Induction of Epithelial-Mesenchymal Transition via Activation of Epidermal Growth Factor Receptor Contributes to Sunitinib Resistance in Human Renal Cell Carcinoma Cell Lines

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ABSTRACT

Sunitinib is widely used for treating renal cell carcinoma (RCC). However, some patients do not respond to treatment with this drug. We aimed to study the association between sunitinib sensitivity and epithelial-mesenchymal transition (EMT) regulation via epidermal growth factor receptor (EGFR) signaling, which is a mechanism of resistance to anticancer drugs. Three RCC cell lines (786-O, ACHN, and Caki-1) were used, and then we evaluated cell viability, EMT regulatory proteins, and signal transduction with sunitinib treatment. Cell viability of 786-O cells was maintained after treatment with sunitinib. After treatment with sunitinib, EGFR phosphorylation increased in 786-O cells, resulting in an increase in the phosphorylation of extracellular signal-regulated kinase, nuclear translocation of β-catenin, and expression of mesenchymal markers. These results suggest that sunitinib increased phosphorylation of EGFR in 786-O cells, but not in ACHN and Caki-1 cells. Caki-1/SN cells, a resistant cell line originated by continuous exposure to sunitinib, displayed increased phosphorylation of EGFR. Cell viability in the presence of sunitinib was decreased by erlotinib, as the selective inhibitor of EGFR, treatment in 786-O and Caki-1/SN cells. Similarily, erlotinib suppressed sunitinib-induced EGFR activation and upregulated mesenchymal markers. Thus, we postulate that resistance to sunitinib in RCC may be associated with EMT caused by activation of EGFR.

Introduction

Treatment of renal cell carcinoma (RCC) has rapidly evolved during the past few years, with the development of several molecular-targeted drugs. Sunitinib is a small-molecule multiple-receptor tyrosine kinase (RTK) inhibitor targeting vascular endothelial growth factor receptors (VEGFRs: VEGFR-1, VEGFR-2, and VEGFR-3), platelet-derived growth factor receptors (PDGFRs: PDGFR-α and PDGFR-β), FMS-like tyrosine kinase 3, and the stem cell growth factor receptor KIT, and rearranged during transfection (Faivre et al., 2007; Roskoski, 2007). A meta-analysis comparing the clinical efficacy among targeted agents for the treatment of metastatic RCC has shown that sunitinib is more effective than other agents (Durán et al., 2013; Leung et al., 2014). Although most patients benefit from tyrosine kinase inhibitor treatment, including sunitinib, some still exhibit early progression. It has been reported that 11–29% of patients treated with VEGF-targeted therapy exhibit progressive disease as the best response (Uemura et al., 2010).

Several recent studies showed that cells that have undergone epithelial-mesenchymal transition (EMT) are relatively resistant to conventional chemotherapies (Hollier et al., 2009; Keitel et al., 2014; Weygant et al., 2015). Sánchez-Tilló demonstrated that the expression of E-cadherin is associated with a high sensitivity to the epidermal growth factor receptor (EGFR) inhibitor gefitinib (Witta et al., 2006; Sánchez-Tilló et al., 2011). EGFR is one of the RTKs that regulate EMT. Several studies have reported a 50–90% increase in EGFR expression in RCC and the association of EGFR expression with cancer progression (Moch et al., 1997; Dördević et al., 2012). The mutagenic or epigenetic inactivation of the von Hippel–Lindau tumor suppressor gene (VHL) has been observed in RCC tumors (Klatte et al., 2007; Shinojima et al., 2007), and the loss of VHL prolongs the activation of EGFR (Wang et al., 2009). However, the relation between EGFR and RCC chemotherapies has not yet been investigated.

Several studies have reported that the activation of collatera pathways via off-targets induced resistance to molecular-targeted agents (Engelman et al., 2007; Zhang et al., 2012). Critical orchestrators at the convergence of EMT pathways, such as the protein kinase B (Akt) mammalian target of rapamycin (mTOR) axis, mitogen-activated protein kinase, β-catenin, and
activator protein-1/SMAD factors, are controlled by several RTKs, including EGFR, VEGFR, fibroblast growth factor receptor, and PDGFR (Huber et al., 2005; Katoh and Katoh, 2006; Paoli et al., 2013). Based on these findings, we speculated that the activation of collateral pathways can be associated with the induction of EMT in sunitinib-refractory RCC cells.

In this study, the relations between sunitinib sensitivity and EMT regulation, which was one of the mechanisms of resistance to anticancer drugs, were investigated. In addition, the activation of collateral pathways associated with the induction of EMT was examined.

**Materials and Methods**

**Chemicals.** Sunitinib malate and Hoechst 33258 were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). Erolitinib monohydrochloride was purchased from LKT Laboratories, Inc. (St. Paul, MN).

**Antibodies.** Rabbit antiphosphorylated EGFR at tyrosine 1068, rabbit anti-EGFR, antiphosphorylated extracellular signal-regulated kinase (Erk) 1/2, rabbit anti-Erk 1/2 antibodies, rabbit antiphosphorylated p70 S6 kinase, rabbit anti-p70 S6 kinase antibodies, rabbit antiphosphorylated Akt at serine 473, rabbit anti-Akt, rabbit anti-vimentin, rabbit anti–β-catenin, and anti-rabbit horseradish peroxidase (HRP)-conjugated IgG were purchased from Cell Signaling Technology (Boston, MA). Mouse anti-E–cadherin was purchased from BD Biosciences (San Jose, CA). Rabbit anti–β-actin and mouse antifibronectin were obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO).

**Cells and Cell Culture.** 786-O and ACHN cells, derived from human RCC, were purchased from the Joint Committee of the Regional Metrology Organizations and Bureau International des Poids et Mesures (Sèvres, France). 786-O and Caki-1 cells were maintained in RPMI 1640 medium (Sigma-Aldrich Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. ACHN cells were maintained in Eagle’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Each cell line was seeded into culture flasks grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C and subcultured with 0.05% trypsin/0.02% EDTA (Life Technologies, Carlsbad, CA). For the development of sunitinib-resistant cells, Caki-1 cells were continuously exposed to 1 μM of sunitinib and designated as Caki-1/SN.

**WST-8 Colorimetric Assay.** The cell viability of 786-O, ACHN, and Caki-1 cells after exposure to indicated agents was assessed by the WST-8 assay using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) as previously described (Yamamoto et al., 2014). Cells (2 × 10⁴/well) were seeded onto 96-well plates and precultured for 24 hours. The medium was replaced with one containing various concentrations of these reagents or a dimethylsulfoxide control vehicle (the final concentration of dimethylsulfoxide did not exceed 0.1%). Cells were incubated with indicated concentrations of sunitinib. Cell viability was determined using a WST-8 colorimetric assay. Dunnett’s test was used to compare cell viability among different cell lines. Each bar represents mean ± S.D. (n = 4).

**Western Blotting.** Western blotting was performed as previously described (Yamamoto et al., 2014). Briefly, proteins in the total cell lysate were extracted from cells by using cell lysis buffer in addition to 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. Proteins were separated using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred on a nitrocellulose blotting membrane (Amersham Protran NC; GE Healthcare, Buckinghamshire, UK). Subsequently, the blot was blocked in a wash buffer solution (10 mM Tris, pH 7.5; 150 mM NaCl; and 0.05% Tween 20) containing 5% skim milk. The membrane was soaked overnight in a wash buffer containing specific primary antibodies, followed by incubation with horseradish peroxidase–conjugated secondary antibodies for 1 hour. Antibody-bound proteins were visualized by treating the membrane with an enhanced lumino-based chemiluminescent method, which was freshly prepared just before detection. Finally, blot images were acquired using ChemiStage 16-CC (KURABO Industries Ltd., Osaka, Japan). Wherever indicated, the membranes were stripped and reprobed with a different antibody. The intensities of protein bands for the densitometric assay were determined using Image J software (KURABO Industries Ltd., Osaka, Japan).

**Immunofluorescence Imaging and Cytometric Analysis.** Cells were fixed with 2% paraformaldehyde for 10 minutes at room temperature and blocked in 5% bovine serum albumin. The cells were incubated with anti–β-catenin and anti-EGFR antibody, followed by incubation with Cy3-conjugated anti-rabbit IgG (Jackson Immuno Research, West Grove, PA) and 1 μg/ml Hoechst 33258 for staining the nuclei. Visualized on an IN Cell Analyzer 2000 (GE Healthcare, Little Chalfont, UK), image acquisition was configured to yield at least 1000 cells per replicate well. Cytometric analysis was performed with IN Cell Analyzer Workstation version 3.2. β-catenin nuclear entry and EGFR intracellular entry were determined by counting the granules of red fluorescence using the membrane translocation analysis module. For this application note, data from the Hoechst channel were used to segment the nuclei and define a collar to sample the cell area. Plasma membrane-associated ruffles were segmented from the Cy3 channel. The mean inclusion count per cell in the nucleus or cytoplasm area was measured. The representative values of β-catenin nuclear translocation and EGFR intracellular translocation are shown as mean ± S.D.

**Results**

**Effects of Sunitinib on Cell Viability in Various RCC Cell Lines.** The sensitivity of 786-O, ACHN, and Caki-1 cells to sunitinib was analyzed. As shown in Fig. 1, treatment with sunitinib decreased cell viability of ACHN and Caki-1 cells in a dose-dependent manner. However, there was no decrease in cell viability of 786-O cells at the range of the experimental dose of sunitinib used in this study. We found that 786-O cells had a lower sensitivity to sunitinib compared with the other cell lines.

**Effects of Sunitinib Treatment on the EMT Profile in RCC Cell Lines.** EMT regulatory protein expression after...
treatment with sunitinib in each cell line is shown in Fig. 2A. In 786-O cells, the expressions of mesenchymal markers, fibronectin and vimentin, was increased by sunitinib treatment. With respect to ACHN and Caki-1 cells, sunitinib treatment decreased the expression of mesenchymal markers and the re-expression of the epithelial marker E-cadherin. Furthermore, the nuclear translocation of the β-catenin molecule, which regulates the transcription of EMT regulatory proteins, increased after sunitinib treatment in 786-O cells but not in ACHN and Caki-1 cells (Fig. 2B).

**Effects of Sunitinib Treatment on Signal Transduction in RCC Cell Lines.** Figure 3A shows EGFR activity and downstream signal transduction in each cell line after treatment with sunitinib. EGFR Tyr1068 phosphorylation of each cell line decreased after treatment of sunitinib at 24 hours. However, EGFR Tyr1068 phosphorylation and the intracellular localization of EGFR in 786-O cells was increased by sunitinib treatment after 48 hours (Fig. 3A and B), resulting in an increase in Erk phosphorylation. In contrast, the phosphorylation of EGFR and Erk in ACHN and Caki-1 cells was stably inhibited by sunitinib treatment after 48 hours. Treatment with sunitinib inhibited the phosphorylation of P70s6k and Akt in ACHN and Caki-1 cells, whereas the phosphorylations of P70s6k and Akt were conserved in 786-O cells.

**Effects of Sunitinib Treatment on the EMT Profile and Signal Transduction in Caki-1/SN Cells.** A sunitinib-resistant cell line was established by continuously exposing Caki-1 cells to 1 μM sunitinib. These were termed Caki-1 cells. Sensitivity to sunitinib in Caki-1/SN cells decreased compared with sensitivity in Caki-1 cells (Fig. 4A; Table 1). Sunitinib treatment increased the expression of E-cadherin in Caki-1 cells but not in Caki-1/SN cells. Furthermore, sunitinib treatment decreased the expression of vimentin and fibronectin in Caki-1 cells, whereas the expression of mesenchymal markers did not change in Caki-1/SN cells (Fig. 4B). In addition, the nuclear translocation of the β-catenin molecule, EGFR Tyr1068 phosphorylation, and intracellular localization in Caki-1/SN cells was markedly increased by sunitinib treatment (Fig. 4, D and E). Compared with Caki-1 cells, Caki-1/SN cells displayed a reduced Erk and P70s6k inhibition after treatment with sunitinib. In contrast, phosphorylation of Akt was inhibited in both cell lines (Fig. 4C). Phosphorylation of EGFR and Erk in Caki-1 cells was not decreased in Caki-1/SN cells (Fig. 4C).

**Effects of Erlotinib on Sunitinib-Induced EGFR Activation in 786-O and Caki-1/SN Cells.** We showed that an EGFR inhibitor (erlotinib) decreased cell viability of 786-O cells treated with sunitinib (Fig. 5A). Moreover,
Sunitinib combined with erlotinib was able to overcome resistance to sunitinib in Caki-1/SN cells (Fig. 5B). In 786-O and Caki-1/SN cells, the expression of fibronectin, vimentin, and phosphorylation levels of EGFR were increased when treated with sunitinib, whereas they were decreased when treated with both sunitinib and erlotinib (Fig. 5C). In 786-O and Caki-1/SN cells, phosphorylation levels of Erk were increased when treated with sunitinib but were decreased when treated with both sunitinib and erlotinib. In 786-O and Caki-1/SN cells, the expression of fibronectin and vimentin, and expression of mesenchymal markers increased after sunitinib treatment in 786-O cells treated with sunitinib. In contrast, re-expression of E-cadherin and downregulation of fibronectin and vimentin were observed in ACHN and Caki-1 cells. Based on these observations, we speculate that EMT may contribute to resistance to sunitinib as well as other anticancer drugs. However, more detailed studies are required to confirm this. Therefore, we focused on the association between the effects of sunitinib treatment on signal transduction and EMT-related factors. PI3K/Akt/mTOR signal transduction has been reported to play an important role in cell proliferation and the regulation of EMT (Shorning et al., 2011; Chang et al., 2013; Dong et al., 2014). In our study, the effects of sunitinib on the phosphorylation of Akt and P70s6k were not evident in 786-O cells (Fig. 3). We thought that this phenomenon results in the constitutive activation of the PI3K/Akt/mTOR pathway via a deficiency in PTEN (Makhov et al., 2012; Muriel López et al., 2012). Nonetheless, phosphorylation of Akt and P70s6k were maintained at steady levels, the nuclear translocation of β-catenin and expression of mesenchymal markers increased after sunitinib treatment in 786-O cells (Fig. 2). For this reason, we speculate that both the deficiency in PTEN and constitutive activation of the PI3K/Akt/mTOR pathway were not associated with sunitinib-induced EMT in 786-O cells.

EGFR signaling is linked to maintenance of the EMT status in cancer cells (Voon et al., 2013). Although sunitinib targets various RTKs, the association between sunitinib and EGFR has not yet been investigated. We found that EGFR Tyr1068

**Discussion**

In the present study, we initially demonstrated that the effects of sunitinib on cell growth differed among the RCC cell lines (Fig. 1). ACHN and Caki-1 cells are wild type, whereas the 786-O cell line is mutated for VHL (Stickle et al., 2005; Ishimaru et al., 2010; Holland et al., 2012). Moreover, 786-O cells are deficient in PDGFR-β, which is the target molecule of sunitinib. In addition, phosphatase and a tensin homolog deleted from chromosome 10 (PTEN) opposes the phosphoinositide 3-kinase (PI3K)/Akt/mTOR pathway and acts as a tumor suppressor. Growth signaling is constitutively activated in PTEN-deficient 786-O cells (Huang et al., 2010; Makhov et al., 2012; Yang et al., 2012; Chang et al., 2013). Accordingly, it is possible that 786-O cells displayed a lower sensitivity to sunitinib than the other cell lines.

Several cancers have been shown to possess an EMT phenotype that is linked with resistance to conventional chemotherapies (Hollier et al., 2009; Keitel et al., 2014; Weygant et al., 2015). In our study, the expression of fibronectin and vimentin and nuclear translocation of β-catenin were increased in 786-O cells treated with sunitinib. In contrast, re-expression of E-cadherin and downregulation of fibronectin and vimentin were observed in ACHN and Caki-1 cells. Based on these observations, we speculate that EMT may contribute to resistance to sunitinib as well as other anticancer drugs. However, more detailed studies are required to confirm this. Therefore, we focused on the association between the effects of sunitinib treatment on signal transduction and EMT-related factors. PI3K/Akt/mTOR signal transduction has been reported to play an important role in cell proliferation and the regulation of EMT (Shorning et al., 2011; Chang et al., 2013; Dong et al., 2014). In our study, the effects of sunitinib on the phosphorylation of Akt and P70s6k were not evident in 786-O cells (Fig. 3). We thought that this phenomenon results in the constitutive activation of the PI3K/Akt/mTOR pathway via a deficiency in PTEN (Makhov et al., 2012; Muriel López et al., 2012). Nonetheless, phosphorylation of Akt and P70s6k were maintained at steady levels, the nuclear translocation of β-catenin and expression of mesenchymal markers increased after sunitinib treatment in 786-O cells (Fig. 2). For this reason, we speculate that both the deficiency in PTEN and constitutive activation of the PI3K/Akt/mTOR pathway were not associated with sunitinib-induced EMT in 786-O cells.

EGFR signaling is linked to maintenance of the EMT status in cancer cells (Voon et al., 2013). Although sunitinib targets various RTKs, the association between sunitinib and EGFR has not yet been investigated. We found that EGFR Tyr1068...
phosphorylation and EGFR intracellular localization recovered and increased after 48 hours of treatment with sunitinib in 786-O cells, whereas a reduction of EGFR Tyr1068 phosphorylation in ACHN and Caki-1 cells was observed after 48 hours (Fig. 3; Fig. 4C). We speculate that sunitinib-induced EGFR inhibition in RCC cell lines is through an indirect mechanism. Sunitinib inhibition of EGFR transactivation may occur by blocking PDGFR activation (Abouantoun et al., 2011). 786-O cells are deficient in PDGFR-β; therefore, EGFR transactivation may not be induced by PDGFR activation. Thus, EGFR phosphorylation in 786-O cells was temporarily inhibited by sunitinib but was recovered and eventually increased after a 48-hour exposure (Fig. 3A). The recovery activation mechanisms of EGFR in 786-O cells are yet unclear.

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**TABLE 1**

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<tr>
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<th>Caki-1 Cells</th>
<th>Caki-1/SN Cells</th>
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<tr>
<td>IC₅₀</td>
<td>2.22 ± 0.028</td>
<td>5.01 ± 0.127**</td>
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**Significantly different from the respective control at P < 0.01.**
we suggest that EMT status in RCC cells treated with sunitinib may be regulated by EGFR activity. Furthermore, with respect to the MEK/ERK pathway, Caki-1/SN cells may display the drug resistance to Raf or MEK inhibitors, as demonstrated by using sorafenib, vemurafenib, and trametinib. Our results showed that erlotinib, which is a selective inhibitor of EGFR, reduced the increase in phosphorylation of EGFR Tyr1068 and Erk and upregulation of mesenchymal markers by sunitinib treatment in 786-O cells (Fig. 5C). In addition, our study showed that cell growth inhibitory effects of sunitinib were enhanced in combination with erlotinib in 786-O cells (Fig. 5A). Decreased sunitinib-induced EGFR activation in combination with erlotinib may inhibit EMT and result in enhanced sunitinib-induced growth inhibition of 786-O cells.

Furthermore, we examined the contribution of EGFR to acquired resistance to sunitinib in RCC cells. For the development of sunitinib-resistant cells, Caki-1 cells were continuously exposed to sunitinib. After 1 month of sunitinib exposure, Caki-1/SN cells had a decreased sensitivity to sunitinib compared with wild-type parental cells (Fig. 4A; Table 1). Further, we examined the activation profiles of EGFR in Caki-1 and Caki-1/SN cells exposed to sunitinib. In the presence of sunitinib, EGFR Tyr1068 phosphorylation and EGFR intracellular localization increased after treatment with sunitinib in Caki-1/SN cells (Fig. 4, C and E). In addition, the combination of sunitinib with erlotinib could overcome resistance to sunitinib in Caki-1/SN cells (Fig. 5B). For these findings, we suggest that the mechanisms of acquired resistance to sunitinib in RCC cells are related to the activation profiles of EGFR. A recent clinical study stated that erlotinib and sirolimus showed better progression free and overall survivals in patients with mRCC after failure of sunitinib or sorafenib treatment (Flaig et al., 2010). Our results support this finding, and we believe that EMT regulation via EGFR is an important factor that influences the sensitivity to sunitinib in RCC cells. In this study, the mechanism of sunitinib-induced EGFR activation in 786-O and Caki-1/SN cells is unknown. EGFR activation mechanisms have various forms. For example, some cytokines, such as transforming growth factor beta, interleukin-8, and tumor necrosis factor alpha, mediate the secretion of heparin-binding EGF-like growth factor and transforming growth factor alpha while inducing EGFR activation (Lappano and Maggiolini, 2011; Maillé et al., 2011; Moreno-Càceres et al., 2014). Reactive oxygen species, which are generated by ammonia and angiotensin II, activate EGFR through a nonligand-dependent pathway (Sorkin, 2001; Ding et al., 2007; Dai et al., 2013). Therefore, additional investigations are necessary to clarify these phenomena.

In conclusion, we demonstrated an association between sunitinib sensitivity in RCC cells and EMT regulation via EGFR signaling. We speculate that targeting the EGFR pathway may
be an effective therapeutic strategy for tumors with enriched EMT properties through sunitinib treatment. Future research focusing on the association between sunitinib and EGFR may improve RCC treatment outcomes and provide a clinical benefit for patients with RCC.

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Authorship Contributions
Participated in research design: Mizumoto, Yamamoto, Nakagawa.
Conducted experiments: Mizumoto.
Wrote or contributed to the writing of the manuscript: Mizumoto, Yamamoto, Takara.

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