Activated MKK3/MYC crosstalk impairs dabrafenib response in BRAFV600E colorectal cancer leading to resistance

Gianluca Bossi (✉ gianluca.bossi@ifo.it)
IRCCS - Regina Elena National Cancer Institute, Rome, Italy  https://orcid.org/0000-0002-2947-1063

Angelina Pranteda
IRCCS - Regina Elena National Cancer Institute, Rome

Valentina Piastra
IRCCS - Regina Elena National Cancer Institute, Rome, Italy

Martina Serra
University of Rome "Tor Vergata,

Federica Lo Sardo
Regina Elena National Cancer Institute  https://orcid.org/0000-0002-2912-079X

Silvia Carpano
IRCCS - Regina Elena National Cancer Institute

Maria Grazia Diodoro
IRCCS - Regina Elena National Cancer Institute

Armando Bartolazzi
St. Andrea Hospital  https://orcid.org/0000-0001-7820-5127

Giovanni Blandino
Regina Elena Cancer Institute, Italy  https://orcid.org/0000-0002-6970-2241

Michele Milella
Università di Verona

Roberta Bernardini

Article

Keywords: Colorectal Cancer (CRC), MKK3 / p38MAPK, MYC, BRAF target therapy.

Posted Date: January 9th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2382452/v1

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Abstract

Colorectal cancer (CRC) patients with BRAF mutations develop resistance to BRAF inhibitors at very early stage. Understanding the mechanisms involved in resistance to BRAF inhibitors is required to develop novel therapeutic opportunities for this subtype of CRC patients.

CRC cells bearing BRAF mutations are mostly sensitive to the abrogation of MKK3, a specific activator of p38MAPKs signaling, suggesting that BRAF alterations might addict CRC cells to the MKK3/p38MAPK signaling. Interestingly, publicly available gene profiling data show significantly higher MKK3 transcript levels in CRC lines with acquired resistance to BRAF inhibitors.

Herein, we investigated the MKK3 roles in the response to BRAF targeting (dabrafenib) with COLO205 and HT29 BRAF\textsuperscript{V600E} CRC lines and derived dabrafenib-resistant (DABR) sublines. We found that in parental cells, but not in DABR cells, dabrafenib reduces MKK3 phosphorylation and induces autophagy and cell death. The MKK3 knock-down induces cell death in DABR cells, whereas MKK3 ectopic expression in parental cells reduces dabrafenib sensitivity. Molecularly, activated MKK3 interacts and co-localizes with MYC, sustaining MYC protein stability and thus preventing the dabrafenib induced effects in CRC DABR cells both \textit{in vitro} and \textit{in vivo}. Overall, we identify a novel molecular mechanism beyond the dabrafenib resistance, shedding lights on an uncovered vulnerability for the development of novel therapeutic opportunities in BRAF\textsuperscript{V600E} CRC.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy and the second leading cause of cancer death worldwide, with more than 1.9 million new CRC cases and 935,000 deaths in 2020 [1]. BRAF mutations are found in about 10% of CRC patients with locally advanced and metastatic disease [2–5], and are associated with negative prognosis, poor response to chemotherapy, and a median survival of fewer than 12 months [6]. FOLFOXIRI (fluorouracil + calcium folinate + oxaliplatin + irinotecan) plus bevacizumab, initially proposed as first-line treatment, is no longer the treatment of choice for BRAF-mutated CRC patients [7]. BRAF inhibitors as monotherapy in CRC was not as effective as it is for melanoma [8–11], due to the feedback activation of the oncogenic EGFR/MAPK pathway [12–15]. Accordingly, compounds that variably target the MAPK pathway are under investigation in several ongoing clinical trials to improve the prognosis of these patients [16, 17]. Therefore, understanding the mechanisms that mediate the response to BRAF targeting agents as well as those involved in acquired resistance can contribute to the development of novel targets to design new therapeutic opportunities.

The Mitogen-Activated Protein Kinase Kinase 3 (MKK3) is a member of the dual-specificity protein kinase group (M KK) that belongs to the mitogen-activated protein kinase (MAPK) signaling pathway. In response to different stimuli and inflammatory cytokines, MKK3 is activated by MKKK proteins (MEKK 1–4) through the phosphorylation of serine (Ser189) and threonine (Thr193) residues [18]. Activated MKK3 phosphorylates specifically p38MAPK family proteins (α,β,γ,δ), regulating a plethora of cellular programs.
such as cell differentiation, proliferation, death, invasion, and response to drugs [18]. We previously identified MKK3 as a required pro-survival factor in several cancer cell lines of different histotypes, including CRC [19–21]. We also reported the MKK3-p38δ MAPK signaling activation as a pro-survival mechanism in the response of CRC cells to 5-fluorouracil, thus suggesting its targeting as a potential therapeutic opportunity [21, 23]. Noteworthy, CRC lines with BRAF^{V600E} mutation resulted highly sensitive to MKK3 inhibition [19, 21], suggesting that BRAF^{V600E} mutation could exacerbate the MKK3 signaling dependency in CRC. Of interest, available repositories data showed higher MKK3 mRNA levels in BRAF^{V600E} CRC lines (COLO205, HT29) with acquired resistance to BRAF inhibitors (https://www.ncbi.nlm.nih.gov/geoprofiles/101541293).

Herein, we investigated the functions of MKK3 in the response to dabrafenib (GSK2118436) with a panel of parental CRC lines and derived resistant sublines (DABR), generated in the laboratory. We demonstrated that dabrafenib drastically abrogates MKK3 phosphorylation and hence its activation, thus inducing autophagy and cell death in parental CRC cells and corroborating the relevance of MKK3-dependent pro-survival signaling in those cells. By contrast, in DABR CRC cells, dabrafenib treatment did not modulate phospho-MKK3, nor did it induce autophagy and/or cell death. However, DABR CRC cells preserve the dependency on MKK3 pro-survival signaling, since cell death was induced upon its depletion. In an opposite and complementary perspective, ectopic expression of MKK3 weakened the response to dabrafenib in parental CRC cells. Mechanistically, we demonstrate that a MKK3/MYC molecular crosstalk is required to sustain resistance to dabrafenib in CRC cells. Specifically, persistent MKK3 activation in DABR cells sustained MYC protein stability through direct protein-protein interaction and co-localization, supporting DABR cells migration in vitro and tumor malignancy in vivo.

Overall, our data identified a novel molecular axis underlying dabrafenib resistance in CRC cells, providing novel insight for the identification of new strategies to treat BRAF^{V600E} mutant CRC patients.

**Materials And Methods**

**Cell Culture.**

ECACC authenticated human CRC lines COLO205 (#87061208) and HT29 (#91072201) were regularly monitored for mycoplasma contamination by PCR [24] and only Mycoplasma-free cells were used for studies. COLO205 and HT29 lines were cultured in RPMI medium (#31870, Gibco Paisley Scotland, UK) and McCoy’s medium (#12-688F, Lonza Burton on Trent, UK) respectively, and supplemented with 10% heat-inactivated FBS (#10099-141, Gibco), 2 mM L-Glutamine (#17-606E, Lonza), 100 U/ml Penicillin/Streptomycin (#DE17-602E, Gibco). All cells were cultured in HEPA-filtered humidified air in 5% CO2 at 37°C.

**Drug resistance.**

COLO205 and HT29 cells were continuously exposed to dabrafenib (GSK2118436, S2807, Selleckchem) (Fig. 1A), and drug renewed every three days, to generated DABR sublines.
Engineering inducible MKK3 depletion.

Cells were engineered as described [19–21] with a TET-inducible lentiviral-based system, carrying either shRNA sequence specific to MKK3 (sh/MKK3) or control (sh/scr). To induce sh/RNA expression doxycycline 1 µg/ml (#D9891, Sigma Aldrich, Saint Louis, MO, USA) was delivered to the culture media and renewed every three days.

Cell transfection

Twenty-four hours earlier seeded cells (2.0×10⁵/6-well plates) were transduced with empty (pcDNA3), MKK3 (pcDNA3-HA-MKK3) [25] or MYC (pcDNA3-HA-MYC, Invitrogen Carlsbad, CA, USA,) encoding vectors using jetOPTIMUS transfection reagent (Polyplus transfection, NY, USA), or with p38δ-RNAi (100 nM) (VHS40525, Invitrogen) [21], MYC-RNAi (80 nM) si-MYC_1: 5’-GCCACAGCAUACAUCCUGU-3’; si-MYC_2: 5’-GGACUAUCCUGCUGCCAAG-3’; Eurofins MWG) or no-targeting RNAi (AM4611) using Interferin reagent (Polyplus #409 – 10, Illkirch-Graffenstaden, France) following manufacturer's instructions. At specific time points cells were processed for experimental procedures.

Cell proliferation assays.

Twenty-four hours earlier seeded COLO205 (1×10⁴ cells/96-well) or HT29 (5×10³ cells/96-well) parental and DABR cells were challenged with drugs and 48h later MTT (ab211091, Abcam) performed following manufacturer's guidelines. Absorbance (540nm) quantified with Multiskan EX (Thermo Electron Corporation).

Western blotting and antibodies.

Twenty-four hours earlier seeded cells (6.0×10⁵/60mm dishes) were challenged with drugs and harvested at established time points in RIPA buffer (150mM NaCl, 1% Triton X, 0.25% sodium deoxycholate, 0.1% SDS, 50mM Tris/HCl, 20mM EDTA) supplemented with protease inhibitors (#11836153001, Roche, Basel, Switzerland), PMSF (1mM), NaF (50mM), DTT (50mM), and Na3VO4 (1mM). Protein lysates (30µg) were resolved in SDS-PAGE, transferred in PVDF membranes (Immobilon-P #IPVH00010, Merck-Millipore, Burlington, MA, USA), blots blocked in 5% non-fat-dried milk (#M7409, Sigma Aldrich), and incubated overnight at + 4°C with specific primary antibodies: phospho-MKK3/6 (1:1000, #D8E9 Cell Signaling Technology, Danvers, MA, USA); MKK3 (1:1000, #1D10 Sigma-Aldrich; 1:1000, #D4C3 Cell Signaling Technology); phospho-MYC (1:1000, ab185656; Abcam); MYC (1:1000, #D84C12; Cell Signaling Technology; 1:500, #9ED10; Santa Cruz Biotechnology); phospho-p38MAPK (1:1000, sc-166182; Santa Cruz Biotechnology); p38alphaMAPK (1:1000, #9F12; Santa Cruz Biotechnology); p38betaMAPK (1:1000, sc-398546; Santa Cruz Biotechnology); p38deltaMAPK (1:1000, sc-271292; Santa Cruz Biotechnology); LC3I/II (1:1000, ABC929; Sigma-Aldrich); PARP (1:1000, #9542; Cell Signaling Technology); CLEAVED CASPASE-3 (1:1000, #9661; Cell Signaling Technology); CLEAVED CASPASE-9 (1:1000, #20750; Cell Signaling Technology); ACTIN (1:1000, #13E5; Cell Signaling Technology); GAPDH
Bands visualized by HRP-conjugated secondary antibodies (#1721019, #1706516; Biorad, Hercules, CA, USA), ECL (RPN2106; GE Healthcare, Chicago, IL, USA), and quantified by densitometry with ImageJ software.

**Immunoprecipitation.**

Early 72h seeded cells (4.0x10⁶/150mm dishes) were treated 24h with dabrafenib (0.1µM) and harvested with IP-RIPA buffer (1% Triton, 150Mm NaCl, 50Mm Tris/HCl, 1Mm EDTA) supplemented with protease inhibitors + 0.01mM PMSF + 5.0mM NaF + 0.125mM Na3VO4. Protein-G PLUS agarose (sc-2002, Santa Cruz Biotechnology) pre-cleared lysates (0.5 mg/sample) were incubated (1h at 4°C) with specific primary antibodies (MKK3, MYC, p38α/β/Υ/δ MAPK) and further incubated with Protein-G PLUS agarose beads (20µl/sample) overnight at + 4°C with orbital platform. The day after, samples were boiled (5min at 95°C) in IP-loading dye (1% SDS, 25Mm Tris/HCl, 10% Glycerol, 0.010% Bromphenol blu), resolved on SDS/PAGE and blots incubated with phosho-Myc, phospho-MKK3, phospho-p38MAPK specific-antibodies.

**RT-PCR.**

Twenty-four hours earlier seeded (6.0x10⁵/60mm dishes) COLO205 and HT29 parental and DABR cells were treated 24h with dabrafenib (0.1µM), total RNAs extracted with Trizol Reagent (AMBION life technologies, Van Allen Way, Carisbad CA, USA), retro-transcribed (2.0µg) with FastGene Scriptase Basic cDNA-Kit (NIPPON Genetics EUROPE, Duren, Germany), following manufacturer's guidelines, and generated cDNAs analyzed by q-PCR (PCRBIOSYSTEMS 2x # PB20.14) with primers sets:

RT-MKK3-F: 5’-CTACATGGCCCCTGAGA-3’;

RT-MKK3-R: 5’-TCCAGACGTCGGACTTGACAGGAT-3’;

RT-Actin-F: 5’-GCTGCCCTGAGGCAATCTT-3’;

RT-Actin-R: 5’-ATGATGGAGTTGAAGGTAGGTTCGT-3’.

**Immunofluorescence.**

Twenty-four hours earlier seeded (2.0x10⁵/6-well plate) COLO205-parental and -DABR cells, treated 24h with dabrafenib (0.1µM) were fixed with PBS1X + 1.0% formalin (Sigma-Aldrich) (10min at RT), permeabilized in PBS1X + 0.1% TRITON (Sigma-Aldrich), blocked 20min at RT with PBS1X + NaN₃ + 10%FBS (Gibco), incubated 1h at RT with anti-MYC (1:50, D84C12), anti-MEK3 (1:100, sc376627) in PBS1X + NaN₃ + 1.5%FBS, incubated 1h at RT with secondary antibodies anti-Rabbit CY-3 (1:400) and anti-Mouse CY-2 (1:400) in PBS1X + NaN₃ + 5%FBS, nuclei stained with DAPI (Thermo Fisher Scientific) and immunofluorescences analyzed at confocal microscope (LSM880, ZEISS).

**Cell cycle analyses.**
Twenty-four hours earlier seeded cells (5.0x10^4/6-well plate), treated 24h with dabrafenib (0.1µM), were fixed in 70% ethanol + 1mg/ml RNase (PureLINK™, Invitrogen), stained with 5mg/ml PI solution (Sigma-Aldrich), and analyzed with Guava Easycyte 8HT flow-cytometer equipped with Guava Soft 2.1 (Millipore).

**Cell migration assay.**

Twenty-four hours earlier seeded cells (5.0X10^4) on cell-culture-inserts (#353097-Falcon) were fixed with PBS1X + 2% formaldehyde (5min at RT), permeabilized with methanol (Carlo Erba), stained with PBS1X + 0.025% crystal violet, analyzed under optical microscope (20X) and images quantified by ImageJ software. For each sample ten different fields were quantified.

**In vivo studies.**

All experiments were executed at the Animal Technology Station, University of Rome “Tor Vergata” following the EU Directive 2010/63/EU in compliance with institutional guidelines, regulations and authorization by appropriate institutional review board (141/2017-PR dated 02/13/2017). Either exponentially growing COLO205, and -sh/scr or -sh/MKK3, as well as DABR-sh/scr or -sh/MKK3 sublines, were injected subcutaneously (3.0X10^6 cell/mouse) (6 mice/group) in 45-day-old female nude mice (CD1 nu/nu, Envigo, Casatenovo, Italy). After tumor nodule formation (0.2 cm^3), either dabrafenib or vehicle solution was daily delivered (30mg/kg) by oral gavage [26], whereas MKK3 depletion induced by doxycycline (2.0g/l) in drinking water, as reported [27]. Caliper measurements once week estimated the tumor volumes (TV) by the formula: TV=(a x b^2)/2: a and b represents tumor length and width, respectively.

Drug-resistance in vivo was induced in COLO205-sh/MKK3 (3.0X10^6 cell/mouse) xenograft-bearing mice, after tumors nodule formation (0.2 cm^3), by daily treatments with dabrafenib or vehicle solution (control) (6 mice/group). Tumors explanted from euthanized animals were minced and collagenase-digested to generate early-passages cultures.

**Statistical analyses.**

Significance was assessed by Student’s t-test, Fisher’s exact test, or two-way ANOVA. Statistical analysis was performed using GraphPad/PRISM software; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Results**

The acquired resistance to dabrafenib is associated with sustained MKK3 phosphorylation in BRAF V600E CRC cells.

To investigate MKK3 roles in the therapeutic response to BRAF inhibition in BRAF V600E CRC cells, dabrafenib (GSK2118436) was selected for its potent and specific BRAF V600E inhibitory activity as
compared to wild-type BRAF or CRAF, and for the promising results with phase I and II clinical trials [22]. Hence, resistance to dabrafenib (DABR) was established in HT29 and COLO205 CRC lines bearing BRAF\textsuperscript{V600E} mutation, by exposing cells continuously to stepwise increasing concentrations of drug (0.2, 0.5, 1.0, 2.0, 4.0 µmol/L) until the surviving cell fraction reached 90% confluence, about every 15 days (Fig. 1A). Resistance to dabrafenib was well-established with both lines, given significantly higher IC\textsubscript{50} values (> 10 folds) in derived DABR cells when compared to their parental counterparts (Fig. 1B, 1C).

Molecularly, dabrafenib treatments abrogated MKK3 phosphorylation which paralleled reduced p38MAPK activation in both parental COLO205 and HT29 cells (Fig. 1D). In contrast in DABR cells the levels of phospho-MKK3, which were higher than in their parental counterparts in untreated conditions, were not modulated (Fig. 1D). Noteworthy, similarly to MKK3 depletion [19–21], dabrafenib treatments, by hampering MKK3 activation, induced autophagy and cell-death in parental COLO205 cells but not in DABR cells (Fig. 1E). Moreover, dabrafenib treatments induced phospho-MKK3 degradation being rescued by the proteasome inhibitor (MG132) (suppl. Figure 1A), without affecting the mRNA levels (Fig. 1F).

Overall, our results suggest that MKK3 might exert relevant roles in driving the resistance to dabrafenib in tested CRC lines.

**MKK3 sustains resistance to Dabrafenib in CRC lines.**

To assess the relevance of MKK3 in the dabrafenib therapeutic response, we engineered COLO205- and HT29-DABR cells with inducible shRNA lentiviral vectors, as reported [19–21], and evaluated the therapeutic response upon time-dependent depletion of endogenous MKK3. Efficient doxycycline-induced MKK3 abrogation reduced significantly cell-viability in both COLO205-DABR (Fig. 2A) and HT29-DABR cells (suppl. Figure 1B). Conversely, ectopic MKK3 expression significantly hampered the dabrafenib effects on the survival of parental COLO205 cells (Fig. 2B).

Flow-cytometry analyses showed dabrafenib to induce significant subG1 accumulation in parental cells, but not in DABR cells (Fig. 2C, upper and middle panels), whereas MKK3 depletion itself induced significant cell death in DABR cells (Fig. 2C, lower panels), which was enhanced by dabrafenib treatments (Fig. 2C, lower panels). Overall, our results reveal MKK3 as new key player in the resistance to dabrafenib in CRC, and propose MKK3 as a novel vulnerable target to overcome resistance to BRAF inhibition in CRC.

**Activation of p38MAPK isoforms is not involved in MKK3-driven dabrafenib resistance in CRC.**

We previously showed that MKK3 impairs 5-fluorouracil efficacy through the activation of p38\(\delta\) MAPK pro-survival signaling in CRC cells, thus suggesting the targeting of MKK3-p38\(\delta\) MAPK signaling as a potential novel therapeutic opportunity in CRC [21, 23]. Thus, we investigated whether p38MAPK family proteins (\(\alpha, \beta, \gamma, \delta\)) might cooperate in MKK3-driven dabrafenib resistance in CRC. Isoform-specific immunoprecipitations with COLO205-parental and -DABR cells showed consistent phosphorylation of p38\(\delta\) in both parental- and DABR-untreated cells (suppl. Figure 2A), which consistently decreased after dabrafenib treatments in parental cells but not in DABR cells (suppl. Figure 2A), likely due to selective inactivation of MKK3 in dabrafenib-responsive parental cells (Fig. 1D).
might play a role in dabrafenib resistance in CRC cells, we investigated the response to dabrafenib in both COLO205-parental and DABR cells upon depletion of the endogenous protein. In parental cells, the p38δ siRNA significantly inhibited cell survival (suppl. Figure 2B, C), in accordance with previous results [21, 23], and treatment with dabrafenib further reduced cell viability (suppl. Figure 2C). By contrast, in DABR cell, the p38δ depletion affected to a minor extent cell survival that resulted not further impacted by dabrafenib treatment (suppl. Figure 2C). Overall, these results suggest that p38δ isoform activation is not likely to play relevant roles in MKK3-mediated resistance to dabrafenib in CRC models.

**M KK3 complexes with and stabilizes MYC protein, sustaining resistance to dabrafenib in CRC.**

Emerging literature identified MKK3 as a MYC-interacting protein promoting cancer cell proliferation and survival [28], putting forward the disruption of MKK3-MYC protein-protein interaction as a promising strategy to target MYC-driven oncogenic programs [29]. MKK3 was further reported to sustain the MYC-dependent epithelial-to-mesenchymal transition in triple negative breast cancer [30]. Therefore, given the well-known oncogenic activities of MYC and its involvement in various processes of drug resistance including the resistance to EGFR inhibitors in mCRC [31–37], we investigated whether in our experimental setting MYC might play relevant roles in MKK3-driven dabrafenib resistance in CRC. Primary investigations with parental and DABR COLO205 and HT29 cells revealed that, similar to phospho-MKK3, dabrafenib treatment reduced significantly MYC protein levels in parental but not in DABR cells (Fig. 3A).

Consistent with previous reports [28, 29], MKK3 was required for MYC protein stability. This applied to both parental and DABR CRC cells, since inducible MKK3 silencing resulted in reduction of MYC protein levels in both parental and DABR COLO205 (Fig. 3B) and HT29 cells (Suppl. Figure 3A), which undergoes to protein degradation, that unaffected by dabrafenib treatments (Fig. 3C), was rescued by proteasome inhibition with MG-132 (Fig. 3D). Furthermore, MYC knock-down inhibited consistently survival in COLO205-DABR cells (Fig. 3E), whereas ectopic MYC expression reduced significantly the response to dabrafenib in COLO205 when compared to controls (empty vector) (Suppl. Figure 3B).

To further investigate MKK3/MYC crosstalk, immunoprecipitations revealed that phospho-MKK3 complexes with MYC (Fig. 4A, 4B) and that such protein interaction was unaffected by dabrafenib treatments in DABR cells (Fig. 4A). Moreover, MKK3 and MYC co-immunofluorescence revealed a higher protein staining and massive nuclear/cytoplasmic co-localization in DABR than parental cells (Fig. 4B) and, in agreement with results in Fig. 3A, showed that dabrafenib abrogates the MKK3/MYC staining and co-localization in parental but not in DABR cells (Fig. 4B).

Overall, these results suggest that MKK3 sustains MYC protein stability, supporting the MKK3/MYC molecular axis as a novel mechanism involved in dabrafenib resistance in CRC.

**The MKK3/MYC axis sustains migration in CRC-DABR cells.**

Increased motility and invasion have been described as phenotypic features in melanoma cells resistant to BRAF inhibitors [38], whereas MKK3 and MYC proteins were reported as key players involved in cell migration [39, 40]. Accordingly, by cell-migration assays with COLO205 and HT29 parental and DABR
cells, we demonstrate that both DABR sublines showed a significantly higher migration ability when compared to their parental counterparts (Fig. 5A). Moreover, the MKK3/MYC axis is involved in the increased cell motility, since either MKK3 (Fig. 5B) or MYC (Fig. 5C) depletion reduced significantly the number of migrated cells. These results demonstrated that the MKK3/MYC axis activation in DABR cells contributes to increase the CRC cell motility.

**MKK3 sustains CRC-DABR malignancy in vivo.**

To explore the role of MKK3 in dabrafenib resistance with pre-clinical models, we first assessed the effects of either dabrafenib treatment or MKK3 depletion on COLO205 xenograft tumor growth. Significant inhibition of tumor growth was observed during treatment with dabrafenib (Fig. 5D) or upon efficient MKK3 depletion (Fig. 5E, Suppl. Figure 4A) compared to relative controls (Fig. 5D, 5E). Hematoxylin and eosin staining showed diffuse differentiation of tumor cells with normal crypt villous-like structures only in dabrafenib-treated tumors, while extensive areas of necrosis were found in both dabrafenib and MKK3 depleted xenografts (Fig. 5G). Interestingly, and according to in vitro data, MKK3 depletion resulted in significant tumor growth inhibition also in COLO205-DABR xenograft tumors (Fig. 5F). To further corroborate our findings with more clinically relevant experimental models, we induced resistance in vivo by treating COLO205 xenografts bearing mice daily with dabrafenib after tumor nodule formation (0.2 mm³). Dabrafenib significantly delayed tumor growth until the 7th week of treatment (Fig. 6A), followed by a significant and steady increase in tumor volumes reaching those of the vehicle treated group by the 9th week of treatment (Fig. 6A), when tumors were collected (# 2, # 3, # 4) and cultured to generate early cultures. Western blot analysis revealed different degree of MYC activation among the generated cultures when compared to the relative parental and DABR COLO205 cells, with culture #4 showing the highest total and phosphorylated MYC protein level resembling the DABR cells (Fig. 6B). Accordingly, MYC protein levels correlated with: (i) dabrafenib response, revealing culture #4 as the most resistant with respect to the #2 and #3 (Fig. 6C); (ii) hematoxylin-and-eosin staining of tumor sections, showing more aggressive and undifferentiated phenotype in tumor #4 with respect to #2 and #3, which preserved the normal crypt villous-like structures (Fig. 6D). Furthermore, MKK3 depletion reduced significantly cell viability and enhanced dabrafenib therapeutic efficacy in culture #4 (Fig. 6E).

Overall, these results suggest that one of the molecular mechanisms behind dabrafenib resistance in CRC involves the hyper-activation of MKK3 that, likely through enhanced MYC protein activity and stability, contributes to preventing dabrafenib-induced autophagy and cell death in CRC cells. Therefore, MKK3 could constitute a novel valid vulnerability to be targeted to improve therapeutic opportunities for BRAF-mutated CRC patients.

**Discussion**

BRAF mutations are found in about 10% of CRC patients and are associated with a negative prognosis and a median survival of less than 12 months [6, 41, 42]. BRAF inhibitors as monotherapy in CRC was not as effective as melanoma [8–11]. Therefore, understanding the mechanisms that mediate the response to BRAF targeting agents as well as those involved in acquired resistance can contribute to the
development of novel targets to design new therapeutic opportunities to address this still unmet clinical need.

Herein we investigated whether MKK3 could play relevant roles in the response and acquired resistance to BRAF inhibition in CRC cells. Characterization of mutant BRAF CRC lines (HT29 and COLO205) with established resistance to dabrafenib, along with their parental counterparts, showed MKK3 as relevant player in dabrafenib response in CRC, as dabrafenib abolishes phosphorylated MKK3 protein only in the parental cells, while remaining high in DABR cells. Interestingly, our results demonstrated that dabrafenib, similarly to MKK3 knockdown [19–21], induces autophagy and cell death in tested CRC cells, supporting the relevance of MKK3 in the mutated BRAF pro-oncogenic signaling in CRC cells. Indeed, the ectopic MKK3 expression weakens dabrafenib therapeutic efficacy in vitro. When investigating the molecular mechanisms involved, we found that the p38MAPK family proteins are not involved in MKK3-driven dabrafenib resistance. MKK3 has been recently reported to interact and stabilize MYC oncoprotein [24], and due to its involvement in several mechanisms of drug resistance including the resistance to EGFR inhibitors in mCRC [38], we investigated the potential MKK3/MYC axis involvement in driving dabrafenib resistance in CRC. Interestingly, MYC followed similar modulations as phospho-MKK3 upon dabrafenib treatment in parental and DABR cells. As proof of the MKK3/MYC axis contribution in dabrafenib resistance, we show that MKK3 silencing reduced MYC protein levels, by inducing MYC protein degradation. Furthermore, MYC silencing reduced significantly DABR cell survival, whereas either MKK3 or MYC ectopic expression reduced dabrafenib efficacy in parental cells. We found MKK3 complexes and co-localized to MYC, and that dabrafenib abolished the crosstalk in parental cells, by inducing MKK3 and MYC protein degradation. Conversely, in DABR cells, the exacerbated MKK3 activation maintained MYC protein stability and protein co-localization.

Overall the results shed lights on a novel role of MKK3 providing a functional implication of the MKK3/MYC molecular interaction previously reported [24], as the activated MKK3/MYC crosstalk constitutes a novel mechanism behind the inefficacy of BRAF inhibitors in CRC mutated BRAF (Fig. 7), and thus suggesting a novel vulnerability to be targeted for the development of novel therapeutic opportunities in BRAF-mutated CRC. Indeed, MKK3 targeting could constitute an intriguing new strategy to turn-off the undruggable MYC-driven oncogenic programs [29, 45] in BRAF-mutated CRC.

Declarations

Disclosure of Potential Conflicts of Interest. No potential conflicts of interest to disclose.

Other notes about the manuscript as a whole.

CONFLICT OF INTEREST

All authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
AVAILABILITY OF DATA AND MATERIALS

All relevant data are available from the authors upon request.

ACKNOWLEDGEMENTS

This work was financially supported through funding from AIRC - IG 2016 (ID. 18449 project), LILT 5X1000 year 2019, and the institutional “Ricerca Corrente” granted by the Italian Ministry of Health to G. Bossi. We thank Dr. G. Fontemaggi for providing us with MYC-targeting stealth RNAi and MYC-specific antibodies, and Dr. E. Rogges for xenograft tumors staining.

References


\section*{Figures}
Resistance to dabrafenib fosters MKK3 activation in CRC cells. A. Schedule of treatment adopted to induce resistance to dabrafenib in COLO205 and HT29 CRC lines; COLO205 (B) and HT29 (C) parental and resistant (DABR) cells were challenged with the indicated doses of dabrafenib and effects on survival and proliferation were assessed 48h later by MTT assay. Data were quantified with respect to controls (untreated cell) set to 1.0 and reported as mean ± SD. Result from experiment performed in technical
triplicate representative of three independent experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student’s t-test: **p < 0.01; ***p < 0.001, ****p < 0.0001. 

D, E. Parental and DABR cells were treated with dabrafenib or left untreated, and 24h later protein lysates were analyzed by western blot with the indicated antibodies. More relevant bands from the same filter at same exposure length are reported. Densitometry was performed with ImageJ software and relative band intensity was normalized to actin and quantified with respect to untreated cells set to 1.0; 

F. Parental and DABR COLO205 and HT29 cells were treated with dabrafenib or left untreated, then 24h later MKK3 mRNA levels were assessed by qPCR. GAPDH was adopted as reference gene. These experiments were performed in technical replicate. Significance was analyzed using unpaired Student’s t-test: n.s. not significant.
MKK3 hampers dabrafenib therapeutic efficacy in CRC cells. A. Upper panel: COLO205-DABR sh/scr and sh/MKK3 sublines were treated with doxycycline (1µg/ml) and protein lysates were analyzed 144h later by western blot with the indicated antibodies; Lower panel: cells were treated 144h with doxycycline (1µg/ml) and for last 48-hour with dabrafenib (0.1 mM), and effects on cell survival were assessed by MTT assay. Result from experiment performed in technical triplicate representative of three independent
experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student's t-test: **p < 0.01; B. Upper panel: COLO205 parental cells were transiently transfected either with empty (pcDNA3) or cDNA encoding MKK3-HA tagged (pDNA3HA-MKK3) vectors, and 48h later protein lysates were analyzed by western blot with the indicated antibodies; Lower panel: cells were treated with dabrafenib and effects on cell survival were assessed 48h later with MTT assay (lower panel). Data were quantified with respect to controls (untreated cell) set to 1.0 and reported as mean ± SD. Result from experiment performed in technical triplicate representative of three independent experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student's t-test: **p < 0.01; C. Left panels, flow cytometry analyses were performed with COLO205 parental cells untreated or treated with dabrafenib (DAB) (24h) or COLO205-DABR sh/scr and sh/MKK3 sublines after 144h doxycycline (1ug/ml) untreated or treated with DAB (24h). Right panels, results are reported as mean ± SD of three independent experiments (biological replicates) with similar results. Significance was analysed two-tailed unpaired Student's t-test: n.s. not significant; *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3

MKK3 counteracts dabrafenib effects on MYC protein stability in CRC cells driving resistance. A. COLO205 and HT29 parental and DABR cells were plated and treated with dabrafenib, and 24h later protein lysates were analyzed by western blot with the indicate antibodies; B. COLO205 parental and DABR sh/scr and sh/MKK3 sublines were treated with doxycycline (1mg/ml) for the indicated times (h) and protein lysates were analyzed by western blot with the indicated antibodies; C. COLO205-DABR
sh/scr and sh/MKK3 sublines were treated with doxycycline (1mg/ml) for 96h, with the addition of dabrafenib at the indicated concentrations for the last 24h; then, protein lysates were analyzed by western blot with the indicated antibodies; D. COLO205-DABR sh/scr and sh/MKK3 sublines were treated 144h with doxycycline (1ug/ml) and co-treated the last 4h with MG132 (5.0 mM), then protein lysates were analyzed by western blot with the indicated antibodies; E. Upper panels: COLO205-DABR cells were transiently transfected with ctr siRNA or siMYC#1 or siMYC#2 siRNAs (80 nM) and 72h later protein lysates were analyzed by western blot with the indicated antibodies. More relevant bands from the same filter at the same exposure length are reported. Lower panel: effects on cell proliferation were assessed by MTT assay. Results were quantified with respect to controls (si/CTR) set to 1.0 and reported as mean ± SD. Data from experiment performed in technical triplicate representative of three independent experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student's t-test: **p < 0.01; ***p < 0.001
Dabrafenib abrogates MKK3-MYC protein-protein interaction and co-localization in parental but not DABR cells. A. Protein lysates from COLO205 parental and DABR cells, untreated or treated 24h with dabrafenib, were immunoprecipitated either with anti-MKK3 or anti-MYC antibody and immuno-complexes revealed by western blot with the indicated antibodies. More relevant bands from the same filter at same exposure length are reported. B. Immunofluorescences were performed with the indicated antibodies with COLO205...
parental and DABR cells, either untreated or treated for 24 hours with dabrafenib. Immune-stained cells were analysed at the confocal microscope (LSM 880, ZEISS). Representative data from three independent experiments with similar results are reported.

Figure 5
**MKK3-driven MYC activation promotes cell migration and sustains tumour malignancy.** Migration assays were performed by transwell migration assay with: A. COLO205 and HT29 parental and DABR cells; B. HT29 parental and HT29 DABR cells with or without doxycycline-inducible depletion of endogenous MKK3 (96h, 1 μg/ml); C. COLO205-DABR cells with or without siRNA-mediated endogenous MYC depletion. Twenty-four hours later, all inserts were stained with crystal violet and analyzed by optical microscope at 20x magnification; acquired images were quantified by ImageJ software. For each sample total cells /field from ten different fields were quantified and results expressed as mean ± SD. Data from experiment performed in technical triplicate representative of three independent experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student’s t-test: *p < 0.05, **p < 0.01; ****p < 0.0001; D-G. Xenografts were generated subcutaneously with COLO205 (D), COLO205 sh/scr and sh/MKK3 (E), and COLO205-DABR sh/scr and sh/MKK3 (F) sublines (6 mice / group); after tumors nodule formation (0.2 mm$^3$), animals were treated either with dabrafenib (30mg/kg) or vehicle solution (D), or doxycycline (2.0 g/l) (E, F). Gray lines show the windows of treatments. Tumour growth was followed by caliper measurements once a week, results are reported as means ± S.D. Significance was analyzed using two-way ANOVA: *p < 0.05, **p < 0.01; G. Representative haematoxylin-and-eosin staining with a subset of explanted tumors are reported. Red and black arrows show respectively the normal crypt villous like structures and necrosis areas.
Clinically relevant experimental model of induced resistance to dabrafenib in vivo corroborates the MKK3/MYC crosstalk relevance. A. Xenografts were generated subcutaneously with parental COLO205 sh/MKK3 subline, after tumors nodule formation (0.2 mm³), dabrafenib (30mg/kg) or vehicle solution (6 mice / group) was delivered for the indicated time (gray line), and tumor growth followed by caliper measurements once a week. Early-passage cultures were established from subset of explanted tumors.
(#2, #3, #4) and: B. Protein lysates analyzed by western blot with the indicated antibodies; C. Response to dabrafenib assessed by MTT assay following 48h of treatment; D. Representative haematoxylin-and-eosin staining of explanted tumors; E. Established early-passage culture #4 was treated or untreated with doxycycline (1.0 mg/ml) and: i) protein lysate analyses by western blot 96h later with the indicated antibodies (upper panel); ii) treated for further 48h with dabrafenib at the indicated concentrations and effects on cell survival assessed by MTT assay (lower panel). Results are reported as mean ± SD. Data from experiment performed in technical triplicate representative of three independent experiments (biological replicates) with similar results are reported. Significance was analyzed using unpaired Student’s t-test: **p < 0.01; ****p < 0.0001.

Pranteda et al., Figure 7

Figure 7

The MKK3 / MYC molecular axes in the established dabrafenib resistance in CRC cells. In CRC cells responsive to dabrafenib, inhibition of mutant BRAF reduces the activation of the underlying canonical pathways (MEK – ERK) and the newly identified MKK3 effector, inducing autophagy and cell death. In CRC cells with established resistance to dabrafenib, exacerbated activation of the MKK3 / MYC axis contributes to supporting the survival of CRC DABR cells.

Supplementary Files

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