Oncogenic Kras Promotes Chemotherapy-Induced Growth Factor Shedding via ADAM17

Sandra Van Schaeybroeck1, Joan N. Kyula1, Audrey Fenton1, Catherine S. Fenning1, Takehiko Sasazuki2, Senji Shirasawa3, Daniel B. Longley1, and Patrick G. Johnston1

Abstract

Oncogenic mutations in Kras occur in 40% to 45% of patients with advanced colorectal cancer (CRC). We have previously shown that chemotherapy acutely activates ADAM17, resulting in growth factor shedding, growth factor receptor activation, and drug resistance in CRC tumors. In this study, we examined the role of mutant Kras in regulating growth factor shedding and ADAM17 activity, using isogenic Kras mutant (MT) and wild-type (WT) HCT116 CRC cells. Significantly higher levels of TGF-α and VEGF were shed from KrasMT HCT116 cells, both basally and following chemotherapy treatment, and this correlated with increased pErk (phosphorylated extracellular signal regulated kinase)1/2 levels and ADAM17 activity. Inhibition of Kras, MEK (MAP/ERK kinase)1/2, or Erk1/2 inhibition abrogated chemotherapy-induced ADAM17 activity and TGF-α shedding. Moreover, we found that these effects were not drug or cell line specific. In addition, MEK1/2 inhibition in KrasMT xenografts resulted in significant decreases in ADAM17 activity and growth factor shedding in vivo, which correlated with dramatically attenuated tumor growth. Furthermore, we found that MEK1/2 inhibition significantly induced apoptosis both alone and when combined with chemotherapy in KrasMT cells. Importantly, we found that sensitivity to MEK1/2 inhibition was ADAM17 dependent in vitro and in vivo. Collectively, our findings indicate that oncogenic Kras regulates ADAM17 activity and thereby growth factor ligand shedding in a MEK1/2/Erk1/2-dependent manner and that KrasMT CRC tumors are vulnerable to MEK1/2 inhibitors, at least in part, due to their dependency on ADAM17 activity. Cancer Res; 71(3); 1071–80. ©2010 AACR.

Introduction

Ras GTPases act as critical "on-off" switches in cellular growth and survival (1). Ras is activated by several receptor tyrosine kinases (RTK) and is regulated by guanine nucleotide releasing factors (GEF) and GTPase-activating proteins (GAP). Active GTP-bound Ras interacts with a range of effectors to activate multiple downstream pathways, most notably the Raf/MAP (mitogen activated protein kinase) and PI-3 kinase pathways (1, 2). The Ras subfamily consists of 3 proto-oncogenes: c-Harvey (H)-ras, c-Kirsten (K)-ras, and c-Neuroblastoma (N)-ras (3). Ras genes become oncogenic by single point mutations, mainly in codons 12 or 13, which alter the guanine nucleotide–binding region, rendering Ras unresponsive to GAPs and resulting in constitutive activation of Ras and aberrant downstream signaling.

Somatic ras mutations most frequently occur in Kras and arise in approximately 90% of pancreatic cancers (4), 30% of lung cancers (5) and 40% to 45% of colorectal cancers (CRC; ref. 6). Kras and Braf mutations have recently been linked with resistance to anti-EGFR (epidermal growth factor receptor) monoclonal antibodies in advanced CRC (7–9). The development of drugs that inhibit oncogenic Kras in this patient group is therefore of the utmost importance. We have shown previously that chemotherapy acutely activates the protease ADAM17 (a desintegrin and metalloproteinase 17), which results in growth factor shedding, growth factor receptor (GFR) activation, and drug resistance in CRC tumors (10). In this study, we investigated the role of Kras in regulating ADAM17 activity and growth factor shedding. We also investigated the mechanism by which mutant Kras triggers growth factor shedding, in particular, the role of MAPKs in regulating this survival response.

Materials and Methods

Materials

Gefitinib, M880588, and AZD6244 (selumetinib) were obtained from AstraZeneca. PD98059 from Cell signaling, UO126 from Promega, and cetuximab from Merck. The vectors expressing HA-tagged Erk2K52R and HA-ADAM17 were
obtained from Drs. Piero Crespo (University of Cantabria, Cantabria, Spain) and Atanasio Pandiella (University of Salamanca, Salamanca, Spain), respectively (11).

**Cell culture**

All CRC cells were grown as previously described (10). Following receipt, cells were grown up, and as soon as surplus cells became available, they were frozen as a seed stock. All cells were passaged for a maximum of 2 months, after which new seed stocks were thawed for experimental use. All cell lines were tested for mycoplasma contamination at least every month. WiDR (2009)/LS174T (2008)/SW620 (2008)/RKO (2001)/HT-29 (2001)/Caco-2 (2005) cells were obtained from the American Type Culture Collection [ATCC; authentication by short tandem repeat (STR) profiling/karyotyping/isoenzyme analysis] and maintained in Dulbecco’s modified Eagle’s medium (DMEM). LoVo (2004) cells were obtained from the European Collection of Cell Cultures (ECACC; authentication by isoenzyme analysis/multilocus DNA fingerprinting/Multiplex PCR) and maintained in DMEM. HCC2998 cells were obtained from the National Cancer Institute-Frederick Cancer DCT Tumour Repository [October 2008; authentication by SNP arrays, oligonucleotide-base HLA typing, karyotyping, and STR (May 2007)] and maintained in RPMI 1640. LIM2405 cell line, established in 1992 (12), was a gift from Dr. Whitehead (Ludwig Institute of Melbourne and Vanderbilt University, Nashville, TN) and was grown in RPMI. Cell line was tested for morphology/growth rate/response to mitogens/xenograft growth/expression of brush-border and mucin-related antigens/mutational analysis (12, 13). HCT116, HKH-2, and HKe-3 CRC cells, provided by Senji Shirasawa in August 2008, were maintained in DMEM and properties of these cells—morphology/soft agar cloning efficiency/tumorigenicity/c-myc expression (14), apical-basal cell polarity/proliferation in 2- and 3-dimensional cultures, gene expression profiles (15), ras synthetic lethal interaction (16), and response to mTOR inhibitors (17)—were published. We confirmed their Kras mutational status by pyrosequencing and sequencing (April 2010).

**In vivo studies**

**In vivo** studies were conducted as previously described (10). Mice received vehicle (methocel/polysorbate buffer) or AZD6244 25 mg/kg/bid p.o. Each treatment group contained 10 animals.

**Cell viability assay**

Cell viability assays were done as previously described (18). IC50 was calculated using Prism software package. Representative results of at least 3 independent experiments are shown.

**Flow cytometric analysis and cell death measurement**

Flow cytometry was done as previously described (18). Representative results of at least 3 independent experiments are shown.

**Western blotting**

Western blot analysis was carried out as previously described (18). Anti-phospho-Erk1/2 (Thr202/Tyr204; Santa Cruz Biotechnology), anti-Kras (Calbiochem), anti-PARP (ebioscience), and anti-HA probe (Santa Cruz Biotechnology) mouse monoclonal antibodies were used in conjunction with a horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (Amersham). Anti-caspase-3 (Cell Signaling), anti-caspase-9 (Cell Signaling), anti-phospho-Akt (Ser473; Cell Signaling), anti-Akt (Cell Signaling), anti-Erk1/2 (Santa Cruz Biotechnology), anti-phospho-MEK (MAP/ERK kinase)1/2 (Cell Signaling), and anti-MEK1/2 (Cell Signaling) rabbit polyclonal antibodies were used in conjunction with an HRP-conjugated anti-rabbit secondary antibody (Amersham). Equal loading was assessed using β-tubulin (Sigma), β-actin (Sigma), or GAPDH (Biogenesis) mouse monoclonal primary antibodies.

**siRNA transfections**

Kras, Erk, and SC (scramble control) siRNAs were obtained from Dharmacon. siRNA transfections were done as previously described (18).

**ELISA and ADAM17 activity**

VEGF and TGF-α ELISA and ADAM17 activity assay was carried out as previously described (10, 18).

**Statistical analysis**

Two-way ANOVA test was used to determine the significance of change in levels of apoptosis between different treatment groups. All changes in levels of apoptosis that are described as significant had P < 0.05 values (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The nature of the interaction between AZD6244 and chemotherapy was determined by calculating the combination index (CI) according to the method of Chou and Talalay (19).

**Results**

**An activating mutation in Kras results in increased ADAM17 activity and ligand shedding in CRC cells**

To investigate the role of Kras mutations in regulating basal and chemotherapy-induced growth factor shedding, we used the HCT116 CRC cell line, which carries an endogenous activating KrasG13D point mutation and an isogenic HKH-2 clone with a disrupted KrasG13D allele (Fig. 1A, top; ref. 14). KrasMT HCT116 cells were significantly more resistant to the HER1 inhibitor gefitinib and the dual HER1/HER2 inhibitor M880588, with the IC50 doses at 72 hours approximately 5 and 7.5 times higher, respectively, than in the KrasWT cell line (Supplementary Table S1). In addition, treatment with gefitinib, M880588, and the clinically used EGF monoclonal antibody cetuximab resulted in significantly increased levels of apoptosis in the HKH-2 cell line compared with the HCT116 cell line (Supplementary Fig. S1, left). Similar results were obtained in the HK-3 cell line, a second HCT116-derived cell line containing KrasWT (Supplementary Fig. S1, right). Given the clinically established role of Kras mutations in determining sensitivity to HER-targeted therapies (7, 8), these results indicate that the cell lines are a clinically relevant model system.
Notably, we found significantly higher basal levels of TGF-α (18-fold higher) and VEGF (3-fold higher) in the culture medium of HCT116 cells than in the Kras WT isogenic cell line (Fig. 1A, bottom). Moreover, when HCT116 and HKH-2 cells were treated with 10 μmol/L 5-FU for 12 hours, a significant increase in TGF-α and VEGF shedding was observed in the HCT116 cell line but not in the HKH-2 cells (Fig. 1A). As we have previously shown that ADAM17 regulates not only TGF-α shedding but also VEGF shedding following chemotherapy treatment (10), we next directly investigated the effect of mutant Kras in regulating ADAM17 activity in the Kras MT/Kras WT paired model (Fig. 1B). Consistent with the results of the TGF-α and VEGF assays, we found significantly higher constitutive ADAM17 activity levels in HCT116 cell line than in HKH-2 cells (Fig. 1B, left). Furthermore, treatment with 5-FU resulted in a significant increase (1.6-fold) in ADAM17 activity in the Kras MT HCT116 cell line but not in the Kras WT daughter cell line (Fig. 1B, left). As no difference in total ADAM17 expression levels was observed between the two cell lines (Fig. 1B, right), these results indicate that oncogenic Kras regulates ADAM17 activity and growth factor shedding in CRC.

**Oncogenic Kras regulates TGF-α ligand shedding via a MEK1/2-dependent mechanism**

Using a human phospho-kinase array, we found higher basal levels of phosphorylated Erk1/2 in the Kras MT HCT116 cell line than in the Kras WT HKH-2 clone (data not shown). To investigate the involvement of mutant Kras and its downstream effector MEK1/2 in growth factor shedding, we used a gene-specific siRNA directed against Kras and small molecule MEK inhibitors. Kras siRNA downregulated Kras and decreased Erk1/2 activity at a concentration of 25 nmol/L in both cell lines (Fig. 2A; data not shown). Silencing Kras completely attenuated 5-FU–induced TGF-α shedding in HCT116 cells but had no effect on TGF-α ligand shedding following 5-FU treatment in HKH-2 cells (Fig. 2A, left). Of note, basal TGF-α shedding was not significantly affected by Kras silencing. Moreover, we found that 3 different MEK inhibitors—AZD6244 (20), PD98059 (21), and UO126 (22)—significantly abrogated 5-FU–induced TGF-α shedding in HCT116 cells (Fig. 2B and Supplementary Fig. S2A and B). MEK inhibition also decreased constitutive TGF-α shedding in HCT116 cells (Fig. 2B and Supplementary Fig. S2A and B). Similar effects were noted following treatment with SN-38 (the active metabolite of irinotecan; Supplementary Fig. S3A).
These studies were extended into 5 more CRC cell line models, the KrasMT (G13D) LoVo cell line, the KrasMT (G12D) LS174T cell line, the KrasMT (G12V) SW620 cell line, the BrafMT (V600E) LIM2405 cell line, and the KrasWT/BrafWT CACO-2 cell line. Similar to the data obtained with the HKH-2 KrasWT clone, we found that both constitutive and 5-FU–induced TGF-α shedding in HKe-3 cells were lower than those in the KrasMT parental HCT116 cells (Supplementary Fig. S3B). Constitutive TGF-α levels in KrasMT LoVo, LS174T, SW620, and BrafMT LIM2405 varied between 12 and 140 pg/mL but were significantly higher than in the KrasWT CACO-2 cell line (Fig. 2C and Supplementary Fig. S3C). Furthermore, a significant increase in TGF-α shedding was observed following 5-FU treatment in the KrasMT LoVo, LS174T, and SW620 and BrafMT LIM2405 cell lines (Fig. 2C and Supplementary Fig. S3C). Importantly, MEK inhibition abrogated basal and 5-FU–induced TGF-α shedding in the KrasMT and BrafMT cell lines. In contrast, basal TGF-α shedding was low in the KrasWT/BrafWT CACO-2 cell line and was unaffected by 5-FU or AZD6244 treatment (Fig. 2C).

To investigate whether the downstream MEK1/2 effector Erk1/2 plays a role in regulating TGF-α shedding, siRNA and dominant-negative approaches were used. Similar to the effect of Kras silencing, transfection of dominant-negative ErkK52R resulted in complete inhibition of TGF-α ligand shedding following 5-FU treatment but had no effect on basal TGF-α ligand shedding (Fig. 2D). Similar results were obtained using Erk siRNA (data not shown).

**Oncogenic Kras regulates ADAM17 activity via a MEK1/2-dependent mechanism in vitro and in vivo**

Collectively, these data indicate that oncogenic Kras regulates TGF-α ligand shedding and that this process is mediated via MEK1/2 and Erk1/2. We next determined whether 5-FU–induced increases in ADAM17 activity were...
similarly regulated. In HCT116 cells cotreated with a MEK inhibitor (UO126) or transfected with either Kras- or Erk-targeted siRNAs, 5-FU–induced activation of ADAM17 was abrogated (Fig. 3A). This result indicates that chemotheraphy-induced activation of ADAM17 in KrasMT HCT116 cells is Kras, MEK, and ERK dependent and is consistent with ADAM17 regulating 5-FU–induced TGF-α shedding, as we recently reported (10).

To investigate the in vivo relevance of these findings, we assessed the effect of AZD6244 on the growth of KrasMT HCT116 and KrasWT HKH-2 xenografts and the effect of MEK inhibition on tumor ADAM17 activity and TGF-α shedding. Consistent with the findings of others (14), we found that the HKH-2 KrasWT cells did not grow well in vivo, indicating the requirement for oncogenic Kras for tumor growth (data not shown). Notably, very significant growth retardation of the KrasMT HCT116 CRC xenografts was observed in mice treated with AZD6244 (Fig. 3B, left). Western blot analysis confirmed that phosphorylated extracellular signal regulated kinase (pErk)1/2 was inhibited

---

**Figure 3.** Effect of AZD6244 on ADAM17 activity in vitro and in vivo. A, ADAM17 activity in HCT116 and HKH-2 cells transfected with 25 nmol/L Kras, 10 nmol/L Erk1/2, or 25 nmol/L SC siRNAs or preincubated with 5 µmol/L UO126 for 12 hours, followed by treatment with 5-FU for 24 hours. B, left, growth rate of HCT116 in BALB/c SCID mice. Mice received either vehicle or AZD6244 p.o. for 12 days. Differences in growth were determined using Student’s t test and by calculating subsequent P values. ***, P < 0.001. Right, Western blot analysis of pErk1/2, Erk1/2, and cleaved caspase-3 in HCT116 vehicle- and AZD6244-treated xenografts. C, TGF-α levels in serum from mice bearing HCT116 tumors, 12 days following treatment with vehicle or AZD6244 (left) and normalized for tumor volume (right). D, ADAM17 activity in HCT116 tumor lysates, following 12 days of treatment with vehicle or AZD6244.
following AZD6244 treatment in HCT116 xenografts, showing the on-target effects of the inhibitor (Fig. 3B, right). In addition, treatment with AZD6244 resulted in significantly reduced levels of circulating human TGF-α in mice with KrasMT HCT116 xenografts (Fig. 3C, left). This was also the case when human TGF-α levels were adjusted to take into account the larger sizes of the tumors in the vehicle-treated mice (Fig. 3C, right). Importantly, AZD6244 treatment resulted in significant inhibition of ADAM17 activity levels in HCT116 tumors (Fig. 3D). Moreover, caspase-3 cleavage was observed in HCT116 tumors following treatment with AZD6244, indicative of apoptosis induction (Fig. 3B, right). Taken together, these results indicate that oncogenic Kras induces TGF-α shedding by regulating ADAM17 activity through MEK1/2 and Erk1/2. These data also suggest that MEK1/2 inhibition abrogates Erk1/2 signaling and thereby blocks ADAM17-mediated shedding of prosurvival growth factors (Fig. 4A).

Figure 4. Role of mutant Kras in regulating response to MEK1/2 inhibition. A, schematic overview of MEK/ERK/ADAM17 and GFR survival signaling. Top, KrasMT cells exhibit strong activity of the MEK/ERK/ADAM17 survival pathway (bold font and arrows), particularly in the context of chemotherapy treatment. AZD6244 inhibits pERK, the direct substrate of MEK, and switches off ADAM17 survival signaling through GFR and potentially through other cytokines such as TNF-α. Signaling downstream of mutant Kras occurs independently of signaling through GFR (dotted line). Bottom, KrasWT cells are less dependent on the MEK/ERK/ADAM17 pathway for survival, and Kras signaling is highly GFR dependent (bold line). B, flow cytometric analysis of apoptosis in AZD6244-treated HCT116 and HKH-2 cells. C, pERK1/2, Erk1/2, PARP, and cleaved caspase-3 and caspase-9 expression in HCT116 and HKH-2 cells treated with increasing concentrations of AZD6244 for 24 hours. D, HCT116 cells were exposed to AZD6244 alone or in combination with 50 ng/mL rhTGF-α for 48 hours and apoptosis was assessed by flow cytometry.
Role of mutant \textit{Kras} in regulating response to MEK1/2 inhibition

These \textit{in vivo} results suggest that \textit{Kras}\textsuperscript{MT} CRC cells are highly sensitive to MEK1/2 inhibition, so we next directly compared the effects of MEK1/2 inhibition on apoptosis in \textit{Kras}\textsuperscript{MT} and \textit{Kras}\textsuperscript{WT} HCT116 cells. The \textit{Kras} mutant HCT116 was significantly (8-fold) more sensitive to MEK inhibitor-induced apoptosis than the \textit{Kras}\textsuperscript{WT} cell line (Fig. 4B). Furthermore, AZD6244 treatment resulted in higher levels of PARP cleavage and caspase-3 and caspase-9 activation in \textit{Kras}\textsuperscript{MT} HCT116 cells than in the \textit{Kras}\textsuperscript{WT} daughter cell line (Fig. 4C). Similar data were obtained following treatment with other MEK inhibitors (Supplementary Fig. S4A and B). Addition of exogenous TGF-\alpha resulted in significant decreases in apoptosis induction in AZD6244-treated \textit{Kras}\textsuperscript{MT} HCT116 cells, suggesting that abrogation of TGF-\alpha shedding following MEK inhibition (Figs. 2 and 3) is an important mechanism of apoptosis induction in response to these agents (Fig. 4D).

The effect of MEK1/2 inhibition on chemotherapy-induced apoptosis was also determined. Although significant increases in apoptosis were observed when AZD6244 was combined with 5-FU in both HCT116 and HKH-2 cells, the enhancement of apoptosis was significantly greater in the \textit{Kras}\textsuperscript{MT} HCT116 cells (Fig. 5A). We extended these studies to include the panel of \textit{Kras}\textsuperscript{MT} (LS174T, SW620, LoVo, and HCC2998) and \textit{Braf}\textsuperscript{MT} (HT-29, WiDr, LIM2405, and RKO) CRC cell lines models. A supra-additive/synergistic increase in apoptosis was observed when \textit{Kras}\textsuperscript{MT} or \textit{Braf}\textsuperscript{MT} CRC cells were cotreated with 5-FU and AZD6244 (Fig. 5B and Supplementary Fig. S5A). To evaluate the level of synergy between AZD6244 and 5-FU, we used the method of Chou and Talalay and calculated CI values.

Role of \textit{Kras} in regulating response to MEK1/2 inhibition

These \textit{in vivo} results suggest that \textit{Kras}\textsuperscript{MT} CRC cells are highly sensitive to MEK1/2 inhibition, so we next directly compared the effects of MEK1/2 inhibition on apoptosis in \textit{Kras}\textsuperscript{MT} and \textit{Kras}\textsuperscript{WT} HCT116 cells. The \textit{Kras} mutant HCT116 was significantly (8-fold) more sensitive to MEK inhibitor-induced apoptosis than the \textit{Kras}\textsuperscript{WT} cell line (Fig. 4B). Furthermore, AZD6244 treatment resulted in higher levels of PARP cleavage and caspase-3 and caspase-9 activation in \textit{Kras}\textsuperscript{MT} HCT116 cells than in the \textit{Kras}\textsuperscript{WT} daughter cell line (Fig. 4C). Similar data were obtained following treatment with other MEK inhibitors (Supplementary Fig. S4A and B). Addition of exogenous TGF-\alpha resulted in significant decreases in apoptosis induction in AZD6244-treated \textit{Kras}\textsuperscript{MT} HCT116 cells, suggesting that abrogation of TGF-\alpha shedding following MEK inhibition (Figs. 2 and 3) is an important mechanism of apoptosis induction in response to these agents (Fig. 4D).

The effect of MEK1/2 inhibition on chemotherapy-induced apoptosis was also determined. Although significant increases in apoptosis were observed when AZD6244 was combined with 5-FU in both HCT116 and HKH-2 cells, the enhancement of apoptosis was significantly greater in the \textit{Kras}\textsuperscript{MT} HCT116 cells (Fig. 5A). We extended these studies to include the panel of \textit{Kras}\textsuperscript{MT} (LS174T, SW620, LoVo, and HCC2998) and \textit{Braf}\textsuperscript{MT} (HT-29, WiDr, LIM2405, and RKO) CRC cell lines models. A supra-additive/synergistic increase in apoptosis was observed when \textit{Kras}\textsuperscript{MT} or \textit{Braf}\textsuperscript{MT} CRC cells were cotreated with 5-FU and AZD6244 (Fig. 5B and Supplementary Fig. S5A). To evaluate the level of synergy between AZD6244 and 5-FU, we used the method of Chou and Talalay and calculated CI values.

Role of ADAM17 in determining response to MEK1/2 inhibition

To directly investigate the role of ADAM17 in determining sensitivity to the MEK1/2 inhibitor AZD6244, we developed an ADAM17-overexpressing HCT116 cell line model (c4), which we have previously shown to have 40-fold higher TACE activity and 4-fold higher constitutive TGF-\alpha ligand shedding than an empty vector control cell line (10). We found that stable overexpression of ADAM17 significantly reduced the levels
of apoptosis following AZD6244 treatment by flow cytometry and Western blotting analysis of PARP cleavage (Fig. 6A and B, left). Similar effects were noted following treatment with UO126 (Fig. 6A, right). Furthermore, we found that the IC_{50} doses of AZD6244 and UO126 were approximately 10- and 5-fold higher, respectively, in the ADAM17 cl4 cell line than in the EV cell line (Table 1). Moreover, the reduced sensitivity to MEK1/2 inhibition in the ADAM17-overexpressing daughter cell line correlated with increased pAkt activity in this cell line (Fig. 6B, left). AZD6244 treatment resulted in an accumulation of catalytically inactive pMEK1/2, an effect that may be due to abrogation of negative feedback between Erk1/2 and Braf and which has been described previously (Fig. 6B; refs. 23, 24). To rule out cell line–specific effects, KrasMT LoVo cells were transiently transfected with ADAM17 and cells were evaluated for their response to AZD6244 treatment. Similar to the data obtained with ADAM17 cl4 clone, we found reduced levels of PARP cleavage in ADAM17-transfected LoVo cells (A4) treated with AZD6244 compared with control cells (Fig. 6B, right).

Moreover, the protective effect of ADAM17 overexpression again correlated with reduced pAkt downregulation following AZD6244 treatment. Notably, ADAM17 overexpression significantly inhibited the level of apoptosis and synergistic interaction obtained when AZD6244 was combined with 5-FU.

Table 1. EV and AD17 cl4 cells treated with increasing doses of AZD6244 or UO126 for 72 hours and cell viability determined by MTT assay

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC_{50}, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV</td>
<td>AD17 cl4</td>
</tr>
<tr>
<td>AZD6244</td>
<td>0.33</td>
</tr>
<tr>
<td>UO126</td>
<td>1.88</td>
</tr>
</tbody>
</table>
treatment in HCT116 cells (Fig. 6C). In addition, inhibiting TGF-α using a siRNA approach was insufficient to reverse the protective effect of ADAM17 on AZD6244-induced apoptosis (data not shown). This is in keeping with our previous findings that ADAM17 is a key "shedase" for multiple growth factors (e.g., TGF-α, IGF-1, VEGF) and activator of their respective prosurvival receptors (10). Importantly, overexpression of active ADAM17 in vivo very significantly attenuated the anti-tumor activity of AZD6244 treatment (Fig. 6D). Collectively, these data indicate that ADAM17 activity regulates the sensitivi-
ty of CRC tumors to AZD6244 treatment and further confirm that inhibition of ADAM17 activity is a key mecha-
nism of apoptosis induction following MEK1/2 inhibition in KrasMT CRC cells.

Discussion

Use of the EGFR-targeted monoclonal antibody cetuximab in first- and second-line treatment of advanced CRC patients has resulted in increased objective responses and progression-
free survival; however, this benefit is limited to patients with KrasWT tumors (25, 26). Therefore, the identification of therapeutic strategies to target KrasMT tumors is urgently needed in this disease. We have found that chemotherapy acutely activates ADAM17, which results in growth factor shedding, GFR activation, and drug resistance in CRC tumors (10). In light of this, we investigated the role of Kras mutations in regulating growth factor shedding and ADAM17 activity.

Both constitutive and 5-FU–induced TGF-α and VEGF shedding were significantly higher in the KrasMT cell line, and this correlated with enhanced ADAM17 activity. Gango-
rosa and colleagues have previously shown that oncogenic Kras(12V) and Hras(12V) could result in increased mRNA transcripts for TGF-α and secreted TGF-α protein in RIE-1 cells (rat small intestine cells; 27). However, the present study is the first to suggest that oncogenic Kras controls chemother-
apy-induced growth factor shedding via the regulation of ADAM17 activity. We found that the increase in chemother-
apy-induced TGF-α shedding in the KrasMT HCT116 cell line was completely abolished when MAP kinase signaling was inhibited at the level of Kras, MEK, or ERK. Furthermore, treatment with MEK1/2 inhibitors also resulted in significant decreases in basal TGF-α shedding. Moreover, we found that MEK1/2 inhibitors blocked 5-FU–induced TGF-α shedding in a panel of cell lines, with KrasG12D mutation, KrasG12D mutation, or Braf (V600E) mutation, indicating that these effects are not dependent on the type of Kras mutation and are also applicable to cells carrying a mutation in the down-
stream Braf kinase. Similar to the data obtained in the KrasWT HCT116 clone, we found that TGF-α shedding was unaffected by 5-FU or AZD6244 treatment in the KrasWT/BrafWT CAPO-
2 cell line. Importantly, we further found that Kras silencing and MEK1/2 or Erk1/2 inhibition completely abrogated 5-FU–induced increases in ADAM17 activity in HCT116 cells. This is the first study showing that the clinically relevant MEK-
inhibitor AZD6244 abrogates ADAM17 activity in KrasMT CRC xenografts and decreases circulating levels of TGF-α. Taken together, these findings indicate that oncogenic Kras controls chemotherapy-induced growth factor shedding via regulation of an MEK/Erk/ADAM17 signaling axis (Fig. 4A). These results are consistent with previous data showing that Erk could phosphorylate and associate with ADAM17 (11).

Previous reports have shown that KrasWT/BrafWT CRC cells exhibit decreased sensitivity to MEK inhibitors UO126 and CI-1040 or AZD6244 compared with KrasMT cell lines, using soft agar colony formation and MTT assays (28, 29). Having shown key differences in Kras/MEK/Erk/ADAM17 survival signaling between the KrasMT HCT116 and the KrasWT HKH-2 cell lines, we evaluated the effect of MEK inhibition on 5-FU–induced cell death in this model. Using several MEK inhibitors, we found that the HCT116 cell line is highly dependent on MEK for its survival, as MEK inhibition resulted in significant induction of apoptosis. In contrast, the KrasWT cell line was significantly less sensitive to apoptosis induced by MEK inhibitors. Furthermore, when MEK inhibitors were combined with 5-FU, we found a more potent increase in apoptosis in the KrasMT HCT116 cell line than in the KrasWT HKH-2 cell line. In addition, a synergistic activation of apoptosis was observed in vitro when AZD6244 was combined with 5-FU treatment in a broad panel of KrasMT and BrafMT CRC cell lines. Importantly, treatment with AZD6244 resulted in tumor regression and sustained suppression of HCT116 xenografts growth, and this was associated with significantly reduced tumor ADAM17 activity levels, reduced TGF-α levels in serum, and increased apoptosis. One previous study reported that the HCT116 cell line is insensitive to MEK inhibitors (28); however, in agreement with our results, other studies have shown that this cell line is sensitive to MEK1/2 inhibition (29). Moreover, using in vivo models, we have shown that AZD6244 treatment dramatically attenuates the growth of KrasMT HCT116 xenografts.

The importance of ADAM17 activity as a mediator of resistance to AZD6244 treatment was directly shown using ADAM17-overexpressing HCT116 cell lines. We show for the first time that ADAM17 overexpression abrogates sensitivity to AZD6244 treatment alone and in combination with 5-FU treatment. Importantly, overexpression of ADAM17 protected HCT116 xenografts from the growth-inhibitory effects of AZD6244 treatment in vivo. We have previously shown that overexpression of ADAM17 increases activity of not only HER1/3 but also other prosurvival receptors such as IGF-1, VEGF, and Ephrin receptors (10), which may result in enhanced downstream survival signaling and resistance to MEK1/2-targeted agents (Fig. 4A).

In conclusion, we provide evidence that oncogenic Kras controls ADAM17 activity and growth factor shedding following chemotherapy treatment in an MEK/Erk-dependent manner. Furthermore, we have found that KrasMT CRC cells depend on Kras/MEK/Erk/ADAM17 pathway for their survival and that KrasMT CRC tumors are vulnerable to MEK1/2 inhibitors (at least in part) due to their dependency on ADAM17 activity (Fig. 4A). These data suggest that inhibition of MEK1/2 in combination with chemotherapy treatment may represent a promising treatment strategy in KrasMT and BrafMT CRC tumors and provide a preclinical rationale for the ongoing dose finding and phase II study of
AZD6244 in combination with irinotecan in the second-line treatment of patients with *Kras* or *Braf* mutation-positive advanced or metastatic CRC (http://clinicaltrials.gov/ct2/show/NCT01116271). Furthermore, we provide strong evidence that inhibition of ADAM17 activity is a key mechanism of apoptosis induction following MEK1/2 inhibition in *Kras* MT CRC tumors.

**Disclosure of Potential Conflicts of Interest**

P.G. Johnston: directorship Almac Diagnostics, Society Translational Oncology; shareholdings, Fusion Antibodies, Almac Diagnostics; consultancy.

**References**


Roche Pharmaceuticals, Sanofi-Aventis, Chugai Pharmaceuticals; contracted research, AstraZeneca, Amgen. The authors declare no competing interests.

**Acknowledgments**

We thank AstraZeneca for providing us with AZD6244, gefitinib, and erlotinib.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 28, 2010; revised October 29, 2010; accepted December 2, 2010; published OnlineFirst December 10, 2010.
Oncogenic *Kras* Promotes Chemotherapy-Induced Growth Factor Shedding via ADAM17

Sandra Van Schaeybroeck, Joan N. Kyula, Audrey Fenton, et al.


**Updated version**
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-0714

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/12/10/0008-5472.CAN-10-0714.DC1

**Cited articles**
This article cites 29 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/3/1071.full.html#ref-list-1

**Citing articles**
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/71/3/1071.full.html#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.