

ORIGINAL**Regorafenib induces adaptive resistance of colorectal cancer cells via inhibition of vascular endothelial growth factor receptor**

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Abstract : Recently, inhibition of tumor angiogenesis has become an important anti-cancer therapy. Tumor angiogenesis is regulated by multiple signaling pathways, including VEGF and VEGF receptor (VEGF-R), FGF and FGF receptor (FGF-R), and PDGF and PDGF receptor (PDGF-R) pathways. Thus, the antiangiogenic agents, such as regorafenib, simultaneously target those receptors on vascular endothelial cells. In addition to endothelial cells, cancer cells express the three receptors, suggesting that the antiangiogenic inhibitors affect tumor cells. In fact, we previously demonstrated that regorafenib directly acted on human colorectal cancer cells and accelerated their apoptosis resistance and migration capability. Thus, we here elucidated how regorafenib induced the malignant phenotypes in colorectal cancer cells. To identify the responsible receptor among the regorafenib-targeting proangiogenic receptors, we examined the effects of a potent selective inhibitor for VEGF-R, FGF-R or PDGF-R on apoptosis resistance and migration capability. We clarified that blockade of VEGF-R, but not FGF-R and PDGF-R, induced the malignant phenotypes. We confirmed that blocking of VEGF ligands derived from colorectal cancer cells also induced the phenotypes. These results suggest that regorafenib progressed the malignancy via prevention of autocrine and paracrine VEGF signaling in colorectal cancer cells. *J. Med. Invest.* 64 : 262-265, August, 2017

Keywords : angiogenesis inhibitor, regorafenib, VEGF-R, tumor cell aggressiveness

INTRODUCTION

Tumors angiogenesis is aberrant regulation of angiogenesis in solid cancers. It is an important process required for invasion and metastasis (1-4). Although it is not completely clarified, the complex process of tumor angiogenesis is regulated by multiple signaling pathways. The proangiogenic multiple pathways include VEGF and VEGF receptor (VEGF-R), FGF and FGF receptor (FGF-R), and PDGF and PDGF receptor (PDGF-R) pathways in vascular endothelial cells (5). In addition to endothelial cells, tumor cells not only produce VEGF, FGF and PDGF, but also express VEGF-R, FGF-R and PDGF-R, suggesting roles for autocrine and paracrine activation of those signaling pathways in cancer cells (5).

In the advanced solid tumors, recent antiangiogenic chemotherapies have focused on blocking simultaneously the multiple proangiogenic signaling pathways. Several currently available antiangiogenic treatments aim to inhibit VEGF/VEGF-R, FGF/FGF-R and PDGF/PDGF-R pathways. For example, regorafenib is a multiple receptor tyrosine kinase inhibitor (RTKI) that simultaneously inhibits the VEGF-R, FGF-R and PDGF-R (6, 7), suggesting that the multiple RTKIs affect not only vascular endothelial cells but also cancer cells. However, direct effects of the RTKIs on tumor cells remain unclear.

Regorafenib was approved by the FDA in 2012 for the second-

line treatment of metastatic colorectal cancer patients. In a pivotal randomized phase III study of regorafenib treatment, a statistically significant prolongation in overall survival was observed in patients with colorectal cancer who received regorafenib therapy (6, 7). Despite the survival benefit of regorafenib for metastatic colorectal cancer patients, its overall clinical efficacy remains quite limited. Unfortunately, the vast majority of patients who initially respond to the agent will develop resistance (8-10).

The central mechanism of the resistance to multiple RTKIs is considered to be hypoxic stress induced by disruption of tumor angiogenesis (10-12). In addition to hypoxia, we previously demonstrated that regorafenib directly acted on colorectal cancer cells and accelerated their malignant phenotypes, including apoptosis resistance and migration activation, in a hypoxia-independent manner (13).

Thus, the aim of this study is to elucidate how regorafenib induced the malignant phenotypes in colorectal cancer cells. By using a potent selective inhibitor for VEGF-R, FGF-R or PDGF-R, we clarified that blockade of VEGF-R, but not FGF-R and PDGF-R, expressed on colorectal cancer cells induced apoptosis resistance and migration activation.

MATERIALS AND METHODS*Cell culture and treatment*

Human colorectal cancer cell line (HCT116) was maintained in RPMI1640 medium with 5% fetal bovine serum and antibiotics. Based on our previous reports (13, 14), in order to inhibit angiogenic receptors, cells were continuously treated for 14 days with a

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potent selective inhibitor of VEGF-R (KRN633, Selleckchem) at 40 nM, FGF-R (AZD4547, Selleckchem) at 1 nM, PDGF-R (CP-673451, Selleckchem) at 10 nM or vehicle DMSO (0.01%). In order to block VEGF ligands, cells were continuously treated with recombinant soluble VEGF-R1 protein plus soluble VEGF-R3 proteins (250 ng/ml each) (R&D Biosystems) or control bovine serum albumin (500 ng/ml) for 7 days.

For hypoxic culture conditions, cells were incubated at low confluence and 37°C in RPMI1640 medium supplemented with HEPES buffer (pH 7.4) using an AnaeroPack-Kenki 5% anaerobic system in which O₂ was ~0.2% (Sugiyamagen, Japan).

Cell migration assay

Equal numbers (50,000 cells per well) of cells were suspended in 0.25 ml of 1% RPMI1640–FBS without or with regorafenib and placed in the top compartment of a 8 µm pore membrane chambers (BD Biosciences); 0.75 ml of 10% RPMI1640–FBS was added to the bottom compartment. Following 24-h incubation under standard conditions (37°C/5% CO₂), non-migrating cells were scraped from the top compartment, and cells that had migrated to the bottom compartment were fixed and stained using the Hemacolor Rapid staining of blood smear (Merck). Membranes were excised and mounted on a standard microscope slide. The numbers of migrated cells were determined from ten random high-power fields (HPF) visualized at ×200 magnification.

Assessment of apoptosis

Apoptotic cells were assessed by imaging of activated caspase 3/7 using CellEvent caspase-3/7 green detection reagent (Molecular probes), according to the manufacturer's instruction.

Statistical analysis

Results are expressed as means ± S.D. Statistical analyses of data were done using ANOVA and the Scheffé's test. P values < 0.05 was considered significant.

RESULTS

Effect of inhibition of regorafenib-targeting proangiogenic receptors on migration ability

In order to elucidate how a multiple RTKI regorafenib, which inhibits simultaneously VEGF-R, FGF-R and PDGF-R, induced activation of colorectal cancer cell migration (13), we first examined the effect of a single inhibition of the respective receptors on cellular motility. We used a small molecule agent targeting tyrosine kinase of VEGF-R (KRN633), FGF-R (AZD4547) or PDGF-R (CP-673451). As shown in Figure 1, inhibition of VEGF-R markedly activated the migration ability, compared to the DMSO-treated control cells. By contrast, blockade of FGF-R and PDGF-R significantly decreased the ability (Fig. 1).

Effect of inhibition of regorafenib-targeting proangiogenic receptors on apoptosis under hypoxic conditions

We then examined the effect of the single inhibition of VEGF-R, FGF-R or PDGF-R on apoptosis under hypoxic conditions. We chose hypoxic stress, since angiogenic inhibitors induce hypoxia within tumor microenvironment via disruption of tumor neoangiogenesis (1). Inhibition of VEGF-R induced apoptosis resistance, compared to the DMSO-treated control cells (Fig. 2). Blockade of FGF-R significantly increased apoptosis rate, compared to the DMSO-treated control cells (Fig. 2). Under PDGF-R inhibited conditions, the apoptosis rate was similar to the control (Fig. 2).

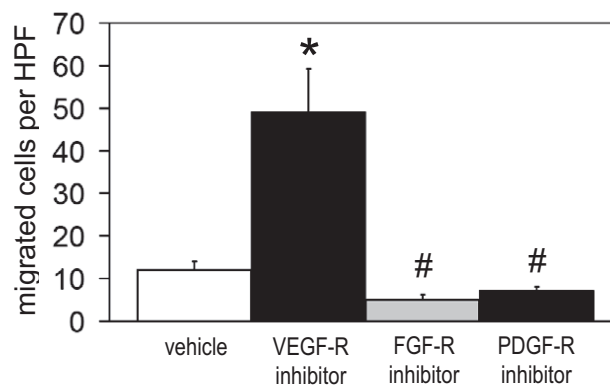


Figure 1. Effect of inhibition of regorafenib-targeting proangiogenic receptors on migration ability of colorectal cancer cells. Cells were treated with a VEGF-R inhibitor (KRN633), FGF-R inhibitor (AZD4547), PDGF-R inhibitor (CP-673451) or vehicle (DMSO). Then cells were used for transwell migration assay in the presence of the respective inhibitor (n=4, means ± SD). *P < 0.01 indicates the increased migration activity compared with the DMSO-treated control cells (vehicle). #P < 0.01 indicates the decreased migration activity compared with the DMSO-treated control cells (vehicle). HPF, high power field.

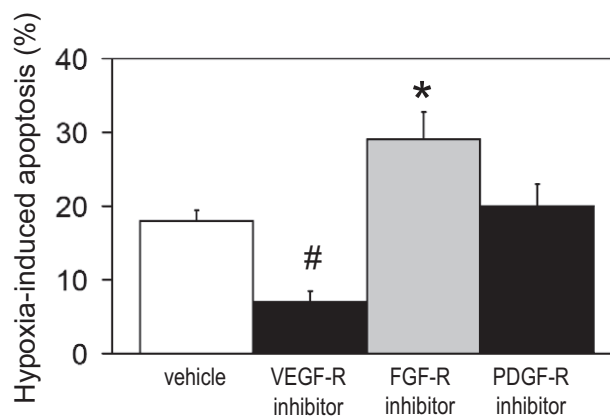


Figure 2. Effect of inhibition of regorafenib-targeting proangiogenic receptors on apoptosis under hypoxic conditions. Cells were treated with a VEGF-R inhibitor (KRN633), FGF-R inhibitor (AZD4547), PDGF-R inhibitor (CP-673451) or vehicle (DMSO). Then cells were exposed to hypoxia (~0.2% O₂) for additional 24 h in the presence of the respective inhibitor. Apoptotic cells were determined by measuring of activated caspase 3/7 (n=4, means ± SD). *P < 0.01 indicates the increased apoptosis rate compared with the DMSO-treated control cells (vehicle). #P < 0.01 indicates the decreased apoptosis rate compared with the DMSO-treated control cells (vehicle).

Effect of blocking of autocrine/paracrine VEGF signals on the malignant phenotypes of colorectal cancer cells

It is previously reported that HCT116 cells express VEGF ligands (VEGF, PlGF, VEGF-B, VEGF-C and VEGF-D) (14, 18). To demonstrate whether blocking of autocrine and paracrine VEGF signaling pathway induced the migration activation and apoptosis resistance in colorectal cancer cells, we blocked all of VEGF ligands using recombinant proteins of soluble VEGF-R1 and soluble VEGF-R3. Soluble VEGF-R1 protein captures VEGF, PlGF and VEGF-B, and soluble VEGF-R3 protein traps VEGF-C and VEGF-D, thus leading to prevention of all VEGF-R activation. As similarly to inhibition of VEGF-R by KRN633 (Fig. 1 and 2), neutralization of

VEGF ligands induced the migration activation and the apoptosis resistance (Fig. 3A and B).

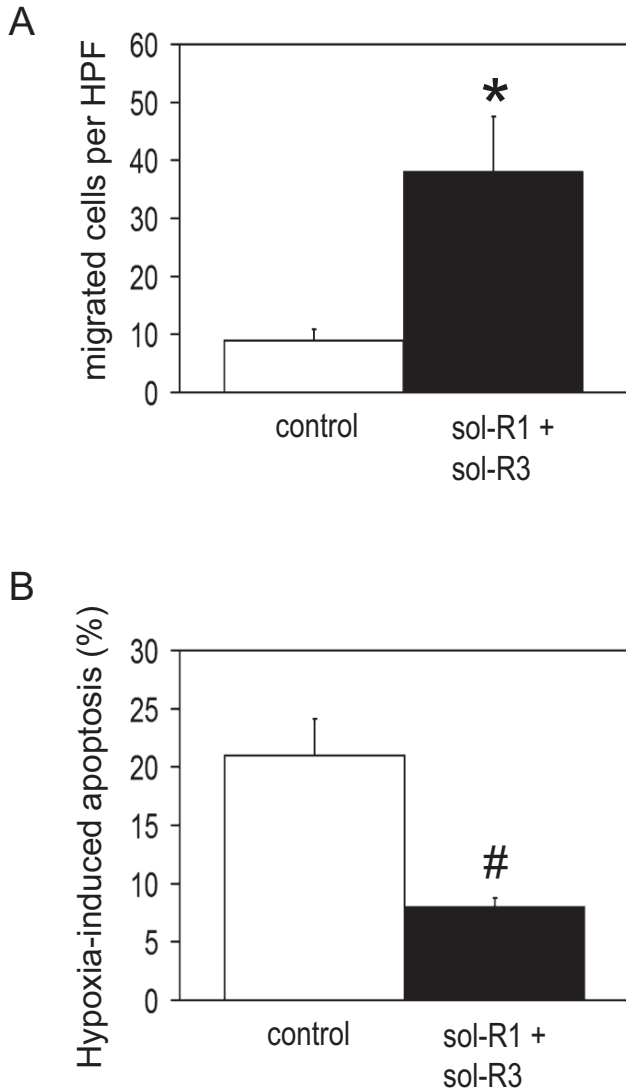


Figure 3. Effect of blocking of autocrine/paracrine VEGF signals on migration ability and on hypoxia-induced apoptosis. Cells were treated with soluble VEGF-R1 and VEGF-R3 recombinant proteins (sol-R1 + sol-R3) or control albumin (control). (A) Then cells were used for transwell migration assay (n=4, means \pm SD). * $P < 0.01$ indicates the increased migration activity compared with the albumin-treated control cells (control). HPF, high power field. (B) The cells were exposed to hypoxia ($\sim 0.2\% O_2$) for additional 48 h in the presence of the respective blocker. Apoptotic cells were determined by measuring of activated caspase 3/7 (n=4, means \pm SD). # $P < 0.01$ indicates the decreased apoptosis rate compared with the albumin-treated control cells (control).

DISCUSSION

In this study, we revealed how a multiple RTKI regorafenib accelerated the malignant phenotypes in colorectal cancer cells. Our results show that inhibition of autocrine and paracrine VEGF/

VEGF-R signaling pathway in colorectal cancer cells induced the phenotypes. By contrast, blockade of FGF-R and PDGF-R repressed the phenotypes, consistent with previous reports (15, 16).

Our results are supported by several reports using VEGF and VEGF-R blockers (14, 17-22). Recently, there is accumulating evidence that several VEGF/VEGF-R targeting antiangiogenic agents act not only on vascular endothelial cells, but also on tumor cells, as cancer cells also express VEGF-R (17). When the agents act on endothelial cells, tumor vascularization is significantly inhibited, leading to tumor regression. By contrast, when act on tumor cells, the aggressiveness of cancer cells is increased (17). For example, a neutralizing anti-VEGF monoclonal antibody (bevacizumab) has been shown to stimulate the expression of tumor invasion associated factors, such as matrix metalloproteases and promoting invasiveness both *in vitro* and *in vivo* in glioblastoma, renal cell carcinoma and colorectal cancer models (14, 18, 19). VEGF-R inhibitors stimulate the expression of genes associated with invasion and metastasis, such as Snail, Slug, Fak and Axl, leading to local invasion and distant metastasis (20-22). By contrast, inhibition of FGF-R and PDGF-R expressed on several cancer cells suppresses the evasive phenotypes of them (15, 16). These reports support our finding that blockade of tumor cell VEGF-R, but not FGF-R and PDGF-R, accelerated aggressiveness of cancer cells. In addition, these results suggest that it might be better not to block the VEGF/VEGF-R signaling in several cancer cells.

Collectively, we conclude that multi RTKI regorafenib induced the malignant phenotypes of colorectal cancer cells via inhibiting of VEGF/VEGF-R pathway. Currently, nine antiangiogenic multiple RTKIs (regorafenib, lenvatinib, nintedanib, cabozantinib, axitinib, vandetanib, sunitinib, sorafenib and pazopanib) are approved by FDA for treatment of patients with several advanced cancers, including colorectal cancer (5). Importantly, all of these approved RTKIs have an inhibitory activity of VEGF-R, suggesting that all the RTKIs possibly activate cancer cell aggressiveness. Thus, it will be needed to clarify precise molecular mechanisms and a complete understanding of the direct effects of the antiangiogenic RTKIs on tumor cells. The elucidation can bring insights into counteracting tumor adaptive refractoriness to the antiangiogenic drugs.

COMPETING INTERESTS-DISCLOSURE

The authors declare no competing interests.

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REFERENCES

1. Bergers G, Hanahan D : Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 8 : 592-603, 2008
2. Ellis LM, Reardon DA : Is there really a yin and yang to VEGF-targeted therapies? *Lancet Oncol* 11 : 809-811, 2010
3. Jayson GC, Kerbel R, Ellis LM, Harris AL : Antiangiogenic therapy in oncology : current status and future directions. *Lancet* 388 : 518-529, 2016
4. Meadows KL, Hurwitz HI : Anti-VEGF therapies in the clinic. *Cold Spring Harb Perspect Med* 2 : a006577, 2012
5. Zhao Y, Adjei AA : Targeting Angiogenesis in Cancer Therapy : Moving Beyond Vascular Endothelial Growth Factor.

- Oncologist 20 : 660-673, 2015
6. Sartore-Bianchi A, Zeppellini A, Amatu A, Ricotta R, Bencardino K, Siena S : Regorafenib in metastatic colorectal cancer. *Expert Rev Anticancer Ther* 14 : 255-265, 2014
 7. Grothey A, Van Cutsem E, Sobrero A, Siena S, Falcone A, Ychou M, Humblet Y, Bouché O, Mineur L, Barone C, Adenis A, Taberero J, Yoshino T, Lenz HJ, Goldberg RM, Sargent DJ, Cihon F, Cupit L, Wagner A, Laurent D ; CORRECT Study Group. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT) : an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet* 381 : 303-312, 2013
 8. Bottsford-Miller JN, Coleman RL, Sood AK : Resistance and escape from antiangiogenesis therapy : clinical implications and future strategies. *J Clin Oncol* 30 : 4026-4034, 2012
 9. Ebos JM, Kerbel RS : Antiangiogenic therapy : impact on invasion, disease progression, and metastasis. *Nat Rev Clin Oncol* 8 : 210-221, 2011
 10. Ribatti D : Tumor refractoriness to anti-VEGF therapy. *Oncotarget* 19 : 46668-46677, 2016
 11. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS : Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 15 : 232-239, 2009
 12. Pàez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Viñals F, Inoue M, Bergers G, Hanahan D, Casanovas O : Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 15 : 220-231, 2009
 13. Tomida C, Yamagishi N, Aibara K, Yano C, Uchida T, Abe T, Ohno A, Hirasaka K, Nikawa T, Teshima-Kondo S : The malignant progression effects of regorafenib in human colon cancer cells. *J Med Invest* 62 : 195-198, 2015
 14. Yamagishi N, Kondo TS, Masuda K, Nishida K, Kuwano Y, Dang DT, Dang LH, Nikawa T, Rokutan K : Chronic inhibition of tumor cell-derived VEGF enhances the malignant phenotype of colorectal cancer cells. *BMC Cancer* 13 : 229, 2013
 15. Appiah-Kubi K, Wang Y, Qian H, Wu M, Yao X, Wu Y, Chen Y : Platelet-derived growth factor receptor/platelet-derived growth factor (PDGFR/PDGF) system is a prognostic and treatment response biomarker with multifarious therapeutic targets in cancers. *Tumour Biol* 37 : 10053-10066, 2016
 16. Katoh M, Nakagama H : FGF receptors : cancer biology and therapeutics. *Med Res Rev* 34 : 280-300, 2014
 17. Simon T, Gagliano T, Giamas G : Direct Effects of Anti-Angiogenic Therapies on Tumor Cells : VEGF Signaling. *Trends Mol Med* 23 : 282-292, 2017
 18. Fan F, Samuel S, Gaur P, Lu J, Dallas NA, Xia L, Bose D, Ramachandran V, Ellis LM : Chronic exposure of colorectal cancer cells to anti-VEGF mAb promotes compensatory pathways that mediate tumor cell migration. *British J Cancer* 104 : 1270-1277, 2011
 19. Lucio-Eterovic AK, Piao Y, de Groot JF : Mediators of glioblastoma resistance and invasion during antivascular endothelial growth factor therapy. *Clin Cancer Res* 15 : 4589-4599, 2009
 20. Huang D, Ding Y, Li Y, Luo WM, Zhang ZF, Snider J, Vandenbeldt K, Qian CN, The BT : Sunitinib acts primarily on tumor endothelium rather than tumor cells to inhibit the growth of renal cell carcinoma. *Cancer Res* 70 : 1053-1062, 2010
 21. Lu KV, Chang JP, Parachoniak MM, Aghi MK, Meyronet D, Isachenko N, Fouse SD, Philips JJ, Cheresch DA, Park M, Bergers G : VEGF inhibits Tumor Cell Invasion and Mesenchymal Transition through a MET/VEGFR2 Complex. *Cancer Cell* 22 : 21-35, 2012
 22. Han KS, Raven PA, Frees S, Gust K, Fazli L, Ettinger S, Hong SJ, Kollmannsberger C, Gleave ME, So AI : Cellular Adaptation to VEGF-Targeted Antiangiogenic Therapy Induces Evasive Resistance by Overproduction of Alternative Endothelial Cell Growth Factors in Renal Cell Carcinoma. *Neoplasia* 17 : 805-816, 2015