cadherin	F: 5'-GAACGCATTGCCACATAC-3' R: 5'-ACCTTCCATGACAGACCC-3'
N-cadherin	F: 5'-TCCTGCTTATCCTTGTGC-3' R: 5'-GTCCTGGTCTTCTTCTCCT-3'
Vimentin	F: 5'-TTGAACGCAAAGTGGAAT-3' R: 5'-AGGTCAGGCTTGGAAACA-3'
GAPDH	F: 5'-GCATCCTGGGCTACACTGAG-3' R: 5'-CCACCACCCTGTTGCTGTAG-3'

Xenograft model

Female BALB/c-nude mice (4–6 weeks old) were obtained from Jinan Pengyue Laboratory Animal Breeding Co., Ltd. All animal experiments were approved by the Ethics and Animal Research Committee of Weifang Medical University. A549 cells were collected, the cell concentration was adjusted to 1.0×10^7/mL with PBS, and 200 μl of cells subcutaneously implanted into the right axillar. When the tumors grew for one week, the mice were randomly divided into 2 groups of 6 animals each. 1 × PBS (0.1 M) or ATP (300 μM) 100 μl was injected into the xenograft tissue twice a week for a total of 8 times [47]. At the end of the experiment, the mice were euthanized by cervical dislocation after intraperitoneal injection with pentobarbital sodium (50 mg/kg) and the transplanted tumor tissues were excised for immunohistochemistry and western blotting analysis.

Statistical analysis

All experiments were repeated at least three times and data are presented as mean ± SD. The data were statistically analyzed with SPSS software, and the statistical significance of differences between the groups were analyzed for statistical significance using Student's t-test and one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

P2X7 receptor expressed in A549 cells is functional

Boldrini et al. [48] found that P2X7 receptor is expressed in NSCLC (squamous cell carcinoma and adenocarcinoma).

It has been found that the clinical outcomes of NSCLC patients with high or low P2X7 receptor expression are significantly different. In order to study the role of P2X7 receptor in NSCLC, we selected A549 cells as a representative NSCLC cell model to detect P2X7 receptor protein expression by western blotting. The result showed that A549 cells expressed P2X7 receptor (Fig. 1a).

And then, to understand whether P2X7 receptor in A549 cells is functional, Fluo-4, AM and YO-PRO-1 staining was performed. First, Fluo-4, AM was used to detect changes in intracellular calcium concentration to evaluate the ion channel function of P2X7 receptor. A549 cells exhibited enhanced intracellular Ca^{2+} fluorescence intensity following P2X7 receptor activation by extracellular ATP (3 mM) or BzATP (300 μM) treatment compared with the control group, and P2X7 receptor antagonist A438079 (100 μM) significantly inhibited ATP or BzATP-induced increases in Ca^{2+} influx (Fig. 1b, c). In addition, the YO-PRO-1 uptake assay was used to evaluate the macropore-forming function by P2X7 receptor. The results showed that ATP (5 mM) or BzATP (500 μM) increased YO-PRO-1 uptake in A549 cells, whereas pretreatment with the P2X7 receptor antagonist A438079 inhibited ATP or BzATP-induced increases in YO-PRO-1 uptake (Fig. 1d-f). Taken together, the above results indicate that P2X7 receptor expressed in A549 cells have ion channel and macropore formation functions.

P2X7 receptor enhances migration and invasion of A549 cells

To understand whether P2X7 receptor plays a role on the biological behavior of A549 cells, the migration and invasion of A549 cells were analyzed using wound healing and transwell assays. The results showed that P2X7 receptor activation by ATP and BzATP enhanced A549 cell migration and invasion compared with the control group, while the use of P2X7 receptor antagonist A438079 or oxATP

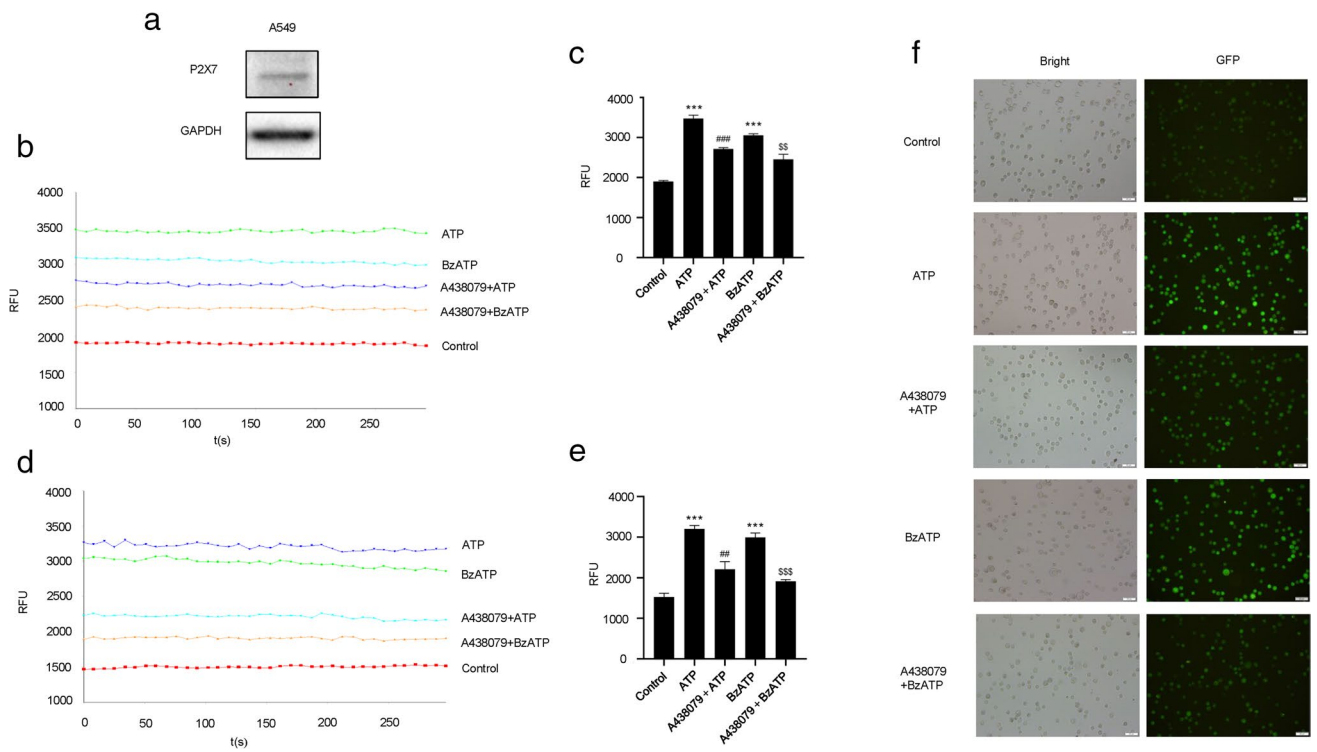

Fig. 1 P2X7 receptor expression in NSCLC cells and activation mediates Ca^{2+} influx and uptake of YO-PRO-1 in A549 cells. (**a**) Western blotting analysis of P2X7 receptor protein expression in A549 cells. (**b**) The fluorescence intensity of intracellular Ca^{2+} in A549 cells was detected by Fluo-4, AM. Treatment with ATP (3 mM) or BzATP (300 μM), or pretreatment with A438079 (100 μM) for 2 h followed by ATP or BzATP. Intracellular Ca^{2+} fluorescence intensity was measured by a multifunctional microplate reader, and relative fluorescence units (RFUs) were measured every 8 s for 5 min. (**c**) Comparison of intracellular Ca^{2+} fluorescence intensity between different treatments. (**d**) The fluorescence intensity of YO-PRO-1 uptake by A549 cells was measured by a multifunctional microplate reader. Treatment with ATP (5 mM) or BzATP (500 μM), or pretreatment with A438079 (100 μM) for 2 h followed by ATP or BzATP. (**e**) Comparison of fluorescence intensity of YO-PRO-1 uptake by different treatments. (**f**) The fluorescence intensity of YO-PRO-1 uptake by A549 cells in different treatment groups was observed under fluorescence microscopy. Data are presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.01$ vs. ATP group; $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.01$ vs. BzATP group

significantly inhibited ATP and BzATP-enhanced cell migration and invasion. (Fig. 2a-f) Together, these findings suggest that P2X7 receptor activation can promote A549 cell migration and invasion.

P2X7 receptor is involved in the regulation of EMT marker expression in A549 cells

It is well-known that EMT is closely related to tumor invasion and metastasis, and in order to further investigate whether P2X7 receptor affects the migration and invasion of A549 cells by regulating EMT, the expression levels of E-cadherin (Fig. 3a), N-cadherin (Fig. 3b) and vimentin (Fig. 3c) were detected by qRT-PCR and western blotting (Fig. 3d-g). The results showed that activation of P2X7 receptor by ATP or BzATP upregulated the expression of N-cadherin and vimentin and downregulated the expression of E-cadherin compared to control, whereas P2X7 receptor antagonists A438079 and oxATP attenuated the modulation of EMT expression by ATP or BzATP. These results suggested that P2X7 receptor may affect the migration and invasion of A549 cells by regulating the expression of EMT.

PI3K/Akt is involved in P2X7 receptor-mediated migration and invasion of A549 cells

To investigate the potential mechanism of P2X7 receptor-mediated migration and invasion of A549 cells, we investigated the role of PI3K/Akt signaling pathway in A549 cells. The results of western blotting analysis showed that ATP and

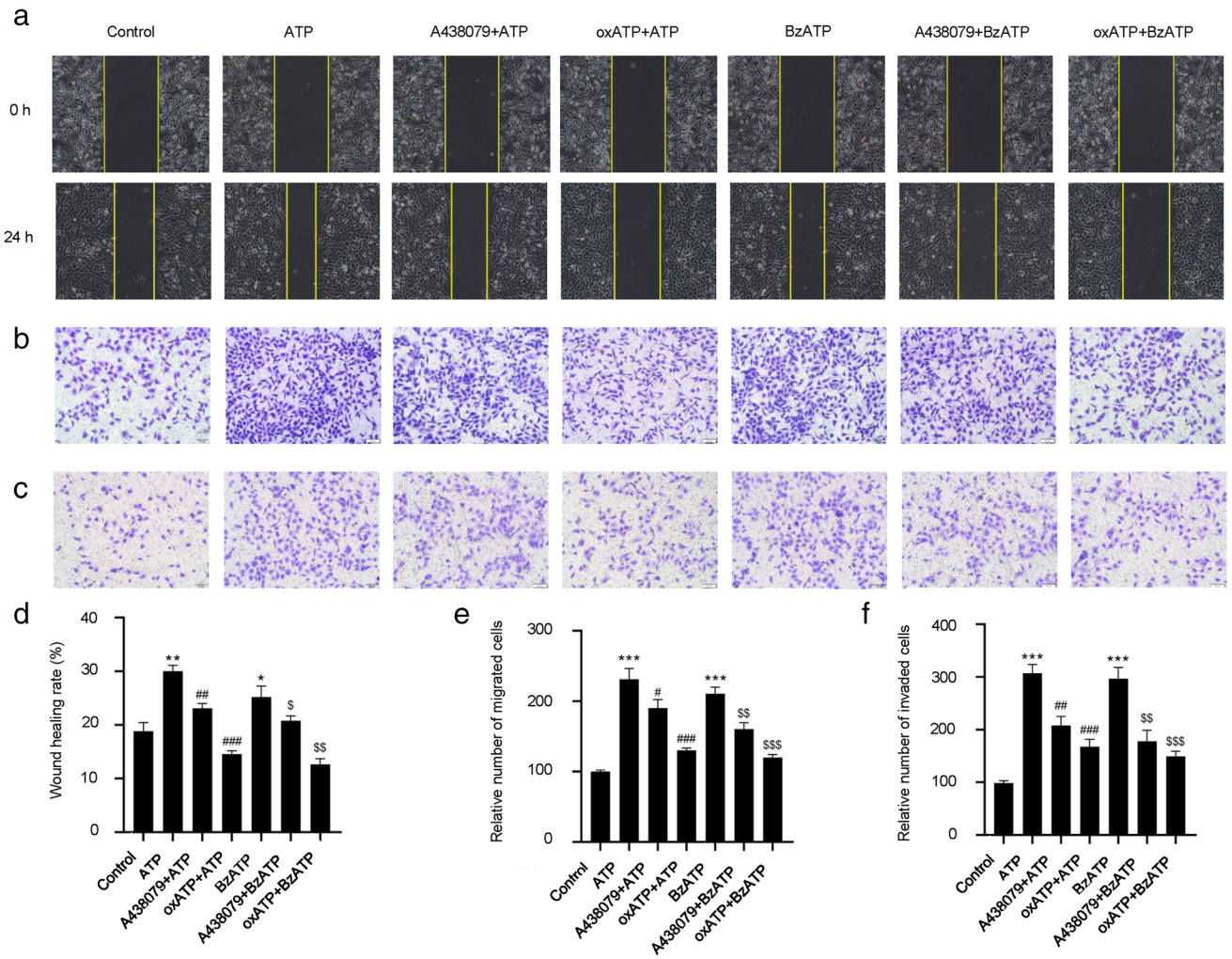

Fig. 2 P2X7 receptor enhances migration and invasion of A549 cells. A549 cells were pretreated with or without the P2X7 receptor antagonist A438079 (100 μM) or oxATP (150 μM) for 2 h and then treated with or without ATP (1 mM) or BzATP (200 μM) for 24 h. (**a, d**) The migration ability of A549 cells was detected by wound healing assay. (**b, e**) Transwell migration assay was used to detect the migration ability of A549 cells. (**c, f**) Transwell invasion assay was used to detect the invasive ability of A549 cells. Data are presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.01$ vs. ATP group; $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.01$ vs. BzATP group

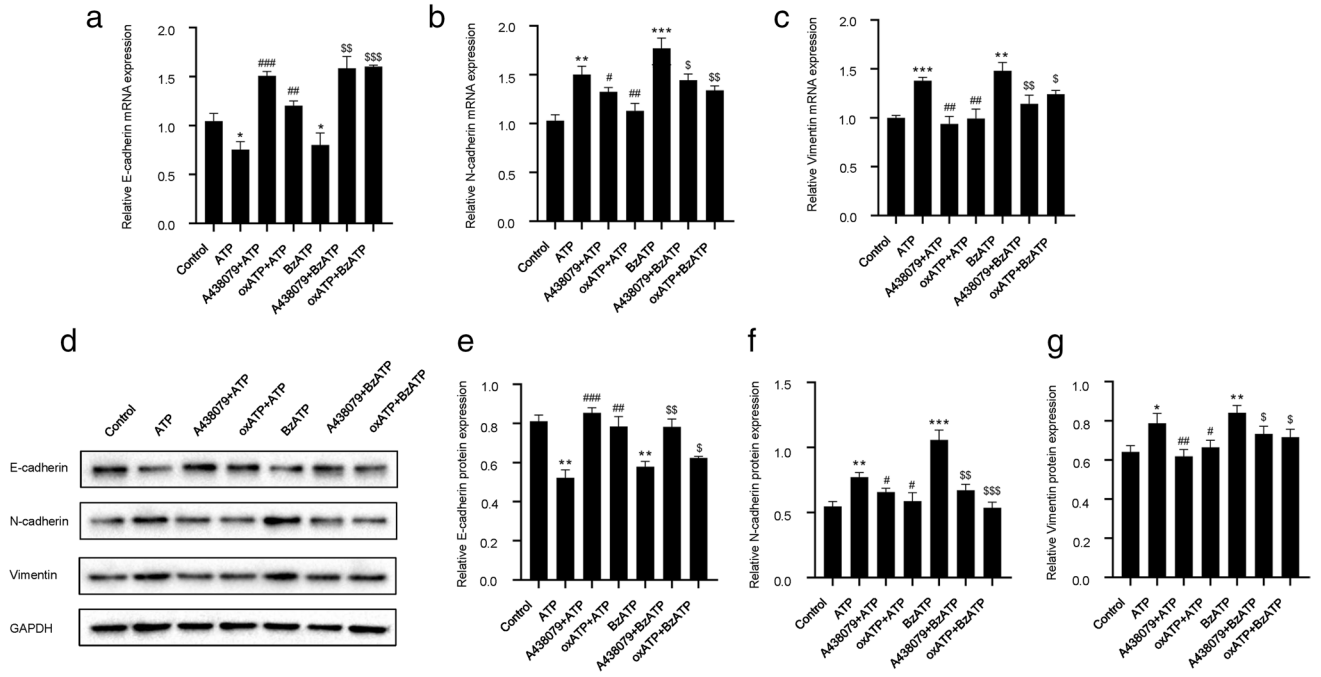

Fig. 3 P2X7 receptor regulates the expression of EMT markers in A549 cells. A549 cells were pretreated with or without the P2X7 receptor antagonist A438079 (100 μM) or oxATP (150 μM) for 2 h and then treated with or without ATP (1 mM) or BzATP (200 μM) for 24 h. (**a-c**) The expression of E-cadherin, N-cadherin and vimentin was detected by qRT-PCR. (**d**) The expression of E-cadherin, N-cadherin and vimentin was detected by western blotting. (**e–g**) The relative protein expression levels of E-cadherin, N-cadherin and vimentin under different treatment conditions were analyzed. Data are presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.01$ vs. ATP group; $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.01$ vs. BzATP group

BzATP induced an increase in p-Akt levels, and conversely, ATP and BzATP-induced p-Akt was significantly reduced using A438079 and oxATP (Fig. 4a, b). These data suggest that the PI3K/Akt signaling pathway is a downstream target of P2X7 receptor activation. In light of the above results, we further investigated the effect of PI3K/Akt signaling pathway on A549 cell migration and invasion. We treated A549 cells using the PI3K/Akt inhibitor LY294002, and the results of wound healing assay (Fig. 4c, d) and transwell assay (Fig. 4e-h) showed that LY294002 significantly inhibited ATP and BzATP-induced cell migration and invasion. Together, the above findings suggest that the PI3K/Akt signaling pathway is involved in P2X7 receptor-mediated A549 cell migration and invasion.

P2X7 receptor induces EMT and PI3K/Akt expression in NSCLC xenografts

To preliminarily investigate the effect of P2X7 receptor on NSCLC migration and invasion *in vivo*, a subcutaneous xenograft model was established. First, the expression of EMT-related proteins E-cadherin, N-cadherin and vimentin in the transplanted tumor tissues was detected, and the results showed that ATP up-regulated the expression of N-cadherin and vimentin and down-regulated the expression of E-cadherin in the transplanted tumor tissues compared with the control group, indicating that P2X7 receptor induces EMT in an *in vivo* NSCLC model (Fig. 5a-c). Next, the expression levels of total Akt and p-Akt proteins in the transplanted tumor tissues were examined using western blotting. The results showed that activation of P2X7 receptor in the ATP group induced an increase in p-Akt protein expression compared with the control group (Fig. 5d, e). The above findings suggest that P2X7 receptor can activate PI3K/Akt signaling pathway in transplanted tumor tissues to induce EMT in NSCLC *in vivo*, consistent with the results of *in vitro* studies.

Discussion

With the in-depth study of purinergic signaling, P2X7 receptor has received much attention in the field of cancer research in recent years because of its involvement in the development and progression of tumors. Many studies have confirmed that P2X7 receptor can regulate tumor cell growth, regulate cell migration and invasion, and P2X7 receptor has become one of the attractive potential therapeutic targets

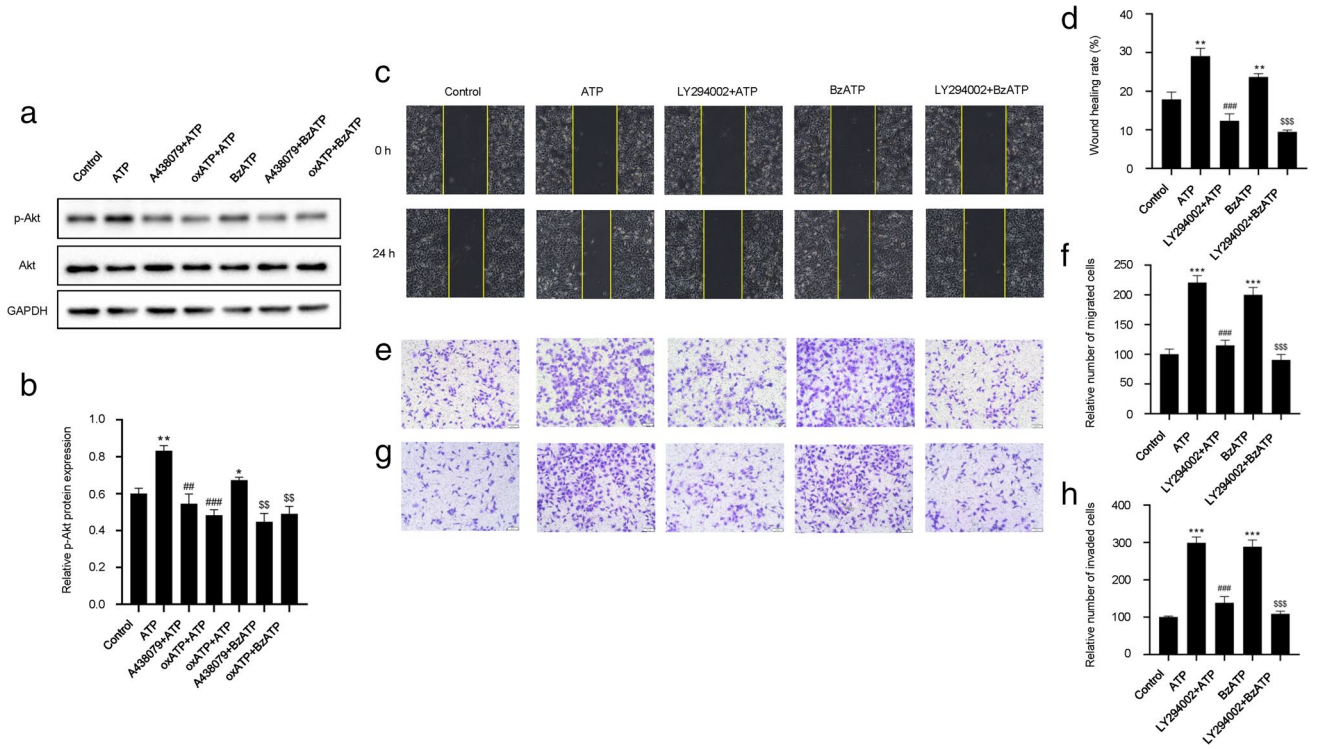

Fig. 4 PI3K/Akt is involved in P2X7 receptor-mediated migration and invasion of A549 cells. A549 cells were pretreated with or without A438079 (100 μM) or oxATP (150 μM) for 2 h and then treated with or without ATP (1 mM) or BzATP (200 μM). (**a**) The protein expression of Akt, p-Akt and GAPDH was detected by western blotting. (**b**) The relative expression levels of p-Akt under different treatment conditions were analyzed. A549 cells were pretreated with or without PI3K inhibitor LY294002 (10 μM) for 1 h and then treated with ATP (1 mM) or BzATP (200 μM) for 24 h. (**c, d**) The migration ability of A549 cells was detected by wound healing assay. (**e, f**) Transwell migration assay was used to detect the migration ability of A549 cells. (**g, h**) Transwell invasion assay was used to detect the invasive ability of A549 cells. Data are presented as the mean ± SD of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.01$ vs. ATP group; $^{\$}P<0.05$, $^{\$\$}P<0.01$, $^{\$\$\$}P<0.01$ vs. BzATP group

closely related to tumors. Currently, little is known about the role of P2X7 receptor in lung cancer, so more detailed studies are needed to reveal the molecular mechanism by which P2X7 receptor mediates lung cancer progression. In the present study, we demonstrated that P2X7 receptor activation promotes EMT, migration and invasion of A549 cells, and promotes EMT in xenograft tumors.

P2X7 receptor has been found to be highly expressed in a variety of tumors, including colorectal cancer [49], breast cancer [50], gastric cancer [51], pancreatic cancer [52], prostate cancer [35], and papillary thyroid cancer [53]. However, unlike most tumors, P2X7 receptor expression is lower in uterine epithelial cancer tissues than in corresponding normal tissues [54]. It is speculated that the expression level of P2X7 receptor may be related to different organs and tumor types. High expression of P2X7 receptor has been found to correlate with clinicopathological features of certain tumors. High P2X7 receptor expression in gastric cancer is closely related to vascular invasion, distant metastasis and TNM stage [55]. In colorectal cancer, high P2X7 receptor expression was significantly correlated with tumor size, lymph node metastasis, and may be an independent indicator of poor prognosis in colorectal cancer patients [56]. Study has confirmed that P2X7 receptor is expressed in NSCLC tissues [48], and the level of P2X7 receptor expression is closely related to clinical outcomes [40]. The expression of P2X7 receptor was not detected in non-cancerous BEAS-2B bronchial epithelial cells, but was detected in human lung cancer cell lines (H292, PC-9, and A549) [36, 37]. In this study, we examined P2X7 receptor expression in A549 cells by immunoblotting. Our findings showed that A549 cells express P2X7 receptor, which is consistent with the previous research results.

The main function of P2X7 receptors is to form ion channels and macropores, which act as bifunctional receptors, and the activation of P2X7 receptors is related to factors such as the concentration, duration, and cell type of ATP stimulation [57]. Recently, it has been found that a different conformational form of P2X7 receptor, called non-porous functional P2X7 receptor (nfP2X7), is widespread in tumors, which cannot form functional pores, but preserves ion channel function, which contributes to

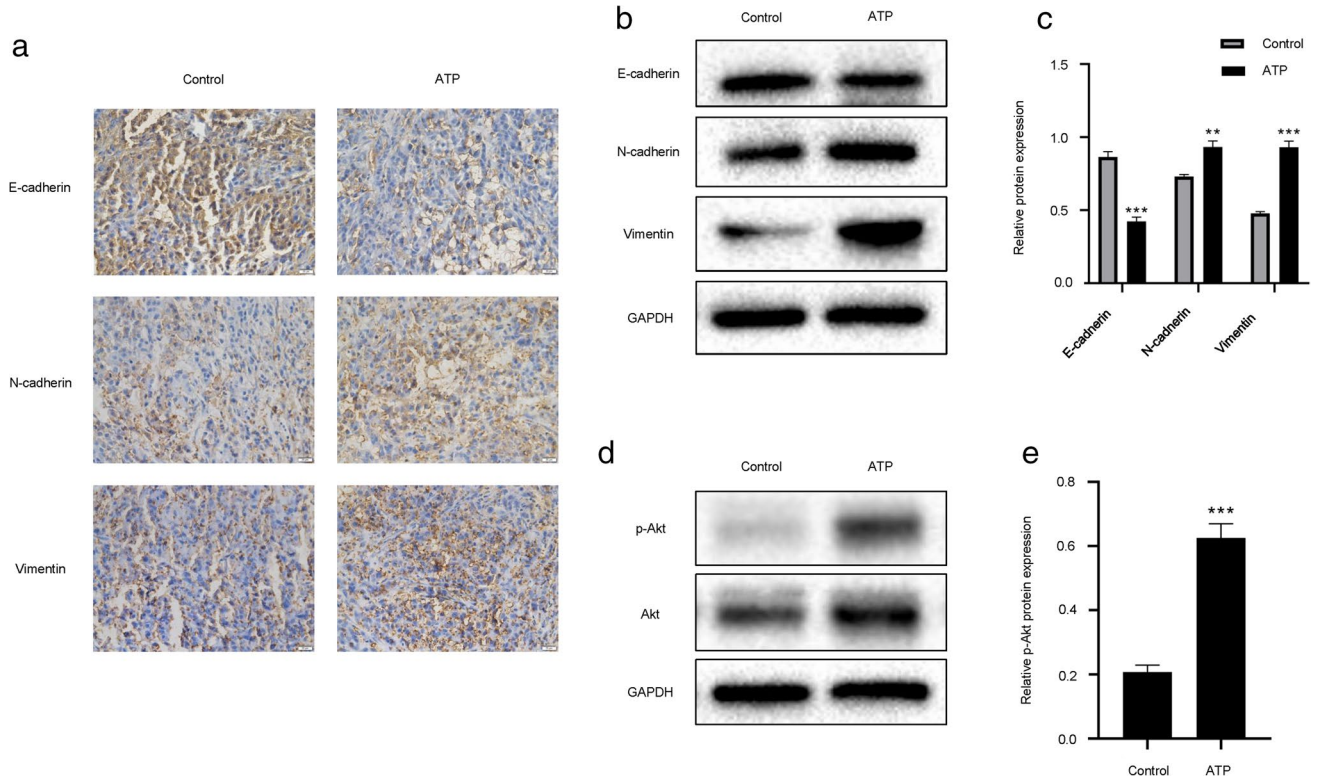

Fig. 5 P2X7 receptor affects the expression of EMT-related markers and the activation of PI3K/Akt signaling pathways *in vivo*. (**a**) Immunohistochemical staining was used to detect the expression of E-cadherin, N-cadherin and vimentin in the transplanted tumor tissues. (**b**) Western blotting was used to detect the protein expression of E-cadherin, N-cadherin and vimentin in the transplanted tumor tissues. (**c**) The relative protein expression levels of E-cadherin, N-cadherin and vimentin under different treatment conditions were analyzed. (**d**) Western blotting was used to analyze the relative expression level of p-Akt in the transplanted tumor tissues. (**e**) The relative protein expression levels of p-Akt under different treatment conditions were analyzed. Data are presented as mean ± SD, $n = 6$ mice/group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control group

tumor cell survival, proliferation, and metastasis [58]. In the present study, we determined whether P2X7 receptors expressed in A549 cells were functional by Flou-4, AM and YO-PRO-1 uptake assays. The results showed that P2X7 receptor caused an increase in intracellular Ca^{2+} influx under transient stimulation with ATP or BzATP; YO-PRO-1 uptake was increased in A549 cells under continuous stimulation with high concentrations of ATP or BzATP, while the P2X7 receptor antagonist A438079 blocked the above effects, indicating that P2X7 receptor expressed in A549 cells has ion channel and macropore function. Our results are similar to those obtained for other tumor cells such as neuroblastoma cells, pancreatic ductal adenocarcinoma cells, glioma cells, etc. [32, 59, 60].

Numerous studies have confirmed that P2X7 receptor plays a key role in the occurrence and development of tumors and is closely related to the growth, invasion and metastasis of tumor cells [61–63]. P2X7 receptor as a tumor promoter in osteosarcoma enhanced cell proliferation, migration and invasion, and increased VEGF release and angiogenesis [64]. P2X7 receptor promoted the proliferation and migration of human glioma cells, but had no significant effect on apoptosis [65]. In colorectal cancer, ATP and BzATP induce P2X7 receptor activation, which promotes cell proliferation and progression through activation of the PI3K/Akt/GSK-3β pathway [66]. P2X7 receptor can activate SK3 potassium channels to enhance breast cancer cell morphological changes and migration, and can also enhance the invasiveness of cysteine cathepsin-dependent cancer cells [67]. Studies have reported that autocrine ATP-P2X7 receptor signaling enhances TGF-β1-induced lung cancer cell migration [36, 37]. Studies have confirmed that extracellular ATP plays an important role in regulating tumor cell motility, migration and invasion [68, 69]. P2X7 receptor is an important receptor mediating ATP pro-invasion [70] and pro-metastasis [71]. The results of Cao et al. [72] further demonstrate that P2X7 receptor is involved in extracellular ATP-induced EMT, migration and invasion of lung cancer cells. In the present study, we investigated the effect of P2X7 receptor on A549 cells migration and invasion, and our results are consistent with most findings that activation of P2X7 receptor by ATP or BzATP enhances migration

and invasion of A549 cells, while P2X7 receptor antagonists attenuate this effect, indicating that activation of P2X7 receptor promotes A549 cell migration and invasion.

EMT is an important process in tumor development and progression and is closely related to tumor migration, invasion and metastasis [42]. Studies have confirmed that EMT plays an important role in NSCLC progression, migration and invasion [72–74]. Extracellular ATP acts as a messenger that activates EMT-inducing signals, alters the levels of EMT-related proteins, and induces changes in the morphology and motility of lung cancer cells [72]. In addition, P2X7 receptor has been found to increase Snail expression and decrease E-cadherin and claudin-1 expression in prostate cancer cells [35]. In colorectal cancer cells, activation of P2X7 receptor significantly increased the expression of vimentin, Snail and fibronectin and decreased the expression of E-cadherin, thereby promoting tumor cell EMT and migration [66]. The above findings suggest that P2X7 receptor can affect the EMT process in tumor cells. In this study, we examined the expression of E-cadherin, N-cadherin and vimentin in NSCLC by *in vitro* and *in vivo* experiments. The results of in vitro experiments showed that ATP or BzATP activated P2X7 receptor to up-regulate the expression of N-cadherin and vimentin, and down-regulate the expression of E-cadherin, while P2X7 receptor antagonist attenuated the regulatory effect of ATP or BzATP on EMT expression. Similar results were observed in preliminary *in vivo* experiments, indicating that P2X7 receptor induces the development of EMT in the tumor xenograft model.

P2X7 receptor can activate multiple signaling pathways (e.g., ERK, Akt, c-Jun N-terminal kinase) to function [75–77]. Among them, the PI3K/Akt signaling pathway plays an important role in tumor progression, and signaling activation can be involved in the regulation of a variety of cellular functions, including cell proliferation and survival, migration, invasion and angiogenesis [78]. The PI3K/Akt pathway is crucial for the up-regulation of P2X7 receptor gene expression in neuroblastoma cells after serum starvation [79]. P2X7 receptor has been found to promote gastric and ovarian cancer cell proliferation by activating Akt and ERK signaling pathways [51, 80]. In colon cancer, on the other hand, P2X7 receptor synergistically regulates cellular autophagy and growth through the PI3K/Akt and AMPK-PRAS40-mTOR pathways, which leads to tumor cell death [81]. In addition, it was found that P2X7 receptor activated Akt pathway to promote the migration and invasion of T47D breast cancer cells [45]. P2X7 receptor can also activate PI3K/Akt and ERK1/2 pathways to enhance invasion and metastasis of prostate cancer cells [35]. Although studies have confirmed that the Akt pathway also plays a key role in NSCLC progression [82], the role of the P2X7 receptor-mediated PI3K/Akt pathway in lung cancer is unknown. In this study, we found that in A549 cells, activation of P2X7 receptor increased p-Akt levels, whereas P2X7 receptor antagonist decreased p-Akt levels. We further found that blocking the PI3K/Akt pathway resulted in a significant reduction in P2X7 receptor-mediated cell migration and invasion. These data suggest that P2X7 receptor enhances migration and invasion of A549 cells through activation of the PI3K/Akt signaling pathway.

In conclusion, this study confirms that P2X7 receptor is expressed in NSCLC cells and plays an important role in the progression of NSCLC cells. Our study shows that P2X7 receptor activation can promote EMT, migration and invasion of A549 cells, and promote EMT and PI3K/Akt expression in transplanted tumors. In addition, we revealed that the PI3K/Akt signaling pathway is required for P2X7 receptor mediated migration and invasion of A549 cells using the PI3K/Akt inhibitor LY294002. Therefore, we suggest that P2X7 receptor may be a potential therapeutic target in NSCLC.

However, there are still some shortcomings and defects in this study, ATP can activate other purinergic receptor subtypes, such as P2X1R and P2YRs and other P2 receptors [83], and *in vivo* studies using only ATP do not fully confirm that it is the P2X7 receptor that plays a role in NSCLC *in vivo*. In addition, it must be pointed out that this is a small pilot study *in vivo*, the effect of P2X7 receptor on NSCLC proliferation and tumor size as well as the effect of P2X7R-specific antagonists need more research and discussion. In the next step, we will apply P2X7 receptor-specific agonists (BzATP) and antagonists (A438079 and oxATP) to deeply investigate the role of P2X7 receptors in NSCLC *in vivo* by comprehensively observing tumor progression. At the same time, P2X7 receptor-specific antagonists were used to establish preclinical animal models, which lay a good preclinical foundation for P2X7R antagonists that may become novel targeted drugs for the treatment of NSCLC patients.

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Author contributions RLZ designed and supervised the study. RLZ, XB, QQL, XXP, XYL, CCQ and YQT performed the experiments. RLZ, XB, QQL, XXP, XYL, CCQ and YQT analyzed the results. RLZ, XB, QQL, XXP drafted the manuscript. RLZ, XB, QQL, XXP revised the manuscript. All authors approved the manuscript submitted.

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Data availability The authors declare that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval All animal experiments were approved by the Animal Care Committee of Weifang Medical University (No 2021SDL124)

and performed according to the guidelines of the National Institutes of Health.

Consent to participate Not applicable.

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Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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