

Title Page

Identifying the ErbB/MAPK Signaling Cascade as a Therapeutic Target in Canine Bladder Cancer

Kathryn E. Cronise, Belen G. Hernandez, Daniel L. Gustafson, and Dawn L.

Duval

Flint Animal Cancer Center, Department of Clinical Sciences, (K.E.C., B.G.H.,
D.L.G., D.L.D.), Cell and Molecular Biology Graduate Program (K.E.C., D.L.G.,
D.L.D.), Colorado State University, Fort Collins, Colorado; University of Colorado
Cancer Center (D.L.G., D.L.D.), Aurora, Colorado

Running Title Page

MAPK and ErbB Inhibition Synergize in Canine Bladder Cancer

Corresponding Author:

Dawn L. Duval; Phone: 970-297-4064; Fax: 970-297-1275; Email:

dawn.duval@colostate.edu; Address: 300 West Drake Road, Campus Delivery

1620, Fort Collins, CO 80523-1620

Number of text pages: 41

Number of tables: 1

Number of figures: 5

Number of references: 47

Number of words in Abstract: 250

Number of words in Introduction: 715

Number of words in Discussion: 1446

Abbreviations:

5' RACE, 5' rapid amplification of cDNA ends; CI, combination index; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FACC, Flint Animal Cancer Center; FBS, fetal bovine serum; IC₅₀, half maximal inhibitory concentration; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MPAS, MAPK pathway activity score; PCR, polymerase chain reaction; PLX7904, 5-(2-

MOL # 115808

Cyclopropylpyrimidin-5-yl)-3-[3-[[ethyl(methyl)sulfamoyl]amino]-2,6-difluoro-
benzoyl]-1*H*-pyrrolo[2,3-*b*]pyridine; RTK, receptor tyrosine kinase; TCC,
transitional cell carcinoma

Recommended section assignment:

Chemotherapy, Antibiotics, and Gene Therapy

Abstract

Transitional cell carcinoma (TCC) of the bladder comprises 2% of diagnosed canine cancers. TCC tumors are generally inoperable and unresponsive to traditional chemotherapy, indicating a need for more effective therapies. BRAF, a kinase in the mitogen-activated protein kinase (MAPK) pathway, is mutated in 70% of canine TCCs. In this study, we use BRAF mutant and wild-type TCC cell lines to characterize the role of BRAF mutations in TCC pathogenesis and assess the efficacy of inhibition of the MAPK pathway alone and in combination with other gene targets as a treatment for canine TCC. Analysis of MAPK target gene expression and assessment of ERK1/2 phosphorylation following serum starvation indicated constitutive MAPK activity in all TCC cell lines. BRAF mutant TCC cell lines were insensitive to the BRAF inhibitor vemurafenib, with IC_{50} values greater than 5 μ M, but exhibited greater sensitivity to a “paradox-breaking” BRAF inhibitor (IC_{50} : 0.2-1 μ M). All TCC cell lines had IC_{50} values less than 7 nM to the MEK1/2 inhibitor trametinib independent of their BRAF mutation status. ERK1/2 phosphorylation decreased after 6-hour treatments with MAPK inhibitors, but rebounded by 24 hours, suggesting the presence of resistance mechanisms. Microarray analysis identified elevated expression of the ErbB family of receptors and ligands in TCC cell lines. The pan-ErbB inhibitor sapitinib synergized with BRAF inhibition in BRAF mutant Bliley TCC cells and synergized with MEK1/2 inhibition in Bliley and BRAF wild-type Kinsey cells. These findings suggest the potential for combined MAPK and ErbB receptor inhibition as a therapy for canine TCC.

Introduction

TCC is the most common bladder cancer in humans and dogs, comprising approximately 4 and 2% of diagnosed malignancies in each species, respectively (Knapp and McMillan, 2013; Siegel et al., 2018). Most canine TCCs are muscle-invasive tumors of intermediate- to high-grade, with metastases present in 15% of patients at diagnosis and 50% at death. Canine TCC tumors are typically located in the trigone of the bladder, preventing complete surgical resection in most cases (Knapp and McMillan, 2013). These tumors are treated with cyclooxygenase inhibitors alone or in combination with cytotoxic chemotherapeutics; however, median survival time is typically less than a year for all treatment options, indicating a need for more effective therapies (Fulkerson and Knapp, 2015; Knapp and McMillan, 2013). Canine and human TCCs share similarities in their molecular markers, sites of metastasis, and response to chemotherapeutic agents (Fulkerson et al., 2017). Muscle-invasive bladder cancer is less common in humans than dogs but has a similar poor prognosis (Knowles and Hurst, 2015).

A major distinction between canine and human TCC is the occurrence of activating BRAF mutations in 70% of canine tumors (Decker et al., 2015; Duval et al., 2014; Mochizuki et al., 2015). BRAF is a serine/threonine protein kinase in the MAPK signaling pathway that regulates cell growth, proliferation, differentiation, and apoptosis (Dhillon et al., 2007). Mutations resulting in dysregulation of the MAPK pathway occur in one-third of human cancers, with activating BRAF mutations identified in 50% of malignant melanomas and at

lower frequencies in colorectal and thyroid carcinomas (Dankner et al., 2018; Dhillon et al., 2007). Ninety percent of activating BRAF mutations in human cancers are valine-to-glutamic acid missense mutations at amino acid 600 (V600E) in the protein's activation loop (Dhillon et al., 2007). This alteration allows BRAF to signal as a monomer independent of upstream RAS activation, resulting in increased MAPK pathway activity (Dankner et al., 2018).

Several small-molecule inhibitors have been developed to target the MAPK signaling cascade. Vemurafenib, an ATP-competitive inhibitor of BRAFV600E, showed promising antitumor activity in humans with late-stage melanoma, with a 48% response rate compared to 5% with standard-of-care dacarbazine (Chapman et al., 2011). Despite vemurafenib's initial success, the majority of these tumors eventually acquired resistance (Sosman et al., 2012). Combination therapies that include both BRAF inhibitors and inhibitors of MEK1/2, BRAF's downstream target, have shown greater success in melanoma than single-agent treatment, leading to FDA-approval of these combinations for the treatment of metastatic melanoma (Ascierto et al., 2016; Long et al., 2017). Unlike melanoma, colorectal tumors are innately resistant to BRAF inhibition (Kopetz et al., 2015). Various resistance mechanisms to BRAF inhibition have been identified in human melanoma and colorectal cancer including up-regulation of receptor tyrosine kinases (RTKs), secondary mutations in RAS, and increased signaling through the PI3K/AKT pathway (Mao et al., 2013; Nazarian et al., 2010).

The discovery of a homologous BRAF-activating mutation in canine TCC identifies a compelling new potential drug target for canine TCC treatment; however, additional *in vitro* evaluation of canine BRAF's function and sensitivity to targeted agents is required. The utility of canine cancers as a model for human cancers has become increasingly widespread. Mouse models are poor predictors of anticancer drug efficacy in human patients. Spontaneous tumors in dogs develop under normal immunosurveillance, share molecular and histological features with human cancers, and undergo the processes of metastasis and drug resistance. Additionally, clinical trials in dogs provide the opportunity to evaluate the efficacy of novel treatments in chemotherapy-naïve patients in a shorter amount of time compared to clinical trials in human patients (Gordon et al., 2009).

In this study, we further characterize BRAF mutations in canine TCC cell lines. We assess the ability of BRAF and MEK1/2 targeted agents to inhibit TCC cell growth and block ERK1/2 phosphorylation as a measure of MAPK pathway activation. We use differential gene expression analysis to determine other potential gene targets for TCC treatment, identifying combined inhibition of the MAPK pathway and the ErbB family of receptors as a therapy with synergistic activity in both BRAF mutant and wild-type TCC cell lines. The results of this study not only identify a novel therapy for canine TCC, but also establish canine TCC's value as a model for human MAPK-driven cancers, where clinical trials in dogs with naturally occurring bladder tumors can inform therapies for human patients.

Material and Methods

Cell lines

Human cell lines were provided by Dr. John Tentler, University of Colorado Anschutz Medical Campus (RKO, HT29, Colo205) or purchased from ATCC (A375) (Manassas, VA). Canine TCC cell lines were provided by Dr. Steve Dow at Colorado State University (Bliley) or Dr. Elizabeth McNeil at Tufts University (Angus1, Kinsey, Tyler1, Tyler2). All cell lines were maintained in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/ml streptomycin, and 1mM sodium pyruvate (Thermo Fisher Scientific, Waltham, MA). Cells were incubated at 37°C in 5% CO₂ and 100% humidity. Canine cell lines were validated using short tandem repeat analysis with the Canine Stockmarks Genotyping Kit (Thermo Fisher Scientific, Waltham, MA) as previously described (Supplemental Table 1) (O'Donoghue et al., 2011).

Sequencing of canine BRAF

Total RNA was extracted from the Bliley TCC cell line using the RNeasy Mini Kit (Qiagen, Germantown, MD) and reverse transcribed to cDNA with the ImProm-II Reverse Transcription System (Promega, Madison, WI). Polymerase chain reaction (PCR) amplification of BRAF was performed using the following primers: forward, 5'-CACCATGGAAGCCCTATTGGACAAGTTTGGT-3'; reverse, 5'-CTTGAAGGCTGCAAATTCTCCGTA-3'. The resulting amplicon was gel extracted with the QIAquick Gel Extraction Kit (Qiagen, Germantown, MD) and cloned into an expression vector using the pcDNA3.2/GW/D-TOPO Expression

Kit (Thermo Fisher Scientific, Waltham, MA). Following transformation into One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific, Waltham, MA), individual clones were isolated and sequenced at the Proteomics and Metabolics Facility at Colorado State University.

5' rapid amplification of cDNA ends (5' RACE) with the SMARTer RACE 5'/3' Kit (Takara Bio, Japan) was performed according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA isolated from the Bliley TCC cell line. 5' RACE PCR using the reverse primer 5'-GATTACGCCAAGCTTTGGCGTGTAAGTAATCCATGCCCTGTGC-3' and SeqAmp DNA Polymerase (Takara Bio, Japan) was performed to obtain a product containing the 5' sequence of BRAF. The 5' RACE PCR product was gel extracted using NucleoSpin Gel and PCR Clean-Up Kit (Takara Bio, Japan) and cloned into the 5' RACE vector with the In-Fusion HD Cloning Kit (Takara Bio, Japan). The resulting construct was transformed into Stellar Competent Cells (Takara Bio, Japan) according to the manufacturer's protocol and individual clones were isolated for sequencing by GENEWIZ (South Plainfield, NJ).

Cell viability assays

Vemurafenib and 5-(2-cyclopropylpyrimidin-5-yl)-3-[3-[[ethyl(methyl)sulfamoyl]amino]-2,6-difluoro-benzoyl]-1*H*-pyrrolo[2,3-*b*]pyridine (PLX7904) are inhibitors of BRAFV600E. Selumetinib and trametinib are inhibitors of MEK1/2. Sapitinib is an inhibitor of ErbB receptors EGFR, ERBB2 and ERBB3. All inhibitors were purchased from Selleck Chemicals (Houston,

TX) and stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored according to the manufacturer's protocol.

Cell lines were plated in 96-well plates at 1,000-5,000 cells/well in 100 μ l of complete media and incubated for 24 hours at 37°C. Serial dilutions of inhibitor or DMSO control were prepared in complete media at a 2X concentration and 100 μ l was added to each well. Plates were incubated at 37°C for 72 hours and cell proliferation was monitored using the IncuCyte ZOOM Live-Cell Analysis System (Essen BioScience Inc., Ann Arbor, MI). Percent confluence at 72 hours was normalized by dividing by confluence at 0 hours, and relative cell number was determined as a fraction of DMSO control. Dose-response curves were fitted in GraphPad Prism (v7) using a non-linear regression of the logarithm of inhibitor concentration versus fraction of control. Half maximal inhibitory concentration (IC_{50}) values were determined as the concentration of inhibitor corresponding to half the fraction of control on the dose-response curves.

For combination treatments, relative cell number was determined similar to single-agent therapies, and fraction affected was determined as a fraction of DMSO control. Combination indices were calculated for each combination using CalcuSyn (v2.11) software. Representative images of combination treatments were obtained using red-labeled cells (NucLight Rapid Red Reagent, Essen BioScience Inc., Ann Arbor, MI). For both single-agent and combination drug sensitivity assays, three to five independent experiments were conducted for

each cell line. Each independent experiment included three technical replicates for each drug concentration and DMSO control.

Western blotting and antibodies

Cells were lysed by sonication in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, and 50 mM NaF) supplemented with protease and phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1mM Na₃VO₄, and 1 ug/ml leupeptin). Lysates were centrifuged at 14,000 rpm to isolate protein fractions and total protein was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Equal amounts of total protein were resolved on 4-20% Criterion TGX Protein Gels (Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF membranes with the TransBlot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) with 5% bovine serum albumin for one hour at room temperature. Blots were incubated overnight at 4°C with primary antibodies diluted in blocking buffer (phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit mAb #4370 (1:1,000), p44/42 MAPK Rabbit mAb #4695 1:1,000, B-Raf Rabbit mAb #14815 (1:1,000), Cell Signaling Technology, Danvers, MA; α -tubulin #T5168 (1:5,000), Sigma-Aldrich, St. Louis, MO). Membranes were washed three times with TBST, incubated with secondary antibody (goat anti-rabbit IgG HRP-conjugated (1:10,000) or goat anti-mouse IgG HRP-conjugated (1:10,000), Bio-Rad Laboratories, Hercules, CA) at room temperature for an hour, followed by three TBST washes. Blots were developed using Clarity

Western ECL Blotting Substrate (Bio-Rad Laboratories, Hercules, CA) and imaged with a Chemi Doc XES+ System (Bio-Rad Laboratories, Hercules, CA).

Microarray analysis

Total RNA was isolated from cell lines with the RNeasy Mini Kit (Qiagen, Germantown, MD) and microarray analysis was performed at the Functional Genomics Facility at University of Colorado Denver Anschutz Medical Campus using GeneChip Canine Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA) (Fowles et al., 2017). Gene expression values were determined following robust multi-array average normalization in R (v3.3) using the 'oligo' package (Halper-Stromberg et al., 2011). Differentially expressed genes were determined using a Benjamani & Hochberg false discovery rate cutoff of 0.05 and a fold change of at least 1.5. If multiple probe sets for a single gene were present, the probe set with the highest variance across samples was used.

MPAS score

MAPK Pathway Activity Scores (MPAS) were calculated as previously described based on expression levels of ten MAPK target genes: *CCND1*, *DUSP4*, *DUSP6*, *EPHA2*, *EPHA4*, *ETV4*, *ETV5*, *PHLDA1*, *SPRY2*, *SPRY4* (Wagle et al., 2018). Briefly, z-scores for MPAS genes were determined across all samples using log₂-transformed expression values. MPAS scores for each sample were calculated as $MPAS = (\text{sum of z-scores for MPAS genes}) / (\sqrt{10})$.

Results

Canine BRAF is homologous to human BRAF

Reverse transcription PCR was utilized to amplify BRAF's coding sequence from the Bliley TCC cell line. The resulting amplicon, corresponding to predicted canine BRAF AA10-772 (ENSCAFP00000005841), exhibited 99% homology to human BRAF AA62-767 (ENSP00000419060).

Since the predicted canine protein was truncated at the amino-terminus compared to human BRAF, 5' RACE was used to determine the N-terminal sequence of canine BRAF, revealing an additional 48 amino acids not present in the predicted canine BRAF sequence (ENSCAFP00000005841) (Supplemental Figure 1). The resulting full length predicted BRAF protein is 763 amino acids and exhibits 98% homology to human BRAF (ENSP00000419060) (Supplemental Figure 2). Our analysis also identified a heterozygous V to E missense mutation at amino acid 596 consistent with previously identified BRAF mutations in TCC tumors (Decker et al., 2015; Duval et al., 2014; Mochizuki et al., 2015). Western blot analysis of BRAF in canine TCC cell lines and the human BRAF V600E A375 melanoma cell line suggests that BRAF is expressed at a similar size and abundance in both humans and dogs (Supplemental Figure 3).

Canine TCC cell lines exhibit constitutive MAPK activity

BRAFV600E mutations result in constitutive MAPK activity in human cancers (Dhillon et al., 2007). A gene expression signature quantifying relative MAPK activity in a variety of human cancers was recently described (Wagle et

al., 2018). A MAPK Pathway Activation Score (MPAS) was calculated based on expression levels of 10 gene targets of the MAPK pathway obtained using Canine 1.0ST arrays. MPAS scores were calculated for five TCC cell lines as well as 30 other canine cancer cell lines in the Flint Animal Cancer Center (FACC) cell line panel (Fowles et al., 2017). TCC cell lines exhibited high MPAS values relative to other canine cancer cell lines, suggesting high MAPK pathway activity in TCC cell lines (Figure 1A,B). To validate these findings, MPAS scores were also calculated using gene expression levels from Canine 2.0 arrays (Fowles et al., 2017). MPAS scores correlated between Canine 1.0ST and Canine 2.0 arrays ($R=0.9263$, $p<0.0001$, Figure 1C).

To determine whether canine TCC cell lines with BRAF mutations exhibit constitutive MAPK pathway activity, cells were cultured for 24 hours in the absence of FBS followed by assessment of ERK1/2 phosphorylation by western blot. Five TCC cell lines were analyzed: three with heterozygous BRAF V596E mutations (Bliley, Tyler1, and Tyler2), one with a heterozygous KRAS G12D mutation (Angus1), and one wild-type for BRAF and KRAS (Kinsey) (Das et al., unpublished data). ERK1/2 phosphorylation was sustained under serum-starved conditions in BRAF and KRAS mutant cell lines (Figure 1D). Interestingly, the Kinsey cell line, which does not harbor any known activating cancer gene mutations in the MAPK pathway, also showed constitutive ERK1/2 phosphorylation.

BRAF mutant canine TCC cell lines are insensitive to vemurafenib, but sensitive to a “paradox-breaking” BRAF inhibitor

The effect of BRAF inhibition on TCC cell proliferation was determined using vemurafenib, an ATP-competitive inhibitor of mutant BRAF. Drug sensitivity assays were also conducted in human BRAF mutant melanoma (A375) and colorectal cancer cell lines (RKO, HT29, Colo205) with varying degrees of sensitivity to vemurafenib (Yang et al., 2012). All TCC cell lines had IC₅₀ values greater than 5 μ M (Figure 2A; Table 1). Canine TCC cell lines were roughly 10- to 100-fold less sensitive than human BRAF mutant A375, Colo-205 and HT29 cancer cell lines and exhibited levels of sensitivity similar to the RKO colorectal cancer cell line (Table 1).

To determine whether vemurafenib inhibits MAPK pathway activity in TCC cell lines, ERK1/2 phosphorylation was assessed following 6- and 24-hour treatments with vemurafenib. ERK1/2 phosphorylation was suppressed at 6 hours in BRAF mutant cell lines, but rebounded by 24 hours (Figure 2B, Supplemental Figure 4). Similarly, colorectal cancer cell lines achieve MAPK pathway reactivation by 24 hours post-treatment, whereas melanoma cell lines maintain pathway suppression (Corcoran et al., 2012). Conversely, vemurafenib treatment increased ERK1/2 phosphorylation in KRAS mutant Angus1 and BRAF/KRAS wild-type Kinsey cells. This response is consistent with paradoxical activation of the MAPK pathway following BRAF inhibition in KRAS mutant and BRAF/KRAS wild-type human cancer cell lines. Mutant BRAF signals as a monomer, but wild-type BRAF requires dimerization with other RAF isoforms. Binding of BRAF inhibitors to wild-type BRAF stabilizes the formation of dimers

resulting in increased MAPK signaling (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010).

Since BRAF mutations in canine TCC cell lines are heterozygous, we wanted to investigate whether TCC insensitivity to vemurafenib could be due to paradoxical MAPK pathway activation as a result of the wild-type copy of BRAF. Thus, sensitivity to the “paradox-breaking” BRAF inhibitor PLX7904 was determined in canine and human cell lines. BRAF mutant canine cell lines had IC₅₀ values ranging from 0.2 to 1 μM, similar to BRAF mutant human cell lines, whereas wild-type cell lines exhibited IC₅₀ values greater than 5 μM (Figure 2C; Table 1). ERK1/2 phosphorylation decreased in BRAF mutant cell lines following a 6-hour incubation with PLX7904, but rebounded by 24 hours, although the extent of rebound was less than that with vemurafenib (Figure 2D, Supplemental Figure 4). ERK1/2 phosphorylation remained unchanged in KRAS mutant Angus1 and increased in BRAF/KRAS wild-type Kinsey cells.

Canine TCC cell lines are sensitive to MEK inhibition

To determine whether MAPK inhibition downstream of BRAF is an effective therapeutic option, we targeted MEK in canine and human cell lines with the selective, allosteric MEK1/2 inhibitors selumetinib and trametinib. Canine TCC cell lines exhibited similar degrees of sensitivity to MEK inhibition as human BRAF mutant cell lines. Canine TCC IC₅₀ values ranged from 18 to 390 nM and 0.1 to 6.2 nM for selumetinib and trametinib, respectively. Human cell lines exhibited IC₅₀ values of 81 to 1,700 nM and 0.3 to 8.6 nM for selumetinib and trametinib, respectively (Figure 3A,C; Table 1). BRAF mutant cell lines in the

Genomics of Drug Sensitivity in Cancer database have median IC₅₀ values of 640 and 19 nM to selumetinib and trametinib, respectively (Yang et al., 2013). Thus, canine TCC cell lines exhibited sensitivities to MEK inhibition similar to human BRAF mutant cell lines. Interestingly, BRAF/KRAS wild-type Kinsey cells were the most sensitive to MEK inhibition of all tested cell lines. This response is supported by Kinsey's MPAS value, which is the fourth highest in the FACC cell line panel. ERK1/2 phosphorylation was blocked in all cell lines after 6 hours of MEK inhibition but, as with BRAF inhibition, showed a degree of rebound by 24 hours indicating reactivation of the MAPK pathway despite MAPK inhibition (Figure 3B,D; Supplemental Figure 4). Combined BRAF and MEK inhibition synergized in BRAF mutant Bliley cells, but not wild-type Kinsey cells (median combination index (CI) of 0.5 versus 1.1, respectively; Supplemental Figure 5).

ErbB signaling is up-regulated in TCC cell lines relative to other canine cancer cell lines

Synthetic lethality has been widely explored as an antitumor strategy. Cancer cells often harbor specific oncogenic alterations that may not be targeted effectively alone but, when targeted in combination with a second gene, elicit a lethal response (O'Neil et al., 2017). Single-agent treatment of canine TCC cell lines with BRAF or MEK inhibitors yielded an initial attenuation of MAPK pathway activity followed by a rebound in pathway activity by 24 hours of treatment. This short-lived response suggests that MAPK inhibition may not be effective as a monotherapy for TCC treatment. Thus, we sought to identify a second gene target that, when inhibited in combination with BRAF, exhibits a synergistic

response. To identify potential candidate targets, we determined genes differentially expressed in TCC cell lines relative to other canine cancer cell lines. To avoid histotype-specific genes, we limited our analysis to 719 cancer-related genes present in the COSMIC database (v83) (Forbes et al., 2017).

Twenty-nine and nine cancer genes were up- and down-regulated in TCC cell lines relative to other cancer cell lines, respectively (Figure 4A, Supplemental Table 2). This analysis revealed up-regulation of genes encoding epidermal growth factor (EGFR) and receptor tyrosine-protein kinase erbB-2 (ErbB2) receptors in TCC cell lines. We then expanded our analysis to all ErbB ligands and receptors present in the KEGG pathway database (hsa04012) (Figure 4B). In addition to up-regulation of genes encoding EGFR and ErbB2 receptors, the ligand epiregulin (EREG) was also up-regulated in TCC cell lines. Additional cancer genes up-regulated in TCC cell lines include *CDH1*, *PPARG*, *NOTCH1*, and *MYC*. Down-regulated genes include *IDH2*, *SMO*, and *ALDH2*.

Pan-ErbB inhibition synergizes with MAPK inhibition in canine TCC

To determine whether ErbB inhibition alone or in combination with MAPK inhibition may be an effective treatment for canine TCC treatment, TCC cell lines were treated with a pan-ErbB inhibitor sapitinib that targets EGFR, ErbB2, and ErbB3 receptors. All TCC cell lines had IC_{50} values greater than 1 μ M (Supplemental Figure 6). Combination treatments were performed to assess the efficacy of pan-ErbB inhibition with MAPK inhibition. Sapitinib treatment synergized with the “paradox-breaking” BRAFV600E inhibitor PLX7904 in BRAF mutant Bliley cells but not in wild-type Kinsey cells (median CI 0.4 versus 1.4,

respectively; Figure 5A,B). Conversely, pan-ErbB inhibition synergized with MEK inhibition in both Bliley and Kinsey cells (median CI 0.7 and 0.5, respectively; Figure 5C,D).

Discussion

Recent studies have identified BRAF mutations in approximately 70% of canine TCC tumors (Decker et al., 2015; Mochizuki et al., 2015). Despite this discovery, little is known about the role that BRAF mutations play in canine TCC development and whether targeting mutant BRAF is a feasible therapy for TCC. In this study, we targeted BRAF and its downstream kinase, MEK, in five TCC cell lines: three BRAF mutant, one KRAS mutant, and one BRAF/KRAS wild-type. MAPK inhibitors suppressed proliferation in TCC cell lines with varying degrees of efficacy, but failed to sustain attenuation of MAPK pathway activity. The ErbB family of receptors was identified as a potential therapeutic target for TCC treatment, and inhibition of ErbB receptors synergized with MAPK inhibition in TCC cell lines. These data demonstrate the potential of ErbB receptor inhibition combined with either BRAF or MEK inhibition as an effective therapy for canine TCC. Additionally, our findings illustrate canine TCC's potential utility as a naturally occurring model for investigating intrinsic resistance mechanisms to MAPK inhibition in human cancers and tailoring treatments to combat the emergence of resistance.

BRAF and KRAS mutant cell lines exhibited constitutive MAPK pathway activity based on expression levels of MAPK target genes (MPAS scores) and sustained ERK1/2 phosphorylation in the absence of FBS. Additionally, the Kinsey cell line, with no known MAPK mutation, also exhibited constitutive pathway activity. This phenomenon occurs in human cancers where ERK1/2 phosphorylation and expression of MAPK target genes do not always correlate

with RAS/BRAF mutation status (Houben et al., 2008; Levidou et al., 2012; Wagle et al., 2018). Another group previously analyzed five different canine TCC cell lines and also showed sustained ERK1/2 phosphorylation in the absence of FBS for all cell lines (Rathore and Cekanova, 2014). Thus, constitutive MAPK activity seems to be a common occurrence in canine TCC, suggesting a possible causative role for the MAPK pathway in canine TCC pathogenesis. In human bladder cancer, BRAF mutations are rare; however, mutations in NRAS/HRAS occur in 6% of tumors and alterations in the RTK/Ras/PI3K pathway are present in 72% of tumors (Robertson et al., 2017; TCGA, 2014).

All five TCC cell lines were insensitive to BRAF inhibition with vemurafenib relative to human BRAF mutant melanoma (A375) and colorectal (Colo205 and HT29) cell lines. In fact, TCC cell lines exhibited IC₅₀ values similar to that of the RKO colorectal cancer cell line, which was previously reported to be insensitive to vemurafenib (Yang et al., 2012). Vemurafenib treatment yielded an initial decrease in MAPK pathway activity in BRAF mutant TCC cell lines, but pathway activity rebounded by 24 hours post-treatment. Human colorectal cancer cell lines also achieve MAPK pathway reactivation following 24 hours of vemurafenib treatment (Corcoran et al., 2012; Yang et al., 2012). Unlike canine TCC and human colorectal cancer cell lines, vemurafenib treatment in human melanoma cell lines maintains suppression of MAPK activation after 24 hours (Corcoran et al., 2012). Vemurafenib induced the same paradoxical MAPK activation in BRAF wild-type TCC cell lines that has been described in human BRAF wild-type cell lines. The mechanism behind paradoxical activation involves increased

transactivation of RAF homo- or heterodimers as a result of vemurafenib binding, causing increased pathway activity (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010).

In melanoma patients, decreased BRAF allelic frequency is associated with a poorer clinical outcome to BRAF inhibition and combined BRAF/MEK inhibition (Lebbe et al., 2014; Stagni et al., 2018). The proposed mechanism behind this response is paradoxical MAPK pathway activation due to a higher wild-type allele frequency. BRAF mutations in the canine TCC cell lines used in this study are heterozygous; thus, we hypothesized that their reduced sensitivity to vemurafenib may be the result of paradoxical activation of the MAPK pathway due to the wild-type copy of BRAF. In support of this hypothesis, when treated with the “paradox-breaking” inhibitor PLX7904 BRAF mutant TCC cell lines were equally as sensitive as BRAF mutant human lines. Further studies in canine and human BRAF mutant cell lines are required to establish the role of BRAF zygosity in sensitivity to BRAF inhibitors.

In this study, canine TCC cell lines were equally sensitive to MEK1/2 inhibition with selumetinib or trametinib compared to BRAF mutant human cell lines. Our group assessed trametinib sensitivity for the entire FACC panel of canine cancer cell lines and found that TCC cell lines were more sensitive than other cancer cell types (Das et al., unpublished data). Analysis of ERK1/2 activation following MEK1/2 inhibition for 6 hours showed complete or reduced pathway inhibition with trametinib and selumetinib, respectively. However, similar to the response with BRAF inhibition, TCC cell lines displayed a rebound

in pathway activity by 24 hours post-treatment. Collectively, these data suggest canine TCC's initial dependence on the RAS-BRAF-MEK signaling axis; however, intrinsic resistance mechanisms are able to bypass pathway inhibition by 24 hours.

In metastatic melanoma patients, tumor regression in response to BRAF inhibition correlates with sustained inhibition of ERK1/2 phosphorylation. Additionally, patients experiencing tumor regression typically had at least 80% inhibition of ERK1/2 phosphorylation following treatment (Bollag et al., 2010). This finding likely explains the lack of success of MAPK inhibition as a monotherapy for colorectal cancer treatment (Kopetz et al., 2015). Similar to the response of colorectal cancer cell lines (Corcoran et al., 2012), the TCC cell lines analyzed in this study showed re-activation of the MAPK pathway by 24 hours following MAPK inhibition, suggesting that long-term treatment with a MAPK inhibitor alone may not be an effective therapy for canine TCC.

Human melanoma and colorectal cancer exhibit acquired and innate resistance to BRAF inhibition, respectively. Mechanisms of acquired resistance in melanoma include BRAF splice isoforms, BRAF amplification, secondary NRAS mutations, CRAF overexpression, MEK1/2 mutations, and increased signaling through RTKs such as IGF-1R (Montagut et al., 2008; Nazarian et al., 2010; Poulikakos et al., 2011; Shi et al., 2012; Villanueva et al., 2010; Wagle et al., 2011). Mechanisms of intrinsic resistance in colorectal cancer include activation of RTKs (EGFR) and increased signaling through the PI3K/AKT pathway (Corcoran et al., 2012; Mao et al., 2013; Prahallad et al., 2012). The

majority of these resistance mechanisms involve re-activation of the MAPK pathway, explaining the increased efficacy in melanoma and colorectal cancer patients with dual inhibition of BRAF and MEK compared to monotherapy (Corcoran et al., 2015; Robert et al., 2015). A similar response was observed in this study where combined inhibition of BRAF and MEK was synergistic in the BRAF mutant Bliley TCC cell line.

To identify potential mediators of resistance to MAPK inhibition in TCC, in addition to determining other drivers that may contribute to TCC pathogenesis, we determined cancer genes that are differentially expressed in TCC cell lines versus other canine cancer cell lines. Genes up-regulated in TCC cell lines include *CDH1*, *PPARG*, *EGFR*, *ERBB2*, *EREG* and *MYC*. EGFR and ERBB2 protein expression is up-regulated in 50% and 8-30% of muscle-invasive human bladder cancers, respectively (Knowles and Hurst, 2015). Additionally, copy number alterations in EGFR (11%), ERBB2 (7%), PPARG (17%), and MYC (13%) are common in human bladder cancer (TCGA, 2014).

Of particular interest to us was the up-regulation of genes encoding ErbB receptors, EGFR and ERBB2, and the ErbB ligand EREG. Previous studies in colorectal cancer cell lines have shown that increased signaling through EGFR and/or ERBB2 allows cells to bypass BRAF inhibition (Corcoran et al., 2012; Prahallad et al., 2012). The mechanism behind the efficacy of combined ErbB and BRAF inhibition in colorectal cancer cell lines involves negative feedback regulation of the MAPK pathway. Increased MAPK signaling results in increased expression of negative regulators such as DUSP and SPRY. DUSP proteins

inactivate ERK1/2 and SPRY proteins inhibit RTK-mediated activation of RAS (Lake et al., 2016). BRAF inhibition results in decreased expression of these negative regulators, thus, relieving negative feedback of the MAPK pathway. In colorectal cancer it was suggested that this decrease in negative feedback results in increased activation of the RTKs EGFR and ERBB2 and subsequent increased MAPK signaling through CRAF (Corcoran et al., 2012; Prahallad et al., 2012).

Similar to the response in colorectal cancer cell lines, MAPK inhibition with either a BRAF or MEK inhibitor synergized with a pan-ErbB inhibitor targeting EGFR, ERBB2, and ERBB3. These results suggest that the ErbB family of receptors and ligands may facilitate resistance to MAPK inhibition in canine TCC. Overall, the findings in this study suggest that dogs with BRAF mutant TCC may benefit from combined treatment with a pan-ErbB inhibitor and a “paradox-breaking” BRAF inhibitor, while dogs with BRAF wild-type TCC may benefit from combined pan-ErbB and MEK inhibition. Clinical trials evaluating the efficacy of these combinations in dogs with TCC may inform treatment modalities in human MAPK-driven cancers.

Acknowledgments

We thank Deanna Dailey for isolating total RNA from FACC cell lines and Rupa Idate for cell line validation.

Authorship Contributions

Participated in research design: Cronise, Hernandez, Gustafson and Duval

Conducted experiments: Cronise and Hernandez

Contributed new reagents or analytical tools: Gustafson

Performed data analysis: Cronise, Hernandez and Duval

Wrote or contributed to the writing of the manuscript: Cronise, Gustafson, and

Duval

References

- Ascierto PA, McArthur GA, Dreno B, Atkinson V, Liskay G, Di Giacomo AM, Mandala M, Demidov L, Stroyakovskiy D, Thomas L, de la Cruz-Merino L, Dutriaux C, Garbe C, Yan Y, Wongchenko M, Chang I, Hsu JJ, Koralek DO, Rooney I, Ribas A and Larkin J (2016) Cobimetinib combined with vemurafenib in advanced BRAF(V600)-mutant melanoma (coBRIM): updated efficacy results from a randomised, double-blind, phase 3 trial. *Lancet Oncol* **17**(9): 1248-1260.
- Bollag G, Hirth P, Tsai J, Zhang J, Ibrahim PN, Cho H, Spevak W, Zhang C, Zhang Y, Habets G, Burton EA, Wong B, Tsang G, West BL, Powell B, Shellooe R, Marimuthu A, Nguyen H, Zhang KY, Artis DR, Schlessinger J, Su F, Higgins B, Iyer R, D'Andrea K, Koehler A, Stumm M, Lin PS, Lee RJ, Grippo J, Puzanov I, Kim KB, Ribas A, McArthur GA, Sosman JA, Chapman PB, Flaherty KT, Xu X, Nathanson KL and Nolop K (2010) Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* **467**(7315): 596-599.
- 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 AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA and Group B-S (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**(26): 2507-2516.

- Corcoran RB, Atreya CE, Falchook GS, Kwak EL, Ryan DP, Bendell JC, Hamid O, Messersmith WA, Daud A, Kurzrock R, Pierobon M, Sun P, Cunningham E, Little S, Orford K, Motwani M, Bai Y, Patel K, Venook AP and Kopetz S (2015) Combined BRAF and MEK Inhibition With Dabrafenib and Trametinib in BRAF V600-Mutant Colorectal Cancer. *J Clin Oncol* **33**(34): 4023-4031.
- Corcoran RB, Ebi H, Turke AB, Coffee EM, Nishino M, Cogdill AP, Brown RD, Della Pelle P, Dias-Santagata D, Hung KE, Flaherty KT, Piris A, Wargo JA, Settleman J, Mino-Kenudson M and Engelman JA (2012) EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib. *Cancer Discov* **2**(3): 227-235.
- Dankner M, Rose AAN, Rajkumar S, Siegel PM and Watson IR (2018) Classifying BRAF alterations in cancer: new rational therapeutic strategies for actionable mutations. *Oncogene* **37**(24): 3183-3199.
- Decker B, Parker HG, Dhawan D, Kwon EM, Karlins E, Davis BW, Ramos-Vara JA, Bonney PL, McNeil EA, Knapp DW and Ostrander EA (2015) Homologous Mutation to Human BRAF V600E Is Common in Naturally Occurring Canine Bladder Cancer--Evidence for a Relevant Model System and Urine-Based Diagnostic Test. *Mol Cancer Res* **13**(6): 993-1002.
- Dhillon AS, Hagan S, Rath O and Kolch W (2007) MAP kinase signalling pathways in cancer. *Oncogene* **26**(22): 3279-3290.

- Duval DL, Hernandez B, Brown J, Lana SE, Page R and Jones KL (2014) 13
Whole exome sequence analysis of canine transitional cell carcinoma of
the bladder. *European Journal of Cancer* **50**, Supplement 6: 11.
- Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, Cole CG, Ward
S, Dawson E, Ponting L, Stefancsik R, Harsha B, Kok CY, Jia M, Jubb H,
Sondka Z, Thompson S, De T and Campbell PJ (2017) COSMIC: somatic
cancer genetics at high-resolution. *Nucleic Acids Res* **45**(D1): D777-D783.
- Fowles JS, Dailey DD, Gustafson DL, Thamm DH and Duval DL (2017) The Flint
Animal Cancer Center (FACC) Canine Tumour Cell Line Panel: a resource
for veterinary drug discovery, comparative oncology and translational
medicine. *Vet Comp Oncol* **15**(2): 481-492.
- Fulkerson CM, Dhawan D, Ratliff TL, Hahn NM and Knapp DW (2017) Naturally
Occurring Canine Invasive Urinary Bladder Cancer: A Complementary
Animal Model to Improve the Success Rate in Human Clinical Trials of
New Cancer Drugs. *Int J Genomics* **2017**: 6589529.
- Fulkerson CM and Knapp DW (2015) Management of transitional cell carcinoma
of the urinary bladder in dogs: a review. *Vet J* **205**(2): 217-225.
- Gordon I, Paoloni M, Mazcko C and Khanna C (2009) The Comparative
Oncology Trials Consortium: using spontaneously occurring cancers in
dogs to inform the cancer drug development pathway. *PLoS Med* **6**(10):
e1000161.
- Halper-Stromberg E, Frelin L, Ruczinski I, Scharpf R, Jie C, Carvalho B, Hao H,
Hetrick K, Jedlicka A, Dziedzic A, Doheny K, Scott AF, Baylin S, Pevsner

- J, Spencer F and Irizarry RA (2011) Performance assessment of copy number microarray platforms using a spike-in experiment. *Bioinformatics* **27**(8): 1052-1060.
- Hatzivassiliou G, Song K, Yen I, Brandhuber BJ, Anderson DJ, Alvarado R, Ludlam MJ, Stokoe D, Gloor SL, Vigers G, Morales T, Aliagas I, Liu B, Sideris S, Hoeflich KP, Jaiswal BS, Seshagiri S, Koeppen H, Belvin M, Friedman LS and Malek S (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* **464**(7287): 431-435.
- Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, Hussain J, Reis-Filho JS, Springer CJ, Pritchard C and Marais R (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* **140**(2): 209-221.
- Houben R, Vetter-Kauczok CS, Ortmann S, Rapp UR, Broecker EB and Becker JC (2008) Phospho-ERK staining is a poor indicator of the mutational status of BRAF and NRAS in human melanoma. *J Invest Dermatol* **128**(8): 2003-2012.
- Knapp DW and McMillan SK (2013) Tumors of the Urinary System, in *Withrow and MacEwen's Small Animal Clinical Oncology* (Withrow SJ, Page RL and Vail DM eds) pp 572-582, Elsevier Saunders, St. Louis, MO.
- Knowles MA and Hurst CD (2015) Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. *Nature reviews Cancer* **15**(1): 25-41.

- Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Maru D, Morris V, Janku F, Dasari A, Chung W, Issa JP, Gibbs P, James B, Powis G, Nolop KB, Bhattacharya S and Saltz L (2015) Phase II Pilot Study of Vemurafenib in Patients With Metastatic BRAF-Mutated Colorectal Cancer. *J Clin Oncol* **33**(34): 4032-4038.
- Lake D, Correa SA and Muller J (2016) Negative feedback regulation of the ERK1/2 MAPK pathway. *Cell Mol Life Sci* **73**(23): 4397-4413.
- Lebbe C, How-Kit A, Battistella M, Sadoux A, Podgorniak MP, Sidina I, Pages C, Roux J, Porcher R, Tost J and Mourah S (2014) BRAF(V600) mutation levels predict response to vemurafenib in metastatic melanoma. *Melanoma Res* **24**(4): 415-418.
- Levidou G, Saetta AA, Gigelou F, Karlou M, Papanastasiou P, Stamatelli A, Kavantzias N, Michalopoulos NV, Agrogiannis G, Patsouris E and Korkolopoulou P (2012) ERK/pERK expression and B-raf mutations in colon adenocarcinomas: correlation with clinicopathological characteristics. *World J Surg Oncol* **10**: 47.
- Long GV, Flaherty KT, Stroyakovskiy D, Gogas H, Levchenko E, de Braud F, Larkin J, Garbe C, Jouary T, Hauschild A, Chiarion-Sileni V, Lebbe C, Mandala M, Millward M, Arance A, Bondarenko I, Haanen J, Hansson J, Utikal J, Ferraresi V, Mohr P, Probst A, Dummer R, Schadendorf D, Wolchok JD, Hodi FS, Robert C, Ribas A, Davies MA, Lane SR, Legos JJ, Mookerjee B and Grob JJ (2017) Dabrafenib plus trametinib versus dabrafenib monotherapy in patients with metastatic BRAF V600E/K-mutant melanoma: long-term

- survival and safety analysis of a phase 3 study. *Ann Oncol* **28**(7): 1631-1639.
- Mao M, Tian F, Mariadason JM, Tsao CC, Lemos R, Jr., Dayyani F, Gopal YN, Jiang ZQ, Wistuba, II, Tang XM, Bornman WG, Bollag G, Mills GB, Powis G, Desai J, Gallick GE, Davies MA and Kopetz S (2013) Resistance to BRAF inhibition in BRAF-mutant colon cancer can be overcome with PI3K inhibition or demethylating agents. *Clin Cancer Res* **19**(3): 657-667.
- Mochizuki H, Kennedy K, Shapiro SG and Breen M (2015) BRAF Mutations in Canine Cancers. *PLoS One* **10**(6): e0129534.
- Montagut C, Sharma SV, Shioda T, McDermott U, Ulman M, Ulkus LE, Dias-Santagata D, Stubbs H, Lee DY, Singh A, Drew L, Haber DA and Settleman J (2008) Elevated CRAF as a potential mechanism of acquired resistance to BRAF inhibition in melanoma. *Cancer Res* **68**(12): 4853-4861.
- Nazarian R, Shi H, Wang Q, Kong X, Koya RC, Lee H, Chen Z, Lee MK, Attar N, Sazegar H, Chodon T, Nelson SF, McArthur G, Sosman JA, Ribas A and Lo RS (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **468**(7326): 973-977.
- O'Donoghue LE, Rivest JP and Duval DL (2011) Polymerase chain reaction-based species verification and microsatellite analysis for canine cell line validation. *J Vet Diagn Invest* **23**(4): 780-785.
- O'Neil NJ, Bailey ML and Hieter P (2017) Synthetic lethality and cancer. *Nat Rev Genet* **18**(10): 613-623.

- Poulikakos PI, Persaud Y, Janakiraman M, Kong X, Ng C, Moriceau G, Shi H, Atefi M, Titz B, Gabay MT, Salton M, Dahlman KB, Tadi M, Wargo JA, Flaherty KT, Kelley MC, Misteli T, Chapman PB, Sosman JA, Graeber TG, Ribas A, Lo RS, Rosen N and Solit DB (2011) RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* **480**(7377): 387-390.
- Poulikakos PI, Zhang C, Bollag G, Shokat KM and Rosen N (2010) RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* **464**(7287): 427-430.
- Prahallad A, Sun C, Huang S, Di Nicolantonio F, Salazar R, Zecchin D, Beijersbergen RL, Bardelli A and Bernards R (2012) Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* **483**(7387): 100-103.
- Rathore K and Cekanova M (2014) Animal model of naturally occurring bladder cancer: characterization of four new canine transitional cell carcinoma cell lines. *BMC Cancer* **14**: 465.
- Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, Lichinitser M, Dummer R, Grange F, Mortier L, Chiarion-Sileni V, Drucis K, Krajsova I, Hauschild A, Lorigan P, Wolter P, Long GV, Flaherty K, Nathan P, Ribas A, Martin AM, Sun P, Crist W, Legos J, Rubin SD, Little SM and Schadendorf D (2015) Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* **372**(1): 30-39.

Robertson AG, Kim J, Al-Ahmadie H, Bellmunt J, Guo G, Cherniack AD, Hinoue T, Laird PW, Hoadley KA, Akbani R, Castro MAA, Gibb EA, Kanchi RS, Gordenin DA, Shukla SA, Sanchez-Vega F, Hansel DE, Czerniak BA, Reuter VE, Su X, de Sa Carvalho B, Chagas VS, Mungall KL, Sadeghi S, Pedamallu CS, Lu Y, Klimczak LJ, Zhang J, Choo C, Ojesina AI, Bullman S, Leraas KM, Lichtenberg TM, Wu CJ, Schultz N, Getz G, Meyerson M, Mills GB, McConkey DJ, Network TR, Weinstein JN, Kwiatkowski DJ and Lerner SP (2017) Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. *Cell* **171**(3): 540-556 e525.

Shi H, Moriceau G, Kong X, Lee MK, Lee H, Koya RC, Ng C, Chodon T, Scolyer RA, Dahlman KB, Sosman JA, Kefford RF, Long GV, Nelson SF, Ribas A and Lo RS (2012) Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun* **3**: 724.

Siegel RL, Miller KD and Jemal A (2018) Cancer statistics, 2018. *CA Cancer J Clin* **68**(1): 7-30.

Sosman JA, Kim KB, Schuchter L, Gonzalez R, Pavlick AC, Weber JS, McArthur GA, Hutson TE, Moschos SJ, Flaherty KT, Hersey P, Kefford R, Lawrence D, Puzanov I, Lewis KD, Amaravadi RK, Chmielowski B, Lawrence HJ, Shyr Y, Ye F, Li J, Nolop KB, Lee RJ, Joe AK and Ribas A (2012) Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. *N Engl J Med* **366**(8): 707-714.

- Stagni C, Zamuner C, Elefanti L, Zanin T, Bianco PD, Sommariva A, Fabozzi A, Pigozzo J, Mocellin S, Montesco MC, Chiarion-Sileni V, De Nicolo A and Menin C (2018) BRAF Gene Copy Number and Mutant Allele Frequency Correlate with Time to Progression in Metastatic Melanoma Patients Treated with MAPK Inhibitors. *Mol Cancer Ther* **17**(6): 1332-1340.
- TCGA (2014) Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**(7492): 315-322.
- Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, Cipolla AK, Wubbenhorst B, Xu X, Gimotty PA, Kee D, Santiago-Walker AE, Letrero R, D'Andrea K, Pushparajan A, Hayden JE, Brown KD, Laquerre S, McArthur GA, Sosman JA, Nathanson KL and Herlyn M (2010) Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **18**(6): 683-695.
- Wagle MC, Kirouac D, Klijn C, Liu B, Mahajan S, Junttila M, Moffat J, Merchant M, Huw L, Wongchenko M, Okrah K, Srinivasan S, Mounir Z, Sumiyoshi T, Haverly PM, Yauch RL, Yan Y, Kabbarah O, Hampton G, Amler L, Ramanujan S, Lackner MR and Huang SA (2018) A transcriptional MAPK Pathway Activity Score (MPAS) is a clinically relevant biomarker in multiple cancer types. *NPJ Precis Oncol* **2**(1): 7.
- Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, Kehoe SM, Johannessen CM, Macconail LE, Hahn WC, Meyerson M and Garraway

- LA (2011) Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* **29**(22): 3085-3096.
- Yang H, Higgins B, Kolinsky K, Packman K, Bradley WD, Lee RJ, Schostack K, Simcox ME, Kopetz S, Heimbrook D, Lestini B, Bollag G and Su F (2012) Antitumor activity of BRAF inhibitor vemurafenib in preclinical models of BRAF-mutant colorectal cancer. *Cancer Res* **72**(3): 779-789.
- Yang W, Soares J, Greninger P, Edelman EJ, Lightfoot H, Forbes S, Bindal N, Beare D, Smith JA, Thompson IR, Ramaswamy S, Futreal PA, Haber DA, Stratton MR, Benes C, McDermott U and Garnett MJ (2013) Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic Acids Res* **41**(Database issue): D955-961.

Footnotes

Funding was provided by Morris Animal Foundation (D16CA-071) to D.L.D. and Shipley University Chair in Comparative Oncology to D.L.G.

Figure Legends

Figure 1. Canine TCC cell lines exhibit constitutive MAPK pathway activity.

(A) Heat map of z-transformed expression values for MPAS genes in the FACC cell line panel. Asterisks (*) indicate TCC cell lines. (B) MPAS values for FACC cell lines. TCC cell lines are shown in red. (C) MPAS values determined using Canine 1.0ST arrays versus those determined using Canine 2.0 arrays (Pearson $r = 0.9263$, $p < 0.0001$). (D) Serum starvation of canine TCC cell lines followed by western blot analysis of ERK1/2 phosphorylation. BRAF mutant cell lines are shown in blue and wild-type are shown in red.

Figure 2. BRAF mutant TCC cell lines are sensitive to “paradox-breaking” PLX7904, but insensitive to vemurafenib.

Cell lines were treated with serial dilutions of (A) vemurafenib or (C) PLX7904 for 72 hours. Relative viability at each dose was determined as a fraction of vehicle control. Three to five independent experiments with three technical replicates were conducted for each cell line. Error bars represent the standard deviation of the fraction of control from combined experiments ($n=3-5$). TCC cell lines were treated with (B) 15 μM vemurafenib or (D) 2 μM PLX7904 for 6 (top) and 24 (bottom) hours and assessed for ERK1/2 phosphorylation via western blot analysis. Western blot analysis of Kinsey cell lysate in (B) was performed on a separate blot. BRAF mutant and wild-type canine cell lines are shown in blue and red, respectively. BRAF mutant human cell lines are shown in green.

Figure 3. Canine TCC cell lines are sensitive to MEK1/2 inhibition.

Cell lines were treated with serial dilutions of (A) selumetinib or (C) trametinib for 72 hours. Relative viability at each dose was determined as a fraction of vehicle control. Three to five independent experiments with three technical replicates were conducted for each cell line. Error bars represent the standard deviation of the fraction of control from combined experiments (n=3-5).. TCC cell lines were treated with (B) 500 nM selumetinib or (D) 25 nM trametinib for 6 (top) and 24 (bottom) hours and assessed for ERK1/2 phosphorylation via western blot analysis. BRAF mutant and wild-type canine cell lines are shown in blue and red, respectively. BRAF mutant human cell lines are shown in green.

Figure 4. Up-regulation of the ErbB signaling cascade in canine TCC cell lines.

Microarray analysis was used to analyze gene expression in the FACC panel of canine cancer cell lines. (A) Cancer genes up- or down-regulated in TCC relative to other canine cancer cell lines using a fold change cutoff of 1.5 and $q < 0.05$. (B) Expression of ErbB ligands and receptors (KEGG pathway hsa04012) in FACC cell lines. Asterisks (*) indicate ErbB genes significantly up-regulated in TCC cell lines. Colors on heat maps represent z-transformed expression values. Color bars above heat maps indicate cell line histotype.

Figure 5. Combined MAPK and ErbB inhibition synergizes in canine TCC cell lines. Cells were treated with serial dilutions of the pan-ErbB inhibitor sapitinib (ERBBI) and (A,B) BRAF inhibitor PLX7904 (BRAFi) or (C,D) MEK1/2 inhibitor trametinib (MEKi), and cell proliferation was monitored for 72 hours. Drug synergy was determined using CalcuSyn software. (A,C) Colors on heat

maps represent combination index (CI) values, where $CI < 1$ is synergistic (green), $CI = 1$ is additive, and $CI > 1$ is antagonistic (red). CI values were determined from three independent experiments ($n=3$). (B,D) Representative images of red-labeled TCC cells 72 hours post-treatment. Images were acquired using IncuCyte ZOOM Live-Cell Analysis System at 10X magnification. Scale bars are 300 μm .

Tables

Table 1. IC₅₀ Values for MAPK Inhibitors in Canine and Human Cell Lines

| | Vemurafenib [μM] ^a | PLX7904 [μM] ^a | Selumetinib [nM] ^a | Trametinib [nM] ^a |
|----------------|--|--|-------------------------------|------------------------------|
| <i>Canine</i> | | | | |
| Angus1 | 19 (7.6 – 48) | 21 (6.6 – 69) | 150 (41 – 530) | 6.2 (1.9 – 20) |
| Kinsey | 20 (9.3 – 45) | 6.1 (5.8 – 6.3) | 18 (13 – 23) | 0.10 (0.019 – 0.56) |
| Bliley | 9.0 (2.8 – 29) | 0.96 (0.43 – 2.1) | 240 (140 – 410) | 1.4 (0.39 – 5.6) |
| Tyler1 | 26 (14 – 49) | 0.52 (0.18 – 1.5) | 140 (47 – 390) | 2.2 (0.55 – 9.0) |
| Tyler2 | 19 (8.4 – 42) | 0.20 (0.072 – 0.58) | 390 (100 – 1,500) | 3.1 (0.94 – 10) |
| <i>Human</i> | | | | |
| A375 | 0.35 (0.23 – 0.54) | 0.11 (0.058 – 0.23) | 81 (55 – 120) | 1.2 (0.72 – 2.0) |
| Colo205 | 0.077 (0.020 – 0.30) | 0.10 (0.020 – 0.54) | 100 (29 – 360) | 0.26 (0.076 – 0.87) |
| HT29 | 0.60 (0.25 – 1.4) | 0.32 (0.075 – 1.3) | 130 (62 – 270) | 0.70 (0.17 – 2.9) |
| RKO | 14 (9.5 – 21) | 2.8 (1.1 – 7.1) | 1,700 (660 – 4,600) | 8.6 (0.96 – 78) |

^a Values shown are the mean and 95% confidence interval of IC₅₀ values determined from three to five independent experiments. Each experiment included three technical replicates for each drug concentration. IC₅₀ values were rounded to two significant figures.

Figures

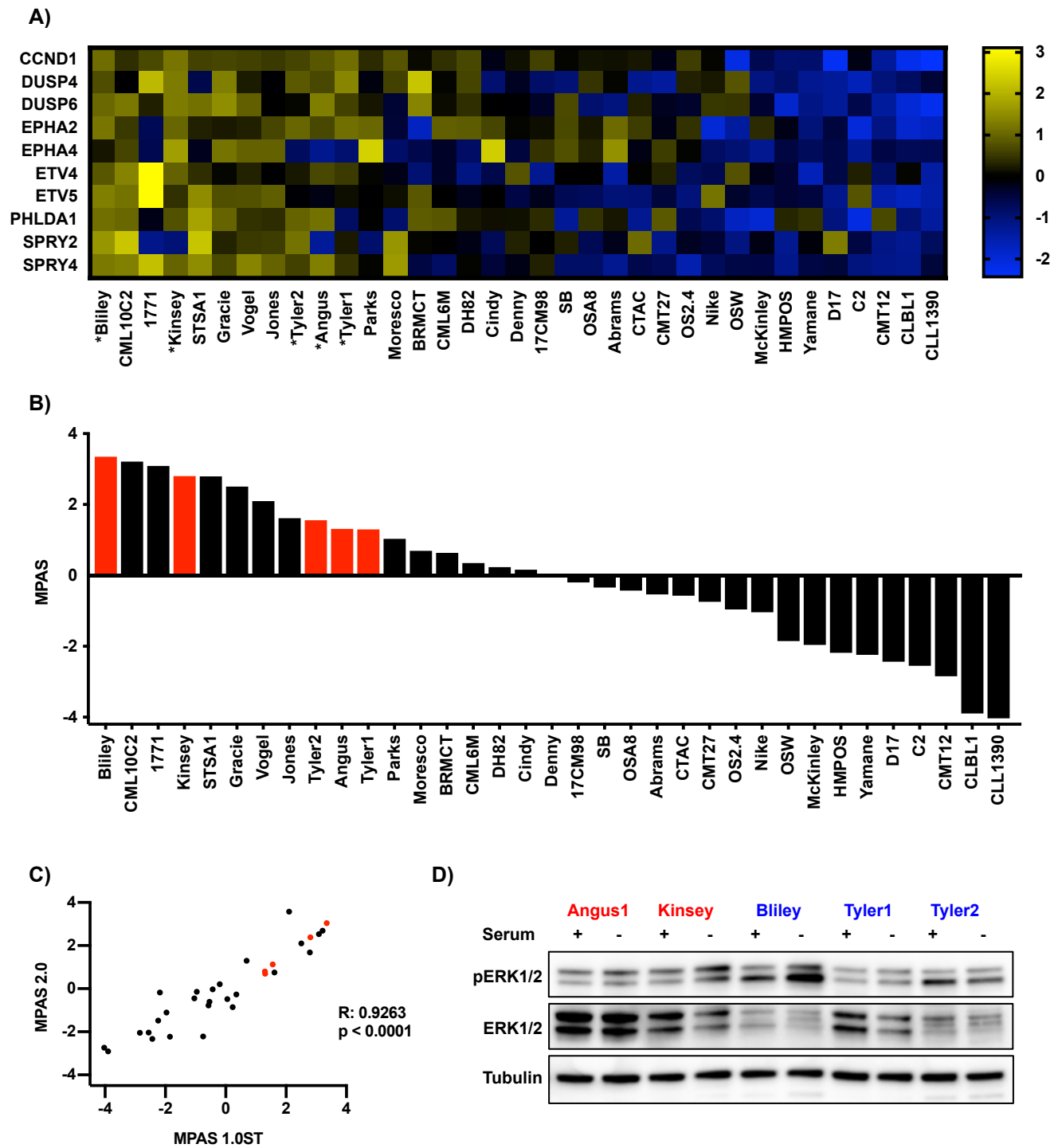


Figure 1

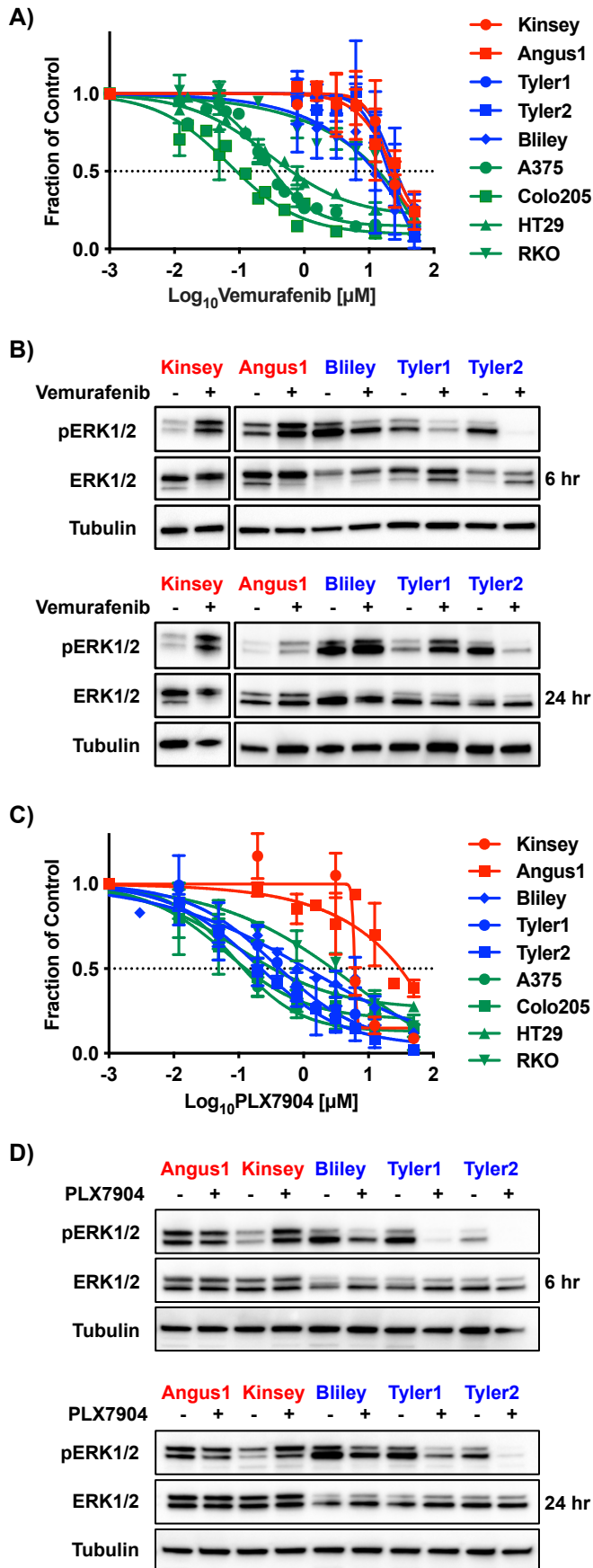


Figure 2

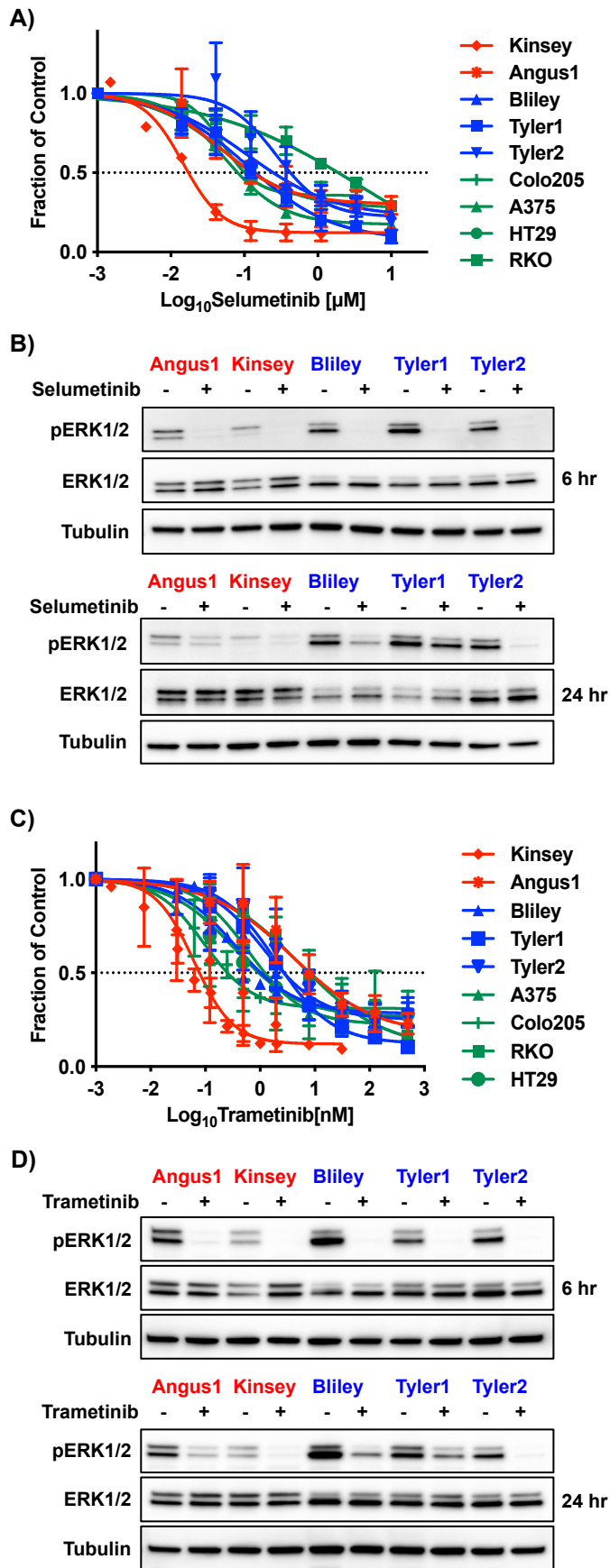


Figure 3

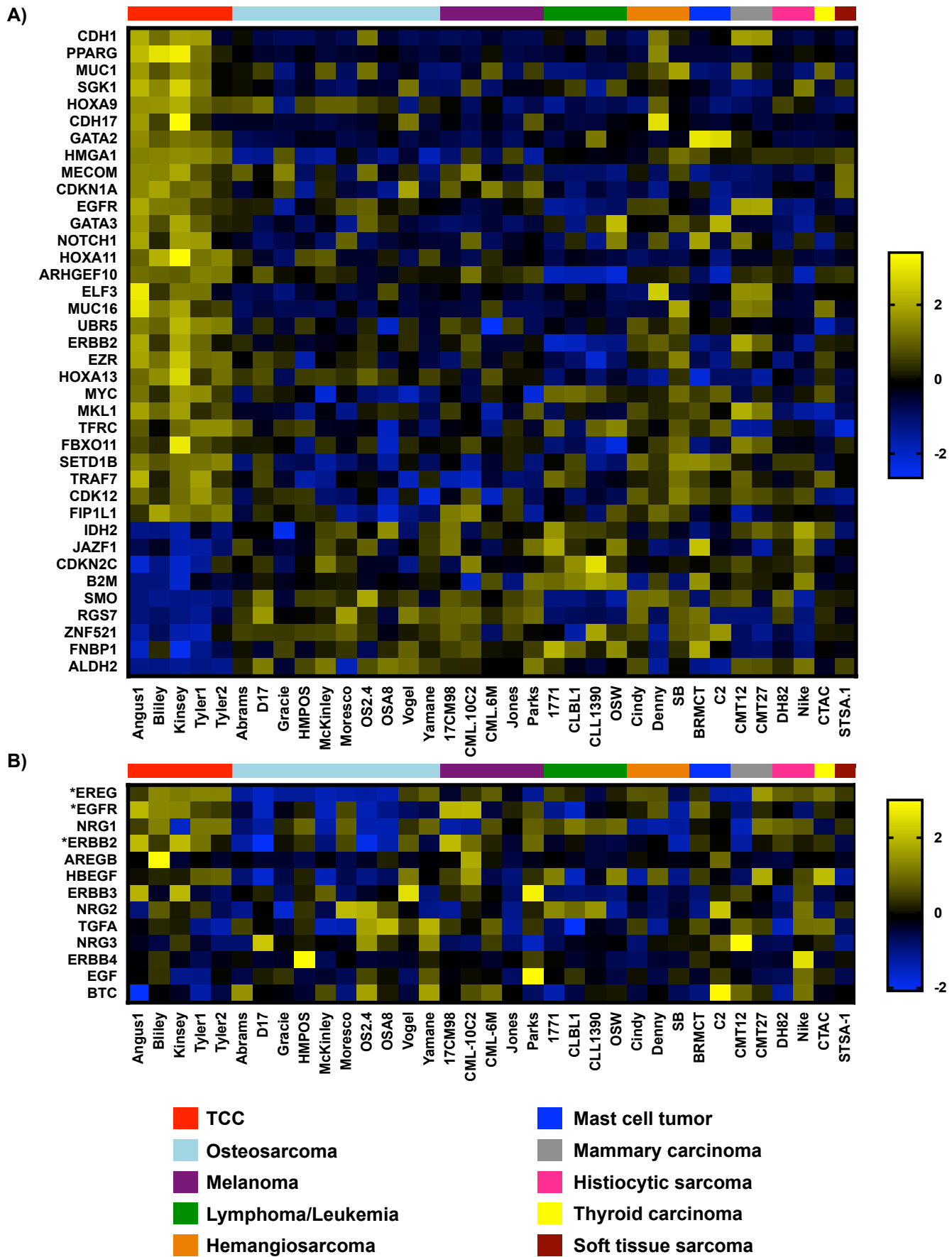


Figure 4

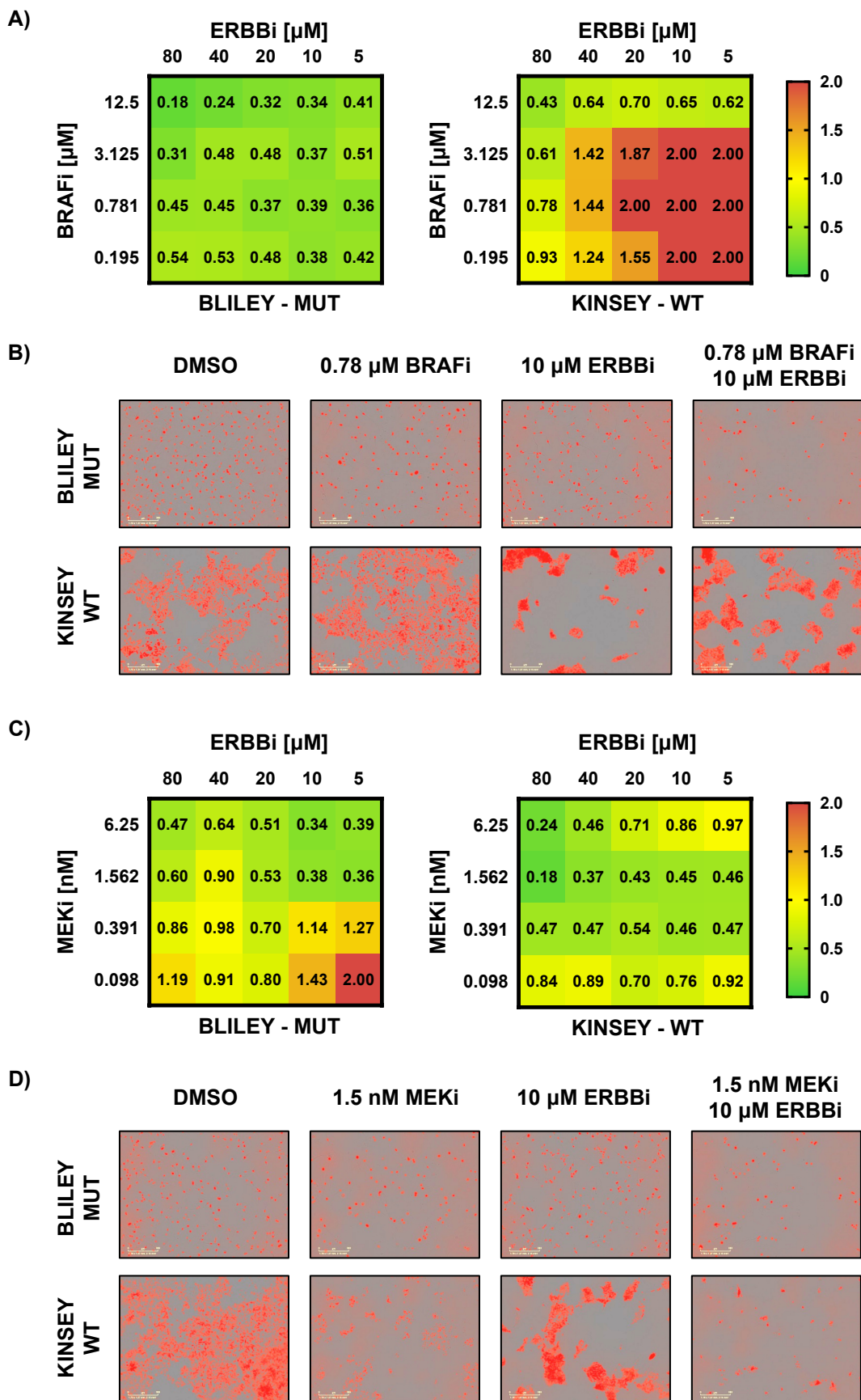


Figure 5