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EGFR inhibition enhances the antitumor efficacy of a selective BRAF V600E inhibitor in thyroid cancer cell lines

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Abstract. BRAF V600E is the most common genetic alteration in thyroid cancer and is indicative of a relatively poor prognosis. A selective inhibitor of BRAF V600E has been proposed as a novel treatment for patients with thyroid cancer exhibiting BRAF V600E mutations. However, this inhibitor has demonstrated a limited therapeutic effect. In the present study, possible adaptive mechanisms of resistance of thyroid cancer cells to the specific BRAF V600E inhibitor, PLX4032, were investigated. MTT assays were performed to determine the anti-proliferative efficiencies and half maximal inhibitory concentration (IC_{50}) of inhibitory treatments. The level of phosphorylated ERK was used to evaluate the activity of the mitogen assisted protein kinase (MAPK) pathway. Flow cytometry was performed to evaluate the rate of apoptosis. The IC_{50} measurements of PLX4032 in K1 and BCPAP cells were 0.550 and 1.772 μM, respectively. Co-treatment with an endothelial growth factor receptor (EGFR) inhibitor decreased the IC_{50} of PLX4032 to 0.206 μM, and prolonged the inhibitory effect of PLX4032 in K1 cells. In cells treated with PLX4032 alone, the MAPK pathway was reactivated after 24 h. However, the addition of an EGFR inhibitor suppressed this reactivation and increased the rate of apoptosis. In summary, the present study demonstrated that thyroid cancer harboring the BRAF V600E mutation was resistant to a selective BRAF inhibitor due to reactivation of the MAPK pathway. Co-treatment with an EGFR inhibitor increased antitumor efficacy and suppressed resistance to the BRAF V600E inhibitor.

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Introduction

The incidence of thyroid cancer has rapidly increased over the past few years and thyroid cancer is now the fifth most common cancer type diagnosed in women in the USA (1,2). Papillary thyroid cancer (PTC) accounts for >80% of thyroid tumors and is associated with a relatively favorable prognosis subsequent to surgical treatment. However, PTC tumors that are difficult to resect and those that metastasize remain a challenge to treat with long-term success (3-5). Examples of such challenging cases include recurrent thyroid cancer and anaplastic thyroid cancer (ATC) (6). It has been reported that recurrence is experienced in 20-30% of patients with thyroid cancer within the first 20 years post-surgery (7). Radioactive iodine and thyrotropin-suppressive therapies are used to treat these patients; however, persistent metastasis and dedifferentiation despite these treatments associate them with poor prognoses (4,5). The 10-year survival rate for patients with recurrent disease is only 10%, and this value has remained unchanged for numerous years due to the limited progress in the available treatments for recurrent thyroid cancer (8).

BRAF mutations have been identified in a variety of human cancer types, including thyroid cancer, malignant melanoma, ovarian tumors and colorectal cancer (CRC) (9,10). Over 40 BRAF mutations have been reported, the most commonly reported being BRAF V600E, which results from a thymine-to-adenosine transformation at position 1799 in exon 15, causing a valine-to-glutamate substitution at residue 600 in the peptide (11). The activity of the BRAF V600E protein is 10-fold greater than that of the wild-type protein and cannot be regulated appropriately (12). The resulting continuous activation of BRAF V600E activates the mitogen assisted protein kinase (MAPK) signaling pathway and promotes tumor progression (12).

The BRAF V600E mutation occurs in 29-83% cases of PTC, the most common subtype of thyroid cancer, and 24% cases of ATC, the most aggressive and lethal subtype of thyroid cancer (13-15). In addition to acting as a biomarker for diagnosis, the BRAF V600E expression is associated with aggressive and iodine-resistant phenotypes of thyroid cancer (14). It has been reported that thyroid cancers harboring the BRAF V600E mutation tend to exhibit other factors indicative of a

poor prognosis, including extra-thyroidal extension, lymph node metastasis, advanced stage, iodine-131 resistance, recurrent disease and distant metastasis (16-21). Even in papillary thyroid microcarcinoma (with diameters <1 cm), the BRAF V600E mutation is associated with extra-thyroidal extension and lymph node metastasis (22,23).

The BRAF V600E protein has been investigated as a therapeutic target in a number of studies and various BRAF V600E inhibitors have been identified (24-26). Sorafenib is a first-generation BRAF V600E inhibitor. However, the mechanism by which the effect of sorafenib is mediated remains unclear due to its ability to inhibit multiple kinases (27,28). Vemurafenib and dabrafenib are selective BRAF V600E inhibitors, which have demonstrated therapeutic activity in phase 1 and 2 clinical trials in patients with BRAF V600E-mutation-induced metastatic melanoma, and they have been approved by the Food and Drug Administration (29). However, these inhibitors are unable to efficiently suppress the progression of other types of cancer and resistance to the inhibitors developed within 6-7 months, even in melanoma (30,31). In CRC, endothelial growth factor receptor (EGFR)-mediated re-proliferation serves an essential role in the resistance to BRAF inhibition (32). It has been reported that overexpression of EGFR results in constitutive activation of the MAPK signaling pathway and promotes cancer cell proliferation, even during treatment with selective BRAF V600E inhibitors (32). In the present study, BRAF V600E was inhibited and downregulation of EGFR was induced in PTC cell lines to determine whether this combined strategy was able to efficiently block cancer cell proliferation, and if it may be a potential novel treatment for thyroid cancer.

Materials and methods

Cell lines and reagents. The K1 and BCPAP cell lines were purchased from Sigma-Aldrich (Merck KGaA Darmstadt, Germany). K1 and BCPAP are papillary thyroid cancer cell lines, and K1 cells are a derivative of the GLAG-66 cell line (33). Both cell lines were passaged for <3 months from stocks generated from the first or second passage of the original cells. The mutation status of these cell lines is reported in Table I. BCPAP cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (FBS), and K1 cells were maintained in RPMI-1640 supplemented with 10% FBS. The cells were maintained at 37˚C in a humidified incubator with 5% carbon dioxide. The BRAF V600E-selective inhibitor PLX4032 was obtained from Plexxikon, Inc. (Berkeley, CA, USA) and the EGFR-selective inhibitor, gefitinib, was obtained from Roche Diagnostics (Basel, Switzerland). Inhibitors were dissolved in DMSO and the stock solutions were stored at -20˚C.

Cellular proliferation assays. Proliferation of K1 and BCPAP cells was evaluated using the MTT assay (Sigma-Aldrich; Merck KGaA). Cells were plated in 96-well microtiter plates at a density of $3x10^3$ cells/well in a volume of 180 μl DMEM with FBS. PLX4032 with or without gefitinib was diluted in media containing 1% DMSO at 10X the final assay concentrations (PLX4032: 0.01, 0.03, 0.1, 0.3, 1 and 3 μM; gefitinib: 0.125 μM). After 24 h of culture, 20 μl each drug dilution was added in triplicate to separate wells. Cells were assayed for proliferation at 24, 48, 72, 96 and 120-h time points. The percentage of inhibition was calculated using the following formula: 100-(mean absorbance of experimental wells/mean absorbance of control wells) x100. The half maximal inhibitory concentration (IC_{50}) values were determined by calculating the regression of plots produced from the logarithms of concentration vs. percentage inhibition, using XLfit software (version 4.2; ID Business Solutions Ltd., Guildford, UK).

Table I. Basic information and genetic profiles of the cell lines.

Cell line	Subtype	BRAF variation	EGFR variation
K1	PTC	GTG-GAG	Wild-type
BCPAP	PTC	GTG-GAG	CAG-CAA

EGFR, endothelial growth factor receptor; PTC, papillary thyroid cancer.

Western blot analysis. Cells were seeded at 70-75% confluence in 6-well plates, 1 day prior to drug treatment. Cells were cultured at the aforementioned drug concentrations and times at 37˚C with 5% CO_2, and were harvested and lysed in 1X cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). After a 20-min incubation on ice, the lysates were centrifuged at 12,000 x g for 15 min at 4˚C to clear insoluble debris. The protein concentrations of the lysates were then determined with a BCA kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and equal amounts of total protein from cell lysates and tumor lysates (10 μg for each lane) were resolved on 4-12% NuPage gradient polyacrylamide gels (Invitrogen; Thermo Fisher Scientific, Inc.) before they were blotted onto polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were first blocked with 5% dry fat free milk for 1 h at room temperature and washed twice with Tris-Buffered Saline containing 0.1% Tween-20 (Affymetrix, Inc.; Thermo Fisher Scientific, Inc.). The blocked membranes were probed with rabbit polyclonal antibodies against human phosphorylated-extracellular regulated kinase (p-ERK1/2) (Cell Signaling Technology, Inc.; cat no. 4370S; dilution, 1:1,000), ERK1/2 (Sigma-Aldrich; Merck KGaA; cat no. M5670; dilution, 1:1,000), β-actin (cat no. sc-130656; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution, 1:2,000) and incubated for 2 h at room temperature. Followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat no. sc-2004; dilution, 1:5,000; Santa Cruz Biotechnology, Inc.). A chemiluminescent signal was generated using Amersham ECL Plus Western Blotting Detection reagents (GE Healthcare, Chicago, IL, USA) and detected with a Fujifilm LAS-3000 imager (Fujifilm, Tokyo, Japan). The densitometry was performed using Multi Gauge 3.0 software (Fujifilm).

Flow cytometry. Cells were seeded in 6-well plates at $4x10^5$ cells/well and incubated for 24 h prior to treatment with PLX4032 and/or gefitinib. A total of five treatment groups were analyzed: PLX4032 monotreatment for 1 day; PLX4032 monotreatment for 5 days; PLX4032 combined with gefitinib

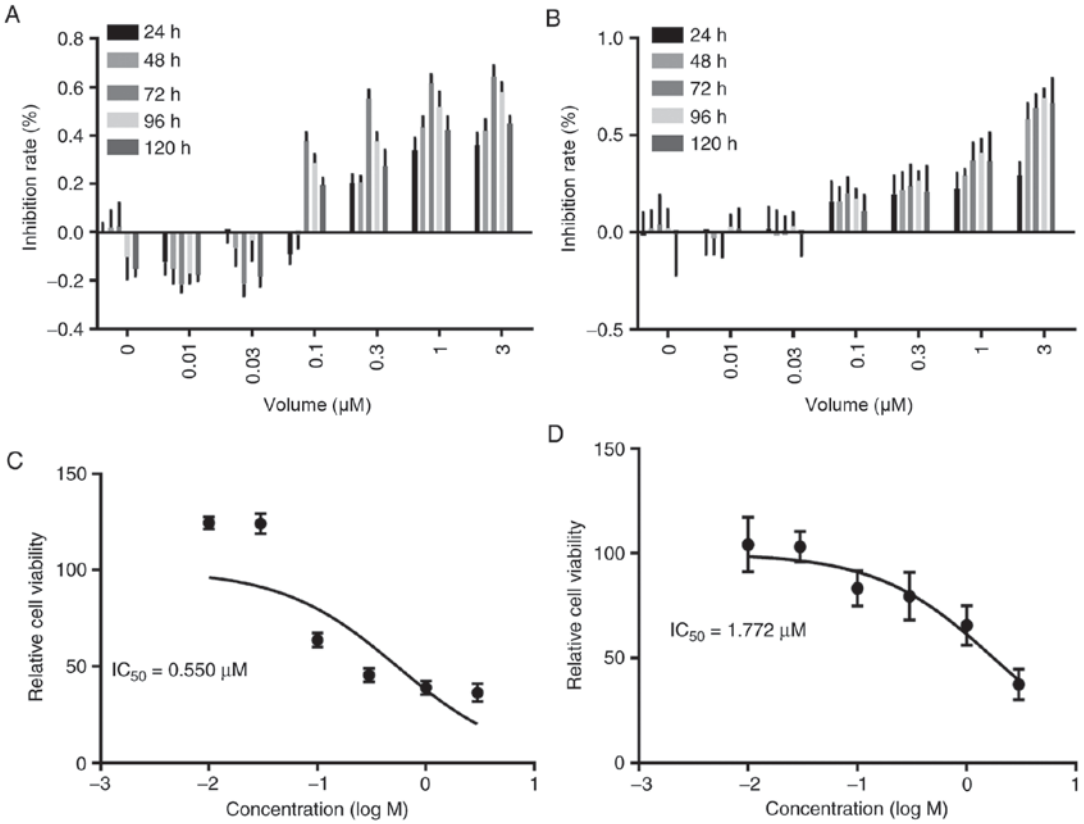

Figure 1. Anti-proliferation efficacy of PLX4032 in K1 and BCPAP cells. K1 and BCPAP cells were treated with PLX4032 at 0.01, 0.03, 0.1, 0.3, 1 or 3 μM for 24, 48, 72, 96 or 120 h. Untreated cells served as a control. (A) PLX4032 inhibition rate (%) in K1 cells. (B) PLX4032 inhibition rate (%) in BCPAP cells. (C) IC_{50} of K1 cells treated with PLX4032 for 72 h. (D) IC_{50} of BCPAP cells treated with PLX4032 for 72 h. IC_{50}, half maximal inhibitory concentration; log M, log(inhibitor) vs. normalized response.

for 1 day; and PLX4032 combined with gefitinib for 5 days. The concentrations of PLX4032 and gefitinib were 0.206 and 0.125 μM, respectively. Untreated cells were used as a control group. Apoptosis was evaluated by measuring the exposure of phosphatidylserine on cell membranes using Annexin V-FITC apoptosis detection kits (Sigma-Aldrich; Merck KGaA). Following the treatments, cell pellets were resuspended in a solution containing 5 μg/ml propidium iodide and 1 μg/ml Annexin V-fluorescein isothiocyanate for 15 min at room temperature in darkness. Subsequently, the cells were assessed by a flow cytometer equipped with CellQuest 5.1 software (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical methods. GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze all data and the results are presented as the mean ± standard error. Significant differences were determined by one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Co-treated with gefitinib and PLX4032 had a stronger anti-proliferation efficacy. To evaluate the anti-proliferation efficacy of PLX4032, the IC_{50} values of PLX4032 were obtained for K1 and BCPAP cells using an MTT assay at different time-points. Both cell lines were most sensitive to PLX4032 at 72 h. The IC_{50} in K1 cells (0.550 μM) was compared with BCPAP cells (1.772 μM) (Fig. 1). It has been reported that the IC_{50} of gefitinib is 0.125 μM for other cancer cell lines (32). The IC_{50} for PLX4032 in K1 cells co-treated with gefitinib was determined at the 72-h time-point. The combination suppressed proliferation more effectively than PLX4032 treatment alone, resulting in a lower IC_{50} of 0.206 μM (Fig. 2).

Reactivation of the MAPK signaling pathway was found in papillary thyroid cancer cell lines. To investigate the mechanism of inhibitor resistance, the activity of the MAPK signaling pathway was examined. ERK is a key enzyme in this pathway; therefore, the phosphorylation of ERK was measured in order to evaluate MAPK pathway activity. K1 and BCPAP cells were treated with PLX4032 (K1: 0.2 μM; BCPAP: 1.772 μM) for 12 h. Western blot analyses indicated that p-ERK levels were low in the PLX4032-treated groups compared with the control groups, suggesting that MAPK activity was suppressed by PLX4032 (Fig. 3). K1 was more sensitive to PLX4032; therefore K1 was used in the following experiments. Treatment of K1 cells with varying concentrations (0, 0.01, 0.03, 0.1, 0.3 and 1 μM) of PLX4032 demonstrated that this inhibition of MAPK was dose-dependent (Fig. 4). Subsequently, K1 cells were treated with 0.550 μM PLX4032 and the p-ERK levels were examined at different time points (0, 6, 24 and 48 h). The lowest level of p-ERK was detected at the 6 h time-point, after

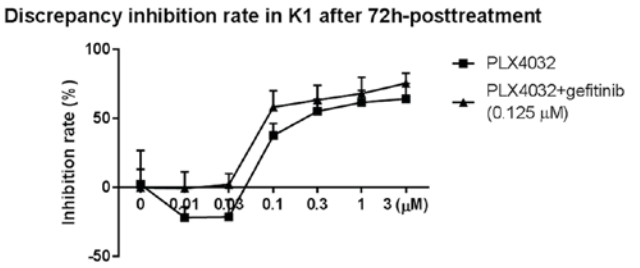

Figure 2. The anti-proliferation efficacy of PLX4032 with or without gefitinib in K1 cells at 72 h. The IC_{50} of PLX4032 in the presence of gefitinib (0.206 μM) was significantly lower than that of PLX4032 alone (0.550 μM) ($P<0.05$). IC_{50}, half maximal inhibitory concentration.

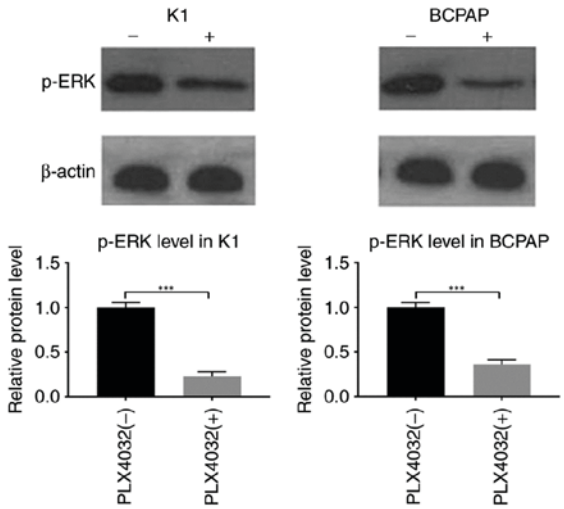

Figure 3. Effects of acute treatment with PLX4032 on the K1 and BCPAP cell lines. p-ERK was used to evaluate MAPK activation. Protein level was quantified relative to β-actin. Following 12 h treatment, p-ERK was significantly decreased in K1 and BCPAP cells ($P<0.05$). p-, phosphorylated; ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase. ***$P<0.05$.

which the p-ERK levels increased, indicating reactivation of the MAPK signaling pathway (Fig. 5).

Co-treated with gefitinib and PLX4032 promoted apoptosis. Flow cytometric analysis of apoptosis indicated that apoptosis was elevated in cells cultured with PLX4032 for 1 day compared with cells in the control group. However, apoptosis in the treated group decreased from day 1 to 5, indicating a decrease in the antitumor activity of PLX4032. Apoptosis of K1 cells treated with PLX4032 and gefitinib was significantly elevated compared with the control group and the PLX4032 monotreatment group ($P<0.05$). In the co-treated group, there was no significant difference between the level of apoptosis on days 1 and 5, which indicates continued suppression over time (Fig. 6). Additionally, the combined treatment with an EGFR inhibitor was able to increase the antitumor efficacy of BRAF V600E inhibitors and suppress drug resistance in PTC cells (Fig. 7).

Discussion

The BRAF V600E mutation has been reported in numerous types of cancer, including thyroid cancer, CRC and

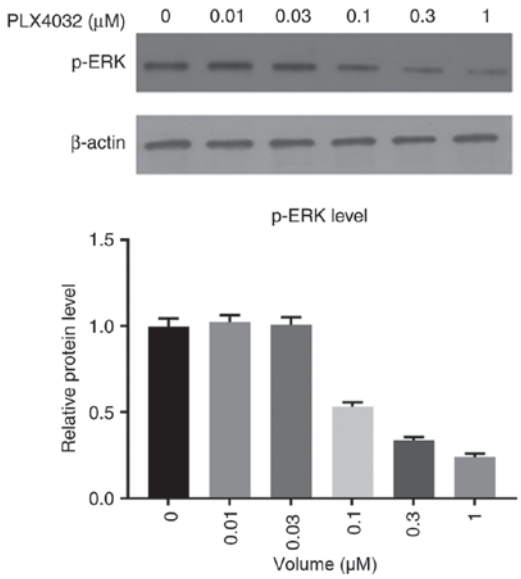

Figure 4. MAPK pathway activation in PLX4032-treated cells. K1 cells were treated with the indicated concentrations of PLX4032 for 12 h. Untreated cells served as a control group. Protein level was quantified relative to β-actin. PLX4032 significantly p-ERK expression at concentrations greater than 0.1 μM, and this inhibition was dose-dependent. MAPK, mitogen-activated protein kinase; p-, phosphorylated; ERK, extracellular signal-related kinase.

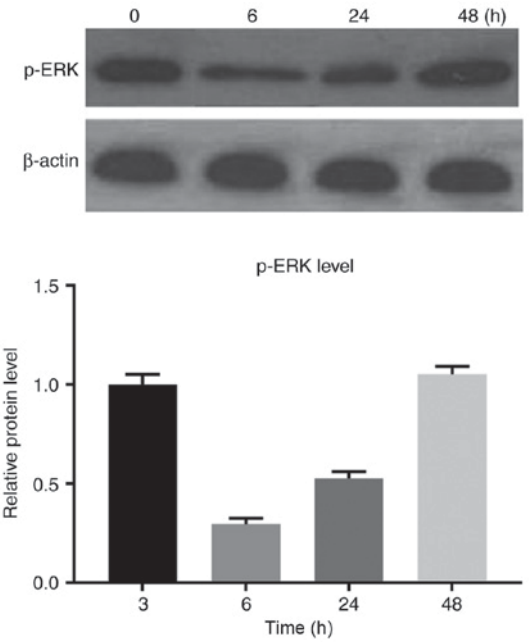

Figure 5. Time-course study of MAPK pathway activation in PLX4032-treated cells. The highest level of inhibition was observed at 6 h, after which the MAPK pathway was reactivated. Untreated cells (0 h) served as a control. Protein level was quantified relative to β-actin. MAPK, mitogen-activated protein kinase; p-, phosphorylated; ERK, extracellular signal-related kinase.

melanoma (9,10). BRAF V600E is an oncogene, and serves an important role in tumorigenesis and cancer progression. The protein encoded by BRAF V600E is >10-fold more active compared with the wild-type protein and is not regulated by normal feedback mechanisms (12). BRAF V600E can continuously activate the MAPK signaling pathway, thus promoting tumor proliferation, invasion and metastasis (34). BRAF

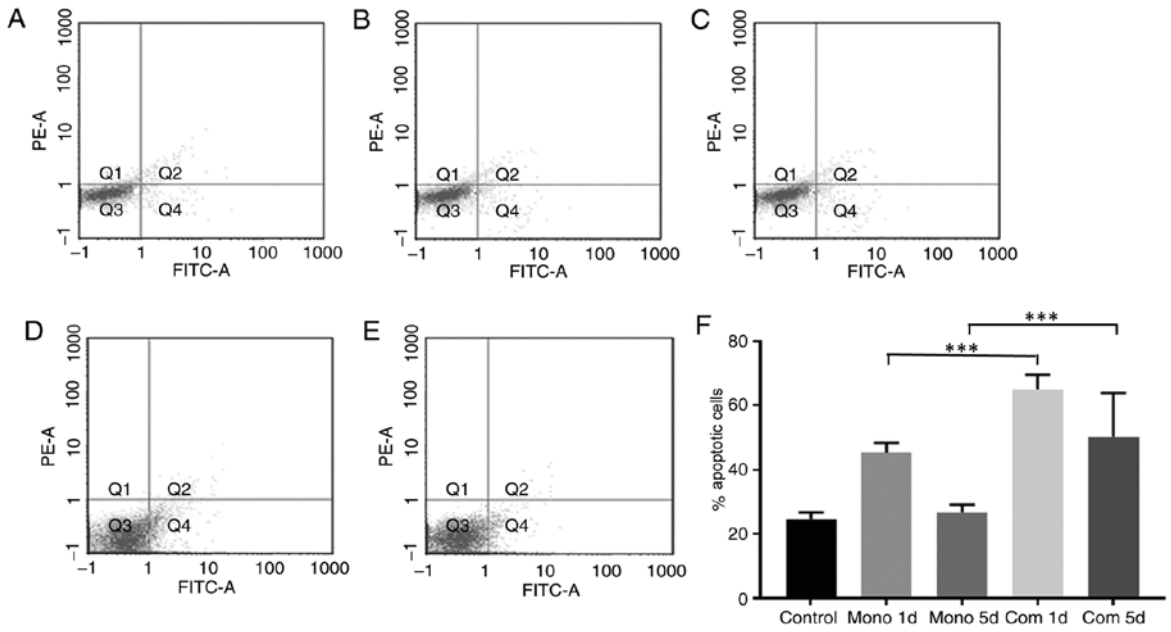

Figure 6. Rate of apoptosis in cells treated with PLX4032 and/or gefitinib. PLX4032-treated K1 cells demonstrated elevated levels of apoptosis compared with control cells after 1 day of treatment (P<0.05). However, there was no significant difference after 5 days of treatment. The combination of gefitinib and PLX4032 induced apoptosis more effectively than PLX4032 alone (P<0.05). The treatment groups were as follows: Mono 1d, PLX4032 (0.550 μM) treatment for 1 day; Mono 5d: PLX4032 (0.550 μM) treatment for 5 days; Com 1d, PLX4032 (0.206 μM) combined with gefitinib (0.125 μM) treatment for 1 day; Com 5d, PLX4032 (0.206 μM) combined with gefitinib (0.125 μM) treatment for 5 days. (A) No treatment; (B) PLX4032 monotreatment for 1 day; (C) PLX4032 monotreatment for 5 days; (D) PLX4032 combined with gefitinib for 1 day; (E) PLX4032 combined with gefitinib for 5 days, and (F) quantification of apoptotic rates according to treatment group. PE-A, phycoerythrin-A; FITC-A, fluorescein isothiocyanate; mono, monotreatment; comb, combined treatment. ***P<0.05.

Figure 7. Proposed mechanistic model for the function of BRAF V600E and EGFR in thyroid cancer cell lines. EGFR, endothelial growth factor receptor; ERK, extracellular signal-related kinase.

V600E mutations occur at different rates in different cancer types, but the presence of the mutation consistently indicates poor prognosis (14,15).

BRAF V600E is one of the most common genetic alterations in thyroid cancer. It occurs in 29-83% of PTCs, which comprise >80% of all thyroid tumors (13). PTCs harboring the BRAF V600E mutation have more aggressive clinicopathological features, including extra-thyroidal extension and lymph node metastasis (13-15). Seeing as there are limited therapies for metastatic thyroid cancer, novel treatments are essential to improve disease outcomes for patients exhibiting this mutation. Inhibitors targeting the BRAF V600E protein have been evaluated in clinical trials. However, the inhibitor response depends on the type of cancer (24,25,30). The selective inhibitor PLX4032 has exhibited beneficial therapeutic effects on metastatic melanoma, but no effect on colon or thyroid cancers (32). Numerous mechanisms to account for the resistance of thyroid cancer to BRAF inhibition have been suggested, including the following: Activation of the RAF/MEK/ERK pathway caused by BRAF alternative splicing; activation of the phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) pathway through hepatocyte growth factor receptor; autocrine neuregulin 1-mediated human epidermal growth factor receptor 3 activation of the PI3K/AKT and RAS/RAF/MEK/ERK pathways; autocrine interleukin-6-mediated activation of the janus kinase/signal transducer and activator of transcription 3 and RAS/RAF/MEK/ERK pathways, and increased autophagy (31,35-37).

In CRC, it has been reported that EGFR overexpression promotes tumor proliferation (32). The present study determined whether PLX4032 combined with the EGFR inhibitor, gefitinib, was able to suppress thyroid cancer cell proliferation. Co-treatment with gefitinib significantly increased the antitumor activity of PLX4032. Frasca *et al* (38) reported that co-treatment with PLX4032 was able to decrease the IC_{50} of gefitinib. These data indicate that PLX4032 (vemurafenib) and gefitinib are able to produce synergistic effects. Vemurafenib and gefitinib are safe for use in patients, and therapeutic regimen combining BRAF and MAPK inhibitors may result in greater efficacy and fewer side effects.

To further understand the molecular mechanism of BRAF inhibitor resistance, activity of the MAPK pathway was examined. In thyroid cancer cell lines, the MAPK pathway was suppressed by PLX4032. This inhibition was the most effective after 6 h of treatment. MAPK activity gradually increased after 6 h, indicating that the pathway was reactivated. Danysh *et al* (39) reported that treatment of PTC cell lines with a selective BRAF inhibitor for 5 months led to the acquisition of resistance to the inhibitor through a spontaneous KRAS G12D mutation. Thus, cancer cells can acquire short-term and long-term resistance to BRAF inhibition. In the present study, combined treatment with gefitinib and PLX4032 continuously suppressed the MAPK pathway, indicating that EGFR serves an important role in the resistance of cells to BRAF inhibition.

As MAPK signaling has an established association with apoptosis, the survival of PLX4032-treated thyroid cancer cells was quantified. Flow cytometry demonstrated that PLX4032 treatment initially increased apoptosis, but this effect declined after 5 days of treatment, indicating that the effect of PLX4032 on cell survival was short-term. Combined treatment with gefitinib significantly increased apoptosis and prolonged this effect (Fig. 7).

In conclusion, PTC cells harboring a BRAF V600E mutation may become resistant to selective BRAF inhibition through reactivation of the EGFR/MAPK pathway. Combined treatment with an EGFR inhibitor is able to increase the antitumor efficacy of BRAF V600E inhibitors and suppress drug resistance in PTC cells.

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Availability of data and materials

All data that were generated or analyzed in this study are included in this manuscript.

Authors' contributions

YJ and CZ conceived and designed the study. CH and YY conducted the experiments. XZ and YL performed the statistical analysis; MG interpreted the statistical analysis, reviewed and made final approval of the version to be published. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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