Published in final edited form as:

Urology. 2011 October ; 78(4): 968.e7–968.e11. doi:10.1016/j.urology.2011.07.1384.

Interleukin-6: A Potential Biomarker of Resistance to Multitargeted Receptor Tyrosine Kinase Inhibitors in Castration-Resistant Prostate Cancer

Alexander Kutikov^{1,*}, Peter Makhov¹, Konstantin Golovine¹, Daniel J. Canter¹, Mohit Sirohi¹, Ryan Street¹, Jay Simhan¹, Robert G. Uzzo¹, and Vladimir M. Kolenko¹

¹Department of Surgical Oncology, Division of Urological Oncology, Fox Chase Cancer Center, Philadelphia, PA

Abstract

Objectives—Clinical experience using tyrosine kinase inhibitors (TKIs) in patients with castration-resistant prostate cancer (CRPC) is starting to mature. In Phase II trials, a heterogeneous response to sunitinib has been noted. Change in serum PSA level has proven unreliable for prediction of CRPC response to TKIs. IL-6, a critical mediator of prostate cancer pathogenesis, has been shown to rise in patients with disease progression. We investigated whether cellular IL-6 production can predict TKI response in both in-vitro and in-vivo models.

Methods—IL-6 mRNA levels and protein expression were examined by RT-PCR and ELISA, respectively. Apoptosis was examined using the TUNEL assay. For in-vivo studies, a CRPC xenograft model in C.B17/Icr-scid mice was employed.

Results—PC-3 and DU-145 CRPC cell lines exhibited a heterogeneous response to sunitinib and pazopanib. Dose dependent reduction of IL-6 was observed in TKI-sensitive DU-145 cells. In contrast, the TKI-resistant PC-3 cells failed to suppress IL-6 secretion. Instead, in the presence of TNF- α , IL-6 rose significantly upon administration of TKIs. Findings of in-vitro experiments were confirmed in an in-vivo mouse model of CRPC.

Conclusions—Sensitivity of CRPC cells to TKIs is heterogeneous. These findings are consistent with results of recently-published Phase II clinical trials using sunitinib in patients with CRPC. A substantial rise in IL-6 occurs both in-vitro and in-vivo in the presence of TKIs in resistant PC-3 cells but not in TKI-sensitive DU-145 cells. These findings suggest that IL-6 may represent a biomarker for TKI resistance in patients with CRPC.

Keywords

prostate cancer; sunitinib; pazopanib; interleukin-6

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*Address correspondence to: Alexander Kutikov, MD, Division of Urological Oncology, Department of Surgical Oncology, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, Phone: 215-728-5342, Fax: 215-214-1734, alexander.kutikov@fccc.edu.

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Introduction

Angiogenic inhibition using small molecule receptor tyrosine kinase inhibitors (TKIs) represent a clinically relevant strategy for patients with advanced tumors such as renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GISTs). Since introduction of these targeted therapies, median survival for patients with metastatic RCC has increased to nearly 24 months.¹ Given their efficacy in RCC and GIST, TKIs are being tested clinically for many other solid tumors.²

Castration-resistant prostate cancer (CRPC) is the second leading cause of cancer death among American men.³ Current treatment options, including docetaxel-based chemotherapy and more recently Sipuleucel-T, have been shown to improve survival by only 2 to 4 months.^{4,5} Furthermore, CRPC is highly vascular and possibly angiogenesis-dependent.⁶ Xenograft models of CRPC in mice have confirmed sunitinib's activity against DU-145 castration-resistant prostate cancer cells.⁷ More recently, initial clinical experience with sunitinib in CRPC patients has been reported.^{8,9} In Phase II trials, a heterogeneous response to sunitinib was noted in both docetaxel resistant and chemotherapy naïve patients with a significant proportion of tumors demonstrating at least partial radiographic response. In the most recent study of subjects with docetaxel-resistant CRPC, 11.1% showed 30% tumor reduction by Response Evaluation Criteria In Solid Tumors (RECIST), while another 44.4% of patients demonstrated quantifiable but less significant tumor response.⁹ Notably, clinical response did not correlate well with decreases in prostate specific antigen (PSA),^{8,9} indicating that PSA is a suboptimal biomarker for CRPC response to TKIs.⁸⁻¹⁰

An appropriate biomarker of TKI response/resistance would be relevant to future clinical trials.¹⁰ Data from a recent Phase II trial in advanced hepatocellular carcinoma suggest that a rise in serum interleukin-6 (IL-6) levels in patients treated with sunitinib strongly correlate with rapid disease progression.¹¹ Furthermore, recent data from a model using Kasumi-1 acute myelogenous leukemia (AML) cells, indicate that IL-6 is able to induce sunitinib resistance through the janus kinase 2(JAK2)/signal transducer and activator of transcription 5 (STAT5) signaling pathway.¹² Because IL-6 is a critical mediator of prostate cancer pathogenesis at the molecular level¹³ and has been shown to rise in patients with disease progression,^{14,15} here we investigate whether cellular IL-6 production can predict TKI response in both an *in-vitro* and an *in-vivo* model of CRPC.

Materials and Methods

Cells and materials

Cell lines were obtained from ATCC (Rockville, MD). The cells were resuscitated and cultured in our laboratory for less than 6 months since resuscitation in RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), gentamicin (50 mg/L), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM).

Reagents

TNF- α was obtained from Sigma (St. Louis, MO). Sunitinib was obtained from LC Laboratories (Woburn, MA). Pazopanib was obtained from Eton Bioscience (San Diego, CA).

Measurement of IL-6

IL-6 levels in cell culture supernatants, cell lysates and plasma samples were determined using an ELISA kit (R&D Systems, Minneapolis, MN). For intracellular IL-6 assessment, cells were lysed in 1% Tween 20/PBS containing a proteinase inhibitor cocktail (Roche

Applied Science). Protein concentrations were measured with BCA protein assay reagents (Pierce, Rockford).

Real Time PCR analysis

Total RNA was isolated from cells using MINI RNA isolation II Kit (Zymo-Research, Orange, CA) and purified using DNA-Free RNA Kit (Zymo-Research). Reverse transcription (RT) of 2 µg RNA was subsequently carried out using 200 units of SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was amplified by real time PCR using IL-6 TaqMan Gene Expression Assay (ID# Hs00174131_m1). GAPDH Gene Expression Assay (ID# Hs99999905_m1) was used as an endogenous control. Each sample was run in triplicate using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Reactions were carried out in an Applied Biosystems 7500 Real-Time PCR System. Analysis of IL-6 expression was carried out using the $2^{-(\Delta\Delta C_T)}$ method ($2^{-\Delta\Delta C_T}$).

Luciferase reporter assay

Cells were transfected with pNF-κB-luc (Stratagene, La Jolla, CA) and either GFP (Clontech, Mountain View, CA) (sunitinib experiments) or pRL-TK (Promega, Madison, WI) (pazopanib experiments) plasmids. GFP and pRL-TK plasmids were used to monitor transfection efficacy. Transfections were performed using TransIT-Prostate transfection kit (Mirus Bio, Madison, WI). Twenty-four hours after transfection, cells were treated with either sunitinib or pazopanib for 3 hour followed by treatment with TNF-α (20 ng/ml) for an additional 4 hours. Samples were assayed for firefly and renilla luciferase activities using the Dual-Glo Luciferase assay System (Promega) and normalized as instructed by the manufacturer. GFP expression was assessed using a Bio-Tek microplate fluorimeter with excitation and emission filters of 485/20 and 528/20 nm respectively.

Measurement of apoptosis

DNA fragmentation was detected using APO-BRDU kit (The Phoenix Flow Systems, Inc., San Diego, CA).

In vivo studies

For *in vivo* experiments, 6 week old male C.B17/Icr-scid mice (n=5 mice per group) were inoculated intraperitoneally with 5×10^6 PC-3 cells using a 27-gauge needle. All animal procedures were done according to local guidelines on animal care and with appropriate institutional certification. Ten days following tumor cell inoculation, animals were given sunitinib p.o. (40 mg/kg) followed by an i.v. injection of TNF-α (0.1 mg/kg) into the tail vein. Three hours after TNF-α injection, blood was collected from the retro-orbital plexus under anesthesia from both experimental and control groups. IL-6 levels in plasma samples were examined by ELISA.

Results

Concomitant treatment with sunitinib and TNF-α resulted in apoptosis in DU-145 cells, while PC-3 cells were resistant (Fig. 1A). A dose dependent reduction of IL-6 levels in sunitinib treated DU-145 cell lines was observed (Fig. 1B). Meanwhile, the sunitinib-resistant PC-3 cells did not only fail to suppress IL-6 excretion in the presence of sunitinib, but co-administration of sunitinib and TNF-α resulted in IL-6 levels rising more than 5-fold when compared to IL-6 levels from cells treated with TNF-α alone (Fig. 1C). Similar results were seen with the TKI pazopanib (Fig. 1D). Higher levels of IL-6 protein were also

documented intracellularly by ELISA when cells were treated with sunitinib or pazopanib in the presence of TNF- α (Fig. 1E).

Despite the rise of IL-6 protein levels in PC-3 cells, both IL-6 mRNA levels (Fig. 2A) and NF- κ B activity are suppressed in both PC-3 cells (Fig. 2B) and DU-145 cells (data not shown). Addition of IL-6 at 100 ng/mL to the cell culture medium did not induce sunitinib resistance in the TKI-sensitive DU-145 cell line (Fig. 3A). Furthermore, addition of neutralizing IL-6 antibody (10-fold excess of concentration sufficient to induce 50% inhibition of biological activity) failed to restore sensitivity of PC-3 cells to sunitinib (Fig. 3B).

In an *in-vivo* mouse model in male C.B17/Icr-scid mice with diffuse peritoneal implants, after injection with TNF- α , statistically significant higher plasma levels of IL-6 were present in mice who received oral sunitinib (40 mg/kg) compared to mice who did not (Fig. 4).

Comment

Castration-resistant prostate cancer cell lines PC-3 and DU-145 respond differently to *in-vitro* sunitinib treatment. Concomitant treatment with sunitinib and TNF- α induced significant apoptosis in DU-145 cells, while PC-3 cells were resistant to such conditions (Fig. 1A). TNF- α was used to simulate *in-vivo* conditions, as TNF- α levels are elevated in prostate cancer tumors *in-situ*¹⁵ and in the serum of CRPC patients,¹⁶ but not in human prostate cancer cell line supernatants.¹⁷ Moreover, TNF- α serum levels appear elevated in patients with metastatic RCC who progress on sunitinib therapy.¹⁸

We demonstrated a dose dependent reduction of IL-6 levels in sunitinib treated DU-145 cell lines (Fig. 1B). Similar reduction in IL-6 levels was observed in the CA-HPV-10 prostate cancer cell line. In contrast, the sunitinib-resistant PC-3 cells failed to suppress IL-6 excretion in the presence of sunitinib. Moreover, when sunitinib was co-administered with TNF- α , IL-6 levels rose more than 5-fold when compared to IL-6 levels from cells treated with TNF- α alone (Fig. 1C). Comparable results were noted with pazopanib (Fig. 1D), which is a novel TKI that is currently in early clinical trials for treatment of CRPC.¹⁹ Similar to the rise of IL-6 in the extracellular supernatant, higher levels of IL-6 protein were observed intracellularly. ELISA revealed rising intracellular IL-6 levels over an 8-hour period with TNF- α stimulation in the presence of either sunitinib or pazopanib (Fig. 1E).

In an attempt to understand the mechanisms involved in IL-6 elevation, we investigated IL-6 mRNA levels and the status of NF- κ B activity in the presence of TKIs. We showed that although protein levels rise in PC-3 cells, both IL-6 mRNA levels (Fig. 2A) and NF- κ B activity, a known regulator of IL-6 synthesis, are suppressed in both TKI-resistant PC-3 cells (Fig. 2B) and TKI-sensitive DU-145 cells.²⁰ Nishioka et al. recently showed that IL-6 expression is induced in AML cells in the presence of sunitinib. The authors present evidence that this finding is due to increased binding of c-Jun to the IL-6 promoter.¹² Induction of IL-6 expression appears to proceed by a different mechanism in CRPC. Unlike in the AML model, IL-6 mRNA levels of CRPC are suppressed in the presence of TKI's (Fig. 2A). These data indicate that IL-6 upregulation in TKI-resistant CRPC occurs at a post-transcriptional level. Furthermore, IL-6 appears to be a marker of TKI resistance and not its cause. Addition of IL-6 to the cell culture medium did not induce sunitinib resistance in the TKI-sensitive DU-145 cell line (Fig. 3A). Furthermore, unlike in the AML model,¹² addition of the neutralizing IL-6 antibody failed to sensitize PC-3 cells to sunitinib-mediated apoptosis (Fig. 3B).

Next, we set out to assess whether results of *in-vitro* experiments could be replicated using the *in-vivo* mouse model of human prostate cancer. Indeed, we were able to reproduce our

findings in male C.B17/Icr-scld mice with diffuse peritoneal implants of PC-3 cells. After injection with TNF- α , statistically significant higher plasma levels of IL-6 were present in mice who received oral sunitinib (40 mg/kg) compared to mice who did not (Fig. 4).

One of the main limitations of this study is the required exogenous administration of TNF- α both *in vitro* and *in vivo*. While the use of TNF- α was necessary to simulate clinical conditions, it could be potentially be a confounding variable despite adequate controls. Furthermore, despite the promising results of these *in vitro* and *in vivo* data, the translation of IL-6 to clinical practice still remains a challenge. Although our experiments appear to establish IL-6 as a marker of treatment efficacy, these findings need to be validated in human studies. Ideally, IL-6 would be used as a serum marker to choose which patients with CRPC are responding to TKI therapy and thus should continue while sparing those patients are not exhibiting a diminution in serum IL-6 further ineffective and potentially toxic therapy.

Conclusions

The sensitivity of CRPC cells to multi-targeted tyrosine kinase inhibitors is heterogeneous. These findings are consistent with recently-published Phase II trials.^{8,9} Here we demonstrate that a substantial rise in IL-6 levels occurs both *in-vitro* and *in-vivo* in the presence of tyrosine kinase inhibitors in the resistant PC-3 cells but not in the TKI-sensitive DU-145 cells. These findings suggest that IL-6 may be a valuable biomarker of resistance to multi-targeted receptor tyrosine kinase inhibitors in patients with CRPC.

Acknowledgments

This work was supported in part by National Institutes of Health Grants RO1 CA108890 and CA134463 (VMK); American Institute for Cancer Research Grant (RGU); Department of Defense, Physician Research Training Award (AK).

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Figure 1.

A) Assessment of apoptosis using the TUNEL assay in PC-3 and DU-145 cells. B) IL-6 protein levels in cell culture supernatant of DU-145 cells treated with sunitinib. C) IL-6 levels in cell culture supernatant of PC-3 cells treated with sunitinib. D) IL-6 protein levels in cell culture supernatant of PC-3 cells treated with pazopanib. E) Intracellular IL-6 protein levels in PC-3 cells treated with either sunitinib and TNF- α (solid line), pazopanib and TNF- α (dashed line), or TNF- α only (heavy dashed line).

Figure 2.

A) IL-6 mRNA levels in PC-3 cells treated with sunitinib. B) The effect of sunitinib on the NF-κB transcriptional activity in PC-3 cells.

Figure 3.

A) Administration of IL-6 failed to rescue DU-145 cells from sunitinib-mediated apoptosis.

B) Administration of IL-6 antibody did not sensitize sunitinib-resistant PC-3 cells to sunitinib and TNF- α . Degree of apoptosis was assessed by the TUNEL assay.

Representative data from one of three experiments is shown.

Figure 4.

Plasma IL-6 levels in a PC-3 xenograft C.B17/Icr-scid mouse model. Ten days after mice were inoculated intraperitoneally with PC-3 cells, animals were given sunitinib p.o. (40 mg/kg) followed by an i.v. injection of TNF- α (0.1 mg/kg). Three hours after TNF- α injection, blood was collected from the retro-orbital plexus. IL-6 levels in plasma samples were examined by ELISA.