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Establishment of a new canine prostate cancer cell line and evaluation of antitumoral effects of BRAF inhibitor and MEK inhibitor combination in canine prostate cancer

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Abstract

Canine prostate cancer (cPC) is a highly invasive and metastatic malignancy in male dogs, often associated with a poor prognosis despite conventional treatments such as surgery, radiotherapy, and chemotherapy. Notably, the BRAF V595E mutation, orthologous to the human BRAF V600E mutation, is present in 61–80 % of cPC cases and may contribute to tumor progression via activation of the MAPK pathway. While BRAF/MEK dual inhibition has shown clinical efficacy in various human cancers with BRAF mutations, its utility in cPC remains unexplored.

In this study, we established a novel cPC cell line (EPC) derived from a canine patient with BRAF V595E-mutant cPC. EPC demonstrated strong proliferative activity *in vitro* and tumorigenicity in immunodeficient nude mice. Molecular analysis confirmed the presence of the BRAF V595E mutation. *In vitro*, EPC exhibited resistance to the BRAF inhibitor dabrafenib, characterized by reactivation of pERK, but this resistance was overcome by the MEK inhibitor trametinib or by combination therapy. The IC₅₀ values for dabrafenib and trametinib in the combination treatment were approximately 28.1 nM and 2.81 nM, respectively, comparable to clinically achievable concentrations.

EPC is the first BRAF V595E-mutant cPC cell line to demonstrate resistance to BRAF inhibition and sensitivity to MEK inhibition. These findings highlight EPC as a valuable model for studying MAPK pathway-mediated drug resistance and support the potential of dabrafenib/trametinib combination therapy as a novel treatment strategy for cPC.

Key Words: BRAF, BRAF inhibitor, Canine prostate cancer, MEK inhibitor

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Introduction

Canine prostate cancer (cPC) occurs in 0.2–0.6% of male dogs^{1,24}, with castrated male dogs having the same or higher prevalence when compared to intact male dogs³³. cPC is a highly invasive and metastatic tumor developed from the prostate gland in male dogs³. Local treatment of cPC includes surgery and radiation therapy. However, the application of surgery and radiation therapy is often limited due to the presence of metastases. Due to the high metastatic rate, the median overall survival time of dogs treated with these local treatments is short, approximately 4–8 months²⁶. Systemic therapies such as COX inhibitors and molecularly targeted drugs have been reported. However, these therapies could not improve prognosis much, and the median overall survival times were less than 1 year^{21,31}.

The v-rapidly accelerated fibrosarcoma (RAF) murine sarcoma viral oncogene homolog B (BRAF) is involved in the regulation of the mitogen-activated protein kinase (MAPK) pathway, along with the associated rat sarcoma (RAS)/RAF kinase/mitogen-activated extracellular signal-regulated kinase (MEK)/extracellular signal-related kinase (ERK) signaling pathway, which is essential for cell proliferation and survival²³. In human cell lines, BRAF mutations were identified in 43 of 530 cancer cell lines including 20 of 34 (59%) melanomas, 7 of 40 (18%) colorectal cancers, 4 of 38 (11%) gliomas, 4 of 131 (3%) lung cancer, 5 of 59 (9%) sarcomas, 1 of 26 (4%) ovarian carcinomas, 1 of 45 (2%) breast cancers and 1 of 7 (14%) liver cancers. BRAF mutations were not found in cancer cell lines derived from 29 neuroblastomas, 10 bladder cancers, 53 leukemias and lymphomas, 11 cervical carcinomas, 11 renal cell carcinomas, 3 pancreatic carcinomas, 3 prostate carcinomas, 6 gastric carcinomas, 7 testicular carcinomas, 3 uterine carcinomas, and 29 other cancers⁸.

Especially in human melanomas, the BRAF V600E mutation is the most common BRAF mutation (80–90%)². The BRAF V600E mutation activates the MAPK pathway and promotes tumor growth¹⁵. Canine BRAF V595E mutation was reported to correspond to the human BRAF V600E

mutation. In 667 canine primary tumors and 38 control tissues, sequencing analysis of the exon 15 of BRAF, mutation hot spot of the gene, revealed that 64 primary tumors (9.6%) had BRAF V595E mutation, with particularly high frequency in prostatic carcinoma (20 / 25, 80%) and urothelial carcinoma (30 / 45, 67%). However, only 6% of melanomas (two mucosal and one cutaneous melanoma) and 17% of melanocytomas were identified²².

Targeted inhibition of the MAPK pathway with BRAF inhibitors improves the progression-free and overall survival of patients with melanoma who have the BRAF V600 mutation^{5,13}. However, a majority of patients develop resistance to the BRAF inhibitors, resulting in a median progression-free survival of 6 to 7 months^{6,14}, because of the reactivation of MAPK pathway^{27,29,36}. A combination of a BRAF and MEK inhibitor, such as dabrafenib and trametinib, delayed the emergence of resistance and improved the rate of progression-free survival in previously untreated patients who had metastatic melanoma with BRAF V600E or V600K mutations as compared with single-agent BRAF inhibition²⁰. Not only for melanoma but also for BRAF V600 mutated thyroid cancer and non-small cell lung cancer, the combination therapy showed the clinical effects^{18,37}. Therefore, BRAF is an important therapeutic target in canine and human cancers, and establishing inhibitor therapy against it in canine cancers may improve treatment outcomes.

This study aimed to establish a new cPC cell line that has BRAF V595E mutation and investigate the effects of BRAF and MEK inhibitors against cPC.

Materials and Methods

Establishment of the EPC cell line and xenografted mice

Spontaneous cPC tissue was obtained from a 12-year-old neutered male that was diagnosed with canine prostate cancer at the Veterinary Medical Centre, The University of Tokyo (VMC-

UT). The owner's written consent for the use of the sample for this study was obtained. The tissue was dissected, digested with collagenase, and washed with phosphate-buffered saline (PBS) 3 to 5 times. After washing, the tumor tissue was suspended in warm RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) mixed with 20% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA), 5 mg/L gentamicin (Sigma-Aldrich Corp., St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO₂. After the second passage, the concentration of FBS was reduced to 10%. The EPC was successfully established after 100 passages. The doubling time (DT) was measured as follows. EPC was plated in 6-well plates at a concentration of 1×10^5 /ml and cultured in RPMI 1640 supplemented with 10% FBS. The number of viable cells was determined every 24 hr, and the doubling time at a logarithmic phase was calculated.

EPC (2.0×10^6 cells in 100 μ l PBS) was injected subcutaneously at the left flank of BALB/cSlc nude mice (6-week-old, $n = 5$) (Japan SLC, Inc., Shizuoka, Japan). Tumor growth was evaluated twice weekly for 7 weeks. After the observation period, xenografted tissues were collected, fixed immediately in 10% neutral-buffered formalin, and embedded in paraffin wax. A morphological comparison between the patient-derived primary tissue and the xenografted tissue was performed by hematoxylin and eosin (H&E) staining. All experimental procedures were approved by the animal care and use committee of The University of Tokyo (approval number: P20–106). The humane endpoints were a tumor diameter of 2 cm, significant weight loss (15% of pre-experiment body weight), and observation of clinical signs by progression of transplanted tumor cells, including decreased activity, loss of appetite, shivering, and respiratory failure.

Cell line culture

A human melanoma SK-MEL28-Luc cell line (MEL28) with the BRAF V600E mutation (Japanese Cancer Research Resources Bank, Osaka, Japan) and MDCK, established from the normal kidney of a dog (Japanese Cancer Research

Resources Bank, Osaka, Japan), were maintained in Eagle's Minimum Essential Medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% FBS and 5 mg/l penicillin-streptomycin (FUJIFILM, Tokyo, Japan). EPC was maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS and 5 mg/l gentamicin (Sigma-Aldrich Corp., St. Louis, MO, USA).

BRAF V595E mutation analysis

Total RNA was extracted from the EPC cell line and reverse transcribed using a transcriptase (ReverTraAce, Toyobo, Osaka, Japan). BRAF V595E and wild-type BRAF gene were amplified with Taqman probe, and signal detection was performed by QuantStudio 3D Digital PCR System (Thermo Fisher Scientific Inc.). The probes specific for wild-type BRAF and BRAF V595E mutation genes were labeled with 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM), respectively. Probe and primer sequences were as follows: wild-type probe_VIC, 5'-TAGCCACAGTGAAATC-3'; mutation probe_FAM, 5'-CCACAGAGAAATC-3'; BRAF forward, 5'-CATGAAGACCTCACA GTAAAAATAGGTGAT-3'; BRAF reverse, 5'-TGGGACCCACTCCATCGA-3'. PCR conditions were as follows: 10 minutes at 96°C, 39 cycles of 60°C for 2 minutes and 98°C for 30 seconds, and 2 minutes at 60°C. Raw data were obtained and analyzed using the QuantStudio 3D Digital PCR system and the QuantStudio 3D Analysis Suite software (Thermo Fisher Scientific).

Reagents and antibodies

Dabrafenib and trametinib used for *in vitro* experiments were obtained from Selleck Chemicals (Houston, TX, USA). These reagents were reconstituted in dimethyl sulfoxide (DMSO) and stored at -80°C . For western blotting, rabbit monoclonal antibodies against total MEK (clone; D1A5), phospho-MEK (clone; 166F8), total ERK (clone; 137F5), and phospho-ERK 1/2 (clone; D13.14.4E) were used (Cell Signaling TECHNOLOGY, Danvers, MA, USA). A mouse monoclonal anti-actin antibody (clone; C4, Merck Millipore, Burlington, MA, USA) was used. The

reactivity against canine molecules was validated against manufacturer specifications or previous reports in all antibodies used in this study. All antibodies were diluted according to their manufacturers' recommendations.

Cell proliferation assays

EPC cells (1,000 cells/well) and MEL28 cells (1,300 cells/well) were cultured in 96 well plates for 2 hr. Drug or vehicle control was added to the wells, and the plates were incubated for 72 hr. After 72 hr of incubation, the cell cytotoxicity was evaluated using a WST-1 assay (TakaraBio, Kusatsu, Japan). The absorbance of WST-1 was measured at 450 nm using a Bio-Rad Microplate Reader Model 550 (Bio-Rad Laboratories, Hercules, CA, USA). The 50% inhibitory concentration (IC_{50}) was estimated with a 4-parameter logistic model using RStudio (ver. 2024.12.1+563)

Western blots

EPC and MEL28 were cultured in 6 well plates for 72 hr. After that, they were treated with dabrafenib (1 μ M), trametinib (0.1 μ M), and a combination of both drugs or vehicle control for 1, 3, 6, and 12 hr. Cells were lysed using RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 5 M EDTA, 1% Triton-X, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with 10 mM NaF, 2 mM Na_3VO_4 , and complete Mini Protease Inhibitor Cocktail (Roche Diagnostics). Protein content was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in TBS-t containing 5% skim milk or bovine serum albumin (Sigma-Aldrich Corp.) for 1 hr at room temperature before incubating with the primary antibodies (1:1,000) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, England, UK; 1:10,000, room temperature) as the secondary antibody for 1 hr. The membranes were developed using the ECL Prime Western Blotting Detection

System (GE Healthcare), and luminescence was captured using an imaging system (ChemiDoc Image Lab, Bio-Rad Laboratories).

Statistical analysis

For the statistical analysis, data were analyzed with Student's t-test, and p -values < 0.05 were considered statistically significant (*, P < 0.05).

Results

Case presentation and establishment of EPC from a cPC case

A 12-year-old, neutered male, wire fox terrier was diagnosed with prostate cancer and underwent a total bladder prostatectomy at VMC-UT (Fig. 1A). No lymph node or other organ metastasis was noted. A surgically resected tumor was submitted to our pathological laboratory for histological diagnosis, and the case was diagnosed with prostate cancer (Fig. 1B). Primary culture was performed from the obtained fresh tumor tissue, and these cells showed stable traits and sustainable proliferation after over 100 passages, and finally established a new canine prostate cancer cell line, EPC.

The morphology of EPC cells was epithelioid and round- to spindle-shaped under a phase contrast microscope (Fig. 1C). Growth studies and population doubling time of the EPC cell line were evaluated and the doubling times of the cell line were calculated to be 17 hr (Fig. 1D). The cells grew densely on the paving stones, indicating the characteristic of an epithelial tumor.

To examine the tumorigenicity of the EPC cell line *in vivo*, EPC was injected subcutaneously into nude mice. As a result, mass formation was observed, and the tumor volume reached 680 mm³ at day 45 (Fig. 1E).

Next, we compared the gene mutation status between EPC and the primary case and validated the BRAF V595E mutation using a digital PCR method, and the BRAF V595E mutation was found in EPC (Fig. 1F).

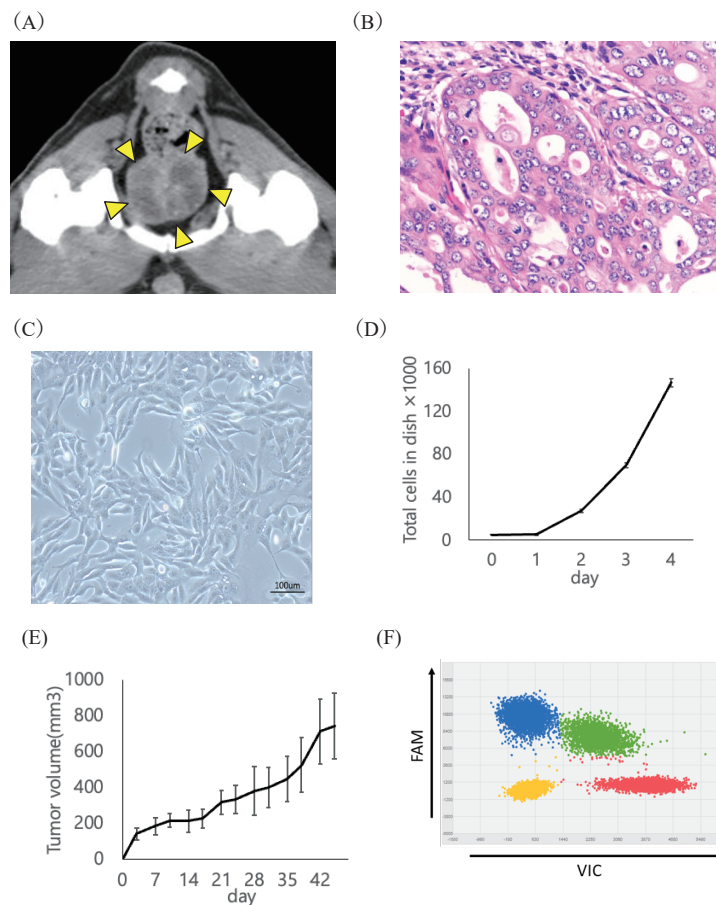


Fig. 1. Establishment of a canine prostate cancer cell line. (A) The axial CT image of the prostate tumor (arrowhead). (B) HE image of the patient's primary tumor tissues. (C) Cell morphology of EPC. (D) *In vitro* cell growth of EPC. (E) *In vivo* tumor growth of EPC. (F) Digital PCR plots of BRAF V595E-mutated EPC. X-axis: VIC, wildtype BRAF gene. Y-axis: FAM, BRAF V595E. The presence of blue and green clusters indicates that a cell line carries BRAF mutation.

Evaluation of antitumor effects of BRAF inhibitor on EPC

Canine BRAF V595E mutation is a homologous mutation to human BRAF V600E mutation, and human BRAF V600E mutation confers constitutive BRAF kinase activity and activates the MAPK pathway. Therefore, we hypothesized canine BRAF V595E mutation also confers constitutive BRAF kinase activity and activates the MAPK pathway in EPC. Then, we evaluated the phosphorylation/activation status of downstream molecules of BRAF in the MAPK pathway and found overexpression of pMEK and pERK, as with a positive control of human BRAF V600E mutated melanoma cell line, MEL28. In contrast, weak phosphorylation was observed in the negative control MDCK cells, a cell line established from the normal kidney of a dog

(Fig. 2A).

Next, to evaluate the anti-tumor effects of a BRAF inhibitor, dabrafenib, was added, and a proliferation assay was performed in EPC. Concentration-dependent growth inhibition was observed in the positive control cell line, MEL28, with IC_{50} less than 10 nM, but EPC showed no growth inhibition at all concentrations even at the maximum concentration of 10000 nM (Fig. 2B). To evaluate the resistance mechanism against the BRAF inhibitor, we evaluated pERK expression at 1, 3, 6, and 12 hr after addition of dabrafenib. pERK was suppressed at 1 to 3 hr after addition, but then pERK expression was re-increased at 6 to 12 hr (Fig. 2C). Thus, EPCs would be resistant to dabrafenib due to reactivation of the MAPK pathway.

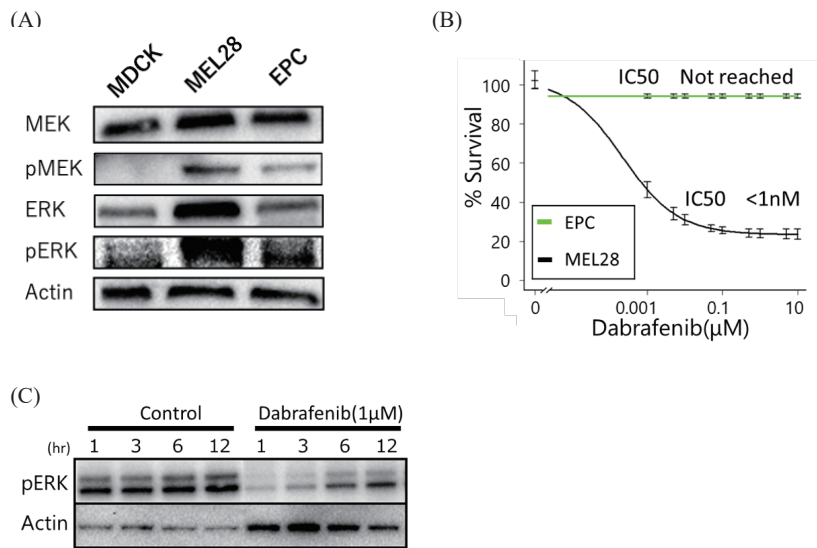


Fig. 2. Evaluation of phosphorylated proteins of the MAPK pathway and effects of BRAF inhibitor. (A) Western blotting of MEK, pMEK, ERK, and pERK on EPC, positive control MEL28, and negative control MDCK. (B) Cell viability of EPC and positive control MEL28 after BRAF inhibitor, dabrafenib treatment. (C) Expression levels of pERK after incubation with 1 μ M of dabrafenib at the indicated exposure time.

Evaluation of antitumor effects of the combination of BRAF inhibitor and MEK inhibitor on EPC

To overcome the reactivation of the MAPK pathway after administration of dabrafenib, we evaluated the combined inhibition activity of BRAF inhibitor and MEK inhibitor in EPC. MEK inhibitor, trametinib, alone showed growth inhibition, with an IC_{50} of 19.6 nM and the combination of dabrafenib and trametinib showed enhanced growth inhibition, with an IC_{50} of 2.81 nM of trametinib and 28.1 nM of dabrafenib (Fig. 3B). Moreover, in EPC, the combination groups of dabrafenib (100 nM) with trametinib (10 nM) and dabrafenib (1000 nM) with trametinib (100 nM) showed a significantly reduced cell viability compared to the trametinib monotherapy group. While dabrafenib as a single agent reactivated pERK, trametinib as a single agent and the combination treatment suppressed the reactivation of phosphorylated ERK and the MAPK pathway (Fig. 3C).

Discussion

In this study, the novel cPC cell line, EPC, was successfully developed. EPC had BRAF V595E mutation, showed good proliferative capacity *in vitro*, and demonstrated engraftability in immunodeficient mice, suggesting its potential for use in *in vivo* experiments. The treatment with BRAF inhibitor, dabrafenib alone, reactivated pERK, indicating resistance to the BRAF inhibitor. However, this reactivation was overcome by a MEK inhibitor, trametinib alone, or the combination treatment.

EPC had BRAF V595E mutation and showed BRAF inhibitor non-sensitive and MEK inhibitor sensitivity *in vitro*. Moreover, *in vivo* tumorigenicity on immunodeficient nude mice. To date, seven cPC cell lines have been reported, including CT1258, Leo, Ace-1, Probasco, LuMa, CHP-1, and CHP-2. BRAF mutations were tested in only 2 cell lines, and the BRAF V595E mutation was detected in CHP-1 and CHP-2, suggesting that EPC is the third cPC cell line with the BRAF V595E mutation. *In vitro*, drug sensitivity assays were performed in 3 cell lines out of 7 cell lines, including Leo, CHP-1, and CHP-2. Leo cell line

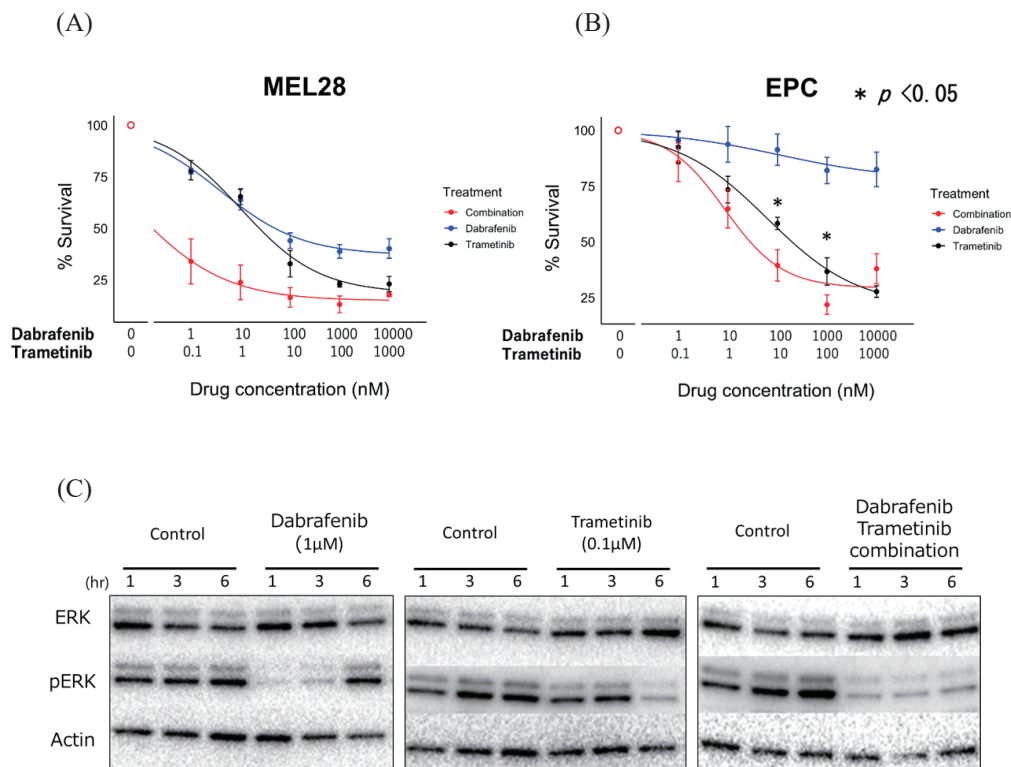


Fig. 3. Evaluation of MAPK pathway phosphoprotein and effects of BRAF inhibitor, ERK inhibitor, and combination of both drugs. (A) Cell viability of positive control MEL28 after BRAF inhibitor, dabrafenib, MEK inhibitor, trametinib, and combination treatments. The cell viability of the 0 % drug (vehicle control) group was defined as 100%. (B) Cell viability of EPC after BRAF inhibitor, dabrafenib, MEK inhibitor, trametinib, and combination treatments. The x-axis indicates drug concentration, and the y-axis represents cell viability. The values are shown as the mean $\pm$ SD. $P < 0.05$; * with Student's t-test, monotherapy (dabrafenib or trametinib only) vs combination. (C) Expression levels of pERK after incubation with 1 μ M of dabrafenib, 0.1 μ M of trametinib, and a combination of both drugs at the indicated exposure time.

showed sensitivity to the proteasome inhibitor (bortezomib) and an HDAC-42 inhibitor³⁵. CHP-1 and CHP-2 cell lines showed sensitivity to MEK inhibitor alone treatments (trametinib, cobimetinib, and mirdametinib), but did not evaluate the anti-tumor effect of BRAF inhibitor and the combination treatments of BRAF inhibitor and MEK inhibitor *in vitro*¹⁹, suggesting that the EPC is the only cell line that demonstrates BRAF inhibitor resistance in BRAF V595E-mutated cPC cell lines. For *in vivo* experiments, all 7 cell lines show *in vivo* tumorigenicity in immunodeficient mice. CT1258 cell line showed tumorigenicity in NOD.CB17-PrkdcScid/J (NOD SCID)¹¹ and Leo, Ace-1, Probasco, LuMa, CHP-1, and CHP-2 cell lines showed tumorigenicity in nude mice^{9,17,30}. Given that the newly established EPC cell line carries the BRAF V595E mutation and possesses

both *in vitro* drug responsiveness and *in vivo* tumorigenicity, it would serve as a valuable model for investigating the molecular pathogenesis of cPC, particularly concerning MAPK pathway alterations and resistance mechanisms for BRAF inhibitors.

Dabrafenib treatment on EPC showed reactivation of pERK and did not show growth inhibition *in vitro*, suggesting EPC would be a useful tool for evaluating the mechanisms of and novel therapies against primary resistance to BRAF inhibitors in canine tumor cells. Reactivation of the MAPK pathway has been reported to occur within hours after BRAF inhibitor treatment in various BRAF-mutated human tumor cells³². Several mechanisms of primary resistance to BRAF inhibitors in BRAF-mutated human malignancies have been

reported. It was reported that the activation of epidermal growth factor receptor (EGFR), which is an upstream signal of the MAPK pathway, after BRAF inhibitor treatment in BRAF V600E mutated colon cancer²⁵⁾. Another report demonstrated that the mesenchymal-epithelial transition factor (c-Met) mediated reactivation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway following BRAF inhibitor treatment in BRAF V600E mutated human thyroid cancer⁴⁾. Therefore, similar resistance pathways observed in human tumors may be related to primary BRAF inhibitor resistance in EPC, and further elucidation of the resistance mechanism is needed as a future study.

We observed that the EPC cell line exhibited resistance to dabrafenib as a monotherapy, while this resistance was successfully overcome by the combination of dabrafenib and trametinib, as evidenced by Western blot analysis. Furthermore, WST-1 assays demonstrated the significant inhibition of cell viability in the combination treatment group compared to the single-agent treatment groups. Consistent with our findings, preclinical studies using BRAF-mutated human colon cancer cell lines have demonstrated that combined inhibition of BRAF and MEK with dabrafenib and trametinib more effectively suppresses MAPK pathway signaling and enhances antitumor efficacy compared to monotherapy⁷⁾. The combination therapy of dabrafenib and trametinib has been clinically approved by the US Food and Drug Administration for the treatment of BRAF V600-mutated melanoma, anaplastic thyroid cancer, non-small cell lung cancer (NSCLC), and, more recently, for unresectable or metastatic solid tumors harboring BRAF V600E mutations, excluding colorectal cancer, reflecting its demonstrated therapeutic benefit in BRAF-mutated human cancers^{10,12)}. These results highlight the importance of treatment strategies targeting the BRAF/MEK pathway, particularly for refractory cPC.

In this study, EPC showed the IC₅₀ values of 28.1 nM of dabrafenib and 2.81 nM of trametinib in the combination treatment group. The blood mean concentration of dabrafenib and trametinib

in humans is reported to be approximately 100 nM and 15 nM, respectively¹⁶⁾. Therefore, although there are differences in species, the IC₅₀ values observed in EPC would be within the therapeutic window, suggesting the combination therapy of dabrafenib and trametinib is a possible therapeutic option for cPC. Moreover, in dogs, a Phase I/II study of vemurafenib, a BRAF inhibitor, has been conducted for BRAF-mutated urothelial carcinoma cases and showed clinical feasibility. In addition, a Phase I study of trametinib, targeting canine histiocytic sarcoma, has also shown its clinical safety^{28,34)}. Overall, the combination therapy of dabrafenib and trametinib would be a possible novel therapy against cPC.

Our study has several limitations. The characteristics of EPC have been validated in this study. However, single-cell cloning for EPC has not been performed yet. Evaluation of the anti-tumor effect of dabrafenib and trametinib was conducted in the only single cPC cell line, EPC. The anti-tumor effects of BRAF/MEK inhibition should also be validated in other canine prostate cancer cell lines harboring the BRAF V595E mutation. The experiment was only conducted *in vitro*, and the effect of the combination therapy *in vivo* remains unknown.

In conclusion, EPC may be a useful cell line for analyzing cPC pathogenesis, including mechanisms of drug resistance, and evaluating the efficacy of novel therapies. The combination therapy with dabrafenib and trametinib has the potential to be a new treatment for cPC, and further clarification of the mechanism of action and *in vivo* efficacy verification are needed for clinical application.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

- 1) Bell FW, Klausner JS, Hayden DW, Feeney DA, Johnston SD. Clinical and pathologic features of prostatic adenocarcinoma in sexually intact and castrated dogs: 31 cases (1970–1987). *J Am Vet Med Assoc* 199, 1623–1630, 1991.
- 2) Bradish JR, Cheng L. Molecular pathology of malignant melanoma: Changing the clinical practice paradigm toward a personalized approach. *Hum Pathol* 45, 1315–1326, 2014. doi: 10.1016/j.humpath.2014.04.001.
- 3) Bryan JN, Keeler MR, Henry CJ, Bryan ME, Hahn AW, Caldwell CW. A population study of neutering status as a risk factor for canine prostate cancer. *Prostate* 67, 1174–1181, 2007. doi: 10.1002/pros.20590.
- 4) Byeon HK, Na HJ, Yang YJ, Ko S, Yoon SO, Ku M, Yang J, Kim JW, Ban MJ, Kim JH, Kim DH, Kim JM, Choi EC, Kim CH, Yoon JH, Koh YW. Acquired resistance to BRAF inhibition induces epithelial-to-mesenchymal transition in BRAF V600E mutant thyroid cancer by c-Met-mediated AKT activation. *Oncotarget* 8, 596–609, 2017. doi: 10.18632/oncotarget.13480.
- 5) Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AMM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364, 2507–2516, 2011. doi: 10.1056/NEJMoa1103782.
- 6) Chapman PB, Hauschild A, Robert C, Larkin JMG, Haanen JBAG, Ribas A, Hogg D, Hamid O, Ascierto PA, Testori A, Lorigan P, Dummer R, Sosman JA, Garbe C, Maio M, Nolop KB, Nelson BJ, Joe AK, Flaherty KT, McArthur GA. Updated overall survival (OS) results for BRIM-3, a phase III randomized, open-label, multicenter trial comparing vemurafenib with dacarbazine in previously untreated patients with BRAF V600E-mutated melanoma. *J Clin Oncol* 30, 8502–8502, 2012. doi: 10.1200/JCO.2012.30.15_suppl.8502.
- 7) Corcoran RB, Dias-Santagata D, Bergethon K, Iafrate AJ, Settleman J, Engelman JA. BRAF gene amplification can promote acquired resistance to MEK inhibitors in cancer cells harboring the BRAF V600E mutation. *Sci Signal* 3, 2010. doi: 10.1126/scisignal.2001148.
- 8) Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JWC, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature* 417, 949–954, 2002. doi: 10.1038/nature00766.
- 9) Elshafae SM, Dirksen WP, Alasonyalilar-Demirer A, Breitbach J, Yuan S, Kantake N, Supsavhad W, Hassan BB, Attia Z, Alstadt LB, Rosol TJ. Canine prostatic cancer cell line (LuMa) with osteoblastic bone metastasis. *Prostate* 80, 698–714, 2020. doi: 10.1002/pros.23983.
- 10) Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, Hamid O, Schuchter L, Cebon J, Ibrahim N, Kudchadkar R, Burris HA, Falchook G, Algazi A, Lewis K, Long GV, Puzanov I, Lebowitz P, Singh A, Little S, Sun P, Allred A, Ouellet D, Kim KB, Patel K, Weber J. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med* 367, 1694–1703, 2012. doi: 10.1056/NEJMoa1210093.
- 11) Fork MA, Escobar HM, Soller JT, Sterenczak

- KA, Willenbrock S, Winkler S, Dorsch M, Reimann-Berg N, Hedrich HJ, Bullerdiek J, Nolte I. Establishing an in vivo model of canine prostate carcinoma using the new cell line CT1258. *BMC Cancer* 8, 240, 2008. doi: 10.1186/1471-2407-8-240.
- 12) Gouda MA, Subbiah V. Expanding the benefit: Dabrafenib/trametinib as tissue-agnostic therapy for BRAF V600E-positive adult and pediatric solid tumors. *Am Soc Clin Oncol Educ Book*, 2023. doi: 10.1200/EDBK_404770.
 - 13) Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, Rutkowski P, Blank CU, Miller WH, Kaempgen E, Martín-Algarra S, Karaszewska B, Mauch C, Chiarion-Sileni V, Martin AM, Swann S, Haney P, Mirakhur B, Guckert ME, Goodman V, Chapman PB. Dabrafenib in BRAF-mutated metastatic melanoma: A multicentre, open-label, phase 3 randomised controlled trial. *Lancet* 380, 358–365, 2012. doi: 10.1016/S0140-6736(12)60868-X.
 - 14) Hauschild A, Grob JJ, Demidov LV, Jouary T, Gutzmer R, Millward M, Rutkowski P, Blank CU, Miller WH, Kaempgen E, Martin-Algarra S, Karaszewska B, Mauch C, Chiarion-Sileni V, Mirakhur BL, Swann RS, Haney P, Goodman VL, Chapman PB. An update on BREAK-3, a phase III randomized trial: Dabrafenib versus dacarbazine in patients with BRAF V600E-positive metastatic melanoma. *J Clin Oncol* 31, 9013–9013, 2013. doi: 10.1200/JCO.2013.31.15_suppl.9013.
 - 15) Hobbs GA, Der CJ, Rossman KL. RAS isoforms and mutations in cancer at a glance. *J Cell Sci* 129, 1287–1292, 2016. doi: 10.1242/jcs.182873.
 - 16) Isberner N, Gesierich A, Balakirouchenane D, Schilling B, Aghai-Trommeschlaeger F, Zimmermann S, Kurlbaum M, Puszekiel A, Blanchet B, Klinker H, Scherf-Clavel O. Monitoring of dabrafenib and trametinib in serum and self-sampled capillary blood in patients with BRAFV600-mutant melanoma. *Cancers (Basel)* 14, 4566, 2022. doi: 10.3390/cancers14194566.
 - 17) Keller JM, Schade GR, Ives K, Cheng X, Rosol TJ, Piert M, Siddiqui J, Roberts WW, Keller ET. A novel canine model for prostate cancer. *Prostate* 73, 952–959, 2013. doi: 10.1002/pros.22642.
 - 18) Khunger A, Khunger M, Velcheti V. Dabrafenib in combination with trametinib in the treatment of patients with BRAF V600-positive advanced or metastatic non-small cell lung cancer: Clinical evidence and experience. *Ther Adv Respir Dis* 12, 2018. doi: 10.1177/1753466618767611.
 - 19) Kobayashi M, Onozawa M, Watanabe S, Nagashima T, Tamura K, Kubo Y, Ikeda A, Ochiai K, Michishita M, Bonkobara M, Kobayashi M, Hori T, Kawakami E. Establishment of a BRAF V595E-mutant canine prostate cancer cell line and the antitumor effects of MEK inhibitors against canine prostate cancer. *Vet Comp Oncol* 21, 221–230, 2023. doi: 10.1111/vco.12879.
 - 20) Long GV, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, Garbe C, Jouary T, Hauschild A, Grob JJ, Chiarion-Sileni V, Lebbe C, Mandalà M, Millward M, Arance A, Bondarenko I, Haanen JBAG, Hansson J, Utikal J, Ferraresi V, Kovalenko N, Mohr P, Probstachai V, Schadendorf D, Nathan P, Robert C, Ribas A, DeMarini DJ, Irani JG, Casey M, Ouellet D, Martin AM, Le N, Patel K, Flaherty K. Combined BRAF and MEK inhibition versus BRAF inhibition alone in melanoma. *N Engl J Med* 371, 1877–1888, 2014. doi: 10.1056/NEJMoa1406037.
 - 21) Maeda S, Motegi T, Iio A, Kaji K, Goto-Koshino Y, Eto S, Ikeda N, Nakagawa T, Nishimura R, Yonezawa T, Momoi Y. Anti-CCR4 treatment depletes regulatory T cells and leads to clinical activity in a canine model of advanced prostate cancer. *J Immunother Cancer* 10, e003731, 2022. doi: 10.1136/jitc-2021-003731.
 - 22) Mochizuki H, Kennedy K, Shapiro SG, Breen M. BRAF mutations in canine cancers. *PLoS One* 10, e0129534, 2015. doi: 10.1371/journal.pone.0129534.
 - 23) Molina JR, Adjei AA. The Ras/Raf/MAPK pathway. *J Thorac Oncol* 1, 7–9, 2006. doi:

- 10.1016/S1556-0864(15)31506-9.
- 24) Obradovich J, Walshaw R, Goullaud E. The influence of castration on the development of prostatic carcinoma in the dog: 43 cases (1978–1985). *J Vet Intern Med* 1, 183–187, 1987. doi: 10.1111/j.1939-1676.1987.tb02013.x.
 - 25) Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, Beijersbergen RL, Bardelli A, Bernards R. Unresponsiveness of colon cancer to BRAFV600E inhibition through feedback activation of EGFR. *Nature* 483, 100–103, 2012. doi: 10.1038/nature10868.
 - 26) Rai SK, Marverti G, Gunnam A, Allu S, Nangia AK. Dabrafenib–Panobinostat salt: Improving the dissolution rate and inhibition of BRAF melanoma cells. *ACS Omega* 8, 18255–18265, 2023. doi: 10.1021/acsomega.3c01881.
 - 27) Rizos H, Menzies AM, Pupo GM, Carlino MS, Fung C, Hyman J, Haydu LE, Mijatov B, Becker TM, Boyd SC, Howle J, Saw R, Thompson JF, Kefford RF, Scolyer RA, Long GV. BRAF inhibitor resistance mechanisms in metastatic melanoma: Spectrum and clinical impact. *Clin Cancer Res* 20, 1965–1977, 2014. doi: 10.1158/1078-0432.CCR-13-3122.
 - 28) Rossman P, Zabka TS, Ruple A, Tuerck D, Ramos-Vara JA, Liu L, Mohallem R, Merchant M, Franco J, Fulkerson CM, Bhide KP, Breen M, Aryal UK, Murray E, Dybdal N, Utturkar SM, Fourez LM, Enstrom AW, Dhawan D, Knapp DW. Phase I/II trial of vemurafenib in dogs with naturally occurring, BRAF-mutated urothelial carcinoma. *Mol Cancer Ther* 20, 2177–2188, 2021. doi: 10.1158/1535-7163.MCT-20-0893.
 - 29) Shi H, Hugo W, Kong X, Hong A, Koya RC, Moriceau G, Chodon T, Guo R, Johnson DB, Dahlman KB, Kelley MC, Kefford RF, Chmielowski B, Glaspy JA, Sosman JA, van Baren N, Long GV, Ribas A, Lo RS. Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discov* 4, 80–93, 2014. doi: 10.1158/2159-8290.CD-13-0642.
 - 30) Simmons JK, Dirksen WP, Hildreth BE, Dorr C, Williams C, Thomas R, Breen M, Toribio RE, Rosol TJ. Canine prostate cancer cell line (Probasco) produces osteoblastic metastases in vivo. *Prostate* 74, 1251–1265, 2014. doi: 10.1002/pros.22838.
 - 31) Sorenmo KU, Goldschmidt MH, Shofer FS, Goldkamp C, Ferracone J. Evaluation of cyclooxygenase-1 and cyclooxygenase-2 expression and the effect of cyclooxygenase inhibitors in canine prostatic carcinoma. *Vet Comp Oncol* 2, 13–23, 2004. doi: 10.1111/j.1476-5810.2004.00035.x.
 - 32) Sos ML, Gordan JD, Shokat KM. Primary resistance to MAPK inhibition in BRAF-mutant cancer is determined by kinetics of feedback release. *Mol Cancer Ther* 12, PR15–PR15, 2013. doi: 10.1158/1535-7163.PMS-PR15.
 - 33) Spada S, England GCW, Vignoli M, Carluccio A, Russo M. Contrast-enhanced ultrasound imaging of prostate gland in neutered dogs. *Animals (Basel)* 11, 559, 2021. doi: 10.3390/ani11020559.
 - 34) Takada M, Kitagawa K, Zhang Y, Bulitta JB, Moirano S, Jones A, Borgen J, Onsager A, Thaiwong T, Vail DM. Population pharmacokinetics, pharmacodynamics and safety properties of trametinib in dogs with cancer: A phase I dose escalating clinical trial. *Vet Comp Oncol* 22, 410–421, 2024. doi: 10.1111/vco.12989.
 - 35) Thudi NK, Shu ST, Martin CK, Lanigan LG, Nadella MVP, Van Bokhoven A, Werbeck JL, Simmons JK, Murahari S, Kisseberth WC, Breen M, Williams C, Chen C, McCauley LK, Keller ET, Rosol TJ. Development of a brain metastatic canine prostate cancer cell line. *Prostate* 71, 1251–1263, 2011. doi: 10.1002/pros.21341.
 - 36) Van Allen EM, Wagle N, Sucker A, Treacy DJ, Johannessen CM, Goetz EM, Place CS, Taylor-Weiner A, Whittaker S, Kryukov GV, Hodis E, Rosenberg M, McKenna A, Cibulskis K, Farlow D, Zimmer L, Hillen U, Gutzmer R, Goldinger SM, Ugurel S, Gogas HJ, Egberts F, Berking C, Trefzer U, Loquai C, Weide B, Hassel JC, Gabriel SB, Carter SL, Getz G, Garraway LA, Schadendorf D. The

- genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov* 4, 94–109, 2014. doi: 10.1158/2159-8290.CD-13-0617.
- 37) Zeng Q, Deng Y, Zhang L, Wang W. Interstitial lung disease associated with combination therapy of dabrafenib and trametinib in metastatic BRAFV600E-mutated poorly differentiated thyroid cancer: a case report and review of the literature. *Int J Clin Pharmacol Ther* 60, 225–231, 2022. doi: 10.5414/CP204184.