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ORIGINAL PAPER

Mechanism of VIPR1 gene regulating human lung adenocarcinoma H1299 cells

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Abstract

The vasoactive intestinal peptide receptor-1(VIPR1) has prominent growth effects on a number of common neoplasms. However, there were contradictions in the effect cross different cancers. We aimed to explore the effect of VIPR1 overexpression on a human lung adenocarcinoma cell line H1299. GEO dataset was used to screen differentially expressed genes in lung adenocarcinoma tissues. The expression of VIPR1 mRNA was determined in the cancer Genome Atlas (TCGA). Immunohistochemical analysis was performed to determine VIPR1 protein expression in lung adenocarcinoma and corresponding adjacent tissues ($n=22$). Fluorescence real-time quantitative PCR detected the expression of VIPR1 in human normal lung epithelial cell line BEAS-2B and lung adenocarcinoma cell line H1299. Overexpression strategies were employed to assess functions of VIPR1 expression on several malignant phenotypes in H1299. The expression of VIPR1 was lower in lung adenocarcinoma tissues than that in adjacent tissues. Compared with the normal lung epithelial cells BEAS-2B, VIPR1 was down-regulated in lung cancer cells H1299 ($P<0.05$). After the overexpression of VIPR1, we found that VIPR1 significantly inhibited growth, migration, and invasion of H1299 cells ($P<0.05$). Our findings point out the tumor suppressor roles of VIPR1 in human LUAD pathogenesis.

Keywords VIPR1 · Lung adenocarcinoma · Proliferation · Migration and invasion

Introduction

Lung cancer, as one of the most common malignancies, has become the leading cause of cancer-related deaths in both male and female all over the world [1]. Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all diagnosed lung cancers. Lung adenocarcinoma (LUAD) is the most common histological subtype of NSCLC [1]. Although there has been significant progress in targeted cancer therapeutics, the treatment of LUAD remains a challenge due to its acquired drug resistance and unknown driving factors [2]. Therefore, new molecular modulators for LUAD need to be identified to provide insight into potential therapeutic targets.

Vasoactive intestinal peptide (VIP), also known as PACAP, is an important neuropeptide that controls lung physiology and mainly functions via two receptor subtypes VAPC1 and VAPC2 [3]. Vasoactive intestinal peptide is a small neuropeptide involved in relaxation of smooth muscle, exocrine, endocrine, and hydration ion flux in lung and intestinal epithelial cells. Research showed that the reduction of VIP may be related to the occurrence of disease and overexpression of VIP could inhibit inflammation reducing acute lung injury of mice [4]. But other studies also found that the immune response and survival rate of VIP knockout mice increased [5]. VIP or VPAC1 receptor antagonist could strengthen the ability of chemotherapy killing breast cancer cells [6, 7] and improve anti-viral immunity [8]. These studies all indicate that VIPR can be used as a tumor diagnosis and treatment target for its potential role in tumor development and progression. Dr. Teddy Moody has summarized the function of VIP/PACAP and its receptor (VPAC1 and VPAC2) in human normal tumors and their role in potential new treatments [9]. Recent study shows that PACAP stimulates the growth of NSCLC cells through activating EGFR [10]. VIP antagonist in vitro inhibits the proliferation of NSCLC and reduces the growth of NSCLC tumors transplanted into nude mice [11]. However, its biological function

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in LUAD remains unclear. In present study, we investigated the effects of VIPR1 overexpression on the growth, migration, and invasion of LUAD cells H1299 to discover their effects on the development of LUAD.

Materials and methods

Materials and reagents

The human normal lung cell line BEAS-2B and human lung adenocarcinoma cell line H1299 cell line were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences; the lentiviral expression vector LV5-EF1a-VIPR1 and the control vector LV5-NC were purchased from Shanghai GenePharma. RPMI-1640 medium was purchased from Sigma, USA. Primary rabbit polyclonal antibody VIPR1 and the second goat anti-rabbit IgG were purchased from Abcam, England. Edu cell proliferation assay kit was purchased from Beijing Solarbio Technology Co., Ltd. Trizol lysate was purchased from Invitrogen, USA, PrimeScript RT kit was purchased from Beijing Baori Medical Science and Technology Co., Ltd., and PCR kit was purchased from Beijing Tiangen Biochemical Technology Co., Ltd. The CCK-8 kit was purchased from Shanghai 7sea Biotechnology Co., Ltd., item number: C008-3. The Transwell chamber was purchased from Millipore, USA.

Bioinformatics analysis

The gene expression data matrix (GSE118370) of lung adenocarcinoma tissue and adjacent tissues was obtained from the National Center for Biotechnology GEO database (https://www.ncbi.nlm.nih.gov/geo/). which included 12 samples. The data analysis was performed with R language using the 'limma' package. The empirically Bayesian method was used to select important differentially expressed genes (DEGs). Finally, DEGs were annotated by the 'annotate' package. The expression level of VIRP1 mRNA and the prognosis survival curve of clinical samples were collected and searched from The Cancer Genome Atlas (TCGA, http://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl) lung cancer database.

Clinical samples

22 paired LUAD and normal lung tissue samples were collected in Second Affiliated Hospital, Zhejiang University School of Medicine. All patients underwent surgical resection in cardio-thoracic surgery of our hospital from July 2017 to January 2018. The gender, age, and LUAD clinicopathological features data collected from all subjects were as follows: gender: 12 males and 10 females; aged from 41 to 79 years, the average age is 64 years; TNM staging: 9 cases in stage I, 7 cases in stage II, 5 cases in stage III, 1 case in stage IV; 13 cases with lymph node metastasis, 9 cases without lymph node metastasis. None of the patient underwent radiotherapy or chemotherapy before surgery, and specimens were diagnosed by histopathological examination after operation. There was no significant difference in age and gender composition between the two groups. All patients have signed informed consent, and this study was approved by The Research Ethics Committee of Second Affiliated Hospital, Zhejiang University School of Medicine.

Cell culture and lentiviral vector construction

The human normal lung cell line BEAS-2B and the lung adenocarcinoma cell line H1299 were cultured in RPMI-1640 medium containing 5% fetal bovine serum (FBS) in an incubator at 5% CO2 and 37 °C. Cells were adherent to the cyathotheca with the density reached 70–80%, digested with 0.25% trypsin and cultured. The cells in logarithmic growth phase were selected for experiment.

To establish the stable overexpression VIPR1 cell line, H1299 cells were transfected using LV5-EF1a-VIPR1 lentivirus (LV5-VIPR1). Cells infected with vector-control sequences were used as controls (LV5-NC). Cells were isolated by 5 μg/mL puromycin for 48 h and treated with 1 μg/mL puromycin. The VIPR1 expression level was confirmed by qRT-PCR. Cells were collected for subsequent experiments.

Immunohistochemistry

Immunohistochemical (IHC) analysis of 44 specimens (22 lung cancer and adjacent tissues) embedded in paraffin was performed using anti-VIPR1 (1:200). After detachment using xylene and different concentrations of ethanol, the sections were mounted using 5% BSA for 20 min. Then, according to the description, the primary anti-VIPR1 and anti-goat anti-rabbit IgG (1:1000) were added. They were colored using DAB, and then counterstained using hematoxylin and mounted. For each slice, 5 fields of view were randomly selected and photographed. The experiment was repeated 3 times.

Real-time fluorescence quantitative PCR

Total RNAs of NSCLC cells were extracted with Trizol. The RNA concentration was measured by UV spectrophotometer, and reverse transcribed into cDNA using the PrimeScript RT kit as a template. The PCR primers for VIPR1 (sense, 5′-TCATCCGAATCCTGCTTCAGA-3′ and antisense, 5′-AGGCGAACATGATGTAGTGTACT-3′); GAPDH (sense,5′-GGAGCGAGATCCCTCCAAAAT-3′

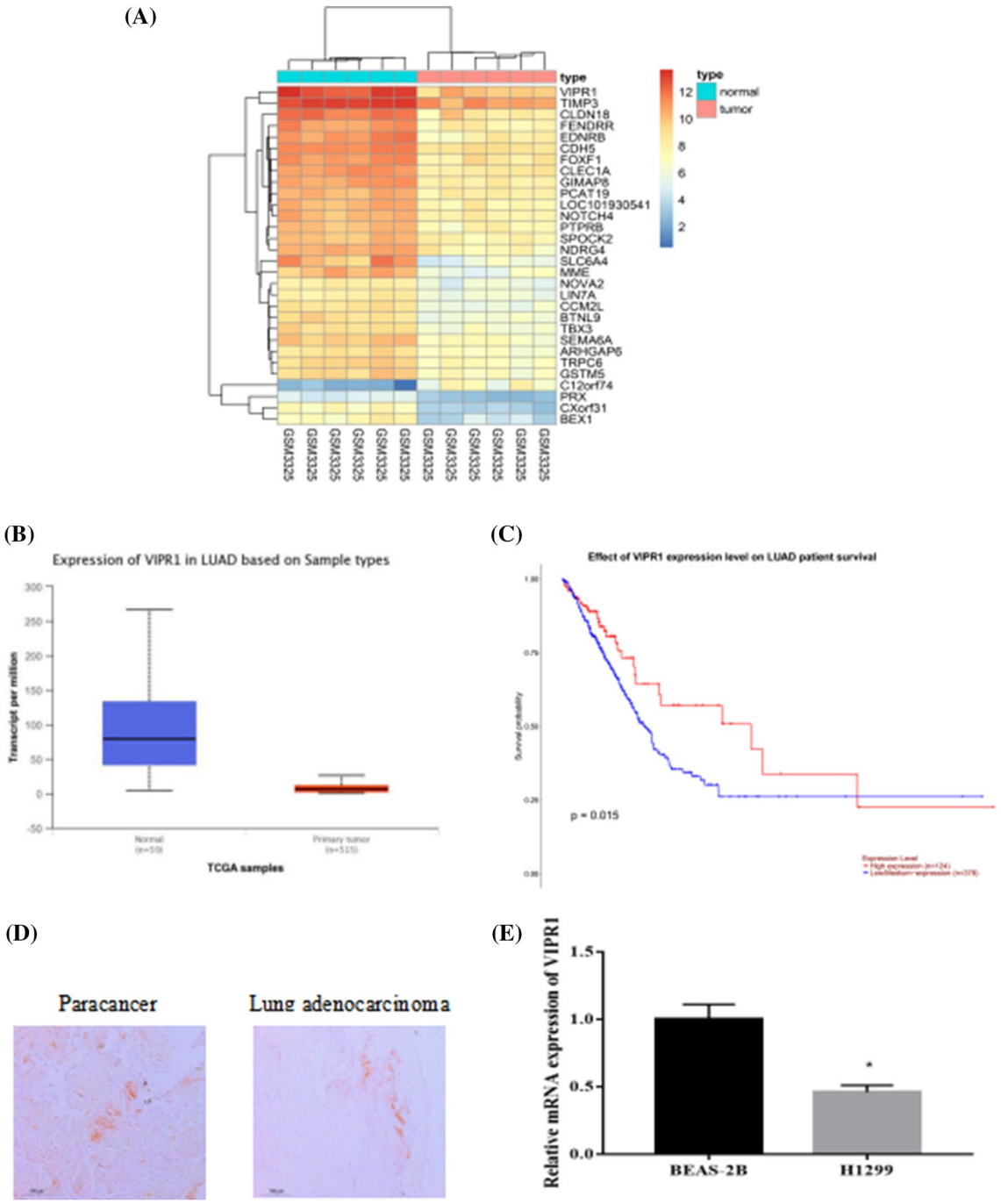

Fig. 1 Low expression of VIPR1 in lung adenocarcinoma. **a** Differential expression heatmap of GSE118370; **b** expression of VIPR1 gene in TCGA lung cancer database; **c** survival curve of VIPR1 gene in TCGA lung cancer database; **d** the expression of VIPR1 in lung adenocarcinoma tissue sections detected by immunohistochemistry; **e** the expression of VIPR1 in the BEAS-2B and H1299 cell lines was detected by qRT-PCR; * indicates $P < 0.05$

and antisense 5′-GGCTGTTGTCATACTTCTCATGG-3′) were designed using primer 5.0. GAPDH served as an internal reference. PCR was carried out according to the procedure in PCR kit. The reaction conditions were pre-denatured at 95 °C for 5 min, 95 °C for 40 s, 57 °C for 40 s, 72 °C for 40 s, 30 cycles, 72 °C for 10 min, and 4 °C for 5 min. The relative transcription level of VIPR1 in H1299 were calculated by $2^{-\Delta\Delta C_t}$. The expression levels of each group of genes were calculated.

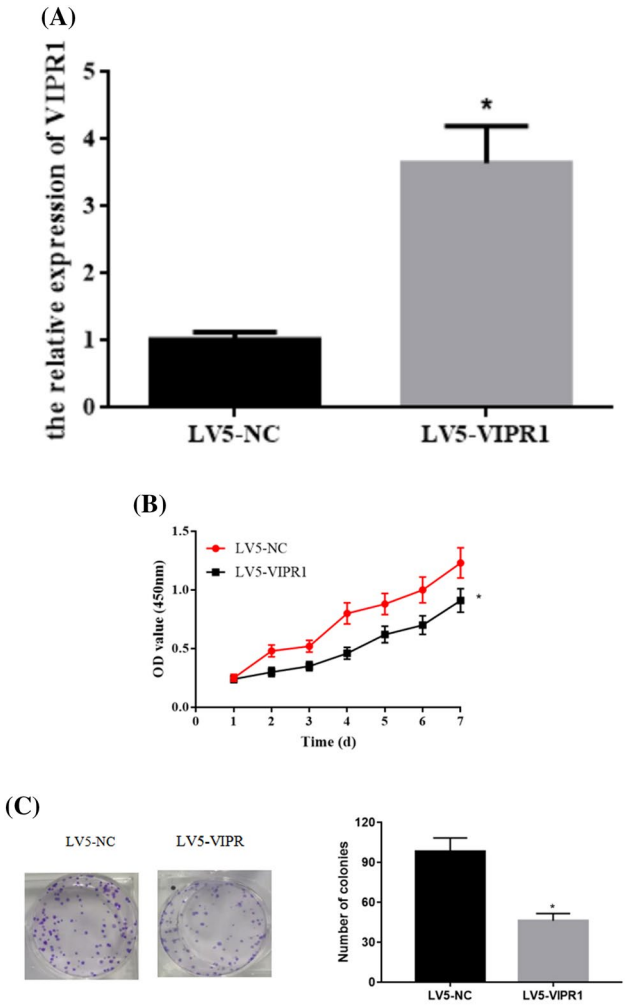

Fig. 2 Up-regulation of VIPR1 inhibits proliferation of H1299 cells. **a** The overexpression efficiency of LV5-VIPR1 on VIPR1 mRNA in H1299 cells; **b** CCK-8 method detected the growth rate of cells; **c** clone formation assay detected proliferation of H1299 cells (×100); * indicates $P < 0.05$

CCK-8 method

Cell viability was monitored using cell counting kit-8 (CCK-8). H1299 cells were seeded in 96-well plates at a density of 3×10^4/cell and cultured overnight at 37° C, 5% CO_2. Approximately 10 µL CCK-8 solution was added to each well and incubated for 4 h at 37 °C. Then, the absorbance at 450 nm was measured using ultraviolet spectrophotometer every other day for 7 days.

Clone formation assay

After transfection, H1299 cells were digested with 0.25% trypsin into individual cells. The cells were suspended in the solution. H1299 cells (2×10^2/well) were culture in 10 mL culture medium for 3 weeks. When macroscopic clones appeared in the culture dish, the colonies were fixed with 4% paraformaldehyde for 15 min and then stained with 0.1% crystal violet stain for 10 min. The colonies were then counted and clone formation rate was calculated. Clone formation rate = (number of clones/number of cells inoculated) × 100%.

Transwell migration and invasion experiment

For the migration experiments, the transiently transfected H1299 cells with the lentiviral vector described above were seeded in a 24-well Transwell chamber with a density of 2×10^5/well and 500 µL of medium containing 10% FBS was added to the lower chamber. After 24-h culturation in the incubator, the non-migrated cells in the upper chamber were wiped with a wet cotton swab. The invaded cells were fixed with 4% paraformaldehyde solution for 30 min, dyed with 0.1% methylrosanilinium chloride solution, and then observed with a microscope. Five fields of view were randomly selected and the number of transmembrane cells was counted. The H1299 cells invasion experiments were using the Matrigel gel, which was placed in the upper chamber according to the manufacturer's protocol. The seeding, staining, and counting of H1299 were performed as before.

Statistical analysis

All data were processed using SPSS 22.0 statistical software and reported as the mean ± standard deviation. All experiments were performed at least in triplicate. Comparisons between groups were determined by a paired *t* test. $P < 0.05$ was considered statistically significant.

Results

VIPR1 is underexpressed in lung adenocarcinoma

In order to investigate the important DEGs in lung adenocarcinoma tissues, we firstly explored the mRNA expression profiles in lung adenocarcinoma tissues. According to the GSE118370 database, 333 DEGs were down-regulated and 206 DEGs were up-regulated in LUAD compared to normal tissue. Top 30 DEGs were selected for cluster heatmap. As shown in Fig. 1a, VIPR1 was significantly down-regulated in LUAD tissues. To confirm the role of VIPR1 in LUAD, the decreased expression of VIPR1 was also confirmed using TCGA LUAD database (Fig. 1b). The Kaplan–Meier survival curve revealed that lower VIRP1

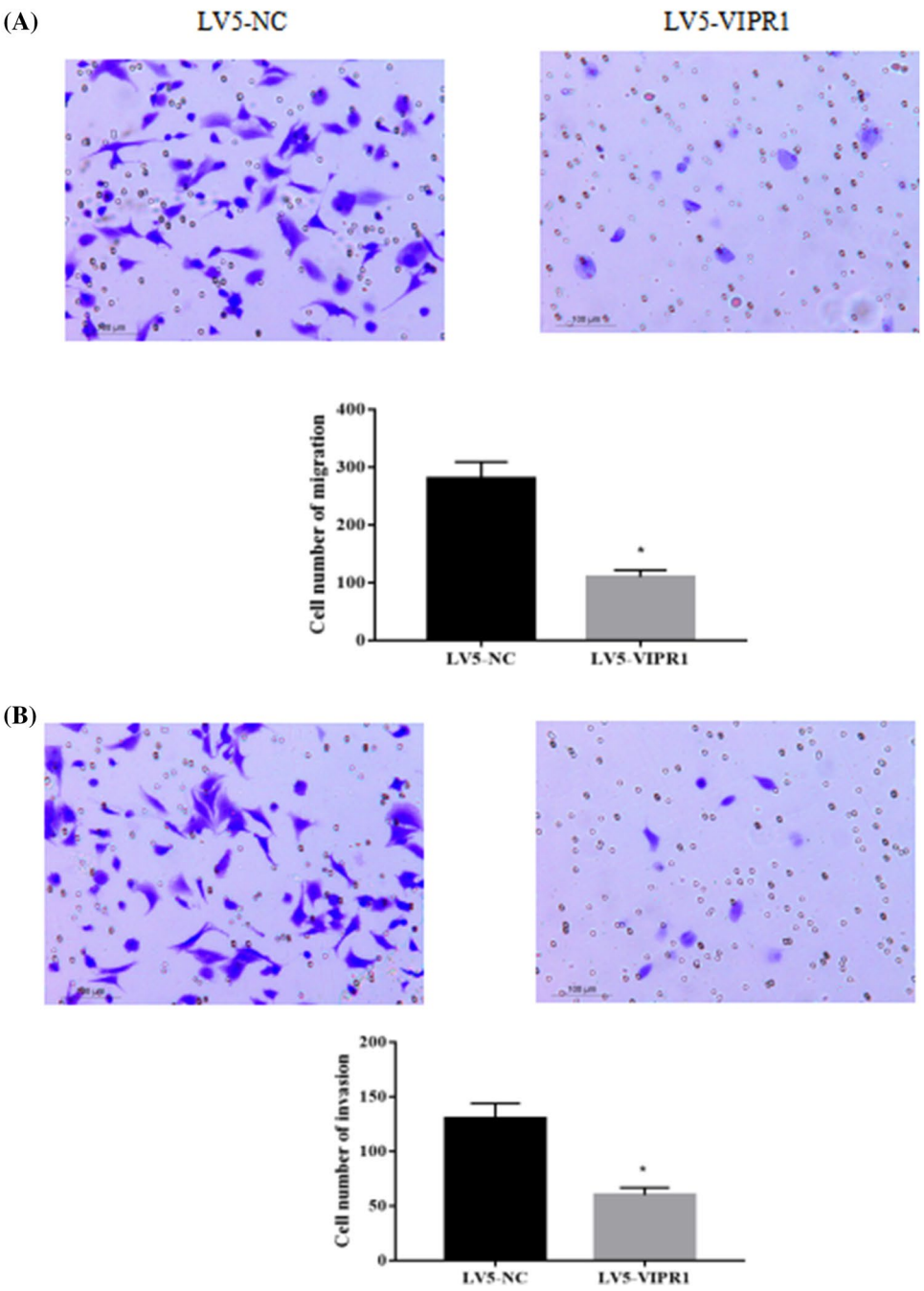

Fig. 3 Up-regulation of VIPR1 inhibits migration and invasion of H1299 cells. **a** Migration ability of H1299 cells in each group detected by Transwell assay; **b** invasive ability of H1299 cells in each group detected by Transwell assay; * indicates $P < 0.05$

expression was associated with a worse overall survival in LUAD patients (Fig. 1c).

We then examined the expression of VIPR1 in LUAD tissues and cell lines. Twenty-two pairs of LUAD tissues and matched adjacent non-tumor tissues were collected and tested for VIPR1 expression with immunohistochemistry. The results showed that the expression of VIPR1 was decreased in LUAD tissue (Fig. 1d). Furthermore, qRT-PCR was used to detect the expression of VIPR1 in human normal lung cell line BEAS-2B and LUAD cell line H1299. As shown in Fig. 1e, the expression of VIPR1 in H1299 cells was significantly down-regulated compared with BEAS-2B cells ($P < 0.05$).

VIPR1 up-regulation inhibits the proliferation of H1299 cells

We further explored the potential functional role of VIPR1 in LUAD H1299 cells. The LUAD cells were transfected with LV5-VIPR1 or LV5-NC, and the qRT-PCR analysis indicated that the expression level of VIPR1 was

significantly overexpression in LV5-VIPR1 cells (Fig. 2a). Subsequent CCK-8 assay showed that the up-regulation of VIPR1 effectively inhibited the growth rate of cells ($P < 0.05$), as shown in Fig. 2b. The cloning ability of H1299 cells was detected by clone formation assay, as shown in Fig. 2c: compared with LV5-NC, the number of clonal cells in the LV5-VIPR1 group significantly decreased ($P < 0.05$).

Up-regulation of VIPR1 inhibits migration and invasion of H1299 cells

The effect of VIPR1 on migration and invasion of H1299 cells was examined by Transwell. Overexpression of VIPR1 observably inhibited the migration and invasion ability of LV5-VIPR1 group compared with LV5-NC group as shown in Fig. 3: Migration and invasion number of cells of LV5-VIPR1 group compared with LV5-NC significantly decreased ($P < 0.05$).

Discussion

VIP is an autocrine growth factor for lung cancer whose receptor exists in many lung cancer cell lines [9]. Some studies have reported that VIPR1 inhibits the growth of several cancers, including prostate cancer [12], lymphoblastoma [13], and medulloblastoma [14], which suggests that VIPR1 may significantly inhibit the growth and development of cancer cells. In the present study, overexpression of VIPR1 was found to reduce the proliferative capacity of H1299 cells. The experimental results are consistent with previous studies, indicating that VIPR1 plays an important role in inhibiting the growth of LUAD cells. It may provide a new target for drug intervention in patients with CAP expressing VIPR1.

Tumor metastasis observed in more than 90% of all tumor deaths is considered to be the leading cause of death in cancer patients. However, the underlying mechanisms remain unclear. Overexpression of VIPR1 has been reported to be associated with a relatively low differentiation of colon cancer, which may be caused by subsequent EGFR activation in cancer cells [15]. In addition, overexpression of VIPR1 in blood vessels and macrophages in tumors may also play a key role in development of invasive cancers [15]. The present study indicates overexpression of VIPR1 inhibited migration and invasion of H1299 cells. In conclusion, our results excavate the role of VIPR1 expression in regulation of lung adenocarcinoma's biological behavior, providing new insights into the pathogenesis of VIPR1-mediated lung adenocarcinoma and a new target candidate for effective lung adenocarcinoma treatment.

Compliance with ethical standards

Conflict of interest We have no conflicts of interest to declare.

Ethical approval The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Second Affiliated Hospital, Zhejiang University School of Medicine.

Informed consent Written informed consent was obtained from individual or guardian participants.

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