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RESEARCH ARTICLE

PP121, a dual inhibitor of tyrosine and phosphoinositide kinases, inhibits anaplastic thyroid carcinoma cell proliferation and migration

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Abstract The tyrosine and phosphoinositide kinases play crucial roles in the regulation of many cancer cell processes including cell survival and cell motility. Anaplastic thyroid carcinoma (ATC) is a rare and deadly type of thyroid cancer, and so far, there are no effective therapeutic compounds for ATC. Herein, we investigate the anticancer activities of PP121, a dual inhibitor of tyrosine and phosphoinositide kinases, in ATC therapy. We found that PP121 is effective at suppressing cell viability, inducing cell apoptosis, and inhibiting cell migration and invasion. The potential anticancer mechanism for PP121 might be its inhibitory effects on phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways in ATC cells. Furthermore, PP121 is effective at suppressing ATC tumor growth in vivo. In summary, our studies suggest that PP121 might be a promising therapeutic compound for ATC treatment, which might shed new light on ATC therapy.

Keywords Anaplastic thyroid carcinoma · PP121 · Tyrosine · Phosphoinositide kinase · Proliferation · Migration

Introduction

Anaplastic thyroid carcinoma (ATC) accounts for about 2 % of thyroid cancers in older adults. It is a deadly type of cancer, and the mean survival time for ATC is usually less than 6 months. ATC grows starting from differentiated thyroid cancer or a benign tumor of the gland. This type of cancer grows very quickly, so it is difficult to treat [1–4]. There are no effective therapeutic reagents and strategies for ATC so far. Therefore, it is very urgent to find novel promising therapeutic targets or compounds for ATC.

Due to the phosphatidylinositol-3-kinase (PI3K) mutant and constitutive activation in ATC, the PI3K/Akt signaling is highly activated, which makes PI3K/Akt pathway a potential therapeutic target in ATC treatment [5–10]. The tyrosine kinases are upstream of PI3K/Akt signaling. All of these signaling molecules play crucial roles in cell proliferation, migration, invasion and tumorigenesis, development, and metastasis of ATC [5–15], so these molecules are promising targets in ATC therapeutics. PP121 is a dual inhibitor of tyrosine and phosphoinositide kinases [16]. Studies on PP121 are quite few, and it is far from being well characterized. Here, we have investigated the potential anticancer activities of PP121 in ATC therapy. Our studies demonstrate that PP121 is effective at suppressing cell viability, inducing cell apoptosis, and inhibiting cell migration and invasion by suppressing PI3K/Akt signaling pathway in ATC cells. Furthermore, PP121 is effective at suppressing ATC tumor growth in vivo.

Materials and methods

Materials

PP121 was purchased from Selleck Chemicals LLC (Huston, TX, USA). Rabbit anti-phospho-Akt (Ser473) (catalog # 4060), anti-phospho-p70S6K (Thr389) (catalog # 9205), anti-phospho-S6 (Ser235/236) (catalog # 2211), anti-S6 (catalog # 2217), and secondary HRP-conjugated antibody (catalog # 7074) were purchased from Cell Signaling Technology. Other reagents and chemicals were purchased from Sigma.

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Cells and cell culture

Human ATC cell lines (CAL62, KAT4) were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM medium with 10 % fetal bovine serum (FBS), supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C in 5 % CO_2.

Cell viability assay

Cells were seeded at a density of 6×10^3 cells per well in 96-well plates. After treatment with PP121 at different concentrations, cell viability was determined by using the CellTiter-Glo assay. The luminescence was measured with a plate reader (Molecular Devices, Sunnyvale, CA).

Transwell assay

Cells were seeded in the upper compartment of 24-transwell Boyden chamber wells (Costar, Bedford, MA) in serum-free media. Six hundred-microliter serum-free media containing 20 μg/ml fibronectin was added to the lower compartment. After treatment with PP121 for 8 h, the cells were fixed and stained with 0.1 % crystal violet. The nonmigrating cells were removed, and the migrated cells were photographed with a microscope, then the cells were lysed with 10 % acetic acid and the absorbance was measured at 595 nm.

Wound-healing assay

Cells were seeded in 96-well plates. Once 100 % confluence was observed, the cell monolayer was scratched with a pipette tip. After being washed for three times, the cells were treated with PP121 for 8 h. Then, the scratches were photographed and the width of scratches was measured.

Cell invasion assay

The upper chamber of 24-transwell Boyden was precoated with 1 mg/ml of Matrigel (BD Biosciences) for 5 h at 37 °C, and then the invasion assay was performed as described in "Transwell assay" above.

Nuclei staining

Cells were seeded on fibronectin precoated glass coverslips, followed by treatments with PP121. Then, the cells were fixed with 4 % paraformaldehyde for 30 min, permeabilized with 0.1 % Triton X-100 for 20 min, and blocked with 5 % normal serum for 30 min. Then, the cells were incubated with Hoechst for 15 min and washed with phosphate-buffered saline (PBS) for three times. The images were taken with a fluorescent microscope.

Flow cytomery

Cells treated with PP121 were harvested by exposure to trypsin, fixed, and stained with propidium iodide by using CycleTEST plus DNA reagent kit (Becton Dickinson). Then, the cells were analyzed for DNA content with the use of a FACSCalibur flow cytometry and CellQuest Pro software (Becton Dickinson).

Western blotting

After treatment with PP121 at different concentrations for 2 h, the cells were harvested and lysed in 20 mM HEPES, pH 7.5, 150 mM NaCl, and 1 % NP-40, supplemented with Complete Mini Protease Inhibitor tablet. After SDS-PAGE separation, the proteins were transferred to nitrocellulose membranes, blocked with 5 % bovine serum albumin at room temperature for 1 h, and incubated with primary antibodies (1:1,000 dilution) at 4 °C overnight. Then, the films were incubated with secondary antibody (1:3,000 dilution) at room temperature for 1 h, and the membranes were developed with chemiluminescence ECL reagent.

Immunohistochemistry

The slides were deparaffinized and hydrated with 100 % ethanol twice for 10 min, 95 % ethanol twice for 10 min, and deionized water for 2 min. For antigen unmasking, slides were placed in a container, covered with 10 mM sodium citrate buffer, pH 6.0, and heated in a steamer for 1 h. The slides were then washed and blocked with 5 % normal goat serum for 30 min. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions. The nuclei were stained with Hoechst for 20 min. The slides were photographed using a fluorescent microscope.

Efficacy studies in mouse xenograft model in vivo

The nud/nud mice were purchased from Slack Company in Shanghai and housed in a BL2 lab. All care and treatment of experimental animals were performed according to the animal care and use committee guidelines. CAL62 cells were harvested and suspended in PBS. Then, the cells were injected into the axillary regions of mice (5×10^6 cells/mouse). When the tumor volumes reached 80 mm^3, the mice were randomly separated to control group and two PP121 groups (n=8 per group), including PP121 low dose (50 mg/kg) and high dose (100 mg/kg) groups (vehicle, 10 % 1-methyl-2-pyrrolidinone and 90 % PEG 300). Tumors were measured every other day with a microcaliper, and the body weight was measured every other day with a scale. The

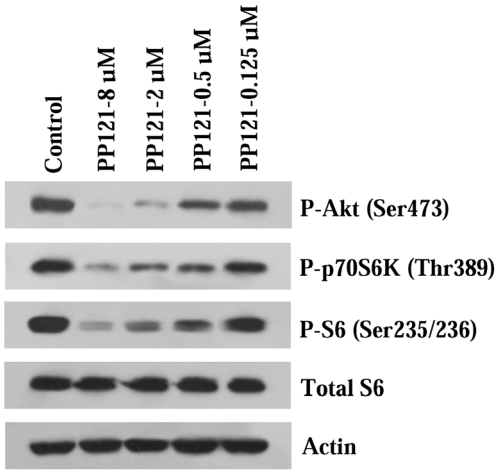

Fig. 1 PP121 inhibits PI3K/Akt signaling pathway in ATC cells. CAL62 cells were treated with PP121 (0.125, 0.5, 2, and 8 μM) for 3 h, followed by Western blot analysis with the indicated antibodies

tumor volumes were calculated with the formula (mm^3) = width × width × length×0.5. After administration for about 2 weeks, the mice were euthanized and tumors were harvested. Tumor cell apoptosis was analyzed using immunohistochemistry (IH).

Statistics

Student's *t* test and analysis of variance (ANOVA) were performed using StatView (SAS Institute, Cary, NC). $P<0.05$ was considered significantly different. The data shown are the mean values of triplicate measurements with error bars corresponding to standard deviation.

Results

PP121 inhibits PI3K/Akt signaling pathway in ATC cells

PP121 is a known dual inhibitor of tyrosine and phosphoinositide kinases [16]. We first examined the effect of PP121 on PI3K/Akt signaling in ATC cells. As shown in Fig. 1, PP121 concentration dependently suppressed PI3K/Akt signaling pathway including phosphorylation of Akt, p70S6, and S6 in CAL62 cells, but no significant changes in total protein levels were observed.

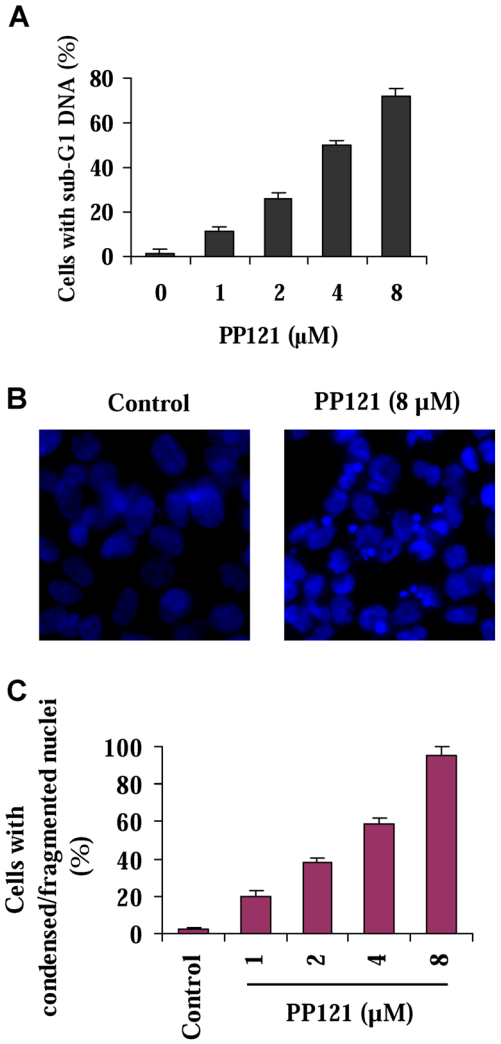

Fig. 3 PP121 induces apoptosis in ATC cells. **a** CAL62 cells were treated with PP121 at the indicated concentrations for 48 h, followed by PI staining and flow cytometry analysis. **b** CAL62 cells were incubated with PP121 for 48 h. The nuclei were stained with Hoechst and analyzed using a fluorescent microscope. The representative images are shown. **c** The number of cells with condensed/fragmented nuclei was quantitated by counting in seven random fields, and the inhibition was calculated

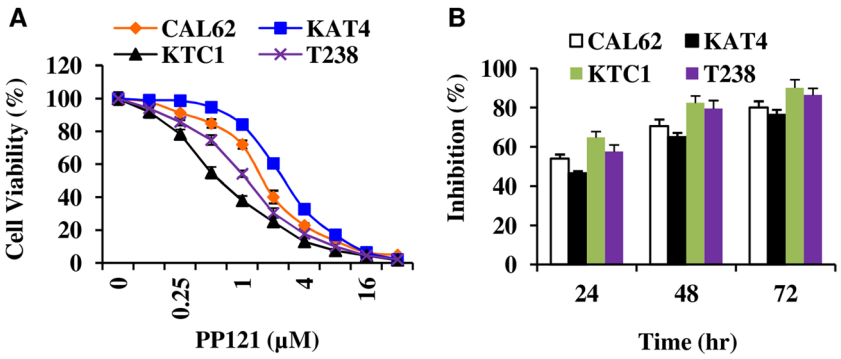

Fig. 2 PP121 suppresses ATC cell viability. **a** CAL62 and KAT4 cells were treated with PP121 (0.125~32 μM) for 48 h, followed by measurement with cell viability assay. **b** CAL62 and KAT4 cells were treated with PP121 (4 μM) for 24, 48, or 72 h, followed by measurement with cell viability assay

PP121 is affective at suppressing ATC cell viability

The tyrosine kinases and their downstream PI3K/Akt signaling pathways play crucial roles in promoting ATC cell survival [5–19]. To explore the potential roles of PP121 in ATC therapy, we first used it for in vitro anti-ATC studies. We examined the effect of PP121 on ATC cancer cell viability, and we found that PP121 significantly reduced viability of ATC cell lines CAL62 and KAT4 in a time- and concentration-dependent manner (Fig. 2a, b).

PP121 induces apoptosis of ATC cells

Since the tyrosine kinases and their downstream PI3K/Akt signaling pathways play critical roles in the inhibition of apoptosis [7–13], we next examined PP121-induced apoptosis in ATC cells. The flow cytometry analysis showed that PP121 caused a significant increase in sub-G1 DNA content in CAL62 cells ($P<0.01$) (Fig. 3a). After treatment with MK-2206, the nuclei of cancer cells exhibited a condensed and fragmented morphology, which is a characteristic for apoptosis (Fig. 3b). This effect is also significant compared to control group ($P<0.01$) (Fig. 3c), suggesting that PP121 could induce dramatic apoptosis in ATC cells.

PP121 is effective at suppressing cell migration and invasion of ATC cells

The tyrosine kinases and their downstream PI3K/Akt signaling pathways play crucial roles in cancer cell motility [20–23]. We next investigated the effect of PP121 on migration of ATC cells. As shown in Fig. 4a, b, PP121 inhibited transwell migration of CAL62 cells. Similar inhibition of cell migration by PP121 was also observed in a wound-healing assay (Fig. 4c, d). Furthermore, PP121 efficiently suppressed cancer cell invasion through Matrigel (Fig. 4e, f).

PP121 suppresses ATC tumor growth in vivo

We next treated animals with PP121 (50 and 100 mg/kg) and examined the effect of intraperitoneal (ip) administration of

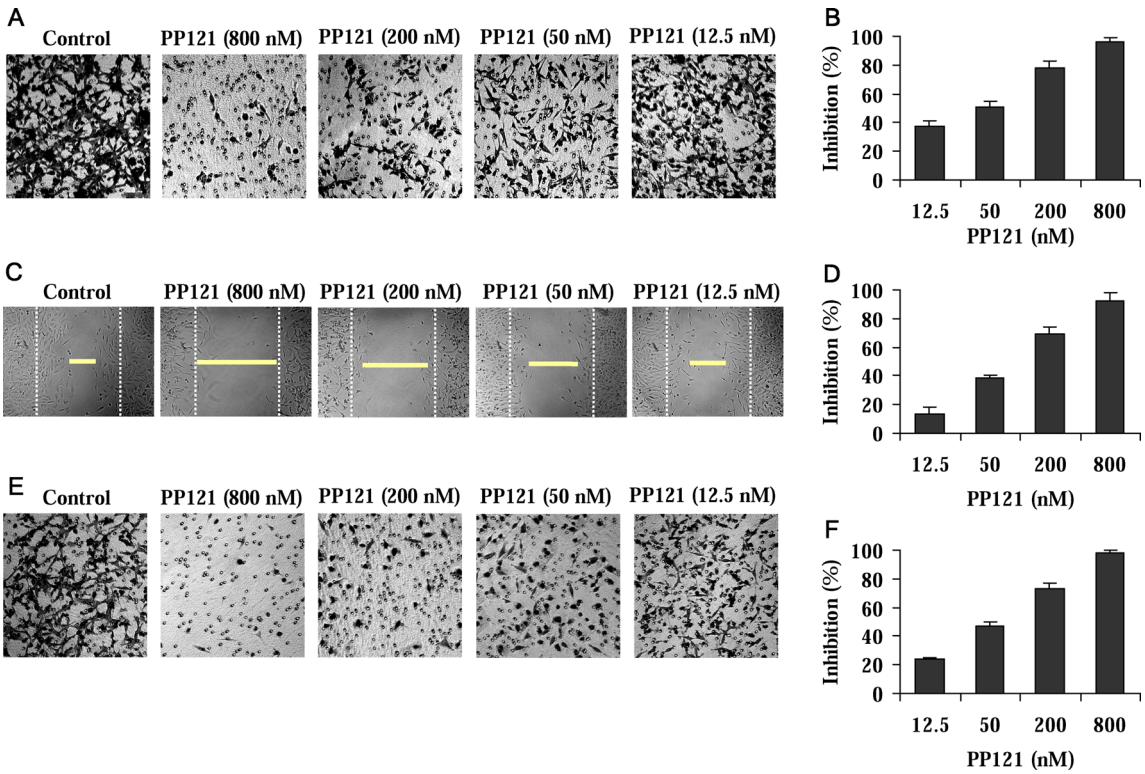

Fig. 4 PP121 inhibits cell migration and invasion in ATC cells. **a** CAL62 cells were treated with PP121 (12.5, 50, 200, or 800 nM) for 8 h. The nonmigrated cells on the upper surface of the filter were removed, and the migrated cells on the lower side were stained and photographed. The representative images are shown, then cells were lysed, and colorimetric determination was made at 595 nm. **b** Quantitation of the inhibition from transwell assay. **c** A scratch was introduced into a monolayer of CAL62 cells, followed by treatment with PP121 (12.5, 50, 200, or 800 nM) for 8 h. The width of wounded cell monolayer was measured in seven random fields, and representative images are shown (*white dashed lines* show the original wound width, *yellow lines* show the final wound width). **d** Quantitation of the inhibition from transwell assay. **e** CAL62 cells were seeded on a Matrigel precoated transwell membrane, and the treatment and analysis are similar with "Transwell assay" described above. **f** Quantitation of the inhibition from invasion assay

PP121 on growth of CAL62 cells in nud/nud mice. The results showed that low-dose PP121 (50 mg/kg) led to an intermediate level of growth suppression, whereas high-dose PP121 (100 mg/kg) exerted a strong inhibition on tumor growth (Fig. 5a). Consistent with the in vitro data, PP121 induced significant apoptosis in tumors as measured using TUNEL and nuclei staining assays (Fig. 5b). In addition, PP121 showed no significant toxicity on mice, and there was no significant loss of weight ($P>0.05$) (Fig. 5c). Taken together, these results showed that PP121 could effectively potentiate apoptosis and enhance suppression of both cell viability in vitro and tumor growth in vivo.

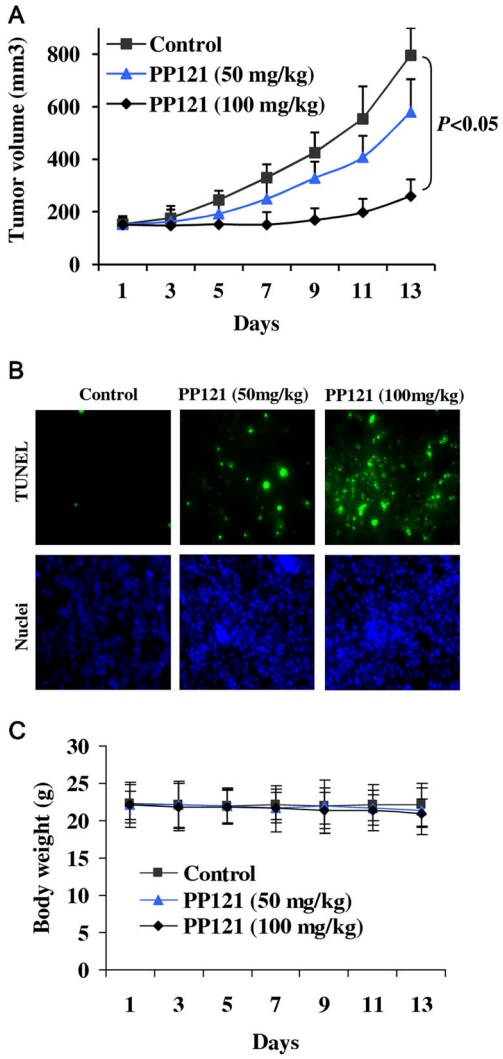

Fig. 5 PP121 suppresses ATC tumor growth in vivo. **a** After inoculation of CAL62 cells, PP121 (50 and 100 mg/kg) was injected to the mice everyday. The tumor volumes were measured every other day, and the tumor volumes are shown. **b** PP121 (50 and 100 mg/kg) induced apoptosis of CAL62 tumor cells in vivo measured with TUNEL assay (*green*), and the nuclei were stained with Hoechst (*blue*). **c** PP121 (50 and 100 mg/kg) had no significant cytotoxic effects on the body weight of mice during the treatments

Discussion

ATC is a rare, typically lethal malignancy in older adults with a very low survival rate. So far, there are no effective therapeutic reagents for ATC [1–4]. So, it is urgent to explore potential therapeutic reagents for ATC. The clinical success of multitargeted kinase inhibitors has stimulated efforts to identify such types of promising anticancer drugs. Tyrosine kinases promote cell survival, proliferation, migration, and invasion and are the targets of frequent oncogenic mutations in tumors. Eight tyrosine kinase inhibitors have been approved for clinical use, and many more are under development.

Upregulation of multiple oncogenic kinases has been demonstrated to be one of the most important underlying mechanisms for resistance generation in cancer therapeutics. Effective therapy for many cancers will require the simultaneous inhibition of multiple oncogenic kinases [24, 25], because tumor cells rapidly develop resistance to inhibitors of individual kinases. It is well known that most of the tyrosine and phosphoinositide kinases play critical roles in both cell viability and cell motility relating to cancer growth and metastasis in vivo. Therefore, molecules that target both tyrosine and phosphoinositide kinases are likely to possess potent antitumor activities including suppressing tumor growth and tumor metastasis.

PP121 is a dual inhibitor of tyrosine and phosphoinositide kinases [16]; however, it is not well studied so far especially in ATC therapy. Here, we have investigated the potential anticancer activities of PP121 in ATC therapy. Our studies demonstrated that PP121 was effective at suppressing cell viability, inducing cell apoptosis, and inhibiting cell migration and invasion. The potential anticancer mechanism for PP121 might be its inhibitory effect on PI3K/Akt signaling pathways in ATC cells. Furthermore, PP121 is effective at suppressing ATC tumor growth in vivo. As a multiple kinase inhibitor, PP121 has significant advantages in Akt highly activated cancers like ATC cancer, which exhibit a higher growth and motility due to the high activation of Akt. The combined inhibition of tyrosine and phosphoinositide kinases by PP121 makes it a promising anticancer drug candidate in therapeutics of both the primary and relapsed ATC tumors. This is the first report about PP121 study in ATC therapy, which might shed new light on ATC therapeutics.

Conflicts of interest None

References

1. Are C, Shaha AR. Anaplastic thyroid carcinoma: biology, pathogenesis, prognostic factors, and treatment approaches. Ann Surg Oncol. 2006;13(4):453–64.

2. Sun C, Xu X, Wang X, et al. Thymic carcinoma with tumor thrombus protruding into the superior vena cava and the right atrium. Thoracic Cancer. 2013;4(3):333–4.
3. Zhang J, Wang H, Tian W, et al. Brown tumor of the rib as a first presentation of primary hyperparathyroidism: report of three cases and literature review. Thoracic Cancer. 2013;4(4):474–8.
4. Wiseman SM, Masoudi H, Niblock P, et al. Anaplastic thyroid carcinoma: expression profile of targets for therapy offers new insights for disease treatment. Ann Surg Oncol. 2007;14(2):719–29.
5. Bruni P, Boccia A, Baldassarre G, et al. PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing activity in thyroid cancer cells is mediated by p27kip1. Oncogene. 2000;19(28):3146–55.
6. Weng LP, Gimm O, Kum JB, et al. Transient ectopic expression of PTEN in thyroid cancer cell lines induces cell cycle arrest and cell type-dependent cell death. Hum Mol Genet. 2001;10(3):251–8.
7. Dahia PL, Marsh DJ, Zheng Z, et al. Somatic deletions and mutations in the Cowden disease gene, PTEN, in sporadic thyroid tumors. Cancer Res. 1997;57(21):4710–3.
8. Gimm O, Perren A, Weng LP, et al. Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue and benign and malignant epithelial thyroid tumors. Am J Pathol. 2000;156(5):1693–700.
9. Ringel MD, Hayre N, Saito J, et al. Overexpression and overactivation of Akt in thyroid carcinoma. Cancer Res. 2001;61(16):6105–11.
10. García-Rostán G, Costa AM, Pereira-Castro I, et al. Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer Res. 2005;65(22):10199–207.
11. Ha HT, Lee JS, Urba S, et al. A phase II study of imatinib in patients with advanced anaplastic thyroid cancer. Thyroid. 2010;20(9):975–80.
12. Zhang J, Wang P, Dykstra M, et al. Platelet-derived growth factor receptor-α promotes lymphatic metastases in papillary thyroid cancer. J Pathol. 2012;228(2):241–50.
13. Kim MJ, Kim SK, Park HJ, et al. PDGFRA promoter polymorphisms are associated with the risk of papillary thyroid cancer. Mol Med Rep. 2012;5(5):1267–70.
14. Malkomes P, Oppermann E, Bechstein WO, et al. Significantly high expression of platelet-derived growth factor (PDGF) in benign nodules of the thyroid: relevance in the development of goitre recurrence? Langenbecks Arch Surg. 2011;396(8):1165–72.
15. Chen KT, Lin JD, Liou MJ, et al. An aberrant autocrine activation of the platelet-derived growth factor alpha-receptor in follicular and papillary thyroid carcinoma cell lines. Cancer Lett. 2006;231(2):192–205.
16. Apsel B, Blair JA, Gonzalez B, et al. Targeted polypharmacology: discovery of dual inhibitors of tyrosine and phosphoinositide kinases. Nat Chem Biol. 2008;4(11):691–9.
17. Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer. 2002;2(7):489–501.
18. Cantley LC. The phosphoinositide 3-kinase pathway. Science (New York, NY). 2002;296(5573):1655–7.
19. Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis. 2004;9(6):667–76.
20. Oka N et al. Galectin-3 inhibits tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by activating Akt in human bladder carcinoma cells. Cancer Res. 2005;65(17):7546–53.
21. Moses SA et al. In vitro and in vivo activity of novel small-molecule inhibitors targeting the pleckstrin homology domain of protein kinase B/AKT. Cancer Res. 2009;69(12):5073–81.
22. Kundra V, Escobedo JA, Kazlauskas A, et al. Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. Nature. 1994;367(6462):474–6.
23. Hirai H, Sootome H, Nakatsuru Y, et al. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther. 2010;9(7):1956–67.
24. Engelman JA et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science. 2007;316:1039–43.
25. Sergina NV et al. Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3. Nature. 2007;445:437–41.