Heliyon 10 (2024) e29055

Contents lists available at ScienceDirect

Heliyon

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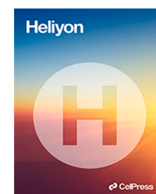

Research article

Synergistic effects of the combination of trametinib and alpelisib in anaplastic thyroid cancer with BRAF and PI3KCA co-mutations

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ARTICLE INFO

Keywords:
Anaplastic thyroid cancer
BRAF
PI3KCA
Dabrafetnib
Trametinib
Alpelisib

ABSTRACT

Background: Anaplastic thyroid cancer (ATC), a rare and aggressive malignancy with a poor prognosis, has shown promise with the approved dabrafenib/trametinib combination for $BRAF^{V600E}$ mutation. Co-occurring PI3KCA mutations, identified as negative prognostic factors in lung cancer with $BRAF^{V600E}$ mutation, emphasize the need to target both pathways. Exploring trametinib and alpelisib combination becomes crucial for ATC.

Methods: A patient-derived xenograft (PDX) and primary cell line were obtained from an ATC patient with BRAF and PI3KCA co-mutation. Individual testing of targeted therapies against BRAF, MEK, and PI3KCA was followed by a combination treatment. Synergistic effects were evaluated using the combination index. Immunoblotting assessed the efficacy, with validation performed using a PDX model.

Results: In this study, the ATC0802 cell line and PDX were established from a refractory ATC patient. NGS revealed BRAF and PI3KCA co-mutations pre- and post-dabrafenib/trametinib treatment. Trametinib/alpelisib combination showed synergy, suppressing both pERK and pAKT levels, unlike monotherapies or BRAF knockdown. The combination induced apoptosis and, in the PDX model, demonstrated superior tumor growth inhibition compared to monotherapies.

Conclusions: The combination of trametinib and alpelisib showed promise as a strategy for treating ATC with co-mutations in BRAF and PI3KCA, both *in vitro* and *in vivo*. This combination offers insights into overcoming resistance to BRAF-targeted treatments in ATC with mutations in BRAF and PI3KCA.

1. Introduction

Anaplastic thyroid cancer (ATC) accounts for approximately 1–2% of all thyroid cancers [1]; however, it is the most lethal malignancy, with a median overall survival of 4–6 months [2]. The BRAF V600E mutation is commonly found in ATC, with a prevalence

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https://doi.org/10.1016/j.heliyon.2024.e29055

Received 19 January 2024; Received in revised form 28 March 2024; Accepted 28 March 2024

Available online 30 March 2024

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of 35–40% [3]. This mutation activates the mitogen-activated protein (MAP) kinase pathway, including the downstream proteins MEK and ERK, leading to cell proliferation and oncogenesis in thyroid tumors [4]. Dabrafenib and trametinib are tyrosine kinase inhibitors (TKIs) effective against BRAF V600E and MEK1/2, respectively, and the combination of dabrafenib and trametinib in ATC was approved based on a single-arm phase 2 study [5]. Our previous study showed that this combination improved the overall survival of patients with ATC [6].

Mutations in the MAPK pathways, including BRAF, KRAS, and NRAS, are often mutually exclusive; however, co-occurring mutations in the MAPK and PI3K-Akt pathways have been reported [7,8]. In a study of BRAF-mutated lung cancer patients treated with dabrafenib and trametinib, those with mutations in the PIK3 pathway had a numerically shorter median overall survival of 5.4 months (range: 3.1–55.2) compared to those without PIK3 pathway mutations [9]. Therefore, the presence of co-mutations in BRAF and PI3KCA may affect an impact on the treatment of ATC that have not been reported to date.

In this study, we established an ATC cell line and a PDX model from a patient with BRAF-mutated ATC that was refractory to

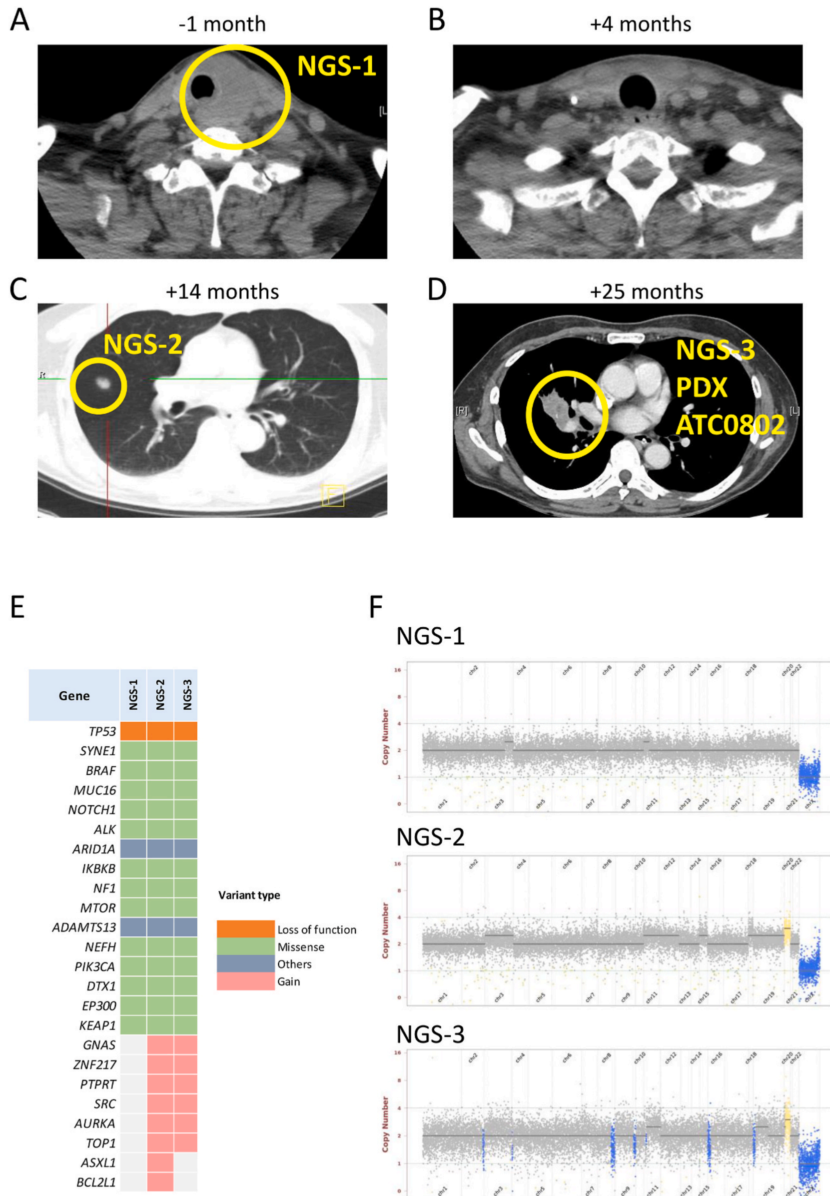

Fig. 1. Imaging findings in an anaplastic thyroid cancer (ATC) patient. The computed tomography (CT) scan showed a tumor in left thyroid (A), a partial response of tumors (B), lung metastasis after discontinuation of dabrafenib and trametinib (C). Although the patient received the targeted therapy again, the ATC recurred (D). NGS-1 to NGS-3 and patient-derived xenograft (PDX), ATC0802 indicate the location of tumor samples obtained for NGS, ATC0802 and PDX model establishment. The genomic profiling of genes (E) and copy numbers (F) in the patient with ATC before and after targeted therapy with dabrafenib and trametinib.

dabrafenib and trametinib. We also identified a novel combination of trametinib and alepesib, a PI3Kα inhibitor, as a potential treatment option for ATC harboring BRAF and PI3KCA co-mutations.

2. Material and methods

2.1. PDX model and cell line establishment

Samples were obtained from a patient with ATC harboring a BRAF mutation who underwent surgical resection for the second occurrence of lung metastasis (Fig. 1). The tumor samples were immediately cut into small pieces (25–30 mm^3) and immersed in antibiotic-containing PBS (200 U/mL penicillin and 200 g/mL streptomycin (1X P/S, Gibco)).

To establish a stable cell line, the tumor tissues were completely minced using sterile scalpel blades. The minced tumor pieces were mixed with 200 units/mL of collagenase IV (Gibco) in DMEM (Gibco) at 37 °C for 2 h for enzymatic dissociation. Single tumor cells were cultured in a complete RPMI-1640 medium (10% FBS, 1X P/S) and passaged until no fibroblasts were present. Based on the collected data, the cells were named ATC0802.

To establish the PDX mouse model, one piece of tissue was implanted subcutaneously in the flank regions of anesthetized 4–6-week-old male NOD/SCID mice (BioLASCO, Taiwan), and body weight and tumor size were monitored once a week. After reaching a diameter of ~1 cm, the xenograft was excised and sub-implanted into subsequent passaged mice for further experiments [10].

The samples were encoded and used according to a protocol approved by the Institutional Review Board of Linkou Chang-Gung Memorial Hospital (Protocol Numbers: IRB # 202101714B0 and 202100148B0).

2.2. Next generation sequencing and whole exome sequencing for ATC tumors

The gene expression profiles of the ATC tumor samples before and after treatment with dabrafenib/trametinib were examined by ACTOnco® (ACT Genomics, Taiwan), an NGS panel sequencing more than 400 genes. Whole-exome sequencing (WES) was performed on ATC0802 by using the Illumina NextSeq platform (Illumina, USA) to targeted 100-fold read depth at Feng Chi Biotech Corporation in Taiwan.

2.3. Proliferation and growth inhibition

ATC0802 cells were seeded in 96-well plates (3×10^3/well) and incubated overnight, and cell viability was assessed with a microplate reader using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Japan) after treatment with dabrafenib, trametinib, or alpelisib for 72 h. The IC_{50} values were calculated using the Prism 8 software.

2.4. Combination index (CI)

To determine the interaction between drugs, the combination index (CI) values were calculated using CalcuSyn (Biosoft, UK) by calculating the growth inhibition results of cells treated with 1/9-, 1/3-, 1-, 3-, and 9-folds of the IC_{50} for single or combination drugs.

2.5. Immunoblotting

ATC0802 cells were lysed in a RIPA buffer supplemented with protease and phosphatase inhibitors. A total of 30 μg of proteins were separated using 10% SDS-PAGE gels and then transferred to the nitrocellulose membranes (Amersham™, GE Healthcare Life Science). After blocking with 5% nonfat milk for 2 h, the membrane was incubated with specific antibodies (Akt, Cell Signaling #4685, 1:1000; p-Akt S473, Cell Signaling #4060, 1:2000; Erk 1/2, Cell Signaling #4695, 1:1000; p-Erk 1/2, Cell Signaling #4370, 1:2000; B-Raf, GeneTex GTX100913, 1:1000; B-Raf (V600E), GeneTex GTX33595, 1:500; GAPDH, GTX627408, 1:10000) overnight at 4 °C and then incubated with a goat anti-mouse/rabbit horseradish peroxidase-conjugated secondary antibody (#115-035-003/111-035-003, 1:5000, Jackson ImmunoResearch Laboratories, USA) at room temperature for 2 h. The blots were visualized using the Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) on a UVP ChemStudio PLUS Touch imager (Analytik Jena AG) [11].

2.6. siRNA transfection

A final concentration of 25 nM siRNA against BRAF and the control sequence was used for transfection with DharmaFECT™ Transfection Reagents (Horizon, UK). The sequences were designed as follows: control siRNA (siNC), sense:5′-UUCUCCGAACGU-GUCACGUTT -3′, antisense:5′-ACGUGACACGUUCGGAGAATT -3′; siBRAF (V600E) #1, sense:5′-GCUACAGAGAAAUCUCGAUdTdT-3′, antisense:5′-AUCGAGAUUUCUCUGUAGCdTdT-3′; siBRAF #2, sense:5′-AGAAUUGGAUCUGGAUCAUdTdT-3′, antisense:5′-AUGAUC-CAGAUCCAAUUCUdTdT-3′.

2.7. Animal experiments

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC, No. 2021092712) of the Chang Gung Memorial Hospital at Linko. Immunodeficient NOD/SCID male mice (BioLASCO, Taiwan) aged five weeks were used in this study. The

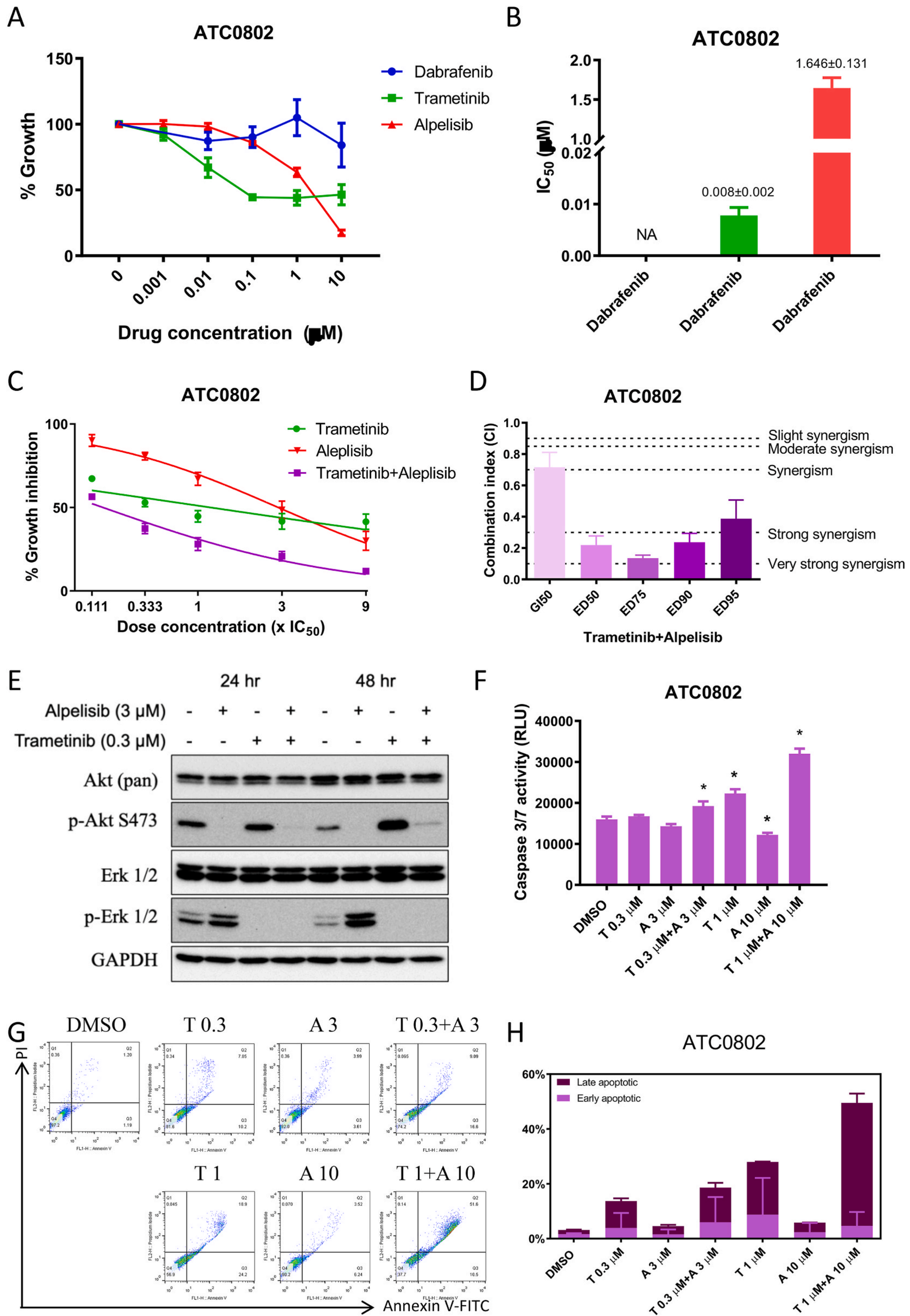

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Fig. 2. Growth inhibition of the combination treatment by trametinib plus alpelisib in ATC0802. The survival curves (A) and half maximal inhibitory concentration (IC_{50}) (B) of alpelisib in ATC0802. (C, D) The combination of trametinib and alpelisib showed a synergistic effect in ATC0802. (E) Immunoblotting of ATC0802 treated with trametinib, alpelisib, and the combination treatment. The caspase 3/7 activity (F) and annexin V assay (G, H) also displayed increased apoptosis significantly after trametinib plus alpelisib treatment. The bar graphs show the mean ± SEM from three independent experiments. *, $p < 0.05$.

tumor tissue (3 mm^3) was subcutaneously embedded in the flank of the mice. When the tumor volume reached an average of 400 mm^3, the mice were randomized into four groups (n = 5) and treated orally as follows: control, trametinib (0.6 mg/kg, 5 d on/2 d off), alpelisib (35 mg/kg, 5 d on/2 d off), and trametinib + alpelisib as above. The tumor size and body weight of the mice were serially measured, and the tumors were removed and weighed after treatment for 9 d.

2.8. Statistical analysis

Data are presented as the mean ± SEM. Student's t-test and two-way analysis of variance (ANOVA) were used for statistical analysis when appropriate, using the GraphPad Prism 8 software. A p-value <0.05 was considered statistically significant.

3. Results

3.1. Clinical course of patients with ATC treated with dabrafenib and trametinib

A 57-year-old male patient was diagnosed with advanced ATC harboring the BRAF V600E mutation and was initially treated with dabrafenib, trametinib, pembrolizumab (100 mg every three weeks for four cycles), and radiotherapy (Fig. 1A). After three months of treatment, a partial response was achieved and targeted therapy was continued (Fig. 1B). However, after 10 months of treatment, the patient developed tracheocutaneous and tracheoesophageal fistulas complicated by a deep neck infection, when a pathological complete response (pCR) was achieved. Targeted therapy with dabrafenib and trametinib was discontinued because of pCR. Lung metastasis was noted three months after the discontinuation of dabrafenib and trametinib (Fig. 1C), and the patient underwent tumor resection and re-initiation of dabrafenib and trametinib. Eleven months later, ATC recurred in the lungs and mediastinal lymph nodes (Fig. 1D) and salvage surgery was performed. A PDX model was established from lung metastasis and named ATC0802. Unfortunately, the tumor recurred in five months after the salvage surgery, progressed rapidly and the patient succumbed to the disease five months after recurrence.

Next-generation sequencing (NGS) using ACTOnco®$^{+}$(ACT Genomics, Taiwan) was used for DNA and RNA sequencing and mutation detection. The data revealed the presence of BRAF V600E and PIK3CA H1047Y mutations before and after treatment with dabrafenib and trametinib (Fig. 1E). Furthermore, copy number gain was observed in BCL2L1, ASXL1, SRC, TOP1, PTPRT, ZNF217, AURKA, and GNAS after the development of resistance to targeted therapy, which may account for drug resistance to dabrafenib and trametinib (Fig. 1E and F).

3.2. Growth inhibition of ATC0802

The ATC0802 cell line was established from the patient with ATC harboring a BRAF mutation who underwent surgical resection for the second occurrence of lung metastasis. ATC0802 underwent whole exome sequencing (WES), verifying the persistence of BRAF and PI3KCA mutations. ATC0802 treated with dabrafenib, trametinib, or alpelisib for 72 h. Although trametinib exhibited the lowest IC_{50} value among the three drugs, alpelisib showed the greatest suppression at high concentrations (Fig. 2A and B).

3.3. Combination of trametinib and alpelisib in ATC0802

The ATC0802 cells were treated with 3-fold dilution of trametinib, alpelisib, or a combination of trametinib and alpelisib, starting from 0.111-, 0.333-, 1-, 3-, and 9-fold IC_{50} for 72 h (Fig. 2C). The combination index was calculated and synergism was observed at ED50, ED75, ED90, and ED95 (Fig. 2D). Immunoblotting analysis showed that trametinib treatment reduced p-Erk expression but increased p-Akt expression in ATC0802 cells, whereas alpelisib treatment decreased p-Akt expression but increased p-Erk expression. However, the combination of trametinib and alpelisib effectively suppressed both p-ERK and p-Akt expression, leading to maximal growth inhibition (Fig. 2E). Moreover, the combination of trametinib and alpelisib induced apoptosis in ATC0802, as indicated by caspase 3/7 activation (Fig. 2F) and annexin V staining (Fig. 2G and H) in cells co-treated with trametinib and alpelisib.

3.4. Knockdown of BRAF by siRNA

As dabrafenib showed minimal activity against ATC0802 cells, two siRNAs were used to knock down BRAF expression in ATC0802 cells. Knockdown of BRAF minimally reduced tumor growth after three days of treatment, indicating that the BRAF mutation is not the major driver mutation for this tumor (Fig. 3A). Similar to the response to trametinib treatment, knockdown of BRAF induced p-Akt expression and reduced p-Erk expression (Fig. 3B).

3.5. Trametinib and alpelisib treatment in the ATC PDX model

ATC0802 tumor tissues were subcutaneously injected into NOD/SCID mice. Four groups, including control, trametinib, alpelisib, and combination therapy, were administered (Fig. 4A). The combination treatment significantly inhibited tumor growth, whereas trametinib alone did not affect tumor size (Fig. 4B and C). Tumor weights were significantly lower in the combination group than in the trametinib-only group, and the *p*-value between the trametinib plus alpelisib and alpelisib-only groups was 0.0557 (Fig. 4D). Trametinib, alpelisib, or combination therapy did not significantly change body weight compared to the control treatment during the study period (Fig. 4E).

4. Discussion

This study investigated a novel combination therapy with trametinib and alpelisib for the treatment of ATC with BRAF and PI3KCA co-mutations. We established a cell line and a PDX model to facilitate *in vitro* and *in vivo* studies. Our results showed a synergistic effect of trametinib and alpelisib, as evidenced by a combination index calculation, leading to the suppression of both MAPK and PI3K/AKT/mTOR pathways, as demonstrated by a reduction in p-Erk and p-Akt levels. These findings suggest that this combination therapy could be a promising approach for treating patients with tumors harboring BRAF and PI3KCA co-mutations.

This cell line and PDX model were established from a patient with ATC harboring BRAF and PI3KCA co-mutations, who was refractory to dabrafenib and trametinib therapy. In contrast, targeted therapy of MEK and PI3KCA using trametinib and alpelisib simultaneously suppressed tumor cell growth (Fig. 5).

Compared with trametinib treatment, knockdown of BRAF-induced p-Akt reduced p-Erk. This highlights the crosstalk between the MAPK/ERK and PI3K/Akt pathways. Crosstalk between the MAPK/ERK and PI3K/Akt pathways is well documented and known to be important in thyroid tumorigenesis [12,13]. Activation of the MAPK/ERK pathway, which is commonly observed in thyroid cancer, can lead to activation of the PI3K/Akt pathway through several mechanisms [14]. The crosstalk between these pathways is complex and still not fully understood; however, it is clear that they are both important in thyroid tumorigenesis and may play a role in resistance to targeted therapies.

After BRAF/MEK inhibition, the PI3K/AKT/mTOR pathway is closely connected to the MAPK pathway and can maintain cell signaling through this alternate pathway, which may explain the resistance to BRAF-targeted therapies in BRAF-mutated cancers [15]. Several clinical studies support this hypothesis. In a phase II study of dabrafenib and trametinib in BRAF-mutated NSCLC, patients whose tumors had concomitant genetic mutations in the PI3K pathway (in addition to BRAF) had a numerically shorter median overall survival rate of 5.4 months compared to 22.7 months in 34 patients with a single BRAF V600E mutation ($p = 0.0660$) [9]. Activation of the PI3K pathway has been associated with decreased response in patients with NSCLC harboring BRAF mutations and progression to BRAF-targeted therapies [16].

However, reports on co-mutations in the BRAF and PI3K pathways are limited. Clinical studies of nine ATC patients treated with dabrafenib plus trametinib did not show efficacy identical to that of PI3K pathway mutations, particularly PIK3CA mutations [17]. Although one patient showed early resistance, another experienced a complete response lasting more than two years. Therefore, PI3K inhibition may be an alternative treatment for patients who progress to BRAF-targeted therapies.

Dabrafenib and trametinib were granted accelerated approval by the U.S. Food and Drug Administration for the treatment of unresectable or metastatic solid tumors with BRAF V600E mutation on June 22, 2022. The approval was based on the results of the BRF117019 (ROAR) (NCT02034110) [5,18,19], NCI-MATCH (NCT02465060) [20], CTMT212X2101 (NCT02124772) [21], and also COMBI-d, COMBI-v [22], and BRF113928 [9] trials. Therefore, this combination is not only a potential treatment for ATC, but may also be applicable to other solid cancers.

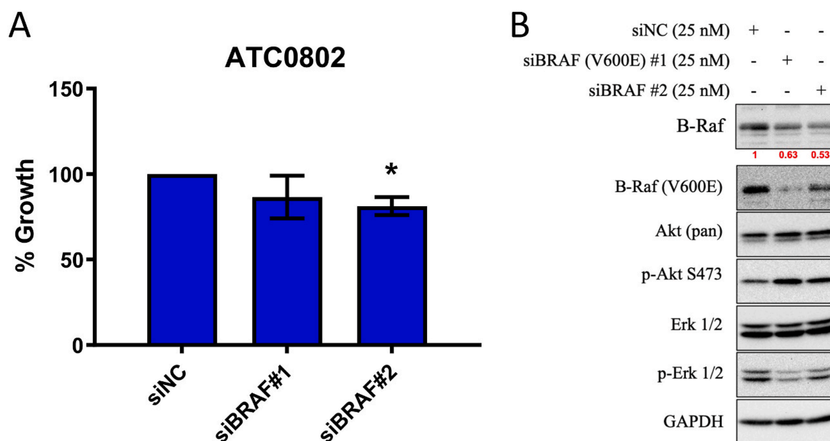

Fig. 3. Knockdown BRAF showed minimal effect on ATC0802 cell survival (A), but induction of Akt phosphorylation and reduction of Erk phosphorylation (B). Numbers below B-raf bands indicate relative band density. *, $p<0.05$.

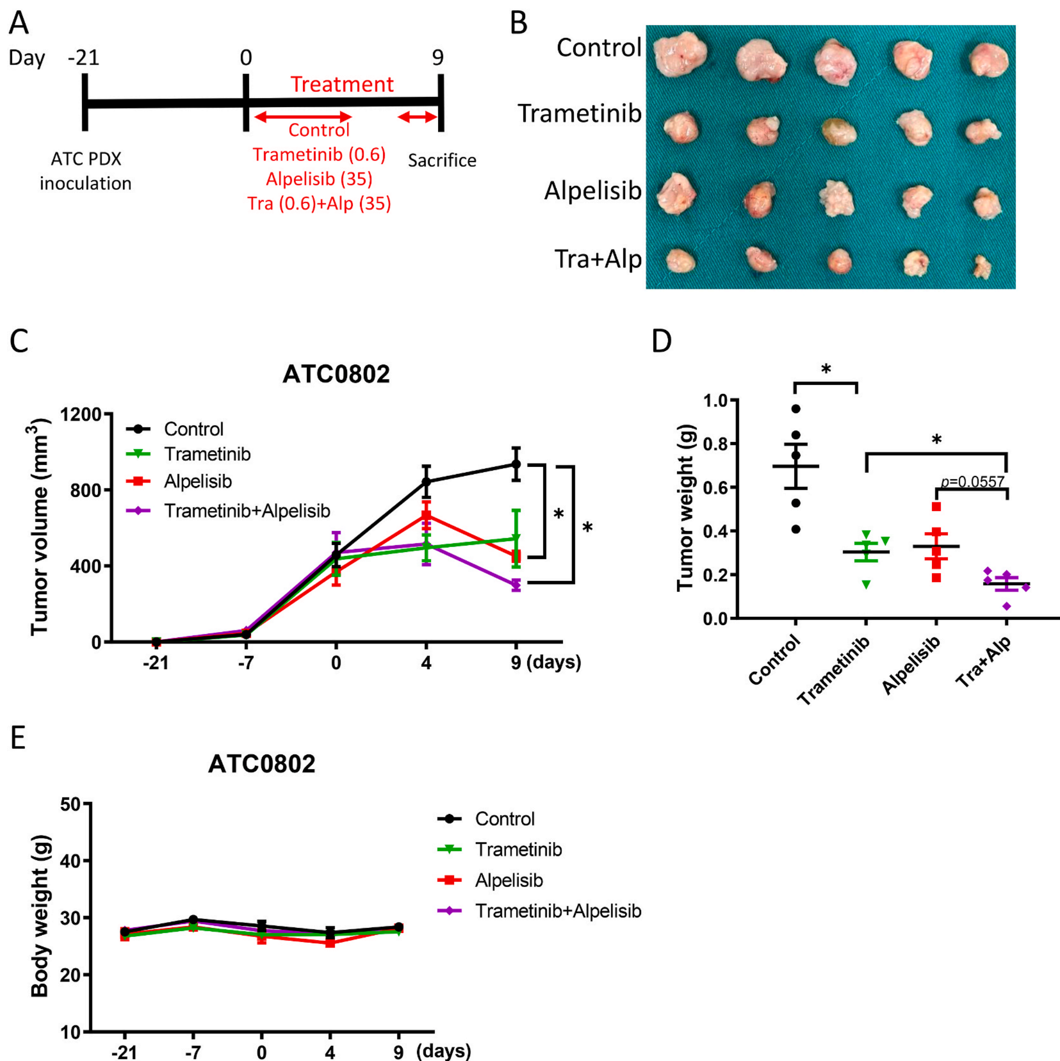

Fig. 4. Trametinib plus alpelisib inhibits tumor formation. (A) Schema showing the experimental timeline. (B) The harvested tumors are shown. (C and D) Tumor volume and tumor weight increased in the control group, but not in the trametinib plus alpelisib group. (E) There were no significant differences in the body weights of xenografted mice treated with different drugs. Data are expressed as mean ± SEM, *, $p < 0.05$.

A notable limitation of this study is its reliance on findings from a single case, potentially limiting the generalizability of the results to all cases of ATC with BRAF and PI3KCA co-mutations, primarily due to the rarity of this specific co-mutation in ATC. Therefore, future research endeavors should prioritize studies with larger sample sizes to enhance the robustness and applicability of the identified genetic landscape.

In conclusion, we successfully established a PDX model in a patient with ATC who harbored BRAF and PIK3CA co-mutations and was refractory to dabrafenib and trametinib. Our results suggest that targeting both mutations may be a promising strategy for the treatment of ATC and other malignancies with BRAF and PIK3CA co-mutations.

Funding

This work was financially supported by grants from Linkou Chang Gung Memorial Hospital (NMRPG3K6201, NZRPG3K0221, NZRPD1M3231, CMRPG3L0911~3, CMRPG3M0521~2, and CMRPG3P0181 to CEW, CMRPG3I0241~3, CORPG3J0251~2, CRRPG3K0011, CRRPG3K0031 and CMRPG3K0711 to CNY) and the National Science and Technology Council (109-2314-B-182-080-MY3 and 111-2811-B-182-017 to CEW).

Ethics statement

This study was reviewed and approved by the Institutional Review Board of Linkou Chang-Gung Memorial Hospital, with the

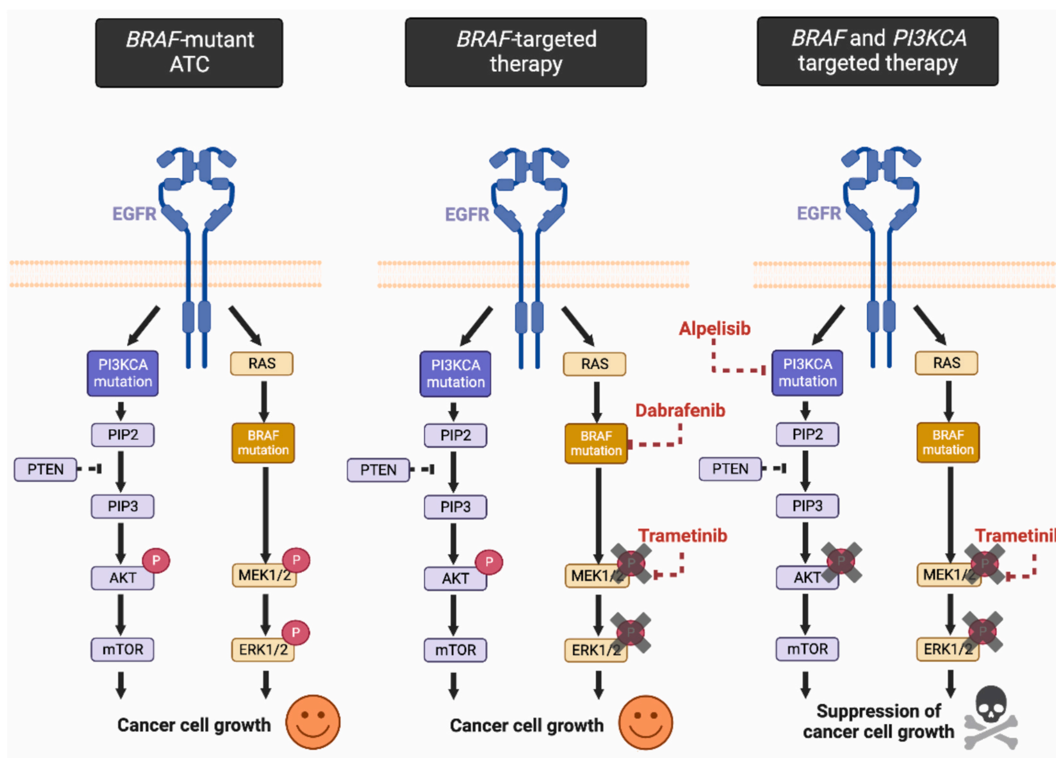

Fig. 5. The diagram shows the mechanism of MEK1/2 inhibitor trametinib plus PI3KCA inhibitor alpelisib for the treatment of ATC bearing BRAF and PI3KCA mutations.

approval IRB#202101714B0 at 2021/10/07 and IRB#202100148B0 at 2021/03/08. All patients (or their proxies/legal guardians) provided informed consent to participate in the study. All patients (or their proxies/legal guardians) provided informed consent for the publication of their anonymised case details and images.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Chiao-Ping Chen: Writing – original draft, Visualization, Investigation. **Shu-Fu Lin:** Visualization, Methodology, Investigation, Formal analysis. **Chun-Nan Yeh:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Wen-Kuan Huang:** Supervision, Methodology, Formal analysis. **Yi-Ru Pan:** Methodology, Investigation, Formal analysis. **Yu-Tien Hsiao:** Visualization, Methodology, Formal analysis. **Chih-Hong Lo:** Methodology, Formal analysis. **Chiao-En Wu:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Chiao-En Wu reports financial support was provided by National Science and Technology Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We thank the Research Specimen Processing Laboratory, Laboratory Animal Center, and Common Laboratory of Chang Gung Memorial Hospital, Linkou, for technical and funding support. This work was financially supported by grants from Linkou Chang-Gung Memorial Hospital (NMRPG3K6201, NZRPG3K0221, NZRPD1M3231, CMRPG3L0911∼3, CMRPG3M0521∼2, and CMRPG3P0181 to CEW, CMRPG3I0241∼3, CORPG3J0251∼2, CRRPG3K0011, CRRPG3K0031 and CMRPG3K0711 to CNY) and the National Science

and Technology Council (109-2314-B-182-080-MY3 and 111-2811-B-182-017 to CEW).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29055.

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