Original Article

Dovitinib (TKI258), a multi-target angiokinase inhibitor, is effective regardless of KRAS or BRAF mutation status in colorectal cancer

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Abstract: Introduction: We aimed to determine whether KRAS and BRAF mutant colorectal cancer (CRC) cells exhibit distinct sensitivities to the multi-target angiokinase inhibitor, TKI258 (dovitinib). Materials and methods: We screened 10 CRC cell lines by using receptor tyrosine kinase (RTK) array to identify activated RTKs. MTT assays, anchorage-independent colony-formation assays, and immunoblotting assays were performed to evaluate the in vitro anti-tumor effects of TKI258. In vivo efficacy study followed by pharmacodynamic evaluation was done. Results: Fibroblast Growth Factor Receptor 1 (FGFR1) and FGFR3 were among the most highly activated RTKs in CRC cell lines. In in vitro assays, the BRAF mutant HT-29 cells were more resistant to the TKI258 than the KRAS mutant LoVo cells. However, in xenograft assays, TKI258 equally delayed the growth of tumors induced by both cell lines. TUNEL assays showed that the apoptotic index was unchanged following TKI258 treatment, but staining for Ki-67 and CD31 was substantially reduced in both xenografts, implying an anti-angiogenic effect of the drug. TKI258 treatment was effective in delaying CRC tumor growth in vivo regardless of the KRAS and BRAF mutation status. Conclusions: Our results identify FGFRs as potential targets in CRC treatment and suggest that combined targeting of multiple RTKs with TKI258 might serve as a novel approach to improve outcome of patients with CRC.

Keywords: Colorectal cancer, FGFR, KRAS, BRAF, Dovitinib (TKI258), multi-target angiokinase inhibitor

Introduction

Colorectal cancer (CRC) is the third-most commonly diagnosed cancer in males and the second-most commonly diagnosed cancer in females worldwide [1]. Moreover, CRC incidence is increasing rapidly in several historically low-risk countries such as countries in Eastern Asia and Eastern Europe [2]. Because conventional anti-cancer drugs are not adequate for improving CRC-treatment outcome, we must understand the molecular biology of colon cancer and identify relevant molecular targets to biologically modulate the cancer.

Numerous inhibitors that target various receptor tyrosine kinases (RTKs) have been confirmed to inhibit tumor survival and angiogenesis in preclinical trial models of CRC [3-5]. Bevacizumab, a humanized monoclonal antibody that targets vascular endothelial growth factor A (VEGF-A) was approved for first- or second-line use in metastatic CRC, in combination with conventional chemotherapy [6-8]. Furthermore, certain epidermal growth factor receptor (EGFR)-inhibiting monoclonal antibodies such as cetuximab and panitumumab showed modest efficacy in monotherapy or combination therapy [9-12]. However, the results of several studies in which these RTK inhibitors were used on CRC patients showed limited effect, implying that more effective therapeutic RTK inhibitors are required.

Fibroblast growth factors (FGFs), which promote angiogenesis and tumor growth by binding to tyrosine kinase. FGF receptors (FGFRs), are reported to be overexpressed in CRC patients...
FGFR genes have been reported to potentially promote tumor growth and invasion in CRC [13, 15, 16], and FGFR signals were implicated in the intrinsic resistance to EGFR inhibitors [17] in non-small cell lung cancer (NSCLC). Given these results, FGFR inhibitors are considered one of the potential RTK inhibitors that can be used to treat CRC patients; this is not only because FGFR is overexpressed in CRC, but also because this treatment might help overcome the resistance to EGFR inhibitors.

TKI258 is an orally active small molecule that potently inhibits the activity of multiple RTKs including FGFRs, platelet-derived growth factor receptors (PDGFRs), and VEGF receptors (VEGFs), which participate in tumor growth, survival, angiogenesis, and vascular development [18] through both direct and indirect mechanisms. Inhibiting multiple angiokinasises - mainly FGFRs - led to the suppression of their downstream signaling including signaling by RAS-RAF-MAPK molecules and PI3K-AKT related molecules that are mainly involved in cell proliferation, cell survival, and tumor invasion [19].

Mutations in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), which occur in approximately 40% of CRC patients, is considered one of the major negative predictive factors in the treatment response in patients receiving EGFR-directed antibodies [20, 21]. In the absence of KRAS mutations, resistance to EGFR inhibitors has been reported to be potentially caused by genetic alterations of molecules related to RAS-RAF-MAPK signaling [19, 22]. Furthermore, mutation in v-raf murine sarcoma viral oncogene homolog B1 (BRAF) appears to mediate cetuximab resistance in the absence of KRAS mutation [23, 24], and. BRAF mutations have been widely reported to occur only in KRAS-negative colon carcinomas, suggesting that BRAF/KRAS activating mutations might be alternative genetic events in CRC [26-28]. Recently, the mutually exclusive BRAF/KRAS mutation status was suggested to be related to RTK-inhibitor sensitivity in CRC. BIBF 1120, another multi-target angiokinase inhibitor showed efficacy when combined with afatinib against CRC with KRAS mutation in vitro [29]. BIBF 1120 also targets FGFR and PDGFR, but mainly targets VEGFR, and no previous studies reported KRAS/BRAF mutant CRC and FGFR inhibitor sensitivity. We surmised that KRAS or BRAF mutation status might affect FGFR inhibitor - TKI258 - sensitivity in CRC.

In this study, to identify better RTK inhibitors that can improve CRC treatment, we determined whether genetic aberration of a novel target's downstream signals might affect the efficacy of an RTK inhibitor. First, we hypothesized that inhibition of FGFR will efficiently suppress tumor growth in CRC, and then we hypothesized that the KRAS and BRAF mutant CRC cell lines will exhibit distinct sensitivities to the TKI258, which mainly targets FGFR. The results of this study could lead to the identification of predictive biomarkers and thus facilitate the selection of CRC patients who are likely to benefit from treatment with the FGFR inhibitor. We investigated the anti-tumor activity of TKI258 in CRC cell lines carrying KRAS or BRAF mutations, in vitro and in vivo, to determine whether the sensitivity of these cells to the inhibitor depends on specific gene alteration.

Materials and methods

Cell lines and cell culture

The 6 human CRC cell lines used in this study - KM12SM, KM-12L4, Colo320DM, SNU-C4, SNU-1235, and SW48 - were with no known KRAS or BRAF mutation. And the 10 human CRC cell lines with any of KRAS or BRAF mutation used in this study - DLD-1, HCT-15, COLO205, SW480, HCT-116, LoVo, WiDr, CaCo2, RKo, and HT-29 - were obtained from American Type Culture Collection (Bethesda, MD). All cell lines were cultured in MEM or RPMI-1640 medium supplemented with 10% FBS (GIBCO), 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified 5% CO2 incubator. Among the 10 CRC cell lines used in this study [25, 30, 31], 5 CRC cell lines had only KRAS mutations (DLD-1, LoVo, SW480, HCT-15, and HCT-116) and 5 CRC cell lines had only BRAFV600E mutations (HT-29, COLO205, RKO, CaCo2, and WiDr). There were no serum starvation or stimulation throughout the experiments.

Receptor tyrosine kinase inhibitor

TKI258 (4-amino-5-fluoro-3-[5-(4-methylpipera-zin-1-yl)-1H-benzoimidazol-2-yl] quinolin-2(1H)-one, formerly known as CHIR258), which targets FGFRs, PDGFRs, and VEGFRs, was
purchased from Eurasian Chemicals Pvt. Ltd. We prepared 10 mM stock solutions of the drug and stored them at -20°C.

**Receptor tyrosine kinase array**

To screen for the activity of specific RTKs, we used the Human Phospho-RTK Array (R&D Systems) according to the manufacturer’s protocols. Briefly, 300 μg of proteins were added to a blocked membrane and incubated at 4°C. After washing, horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibodies (1:2,000) were added to each membrane and incubated. After washing the membranes further, proteins were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham). The activated RTK levels were quantitated using GenePix Pro 4.1 software (Axon Instruments) and normalized using the positive control's intensity in each membrane. RTK signal intensity was measured as the difference between the mean of each RTK's intensity and mean of negative signal intensity. Relative RTK intensity was defined as the RTK signal intensity of each sample divided by the positive signal intensity of each membrane.

**Immunoblotting analysis**

Cells were seeded in 6-well plates and incubated for overnight at 37°C. On the next day, culture media was replaced with serum-free media and the cells were again incubated overnight. Cells were then incubated with the drug (1 μM TKI258 or PBS control) in serum-free media at 37°C for indicated times (0, 0.5, 1, 3, and 6 h). Cells were lysed in NP-40 lysis buffer and proteins were resolved using 6% or 10% SDS polyacrylamide gels and then transferred to PVDF membranes (GE Healthcare) by using electroblotting. The following primary antibodies were used in the experiments: p-FGFR1 (Y653/ Y654) (Santa Cruz Biotechnology), p-Akt (S473), AKT, p-ERK1/2 (T202/Y204), ERK1/2, p-PI3K (Y458/Y199), PI3K, p-4EBP1 (Thr37/46), 4EBP1, p-p70S6K (T389), and p70S6 (Cell Signaling). Anti-GAPDH antibody (Abcam) was used to control for equal loading.

**Cell-viability assay**

Cells were seeded in 96-well plates at a density of 5,000 cells/well and incubated at 37°C. TKI258 was serially diluted using culture media, and various concentrations of the drug were added to each well and incubated for 3 days. MTT (3-[4,5-dimethylthiazol]-2,5-diphenyl tetrazolium) was then added to the medium for 4 h, the medium was removed, the precipitate was dissolved in DMSO, and the absorbance was read at 570 nm. The drug concentrations required to inhibit cell growth by 50% (IC₅₀) were calculated through interpolation of the dose-response curves by using CalcuSyn software (Biosoft).

**Anchorage-independent colony formation assay**

We added 0.6% Noble agar (Difco) as a basement into each well of culture plates. After the agar solidified, cells (3,000 cells/well) were suspended in 0.4% Noble agar mixed in culture media containing 10% FBS and plated in triplicate on top of the basement. Plates were incubated at 37°C for 3 weeks and colonies featuring a diameter > 200 μm were counted and photographed under an inverted microscope.

**Reverse transcription (RT)-PCR analysis**

Total RNA was isolated using the Trizol reagent (Invitrogen) and cDNAs were synthesized using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. RT-PCR reactions were performed using TaqGold DNA polymerase (Invitrogen), and the amplified products were separated on ethidium bromide gels containing 1.2% agarose. The house-keeping gene GAPDH was selected as the positive control. The primer set used for RT-PCR amplification of the gene encoding FGFR1 was the following: forward 5'-CAT CCC CAG AAA AGA TGG AA-3', and reverse 5'-CCT CCC CTG TTC CCA TTA CT-3'.

**Xenograft study**

Female BALB/c nu/nu mice, 6-8-weeks old, were implanted subcutaneously with LoVo cells (2.0 × 10⁷) or HT-29 cells (1.0 × 10⁷) in the flank. Once the tumors could be measured (mean tumor volume of 200-300 mm³), the mice were assigned to 2 groups, the vehicle only group and the TKI258-treated group (n = 4 per group in the case of LoVo cells; n = 6 per group, HT-29 cells). TKI258 was administered orally at 70 mg/kg per day for 4 weeks. Tumor volumes were measured every other day. After
mice were sacrificed, all the organs were thoroughly examined, and formalin-fixed paraffin-embedded tumors were subjected to pathological analysis.

Immunohistochemistry (IHC)

Following deparaffinization and rehydration, tumor sections were treated with an antigen-retrieval solution (DAKO). Endogenous peroxidases were quenched using 3% H$_2$O$_2$. Sections were stained using antibodies against FGFR1 (1:50, Abcam), p-ERK (1:50, Cell Signaling Technology), p-AKT (1:100, Abcam), CD31 (1:100, Abcam), and Ki-67 (1:100, DAKO), which were diluted in an antibody-dilution buffer (DAKO). Staining was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB; DAKO). Lastly, each slide was counterstained with hematoxylin for 5 min, washed with water, mounted, and covered with a coverslip.

TUNEL assay

DNA fragments in tissue sections were detected using a TACS® 2 TdT DAB Kit (Trevigen).
Briefly, the enzyme terminal deoxynucleotidyl transferase (TdT) was used to incorporate digoxigenin-conjugated dUTP at the ends of DNA fragments. The signal of TdT-mediated dUTP nick-end labeling (TUNEL) was then detected using an anti-digoxigenin antibody conjugated with peroxidase. TUNEL-positive cells were counted in 5 random high-power fields per section and are reported as a percentage of positive cells in each cellular compartment.

**Statistical analysis**

The statistical significance of differences between 2 groups of data was assessed using the unpaired t test in SPSS 21.0 software; a p-value of < 0.05 was considered statistically significant.

**Results**

**RTK activity in CRC cell lines**

To screen for RTK activity in KRAS or BRAF mutant 10 CRC cell lines, we used a human phospho-RTK array (Figure 1A). In addition to highly activated EGFR, several other druggable targets such as ErbB2, HGF-R, IGF-IR, FGFR1, FGFR3, and VEGFR3 were also highly activated in CRC cell lines. Various RTKs were more activated in the KRAS mutant cell lines than in the BRAF mutant cell lines (Figure 1B). Specifically, EphA7, Mer, TrkA, and FGFR1 appeared to be...
relatively more activated in KRAS mutant cell lines, whereas EphB2, Insulin R, IGF-IR, and VEGFR3 were relatively more activated in BRAF mutant cell lines (Figure 1C). Because FGFR is considered one of the potential targets in CRC, our results demonstrating that FGFR1 and FGFR3 are highly activated in CRC cell lines provides a strong rationale for treating CRC with an FGFR inhibitor.

**TKI sensitivity based on genetic variations**

Because TKI258 is an ATP-competitive inhibitor belonging to Class III–V RTK inhibitors that are especially sensitive against FGFRs (FGFR1/2/3, with kinase IC\(_{50}\) values of 8-13/21/9-18 nmol/L, respectively [32, 33]), we used TKI258 as a novel tyrosine kinase inhibitor (TKI) to inhibit FGFRs in CRC. First, to test whether the KRAS or BRAF mutation status affected cell viability in response to the FGFR inhibitor, we measured the anti-tumor activity of TKI258 against 16 CRC cell lines (6 KRAS\(^{wt}\)BRAF\(^{wt}\), 5 KRAS mutant cell lines, and 5 BRAF mutant cell lines) (Figure 2). CRC cell lines without any of KRAS or BRAF mutation (KRAS\(^{wt}\)BRAF\(^{wt}\)) were more sensitive to TKI258. We could not tell whether KRAS or BRAF mutation decides sensitivity to TKI258 with this MTT assay result, but there was trend toward KRAS mutant CRC cell...
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90 lines being more sensitive to TKI258 compared to BRAF mutant (IC$_{50}$ (nM): KRAS mutant cell lines: 1274.6 ± 1015.9; BRAF mutant cell lines: 1727.6 ± 867.5). Immortalized normal cell lines (CCD-841COTR and CCD-18CO) were more resistant than CRC cell lines (IC$_{50}$ of 5000 nM, data not shown on figure).

We compared FGFR1 mRNA and protein levels and FGFR1 activity among the CRC cell lines by using RT-PCR and western-blotting analyses; FGFR1 is considered the major target of TKI258. There were no correlation of phosphorylated FGFR1 level and sensitivity to TKI258 measured by MTT assay. The LoVo (KRAS$^{G13D}$) and HT-29 (KRAS$^{wt}$ BRAF$^{V600E}$) cell lines were chosen because phosphorylated FGFR1 was similarly overexpressed in these cells (Figure 3). Assays to determine in vitro anti-tumor effect of TKI258 against LoVo (KRAS$^{G13D}$ BRAF$^{wt}$) and HT-29 (KRAS$^{wt}$ BRAF$^{V600E}$) cell lines are performed. Both cell lines showed a dose-dependent inhibition of cell growth in cell-viability assays (Figure 4A). Whereas LoVo cells were highly sensitive to TKI258 (IC$_{50}$ of 130 nM), HT-29 cells were relatively more resistant to TKI258 treatment (IC$_{50}$ of 2,530 nM). When we used the soft agar colony-formation assays to evaluate the anchorage-independent in vitro anti-tumor activity of TKI258 (Figure 4B, 4C), almost no colony was formed in the case of the KRAS mutant LoVo cell line after adding 500 nM TKI258. However, the BRAF mutant HT-29 cells were resistant to TKI258 and showed only 3% reduction in colony formation relative to control.

Figure 5. Changes in downstream signaling molecules after TKI258 treatment in LoVo and HT-29 cells. KRAS mutant LoVo and BRAF mutant HT-29 cells were treated with 1 μM TKI258 in vitro to evaluate the time-dependent effect on downstream signaling molecules by using western-blotting analysis. PI3K-AKT and RAS-RAF-ERK pathways appeared to be inhibited by TKI258 in LoVo cells, but the PI3K-AKT pathway was not inhibited in HT-29 cells and, in these cells, the RAS-RAF-ERK pathway appeared to be activated. (24 hour treatment data not shown, since cells were damaged due to long serum free status).
Changes in downstream signaling following TKI258 treatment

To evaluate how downstream signaling molecules are affected after drug treatment in time-course, LoVo and HT-29 cells were treated with 1 μM TKI258 in vitro and then examined using western-blotting analysis (Figure 5). TKI258 showed profound, sustained inhibition of FGFR1 phosphorylation after 30-min treatment in the KRAS mutant LoVo cells, but not in the BRAF mutant HT-29 cells. In HT-29 cells, FGFR1 appeared to show increased and sustained activation after 30 min of TKI258 treatment. Analyzing the PI3K-AKT signaling pathways revealed that TKI258 treatment reduced the levels of phosphorylated PI3K, AKT, and P70S6K without altering the levels of phosphorylated 4EBP1 in LoVo cells; however, no alteration in downstream signaling molecules was detected in HT-29 cancer cells. By contrast, TKI258 treatment increased the level of activated ERK in both LoVo and HT-29 cells.

In vivo efficacy and pharmacodynamic marker evaluation using TKI258

To evaluate the anti-tumor effect of TKI258 in vivo, a daily oral dose of TKI258 of 70 mg/kg was administered to mice bearing subcutaneous LoVo or HT-29 human tumor xenografts. Contrasting the in vitro results, no difference in in vivo anti-tumor effect was observed between the 2 xenograft models featuring distinct mutation statuses: TKI258 delayed tumor growth equally in both cell lines compared with the control group (Figure 6). No major toxicity was detected in the treated mice in both groups, and the bodyweights of these mice were not different (data not shown).

Next, when we evaluated the pharmacodynamics of target modulation in the tumors (Figure 7), TKI258 treatment was found to substantially reduce the levels of phosphorylated FGFR1 in both xenografts. Cells positive for phosphorylated AKT were not markedly reduced after the treatment, but cells positive for phosphorylated...
ERK were reduced in the TKI258-treated group. This result differed from the in vitro western-blotting data, which showed that p-ERK staining was not reduced. Large areas of necrosis were also detected in TKI258-treated tumors when compared with control tumors. When CD31 was stained to evaluate the anti-angiogenic activity of TKI258 in vivo, the size and numbers of CD31-positive blood vessels within the tumors were observed to be diminished after the treatment in both xenografts. Furthermore, actively proliferating tumor cells that were identified using Ki-67 staining were markedly reduced in TKI258-treated mice. Moreover, the TUNEL assay used to assess the effect of TKI258 on apoptosis showed no change in the apoptotic index in either xenograft (Figure 8). These results suggest that TKI258 inhibited tumor growth in vivo and that the effect of TKI258 in target-molecule inhibition was similar in KRAS mutant and BRAF mutant cell lines. Taken together, our results suggest that the KRAS and BRAF mutation status does not affect tumor-growth inhibition and changes in target molecules induced by the FGFR inhibitor in vivo.

Discussion

Numerous RTK inhibitors are currently available that target various cancers and these inhibitors are continuing to show clinical success in various cancer treatments. To treat metastatic CRC, anti-EGFR monoclonal antibodies such as cetuximab and panitumumab and anti-angiogenic agents such as bevacizumab and regorafenib are becoming widely used. However, because certain disappointing results such as drug resistance have been obtained, more effective RTK targets are sought for CRC treatment.

We screened 10 KRAS or BRAF mutant CRC cell lines using an RTK array to evaluate which RTKs are up-regulated and to check for novel target-RTK candidates. EGFR was the single most activated RTK, and we also detected other activated RTKs such as HGFR, ErbB2,
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FGFR1, FGFR3, and VEGFR3, which already have inhibitors being used in other types of cancer treatment. Certain RTKs showed varying activity patterns according to KRAS or BRAF mutation status: EphA7, Mer, TrkA, and FGFR1 were highly activated in 5 KRAS mutant cell lines, and EphB2, Insulin R, IGF-IR, and VEGFR3 were highly activated in 5 BRAF mutant cell lines. Among these RTKs, although EphA7 and EphB2 belong to the same ephrin-receptor family, EphA7 was markedly upregulated in KRAS mutant cell lines and EphB2 was upregulated in BRAF mutant cell lines. Ephrin-family proteins are known to affect cytoskeletal and cell-adhesion mechanisms and thus regulate cell position and motility. Interestingly, EphA7, which acts as a tumor suppressor in follicular lymphoma [34], was reported to be down-regulated in CRC relative to normal tissue [35]. The finding for VEGFR3 is also surprising since VEGFR3 is generally associated with lymphangiogenesis. The clinical relevance of these findings in relation to KRAS or BRAF mutation status of CRC remains unknown and further studies are warranted.

FGFR is one of the RTKs that plays key biological roles in cancer, and FGFR has emerged as a potential therapeutic target [36-38]. Recently, FGFR1, FGFR2, or FGFR3 overexpression in CRC was reported to result in increased growth and invasion in preclinical or clinical settings [13, 15, 16, 39]. In this study, 10 CRC cell lines we tested showed overexpressed FGFRs (relative RTK intensity among KRAS-mutant cell lines: FGFR1 > FGFR3 > FGFR2α; and among BRAF-mutant cell lines: FGFR3 > FGFR1 > FGFR2α; almost no FGFR4 was detected in either cell line); this result identifies FGFR as a potential target in CRC treatment. Moreover, considering that tumor progression is mediated not only by the malignant cancer cells themselves but also by surrounding non-malignant stromal cells such as fibroblasts, FGFR is considered an attractive candidate target and several drugs against this RTK family are being developed. Because the initiation and progression of most cancers rarely depend on a single growth factor or a simple genetic alteration, multi-target RTK inhibition might represent an alternative strategy to inhibit complex signaling.

Figure 8. Ki-67 staining and TUNEL assay of xenograft models. Ki-67 staining and TUNEL assay were also performed (A). TKI258-treated mice showed a significant reduction of Ki-67-positive cells in tumors induced by both cell lines (B), but the numbers of TUNEL-positive cells were not statistically different (C). *P < 0.05.
pathways in cancer cells, even if increased toxicity is occasionally detected. Herein, we have described the anti-tumor activities of TKI258, a multi-target angiokinase inhibitor, by inhibiting Class III, IV, and V RTKs, notably those of the FGFR, VEGFR, and PDGFR subfamilies.

We evaluated 16 CRC cell lines for their sensitivity to TKI258 in relation to the KRAS or BRAF mutation status. Our in vitro data showed that TKI258 exhibited significant anti-tumor activity against CRC cell lines without any of KRAS or BRAF mutation, compared to CRC cell lines with any of KRAS or BRAF mutation. Among KRAS or BRAF mutant CRC cell lines, it was hard to tell which mutation type was more sensitive to TKI258, but there was a trend toward KRAS mutant CRC cell lines being more sensitive. In KRAS mutant HT-29 cells, TKI258 inhibited PI3K-AKT activities, whereas these activities in BRAF mutant LoVo cells were not affected by the treatment. ERK appeared to be activated in both cell lines after treatment with TKI258, which suggests that KRAS or BRAF mutation leading to the activation of the RAS-RAF-MAPK pathway might function as a resistance mechanism against the FGFR inhibitor. Studies conducted using EGFR inhibitors also suggested that drug resistance results from the KRAS mutation directly activating RAS-RAF-MAPK downstream signaling [40, 41]. Interestingly, ERK activation peaked at 1 hour after treatment and then appeared to diminish in LoVo cells, where p-ERK levels increased in a time-dependent manner in HT-29 cells. TKI258's ability to inhibit multiple angiokinases might have played a role here. For instance, initial inhibition of FGFR by the drug might have blocked the PI3K-AKT pathway but activated the RAS-RAF-MAPK pathway, and the sequential delayed effects of PDGFR or VEGFR inhibition might have influenced RAS-RAF pathway to inactivate ERK subsequently. Conversely, because KRAS functions not only through the RAS-RAF-MAPK pathway but also through multiple effector-mediated cytoplasmic signaling networks including the PI3K pathway [42], inhibition of the PI3K pathway by the drug might have subsequently affected RAS-RAF-MAPK signaling in KRAS mutant cells. Following TKI258 treatment in the BRAF mutant HT-29 cells, an up-regulation of p-ERK was detected, whereas p-PI3K and p-AKT were repressed. Previously, anchorage-independent growth and tumorigenic growth of BRAF mutation-positive CRC cells were shown to depend on the function of the mutant BRAF and to depend critically on persistent ERK activation [43]. Furthermore, TKI258 might have not altered PI3K pathway in the BRAF mutant HT-29 cells because HT-29 cell lines are known to carry a PI3K-activating E545K mutation, whereas LoVo cells do not exhibit any PI3K mutation.

Because our in vitro data revealed that LoVo cell lines were more sensitive than HT-29 cell lines to TKI258, we expected tumors generated by implanted LoVo cells to have regressed more than tumors generated by the HT-29 cells. However, relative to control, both of the xenografts tested in our study exhibited a significant reduction in tumor growth regardless of the KRAS or BRAF mutation status. Our IHC results were also distinct from the in vitro immunoblotting results after TKI258 treatment: p-ERK-positive cells were reduced but p-AKT-positive cells were similar in both xenografts, whereas the in vitro study showed increased p-ERK in both cell lines but reduced p-AKT in the LoVo cell line considering the crosstalk between the pathways, resulting in drug resistance. Recently, concomitant AKT/mTOR inhibition was suggested to be required for BRAF-mutant CRC [44], and we could talk into account including an mTOR inhibitor as a combination partner in further studies. Treatment with the FGFR inhibitor has been reported to reduce Ki67 staining and increase TUNEL staining in prostate cancer cells and head and neck cancer cells, implying that the inhibitor not only suppressed cell proliferation but also induced apoptosis [45, 46]. In our result, TKI258 treatment inhibited cell proliferation and angiogenesis but did not induce apoptosis. This disparity could be explained related to apoptosis induction as the distinct origins of the tumor and the inhibition of different subclasses of FGFRs.

One explanation of the in vitro and in vivo differences might be from the innate difference of two systems including the delayed effect of the drug, because we used a 4-week treatment in the xenograft study. Another possible explanation is the ability of TKI258 to target multiple RTKs and its effect on the tumor microenvironment: TKI258 targets not only FGFRs but also VEGFR and PDGFR. A key factor of the tumor microenvironment is an abnormal but abundant vasculature, which supplies sufficient nutrients and oxygen for sustenance and ensures...
tumor progression [47]. The VEGFR-inhibiting feature of the multi-target inhibitor TKI258 might play a critical role in equally suppressing proliferation of both KRAS and BRAF mutated tumor cells in vivo; we observed that CD31-stained vessel size and numbers were diminished after treatment with TKI258. In addition, not only VEGFR but also FGFR signaling potentially affects the microenvironment, because one of the well-known direct effects of FGF signaling is the promotion of angiogenesis through endothelial cells and other vascular cells [48]. Hence, TKI258 will show anti-angiogenic effect not only by inhibiting VEGFRs, but also by inhibiting FGFRs.

FGF and FGFRs are considered to act synergistically with the VEGF pathway to promote neo-vascularization, implying one mechanism of adaptive resistance to VEGF inhibitor at least in renal cell carcinoma or pancreatic cancer [49]. Because EGFR and VEGF inhibitors are the only approved RTK inhibitors widely used to treat CRC, TKI258 targeting FGFRs might show promising results among CRC patients who are refractory to EGFR or VEGF inhibitors. Previously, LoVo and HT-29 cells were reported to be resistant to the EGFR inhibitor cetuximab in vivo [50]. However, our results showing that these cell lines are sensitive to TKI258 suggest that the resistance to the EGFR inhibitor might be overcome by using TKI258.

Our results indicate that treatment with the multi-target angiokinase inhibitor TKI258 was effective in KRAS mutant LoVo cells but not in BRAF mutant HT-29 cells in vitro. However, our in vivo studies showed that KRAS and BRAF mutant xenograft tumors were both inhibited by TKI258 treatment, which might have resulted from the angiogenesis-suppressing effect of the multi-target inhibitor TKI258. Moreover, our results revealed the possibility of using TKI258 to overcome resistance to EGFR or VEGF inhibitors in CRC. The results relating to the in vivo effect of the drug would be more compelling if further studies with more cell lines were conducted. In conclusion, FGFR is an effective target for CRC treatment regardless of KRAS or BRAF mutation and our results suggest that combined targeting of multiple RTKs, especially FGFR and VEGFR, might be a novel approach to improve the outcome of CRC patients, possibly among selected patients in whom FGFR is over-expressed or amplified.

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Disclosure of conflict of interest
The authors declare no conflict of interest.

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