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Lenvatinib and everolimus synergistically inhibit neuroendocrine neoplasms by upregulating ITGB4 expression

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Abstract

There are currently few treatment options available for advanced neuroendocrine neoplasms (NENs). When treating metastatic renal cancer, the combination of lenvatinib with everolimus has shown noticeably greater success than monotherapy. Clarifying the synergistic effects of lenvatinib and everolimus on NENs and investigating the underlying processes were the goals of this study. Lenvatinib and everolimus were tested for their synergistic inhibitory effects on NEN cells *in vitro* using CCK-8, colony formation, wound healing, flow cytometry, and tube formation. *In vivo* evaluation of the combined inhibitory effects was conducted using a xenograft mouse model. Western blot analysis was used to look into the main regulatory molecules implicated in this synergy, and clinical case studies were used to quantify the clinical benefits. The combination of lenvatinib and everolimus showed a synergistic anti-tumor impact *in vivo* and suppressed cell proliferation, colony formation, cell migration, angiogenesis, and promoted apoptosis *in vitro*. Notably, in patients with advanced NENs who developed resistance after many lines of therapy (>2nd line), this combination successfully postponed the advancement of their disease. Mechanistically, both medications increased the expression of ITGB4 and inhibited 4EBP1 phosphorylation; their combined inhibitory effects on NENs were lessened when ITGB4 was knocked down. These findings indicate that lenvatinib and everolimus act synergistically to inhibit NEN proliferation and migration and to induce apoptosis *in vitro*, with robust synergistic activity also evident *in vivo*. This synergy appears to be mediated, at least in part, through the upregulation of ITGB4.

Keywords: neuroendocrine neoplasms; lenvatinib; everolimus; 4EBP1; ITGB4

Introduction

Neuroendocrine neoplasms (NENs), including neuroendocrine carcinomas (NECs) and neuroendocrine tumors (NETs), represent a heterogeneous group of malignancies that originate from neuroendocrine cells. These neoplasms are characterized by indolent growth patterns and have the potential for significant metastatic dissemination (1, 2). The management of metastatic NENs poses

considerable challenges owing to their variable clinical behavior and limited treatment options available that provide meaningful long-term control (1). Traditional therapeutic strategies include somatostatin analogs, peptide receptor radionuclide therapy, and cytotoxic chemotherapy (3). However, there has been increasing interest in targeted therapies designed to disrupt tumor-specific signaling pathways.

Lenvatinib is a multi-kinase inhibitor that targets vascular endothelial growth factor receptors (VEGFR1–3), fibroblast growth factor receptors (FGFR1–4), platelet-derived growth factor receptor alpha (PDGFR α), RET, and KIT (4). By inhibiting these pathways, lenvatinib may suppress angiogenesis and tumor proliferation. Recent clinical trials have investigated the efficacy and safety of lenvatinib in patients with advanced NENs, demonstrating an objective response rate of 29.9%, median progression-free survival (PFS) of 15.7 months, and manageable safety profile (5).

Everolimus, an mTOR inhibitor, effectively blocks the mTOR signaling pathway, which is often upregulated in NETs (6, 7). This inhibition impedes cellular growth, metabolism, and survival. A phase III trial demonstrated that everolimus was associated with a 52% reduction in the estimated risk of progression or death among patients with advanced nonfunctional NETs originating from the lung or gastrointestinal tract (8).

Given their complementary mechanisms of action, the combination of lenvatinib and everolimus has emerged as a promising therapeutic strategy for the treatment of advanced tumors. A pivotal phase II trial evaluated the effectiveness of everolimus in conjunction with lenvatinib compared with monotherapy with either agent in patients with metastatic renal cell carcinoma (RCC), whose disease had progressed following prior VEGF-targeted therapy (9, 10). The results from this study indicated that combination therapy exhibited superior efficacy relative to monotherapy.

However, there is a paucity of clinical trial evidence regarding the efficacy of combining lenvatinib and everolimus in the treatment of metastatic NENs. This study aimed to elucidate the synergistic effects of lenvatinib combined with everolimus in NENs by highlighting the relevant clinical evidence, exploring the underlying mechanisms of action, and outlining future research directions within this domain.

Materials and methods

Cell lines and reagents

NCI-H727, a human bronchial carcinoid cancer cell line, and human umbilical vein endothelial cells (HUVECs) were purchased from Procell Biotechnology Company (China). QGP-1, a carcinoembryonic antigen (CEA)-producing human pancreatic islet cell carcinoma cell line, was purchased from Cobioer Biotechnology Company (China). H727 and QGP-1 cells were cultured in RPMI-1640 medium (Gibco, USA), and HUVECs were cultured in DMEM (Gibco) containing 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO₂. The reagents used were lenvatinib (MCE, HY-10981)

and everolimus (MCE, HY-10218), which were dissolved in DMSO.

Cell proliferation assay

H727 and QGP-1 cells were seeded into 96-well culture plates in triplicate at densities of 8×10^3 and 12×10^3 cells per well, respectively. Then, DMSO, lenvatinib (H727: 20 μ M; QGP-1: 30 μ M), everolimus (100 nM), and their combination were used to treat H727 or QGP-1 cells for 5 days. Cell counting kit-8 (CCK-8) reagent (Beyotime Biotechnology, China) was added to each well at 0, 24, 48, 72, 96, and 120 h to assess cell survival and proliferation in three independent experiments according to the manufacturer's instructions. Two hours later, the absorbance was measured at 450 nm.

Combination index (CI) values

H727 and QGP-1 cells were seeded into 96-well plates at densities of 8×10^3 and 12×10^3 cells/well, respectively, with three replicate wells for each group. Cells were treated with different concentrations of lenvatinib (H727: 5, 10, 20 μ M; QGP-1: 7.5, 15, 30 μ M) and everolimus (1, 10, 100 nM) in combination for 2 days (H727) or 3 days (QGP-1). Then, CCK-8 reagent (Beyotime Biotechnology, China) was added to each well, and the plates were incubated for 2 h before OD measurement. The CI values were calculated using CompuSyn software based on the Chou–Talalay method: CI > 1.0 indicates antagonism and CI < 1.0 indicates synergy.

Colony formation assay

H727 and QGP-1 cells were seeded in triplicate into 6-well culture plates at a density of 2×10^3 cells per well and treated with DMSO, lenvatinib (H727: 20 μ M; QGP-1: 30 μ M), everolimus (100 nM), and their combination for 2 weeks. The colonies formed were fixed with 4% paraformaldehyde for 30 min at room temperature and subsequently stained with crystal violet for 15 min. A colony containing more than 50 cells and the total colonies of each well were counted using ImageJ software.

Wound healing assay

6×10^5 H727 cells were seeded into culture inserts. When the cells were overgrown, the culture inserts were moved, and serum-free RPMI-1640 medium with DMSO, 20 μ M lenvatinib, 100 nM everolimus, and their combination was added. Cells were imaged at 0 and 72 h in random five fields using a microscope at 100 $\times$ magnification.

Flow cytometry

For cell cycle analysis, H727 cells were harvested after 48 h of DMSO, lenvatinib (20 μ M), everolimus (100 nM), and their combination treatment and QGP-1 cells (lenvatinib: 30 μ M; everolimus: 100 nM) after 72 h. Cells were fixed with 75% ethanol at 4°C overnight, washed with ice-cold PBS, and then propidium iodide (PI) staining solution was added. After incubation for 30 min at 37°C, the stained cells were analyzed using a flow cytometer (Attune Nxt), according to the user guide of the Cell Cycle Assay Kit (Beyotime Biotechnology). For apoptosis analysis, after the cells were treated in the same way as for the cell cycle analysis, they were trypsinized without EDTA and washed with ice-cold PBS. Cells were stained with Annexin V-FITC and PI dye for 15 min, according to the user guide of Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology), and measured using a flow cytometer (Attune Nxt). The data were analyzed using FlowJo software.

Tube formation assay

Matrigel was added to 24-well culture plates on ice and incubated at 37°C for 30 min until the Matrigel solidified. HUVECs pre-treated with DMSO, lenvatinib (10 μ M), everolimus (1 μ M), and their combination for 24 h were seeded into the culture plates in triplicate at a density of 1.5×10^5 cells per well. After 6 h, the cells were imaged in random five fields using a microscope at 40 $\times$ magnification.

Collection of patient information

We collected four NEN patients to analyze the effect of a combination of two drugs for the treatment of advanced NENs through a retrospective study. Permission for patient information collection was granted by the Ethics Committee of The Fourth Affiliated Hospital of Soochow University.

Xenograft mouse model

5×10^6 H727 cells suspended in 100 μ L (1:1 dilution) cold 1 $\times$ PBS and Matrigel (Corning, USA) were injected subcutaneously into the right flanks of 4-week-old female BALB/c nude mice (Jiangsu GemPharmatech Co., Ltd, China). The volume of the tumor mass was calculated using the following formula: $V (\text{mm}^3) = \pi/6 \times \text{length} \times \text{width}^2$. Lenvatinib and everolimus were dissolved in 2% DMSO, 40% PEG 300, 5% Tween-80, and 53% saline. When the tumors grew to approximately 200 mm^3 , the mice were randomized into four groups: control (300 μ L fixed solvent), lenvatinib (4 mg/kg/day), everolimus (3 mg/kg/day), and combination treatment (lenvatinib: 4 mg/kg/day; everolimus 3 mg/kg/day). In addition, each group contains six mice. The administration was performed via intraperitoneal injection. After 12-day

treatment, the mice were sacrificed. Permission for animal experiments was granted by the ethics committee of The Fourth Affiliated Hospital of Soochow University and was performed according to the Guide for Care and Use of Laboratory Animals.

Western blot

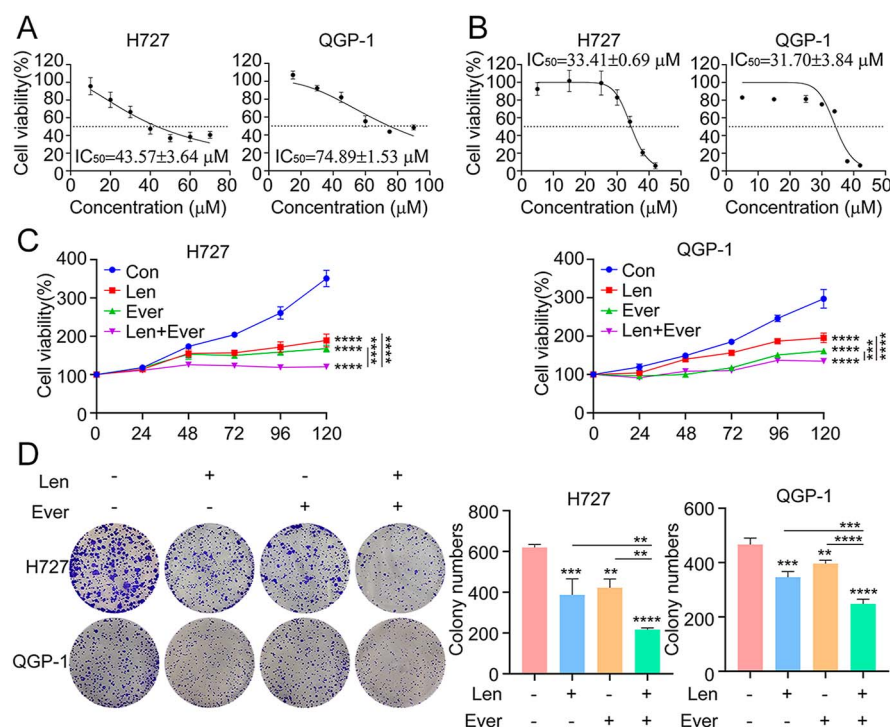
H727 cells were pretreated with DMSO, lenvatinib (20 μ M), everolimus (100 nM), and their combination for 2 days, and QGP-1 cells were pretreated under the same conditions with DMSO, lenvatinib (30 μ M), everolimus (100 nM), and the combination for 3 days. Cells or tumor tissues from xenografts were then lysed in RIPA buffer (Beyotime Biotechnology). A BCA kit (Epizyme Biotech, China) was used to determine the total protein concentration. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Millipore, Germany), and then incubated with primary antibodies at 4°C overnight. The membranes were then washed and incubated with secondary antibodies at room temperature for at least 1 h. Chemiluminescent HRP substrates (Proteintech, China) were used to probe antibody binding. The antibodies used were as follows: anti-Akt (4691, CST), anti-phosphorylated Akt (Ser473) (4060, CST), anti-mTOR (2983, CST), anti-phosphorylated mTOR (Ser2448) (5536, CST), anti-p70 s6k (2708, CST), anti-phosphorylated p70 s6k (Thr389) (9234, CST), anti-4EBP1 (9644, CST), anti-phosphorylated 4EBP1 (Thr37/46) (2855, CST), anti-FGFR4 (8562, CST), anti-LAMC2 (sc-28330, Santa Cruz), anti-ITGB4 (14803, CST), anti-ITGB6 (95153, CST), anti- β -actin (4970, CST), anti-rabbit IgG (7074, CST), and anti-mouse IgG (sc-527346, Santa Cruz, Japan).

RT-qPCR

After H727 cells were treated with DMSO, 20 μ M lenvatinib, 100 nM everolimus, and their combination for 48 h, total RNA was extracted using TRIzol reagent (Invitrogen, USA) and quantified using a NanoDrop Spectrophotometer (Tiangen, China). Subsequently, 1 μ g total RNA was reverse-transcribed using the PrimeScript™ FAST RT Reagent Kit with gDNA Eraser (Takara, Japan). The resulting cDNA was amplified using a real-time PCR detection system (Applied Biosystems, USA) with PowerUp™ SYBR™ Green (Applied Biosystems). qPCR experiments were performed in triplicate, and relative expression was normalized to endogenous actin beta (ACTB) and calculated according to the $\Delta\Delta\text{Ct}$ method. The primer sequences used were displayed in Supplementary Table S1 (see the section on [Supplementary materials](#) given at the end of the article).

Lentiviral infection

We infected H727 cells with lentivirus (Shanghai Genechem Co., Ltd, China) at MOI = 50 and obtained

**Figure 1**

Lenvatinib combined with everolimus showed a stronger anti-tumor efficacy on NEN cells survival and proliferation. (A and B) IC₅₀ values of lenvatinib and everolimus in the H727 cell line on day 2 and in the QGP-1 cell line on day 3, respectively. (C) Cell viability assay conducted to evaluate the inhibitory effects of lenvatinib (H727: 20 µM; QGP-1: 30 µM), everolimus (100 nM), and their combination treatment on the survival of H727 and QGP-1 cell lines. (D) Colony formation assay performed to assess the impact of lenvatinib (H727: 20 µM; QGP-1: 30 µM), everolimus (100 nM), and their combination treatment on the proliferation of H727 and QGP-1 cell lines. Data are presented as the mean ± SD (*n* = 3). ****P* < 0.01, *****P* < 0.001, *****P* < 0.0001. A full color version of this figure is available at <https://doi.org/10.1530/ERC-25-0233>.

several stably transfected cell lines with ITGB4 knockdown and FGFR4 overexpression via shRNA after puromycin screening. The cell lines successfully identified by RT-qPCR and Western blotting were used for subsequent functional rescue experiments.

Statistical analysis

Statistical analysis was performed with data from three independent experiments (*n* = 3, biological replicates). All statistical analyses were performed using Prism 8 software. The data are presented as the mean ± standard deviation (SD). Student's *t*-test was used to analyze the differences between two groups, and one-way ANOVA was used to compare multiple groups. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Results

Lenvatinib combined with everolimus showed a stronger anti-tumor efficacy on NEN cell survival and proliferation

Primarily, we characterized the inhibitory effects and concentration dependency of lenvatinib and everolimus in NEN cells. H727 and QGP-1 cells were treated with lenvatinib or/and everolimus, and cell survival was measured using the CCK-8 assay after 48 hours for H727 and QGP-1 cells, respectively. The results demonstrated that both lenvatinib and

everolimus suppressed NEN cell survival in a dose-dependent manner. The IC₅₀ values for lenvatinib in H727 and QGP-1 cells were 43.57 ± 3.64 µM and 74.89 ± 1.53 µM, respectively (Fig. 1A), while the IC₅₀ values for everolimus in these same cell lines were 33.41 ± 0.69 µM and 31.70 ± 3.84 µM, respectively (Fig. 1B). Based on these findings, along with relevant research (11, 12), we established the treatment concentrations as follows: lenvatinib at 20 µM (H727) or 30 µM (QGP-1) and everolimus at 100 nM.

Furthermore, to evaluate their synergistic effects, the CI values were calculated using CompuSyn software based on the Chou–Talalay method. The CI value for the combination of lenvatinib and everolimus was found to be less than 1.0 in both cell lines studied (Table 1), indicating a synergistic activity of this drug combination in NEN cells.

Table 1 Combination index of the combined effects of lenvatinib with everolimus in NEN cells.

Cell line	Lenvatinib	Everolimus	Fractional affected	CI value
H727	5 µM	1 nM	0.23	0.21011
	10 µM	10 nM	0.25	0.44974
	20 µM	100 nM	0.33	0.55372
QGP-1	7.5 µM	1 nM	0.29	0.61840
	15 µM	10 nM	0.36	0.49895
	30 µM	100 nM	0.44	0.55567

Note: CI < 1.0 indicates synergism, CI = 1.0 indicates additivity and CI > 1.0 indicates antagonism.

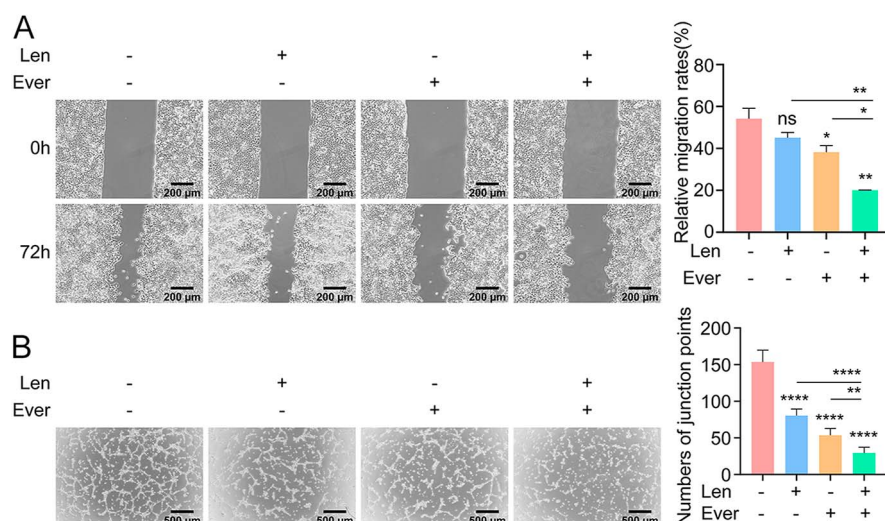


Figure 2

Lenvatinib and everolimus synergistically suppressed H727 cells migration and HUVECs angiogenesis. (A) Wound healing assay conducted to evaluate the effects of lenvatinib (20 μ M), everolimus (100 nM), and their combination treatment on the migration of H727 cells. (B) Tube formation assay performed to assess the impact of lenvatinib (10 μ M), everolimus (1 μ M), and their combination treatment over a 24 h period on the angiogenesis of HUVECs. Data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-25-0233>.

To further investigate the anti-proliferative activity of lenvatinib combined with everolimus, we assessed their survival curves using the CCK-8 assay. The combination exhibited significantly enhanced anti-tumor efficacy compared to either drug alone in NEN cells (Fig. 1C). In addition, colony formation assays were conducted to examine long-term cell proliferation, and the results indicated that co-treatment with lenvatinib and everolimus markedly reduced colony numbers compared with either agent administered alone (Fig. 1D). These data suggest that lenvatinib can potentiate the therapeutic effects of everolimus in the treatment of NENs.

Lenvatinib-everolimus combination synergistically induced apoptosis

To evaluate the potential of the combined treatment in arresting cell cycle and inducing apoptosis, we conducted flow cytometry to analyze the cell cycle status and apoptotic activity in NEN cells treated with lenvatinib and/or everolimus. Cell cycle analysis revealed that no synergistic retardation of the cell cycle was observed with the combined treatment compared to single drug administration (Supplementary Fig. S1A). In terms of apoptosis analysis, the results indicated that both lenvatinib and everolimus had minimal impact on cellular apoptosis when administered individually. In addition, their combination significantly enhanced the population of apoptotic H727 and QGP-1 cells (Supplementary Fig. S1B).

Lenvatinib and everolimus synergistically suppressed cell migration

NEN progression is closely associated with increased cancer cell motility, which contributes to poor prognosis in patients with NENs. However, considering

the biological characteristics of QGP-1 cells, such as their tendency to grow in clusters, slow proliferation, and extremely low migration ability, only H727 cells were selected for this experiment and some subsequent experiments. A wound-healing assay was employed to evaluate whether the combination of lenvatinib and everolimus could induce a synergistic anti-metastatic effect. The results demonstrated that combined treatment significantly inhibited the migration of H727 cells compared to treatment with either everolimus or lenvatinib alone (Fig. 2A).

Lenvatinib and everolimus synergistically suppressed angiogenesis

Previous studies have confirmed that the angiogenesis signaling pathway is over-activated in NENs (13). Therefore, a tube formation assay was used to assess the anti-angiogenic activity of combination treatment involving lenvatinib and everolimus in HUVECs. Notably, the results indicated that treatment with both lenvatinib and everolimus significantly reduced the number of junction points formed during tube formation in HUVECs (Fig. 2B), suggesting that anti-angiogenesis may be one mechanism through which this combination therapy exerts its effects in treating NENs.

Lenvatinib combined with everolimus better suppressed NEN growth in mice

The results presented above confirmed the synergistic inhibitory effects of lenvatinib and everolimus *in vitro*. To further investigate whether these agents also exhibit synergistic effects *in vivo*, we established subcutaneous xenograft tumors using H727 cells in female BALB/c nude mice. Before the formal experiments, a pre-experiment was conducted to determine appropriate dosing.

Clinical doses of lenvatinib (8 mg/day) and everolimus (5 mg/day) in our study were converted to mouse equivalent doses of 1.2 mg/kg and 0.76 mg/kg, respectively, based on body surface area. Dose ranges were tested (lenvatinib: 0, 1, 2, 4, and 8; everolimus: 0, 0.75, 1.5, 3, and 6 mg/kg/day), with two nude mice per group. After continuous intraperitoneal injection for 8 days, lenvatinib at 4 and 8 mg/kg/day and everolimus at 3 and 6 mg/kg/day showed tumor growth inhibition (Supplementary Fig. S2A). In addition, no significant body weight changes occurred (Supplementary Fig. S2B). The lowest effective doses – 4 mg/kg/day for lenvatinib and 3 mg/kg/day for everolimus – were selected for the main study.

In the formal experiment, the animals were randomly assigned to one of four treatment groups: control (300 μ L fixed solvent), lenvatinib (4 mg/kg/day), everolimus (3 mg/kg/day), and combination treatment (lenvatinib: 4 mg/kg/day; everolimus 3 mg/kg/day). Following a 12-day treatment regimen via intraperitoneal injection, tumor growth was moderately inhibited in the single-drug groups. However, significant suppression was observed in the dual-drug group, as evidenced by measurements of tumor growth rate, volume, and weight (Fig. 3A, B, C, D). Importantly, no significant weight loss was recorded in the mice throughout the treatment period (Fig. 3E), indicating

the absence of toxic effects and suggesting that the drug dosages administered were appropriate.

Lenvatinib combined with everolimus for the treatment of NENs exhibited promising clinical benefits

Given that lenvatinib in combination with everolimus has been approved by the FDA for the second-line treatment of advanced RCC and its efficacy and safety have been well established, we conducted a retrospective analysis to evaluate the effectiveness of this combination therapy in four patients with advanced NETs who had previously failed multiple lines of treatment between April 2022 and July 2023. The CT images before and after combination therapy indicated that three out of four patients experienced favorable outcomes (Fig. 3F and Supplementary Table S2).

Patient 1: a 78-year-old male with stage IV mediastinal NET (G2) involving the pulmonary artery, left pleura, pericardium, and bone metastases received first-line etoposide and carboplatin for four cycles and the disease progressed. Subsequently, the patient received capecitabine and temozolomide, but developed severe, intolerable vomiting after temozolomide. The vomiting persisted despite multiple antiemetic agents.

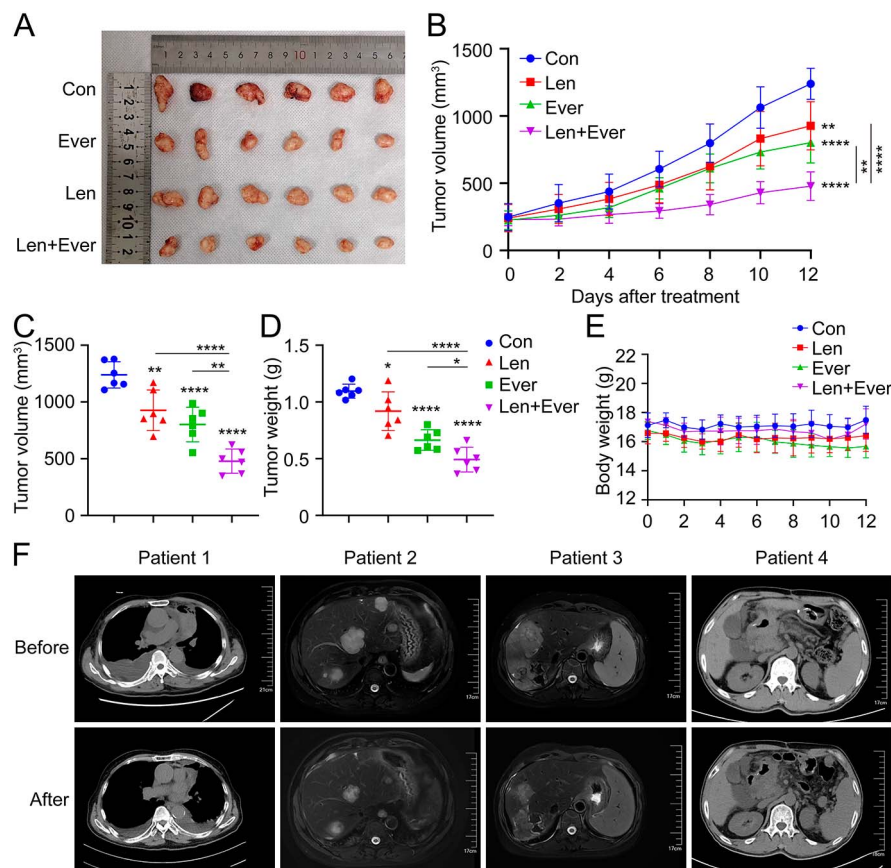


Figure 3

Lenvatinib combined with everolimus better suppressed NEN growth *in vivo*. (A) Photographs depicting subcutaneous H727 xenograft tumors subjected to various treatment regimens. (B) Growth curves illustrating the tumor progression across different experimental groups. (C and D) Measurements of tumor volume and weight at the conclusion of the study, respectively. (E) Changes in body weight of mice throughout the treatment period. (F) CT imaging revealing significant lesion reduction in three out of four patients with advanced NETs who were treated with a combination of lenvatinib (8 mg, daily) and everolimus (5 mg, daily). Data are presented as the mean $\pm$ SD ($n = 6$ mice). * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-25-0233>.

After progression on surufatinib plus toripalimab at 6 months, he started lenvatinib and everolimus, showing symptom relief and a 17% reduction in tumor burden by CT at 20 weeks. Treatment continued beyond progression due to clinical benefit, with a total duration of 13.5 months (Fig. 3F and Supplementary Table S2).

Patient 2: a 61-year-old male with stage IV gastric NEC and liver metastases received 12 months of first-line etoposide, cisplatin, and nivolumab, plus stomach radiotherapy and intermittent microwave ablation of liver lesions. After liver progression, he received albumin-bound paclitaxel and cadonilimab for 2 months without response. He progressed after 1 month on capecitabine and temozolomide, then started lenvatinib and everolimus, achieving a partial response with 36% reduction in target lesions by week 6. Treatment remained effective for 12.2 months before progression (Fig. 3F and Supplementary Table S2).

Patient 3: a 40-year-old woman with stage IV hepatic NEC and metastases to the left cardiophrenic angle, right pararenal, and retroperitoneal lymph nodes progressed after first-line etoposide and cisplatin. She received five prior lines of therapy – temozolomide/capecitabine, fruquintinib/sintilimab, FOLFIRI/sintilimab, and surufatinib/sintilimab – each lasting 2–3 months until radiographic progression. She then started lenvatinib and everolimus, achieving a partial response with 28% reduction in target lesions and symptom improvement by week 12. Progression occurred after 8.1 months (Fig. 3F and Supplementary Table S2).

Patient 4: a 59-year-old man with gastric NEC and abdominal metastases developed severe diarrhea and myelosuppression after two cycles of first-line EP (etoposide and cisplatin). He refused further intravenous chemotherapy and started oral lenvatinib plus everolimus. Disease stability lasted 5 weeks, with PFS of 5.3 months and OS of 11.2 months (Fig. 3F and Supplementary Table S2).

Lenvatinib and everolimus synergistically promoted ITGB4 expression and inhibited FGFR4 expression

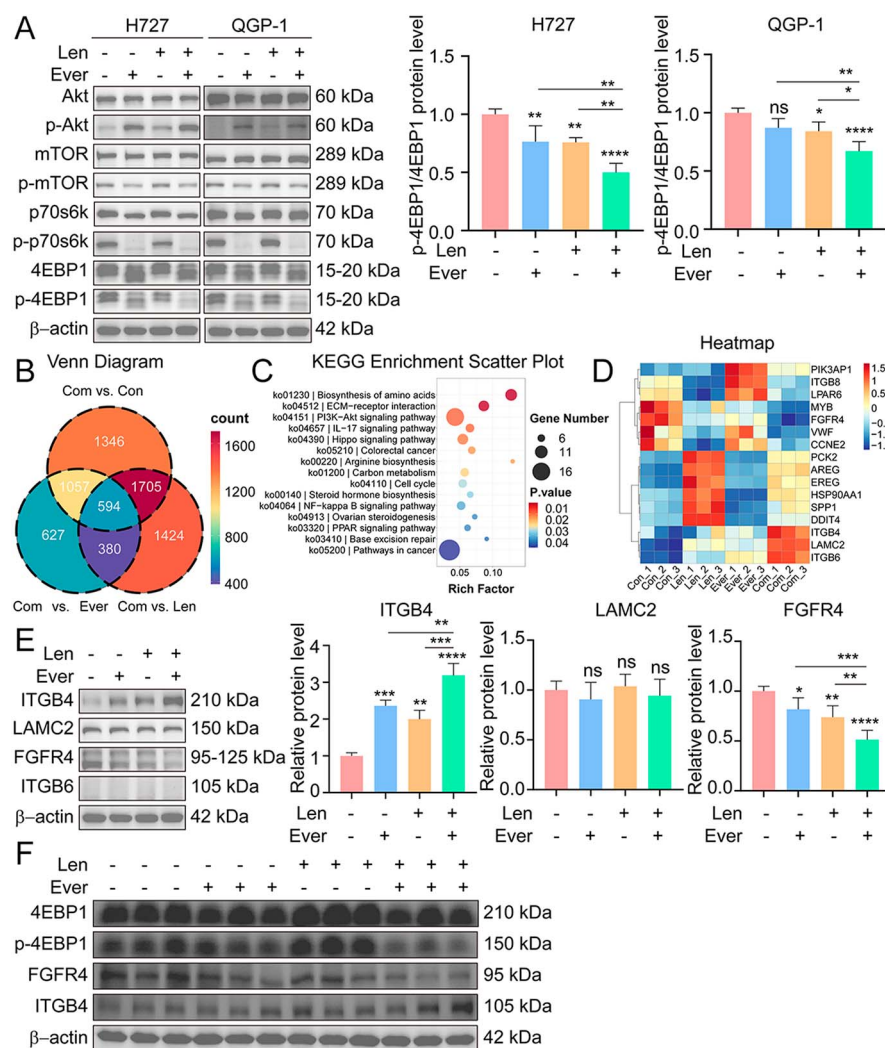
To elucidate alterations in the expression of signal transduction factors following treatment with reagents, we conducted Western blot analyses to detect key proteins within the Akt/mTOR signaling pathway. The results indicated that everolimus significantly suppressed the phosphorylation of mTOR and its downstream effectors p70S6K and 4EBP1. Furthermore, the combined treatment exhibited a synergistic inhibitory effect on the phosphorylation of 4EBP1. In addition, everolimus activated Akt phosphorylation (Fig. 4A). These findings suggest that combination therapy leads to the synergistic inhibition of 4EBP1 phosphorylation.

Next, to investigate the alterations in gene expression levels following drug administration, we performed transcriptome sequencing on H727 cells treated with the control, lenvatinib, everolimus, and a combination of these agents for 48 h. From the comparisons of combination vs control, combination vs lenvatinib, and combination vs everolimus, we determined the intersection of differentially expressed genes. 594 difference genes were found by this approach (Fig. 4B). The PI3K–Akt pathway was one of the top 15 signaling pathways showing notable alterations, according to KEGG enrichment analysis of these 594 differentially expressed genes (Fig. 4C). Then, we employed a heatmap to display the gene expression profiles of the PI3K–Akt pathway (Fig. 4D). We selected genes whose expression levels were either upregulated or downregulated in the combination group compared to the control group, and groups treated with lenvatinib and everolimus for additional confirmation using RT-qPCR and Western blot analysis. The findings showed that whereas FGFR4 had decreased, the mRNA levels of LAMC2, ITGB4, and ITGB6 had increased (Supplementary Fig. S3). Furthermore, our mRNA results were supported by the ITGB4 and FGFR4 protein expression levels. However, there were no discernible results for ITGB6 and no notable alterations in LAMC2 (Fig. 4E). In addition, in line with our Western blot results from H727 cells, combination therapy reduced both FGFR4 expression levels and phosphorylated 4EBP1 while boosting ITGB4 expression in xenograft tumors, as seen in Fig. 4F. According to these findings, ITGB4 or FGFR4 might be important molecules that mediate the synergistic inhibition of NENs when lenvatinib and everolimus are combined.

Knockdown of ITGB4 attenuated the synergistic inhibitory effect of lenvatinib combined with everolimus on H727 cells

In order to comparatively analyze the expression discrepancies of ITGB4 between NENs and normal tissues, we initially detected the expression of ITGB4 in normal lung epithelial cells, namely BEAS-2B cells, and H727 cells. The findings demonstrated a reduction in the expression level of ITGB4 in H727 cells (Supplementary Fig. S4A). Then, we analyzed the whole-exome sequencing data (GSE108055) from the Asiedu team (14), and the results indicated that the expression of ITGB4 was decreased in typical carcinoids, atypical carcinoids and small cell lung cancer (Supplementary Fig. S4B). These results imply that ITGB4 might exert a tumor-suppressive function in NENs.

To elucidate the biological significance of the upregulation of ITGB4 expression following the synergistic action of lenvatinib and everolimus in H727 cells, we used shRNA interference lentivirus to establish stably transformed cell lines with knocked down ITGB4 expression and conducted functional rescue experiments. RT-qPCR and Western blot analyses were performed to assess the knockdown efficiency of ITGB4

**Figure 4**

Lenvatinib and everolimus synergistically promoted ITGB4 expression and inhibited FGFR4 expression. (A) Key phosphorylated proteins in the Akt/mTOR signaling pathway detected via Western blot analysis following treatment with lenvatinib (H727: 20 μM; QGP-1: 30 μM), everolimus (100 nM), and their combination. (B) Venn diagram illustrating the differential genes identified in comparisons of combination vs control, combination vs lenvatinib and combination vs everolimus. (C) KEGG enrichment scatter plot displaying the differential genes that intersected within the Venn diagram. (D) Heatmap depicting the expression levels of differential genes involved in the PI3K-Akt signaling pathway. (E) Western blot analysis to assess the expression levels of LAMC2, ITGB4, ITGB6, and FGFR4 in H727 cells treated with lenvatinib (20 μM), everolimus (100 nM), and their combination over a period of 48 h. (F) Expression levels of proteins 4EBP1, FGFR4, and ITGB4 evaluated in subcutaneous xenograft tumors derived from mice. Data are presented as the mean ± SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-25-0233>.

(Fig. 5A and B). The ability of the combination treatment with lenvatinib and everolimus to inhibit H727 cell proliferation was partially diminished upon knockdown of ITGB4 expression via shRNA (Fig. 5C and D). Consistently, knockdown of ITGB4 also partially weakened the synergistic inhibitory effect of lenvatinib and everolimus on the migration of H727 cells (Fig. 5E).

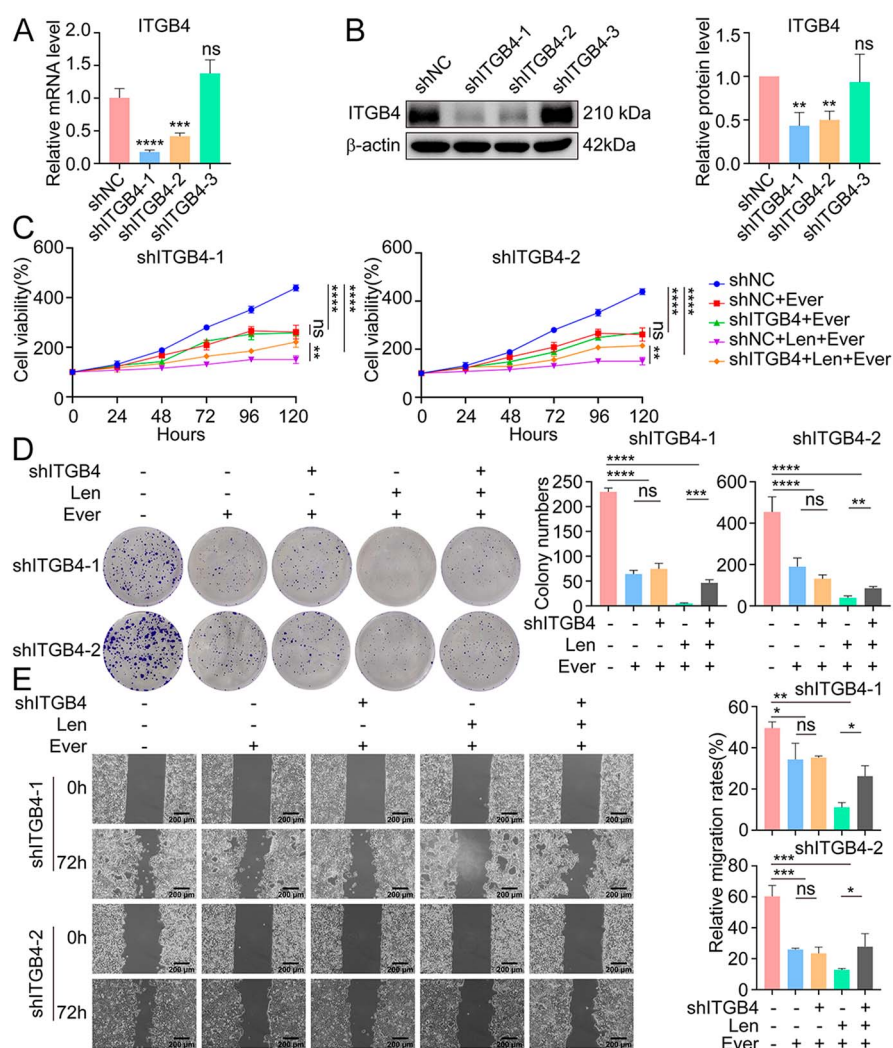
In addition, we constructed FGFR4 overexpression cell lines using a lentiviral infection technique and evaluated overexpression efficiency through qPCR and Western blot analysis (Supplementary Fig. S5A and B). Results from CCK-8 assays indicated that FGFR4 overexpression did not influence the synergistic inhibitory proliferative effects of lenvatinib and everolimus on H727 cells (Supplementary Fig. S5C).

Discussion

Currently, advanced NENs exhibit significant heterogeneity and rapid progression, with limited

therapeutic options and poor efficacy. Lenvatinib and everolimus work well together as monotherapy for backline treatment, according to a number of clinical investigations. Acquired resistance is common, the response duration is short, and the objective remission rate is still poor. Although lenvatinib and everolimus combination therapy has demonstrated a significant improvement in efficacy when compared to monotherapy as a post-line treatment for metastatic renal cancer, little is known about their function and underlying processes in NENs.

For the first time, our work provides proof of the multifaceted synergistic anti-tumor actions of lenvatinib and everolimus on NENs. By using both *in vitro* tests and subcutaneous xenograft models with nude mice, our results validate that this medication combination inhibits NENs in a synergistic manner. Mechanistically, this synergistic impact might result from combined blockage of the PI3K/AKT/mTOR and VEGF/FGFR signaling pathways, which target several receptors, including VEGFR1–3, FGFR1–4, PDGFR, KIT,

**Figure 5**

Knockdown of ITGB4 attenuated the synergistic inhibitory effect of lenvatinib combined with everolimus on H727 cells. (A and B) Validation of ITGB4 knockdown efficiency performed using RT-qPCR and Western blot analysis, respectively. (C) H727 cells transfected with ITGB4 shRNA and subsequently treated with lenvatinib (20 μ M), everolimus (100 nM), or a combination. A CCK-8 assay was conducted to assess the proliferation of H727 cells. (D) H727 cells exhibiting ITGB4 knockdown were subjected to treatment with lenvatinib (20 μ M), everolimus (100 nM), or the combination. A colony formation assay was performed to evaluate cell growth. (E) Following the knockdown of ITGB4 via shRNA, H727 cells were treated with lenvatinib (20 μ M), everolimus (100 nM), or their combination. A wound healing assay was executed to analyze cell migration. Data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-25-0233>.

and RET. It is known that autophosphorylation occurs inside the structural domain of receptor tyrosine kinases when VEGF ligands specifically bind to VEGFR on vascular endothelial cells. Important signaling cascades, such as phospholipase C γ (PLC), Ras/MAPK pathway activation, and PI3K/AKT, are then started by this activation (15). Similarly, the Ras/MAPK and PI3K/AKT signaling pathways are also activated when FGF binds to the extracellular structural domain of FGFR (16). Moreover, RET receptor activation depends on ligand-binding interactions with co-receptors, in which the tyrosine residues of the receptor undergo trans-autophosphorylation. This can attract bridging proteins and activate regulatory networks that control cell growth, proliferation, survival, and differentiation through the RAS/MAPK or PI3K/AKT pathways (17). Lenvatinib and everolimus mostly affected the PI3K/AKT/mTOR pathway, with little impacts on the PLC and Ras/MAPK pathways, according to the findings of the KEGG pathway enrichment study (Fig. 4C). Lenvatinib is a multi-target tyrosine kinase inhibitor that blocks receptors, such as VEGFR, FGFR, and

RET, while also inhibiting PI3K signaling from an upstream position. Conversely, everolimus acts as an inhibitor specific to mTORC1 that directly disrupts the phosphorylation of downstream effector molecules, including 4EBP1 and S6K. According to earlier research, everolimus may only partially block mTORC1 and its downstream targets, including 4EBP1 (18). In contrast, lenvatinib may improve everolimus's ability to inhibit 4EBP1 from an upstream standpoint. Mohan *et al.* showed that eIF4E is activated by the phosphorylation of 4EBP1, which is facilitated by the FGFR and PI3K signaling pathways (19). As a result, lenvatinib and everolimus work in tandem to block 4EBP1 phosphorylation, which in turn affects biological processes, such as cell migration and proliferation in NENs.

ITGB4, a member of the integrin family, shows markedly varied expression levels in various tumor types. Prostate and basal cell carcinomas express it at modest levels, whereas colorectal, lung, oral squamous cell, glioma, and pancreatic malignancies express it at high levels

(20, 21, 22, 23, 24, 25). The majority of research has shown a favorable correlation between the development of malignant tumors and overexpression of ITGB4. However, some studies have also indicated that ITGB4 can promote cell death. Treatment of cells with apoptosis-inducing agents resulted in an increased expression level of ITGB4, whereas knockdown of ITGB4 significantly inhibited apoptosis induced by these agents (26, 27). Moreover, in a human gastric cancer model employing immunodeficient mice, high levels of ITGB4 expression were found to promote apoptosis (28). According to Bachelder *et al.*, ITGB4 can activate p53 and cause p53-dependent apoptosis in cancer cells that express p53 in the wild type (29). It has been demonstrated that ITGB4 interacts with RTKs, such as ErbB2, EGFR, and Met, and that the interaction between integrins and RTKs can drive downstream signaling pathways (30). By selectively binding to and blocking the phosphorylation of ITGB4, FLRT2 delays endothelial cellular senescence through the mTOR2/AKT/p53 signaling pathway; as found by Hwang *et al.*, the cellular senescence caused by loss-of-function mutations in FLRT2 was considerably reduced by inhibition of ITGB4 (31). We hypothesized that in NENs, ITGB4 acts as a tumor suppressor. After receiving lenvatinib and everolimus together, there was a rise in the expression of the tumor suppressor protein ITGB4, which further triggered cell death. However, when ITGB4 was knocked down, its tumor-suppressive activity was lessened, which in turn lessened the combined inhibitory effect of lenvatinib and everolimus on H727 cells. However, more research is needed to determine the precise method by which lenvatinib and everolimus increase the expression of the ITGB4 protein.

This study also shows that lenvatinib and everolimus synergistically suppress FGFR4 expression. FGFR4, a receptor tyrosine kinase, has an extracellular ligand-binding domain, transmembrane domain, and intracellular kinase domain. Genetic alterations causing FGFR pathway overactivation are linked to breast, liver, lung, gastric, and uterine cancers (32). FGFR4 overexpression promotes FRS2–GRB2 complex formation, activating downstream signals and driving liver cancer progression (33). He *et al.* found that FGFR4 activates the PI3K/AKT/HIF1 α pathway, enhancing HOXB5 binding to its promoter, upregulating its own expression, and forming a FGFR4–HOXB5–FGFR4 positive feedback loop in liver cancer cells (34). We propose that lenvatinib directly inhibits FGFR4 while everolimus blocks PI3K/AKT signaling, together enhancing FGFR4 downregulation. Since FGFR4 promotes tumor growth, its reduction helps inhibit proliferation. However, forced FGFR4 overexpression does not reverse the drugs' synergistic effect. This is likely because i) elevated FGFR4 levels remain susceptible to direct inhibition by lenvatinib and ii) everolimus blocks mTOR downstream of RTKs, preventing signal transmission even with FGFR4 overexpression. Although FGFR4 is modulated by the

combination, its exact role in the synergy requires further study using genomics and proteomics.

Conclusion

This study demonstrates the synergistic inhibitory effect of lenvatinib and everolimus in NENs through multiple *in vitro* experiments, including cell proliferation, migration, and apoptosis, and *in vivo* models and clinical patient data. Furthermore, ITGB4 is identified as a potential key regulatory molecule underlying this synergistic interaction. However, several limitations should be acknowledged. Due to the rarity of NENs and the scarcity of available established cell lines, there is currently no mature tumor model system. As a result, this study mainly relies on lung-derived NEN cells, while most clinical cases originate from the digestive system, and the difference in tissue sources may affect the universality of the results. In the future, it is necessary to further collect relevant experimental data of digestive system NEN cells to verify and expand the findings of this study.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-25-0233>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the work reported.

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