A SORAFENIB INDUCED MODEL OF GLOMERULAR KIDNEY DISEASE

A. Stavniichuk [Ph.D. stud.], O. Savchuk, Dr. Sc.
Taras Shevchenko National University, Kyiv, Ukraine

Abdul Hye Khan, PhD, Wojciech K. Jankiewicz [student], John D. Imig, PhD
The Medical College of Wisconsin, Milwaukee, WI, USA

Abstract

Glomerular injury and proteinuria are important pathophysiological features of chronic kidney disease. In the present study, we provide data on a glomerular injury model that was developed using the cancer chemotherapy drug sorafenib. Sorafenib is a tyrosine kinase inhibitor that acts via the vascular endothelial growth factor (VEGF) signaling pathway and is widely used to treat a variety of cancers. On the other hand, sorafenib causes serious renal side effects in patients including the development of chronic kidney disease. The current study aimed to utilize the nephrotoxic property of sorafenib to develop a rat model for chronic kidney disease. We demonstrate that rats administered sorafenib for 8 weeks along with a high salt diet (8% NaCl enriched) develop hypertension (80mmHg higher systolic blood pressure), proteinuria (75% higher), and 4-fold higher glomerular injury compared to vehicle-treated normal control rat. Sorafenib induced glomerular injury was associated with decreased (20–80% lower) renal mRNA expression of key glomerular structural proteins such as nephrin, podocin, synaptopodin, and podoplanin compared to vehicle-treated normal control rat. Renal cortical endothelial-to-mesenchymal transition (EndoMT) was activated in the sorafenib induced glomerular injury model. In the sorafenib treated rats, the renal EndoMT was evident with 20% lower mRNA expression of an endothelial marker WT-1 and 2 to 3-fold higher expression of mesenchymal markers Col III, FSP-1, α-SMA, and vimentin. In conclusion, we developed a rat pre-clinical chronic kidney disease model that manifest glomerular injury. We further demonstrate that the glomerular injury in this model is associated with decreased renal mRNA expression of key glomerular structural proteins and an activated kidney EndoMT.

Keywords
sorafenib; vascular endothelial growth factor; glomerular injury
Introduction.

Angiogenesis inhibition is a cancer chemotherapeutic approach utilizing either monotherapy or combination chemotherapy and has become a standard treatment for several types of cancers. Anti-angiogenic drugs are particularly effective against solid tumors, such as metastatic renal cell carcinoma (mRCC), non-small cell lung carcinoma, gastrointestinal stromal tumors (GIST), and colorectal carcinoma [20]. One of the main angiogenic growth factors that is targeted to treat cancer is vascular endothelial growth factor (VEGF) and VEGF receptors [6]. Several multi-targeted kinase inhibitors (MTKIs) sorafenib, sunitinib, and pazopanib were approved for the treatment of metastatic renal cell carcinoma (mRCC) and several other cancers [14,20]. MTKIs are small molecules that target the VEGF receptor VEGFR-2, the platelet-derived growth factor (PDGF) receptor, RAS, and c-KIT [4].

MTKI medications have expanded to many different solid tumors, with ongoing clinical trials with newer formulations [8]. Although very effective in treating mRCC, GIST and non-small cell carcinoma, the MTKIs such as sorafenib have several limiting serious side effects. The most common and serious side-effects of VGEF-targeted therapies are hypertension including salt-sensitive hypertension, kidney dysfunction with proteinuria, glomerular and renal tubular injuries [4].

In the present study, we have investigated the MTKI sorafenib on the kidney and glomerulus. We demonstrate that sorafenib in combination with a high sodium diet (8% NaCl) causes marked glomerular injury that is associated with a decrease in key glomerular proteins essential for glomerular structure and function. Our data also demonstrate a critical contribution for endothelial-to-mesenchymal transition (EndoMT) in sorafenib-induced kidney and glomerular injury. We propose that sorafenib can be utilized as a glomerular kidney disease model to study pathophysiology during progressive chronic kidney disease.

Material and methods.

Animal Groups

All experiments in this study were approved and carried out according to the guidelines of the Institutional Animal Care and Use Committee, Medical College of Wisconsin, Milwaukee, USA. Eight-twelve weeks old male Sprague–Dawley rats weighing 200–225 g (Charles River, MA, USA) received an 8% NaCl diet and administered vehicle or sorafenib (20mg/kg/d, p.o.) for 8 weeks. The sorafenib dose was chosen based on a previous rat study [21]. A schematic of the experimental protocol is shown in Figure 1.

Blood pressure and urine collection were done at baseline and then on days 28 and 56 of the experimental protocol. Urine samples were used for biochemical analysis to measure protein and creatinine. Blood and kidney tissues were collected at the end of the 56 day protocol. Kidney tissues were processed for histology and gene expression analysis.
Blood Pressure Measurements

Systolic blood pressure measurements of vehicle and sorafenib administered rat groups were carried out by tail-cuff plethysmography (IITC Life Science Inc., Woodland Hills, CA, USA). Blood pressure measurement was done at baseline, day 28, and day 56 of the experimental protocol.

Urinary Biochemical Analysis

Urine samples collected at baseline, day 28, and day 56 were used for protein measurement using a commercially available kit (Cayman, Ann Arbor, MI, USA). Creatinine levels in urine were measured using a commercially available kit from Cayman (Ann Arbor, MI, USA). Urinary protein and creatine values were used to calculate their urinary excretion over a 24-hour period. The ratio for urinary protein and creatinine excretion was calculated as a proteinuria index.

Histological Analysis

At the end of the 8-week experimental protocol, rats were killed for kidney tissue collection. The kidney was removed, decapsulated, cut into sections and immersion fixed in 10% neutral buffered formalin for 48 hours. Fixed kidney samples were embedded in paraffin and cut into 4μm sections. Paraffin embedded kidney slices were finally de-paraffinized, rehydrated, and stained with Periodic Acid Schiff (PAS) staining. A semi-quantitative evaluation of glomerular injury was obtained from the PAS stained kidney sections. For each kidney sample, 100 glomeruli were examined to obtain the glomerular injury score as previously described [13]. Each glomerulus was graded from 1 to 4 as follows; grade 1, normal glomerulus identified by light microscopy; grade 2, involvement of sclerosis in up to 1/3 of the glomerulus; grade 3, involvement of sclerosis in 1/3 to 2/3 of the glomerulus; and grade 4, 2/3 involvement or global sclerosis. The extent of glomerular injury is expressed as glomerular injury index [13].

Kidney Gene Expression Measurements

Kidney cortical mRNA expression for several glomerular proteins (nephrin, podocin, synaptopodin, and podoplanin) were carried out using Real Time-PCR (RT-PCR). RT-PCR was also used to determine the gene expression for endothelial cell marker Wilms Tumor 1 (WT-1) and several mesenchymal markers fibronectin, collagen III (Col III), α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (FSP-1) and vimentin. Messenger RNA (mRNA) was prepared from each kidney cortical sample using RNeasy Mini Kit (QIAGEN, CA, USA). The mRNA samples were quantified spectrophotometrically and cDNA was synthesized from 1μg of total RNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Gene expression was quantified by iScript One-Step RT-PCR Kit with SYBR green using the MyiQ™ Single Color RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Dissociation curve analysis was carried out with iQ5 Optical System Software, Version 2.1 (Bio-Rad Laboratories, Hercules, CA, USA), and each amplified sample analyzed for homogeneity. During RT-PCR, the denaturation of the cDNA was done at 95°C for 2 min followed by 40 cycles used at 95°C for 10s and at 60°C for 30s.
All mRNA samples were run in triplicate and fold change in gene expression was compared to controls determined by comparative threshold cycle (Ct) method. Expression levels of the gene of interest were determined by normalizing Ct values to two housekeeping genes.

**Statistical Analysis**

All data are expressed as mean ± S.E.M. GraphPad Prism® Version 4.0 software was utilized statistical analysis (GraphPad Software Inc, La Jolla, CA, USA). Two-tailed unpaired Student’s t-test was applied to determine statistical significance between groups. P value of <0.05 was deemed significant.

**Results and discussion**

**Sorafenib causes hypertension and proteinuria**

Hypertension is the most common cardiovascular event that occurs with VEGF-signaling pathway inhibitors [2]. Sorafenib induces hypertension in around one-quarter of the patients [27]. In agreement with this finding, data in the present study demonstrate that sorafenib treatment along with a high salt diet resulted in a marked hypertension. Systolic blood pressure increased by 80 mmHg in rats administered sorafenib compared to normal control-vehicle rats by the end of the 8-week protocol (Figure 2A).

Evidence indicates that VEGF inhibition causes hypertension as a consequence of decreased nitric oxide production [11]. Another proposed mechanism by which VEGF-signaling pathway inhibition leads to hypertension is that inhibition of the VEGF-signaling pathway increases the production of endothelin-1, a potent vasoconstrictor [2]. It is important to note that sorafenib is known to cause salt-sensitivity [26], hence, it is very likely that the placing the sorafenib treated rats on high salt diet is also played an important pathophysiological role in the development of hypertension and kidney injury assessed from proteinuria.

In relation a role of salt sensitivity in VEGF inhibition associated hypertension and kidney injury, in normotensive Sprague-Dawley (SD) rats, the tyrosine kinase inhibitor SU5416 induced salt-sensitive hypertension and kidney injury. In a study performed in normotensive Wistar–Kyoto rats exposed to a low dose of sunitinib, a similar VEGF inhibitor like sorafenib, it is found that a high salt diet augmented the rise in blood pressure and proteinuria [16]. Different mechanisms may account for the salt sensitive hypertension during VEGF inhibition. As suggested by Gu et al, the VEGF inhibition–induced decrease in NO production by renal proximal tubular cells may impair the pressure-natriuresis response because of impaired vasodilatation in the vasa recta [10]. Additionally, it is suggested that the VEGF inhibition can activate endothelin system and contribute to salt-sensitive hypertension [19].

In the present study, our data demonstrate that the sorafenib treated rats develop marked proteinuria with a 40-fold higher urinary protein excretion compared to normal control-vehicle rats (Figure 2B).
Like hypertension, development of proteinuria is another common side effect associated with VEGF inhibitors including sorafenib [5,12]. Other findings substantiate that VEGF inhibition by drugs like sorafenib causes damage to the glomerular filtration system and leads to proteinuria [12].

**Sorafenib causes glomerular injury**

In the present study, we demonstrate that rats administered sorafenib in combination to a high sodium diet develop marked 4-fold higher glomerular injury characterized by glomerular sclerosis, mesangiolysis, and glomerular capillary injury (Figure 3AB).

These findings corroborate those of several earlier studies that described glomerular injury during VEGF inhibition [3,22]. Not only others, in an earlier study, we demonstrated that sorafenib treatment along with high salt diet caused marked glomerular injury in rats [21]. The glomerulus is a highly specialized filtration apparatus with selective permeability that allows free passage of water and solutes, but not protein. The permselectivity of the glomerular filtration barrier restricts protein passage into Bowman’s space. Glomerular barrier breakdown and loss of permselectivity leads to proteinuria, which is common in renal diseases. The current study found increased urinary protein and glomerular barrier breakdown in rats administered sorafenib. Although the details of glomerular filtration permselectivity and barrier remain unknown, it is clear that the glomerular epithelial cell and podocyte are important glomerular filtration barrier components. Podocyte injury is frequently involved in the pathogenesis of glomerular diseases [24]. Podocyte damage can be the result of changes in individual podocyte-associated proteins including those that assemble and stabilize the slit diaphragm and those that anchor the foot process to the glomerular basement membrane. In the present study, we evaluated kidney cortical mRNA expression of these proteins including nephrin, podocin, synaptopodin, and podoplanin. Our findings demonstrate that in rats administered sorafenib there is 20–80% lower mRNA expression of glomerular proteins compared to normal control-vehicle rats (Figure 3C–F). It has been proposed that decreased expression of glomerular proteins could be linked to decreased VEGF signaling that occurs with sorafenib administration. Indeed, VEGF is crucial to the maintenance of normal renal function, and both VEGF over- and under-expression can disrupt normal glomerular function. The interaction between VEGF generated by podocytes and VEGFR-2 on glomerular endothelial cells is necessary to maintain glomerular slit diaphragm barrier integrity [8,9,15]. Several studies underscored the importance of VEGF signaling in kidney health. Selective depletion of one VEGF allele in podocytes in mice leads to down regulation of the slit-diaphragm protein nephrin, resulting in proteinuria and structural and functional glomerular damages [7,25].

**Kidney injury in sorafenib-induced model is associated with EndoMT**

Endothelial-to-mesenchymal transition (EndoMT) is a subtype of epithelial–mesenchymal transition. EndoMT is a novel source for myofibroblasts and contributes importantly to fibrosis and chronic kidney disease progression. During EndoMT, endothelial cell progressively changes their endothelial phenotype into a mesenchymal phenotype resulting in the loss of specific endothelial markers like WT-1 and gain in mesenchymal markers, such
as α-SMA or FSP-1 [1,23]. Previous findings indicate that EndoMT plays a critical role in glomerular injury and results in albuminuria during diabetic nephropathy. The findings described in this study and several other studies demonstrate a role EndoMT in causing podocyte damage leading to glomerular injury [17,18,28]. In the present study, we demonstrate that sorafenib administration caused proteinuria and glomerular injury in rats. Considering a role of EndoMT in glomerular injury we investigated if EndoMT contributes to sorafenib-induced glomerular injury. The current findings reveal that sorafenib administration to rats decreased renal cortical endothelial cell marker WT-1 mRNA expression by 20% and increased mesenchymal marker Col III, FSP-1, α-SMA and vimentin mRNA expression by 2–3 fold when compared to normal control-vehicle rats (Figure 4A–F).

These results indicate a potential contribution for EndoMT on glomerular endothelial cells to glomerular injury caused by sorafenib.

**Conclusion.**

VEGF signaling inhibition by sorafenib administration resulted in hypertension and kidney injury. Sorafenib administration induced glomerular injury was associated with increased EndoMT and decreased glomerular barrier proteins. Accordingly, our findings establish sorafenib administration as a glomerular disease model in which glomerular injury is associated with decreased key glomerular structural proteins and activated kidney EndoMT.

**References (Scopus)**

1. Asada N, Takase M, Nakamura J, et al. Dysfunction of fibroblasts of extrarenal origin underlies renal fibrosis and renal anemia in mice. J Clin Investig. 2011;121(10):3981–90. [PubMed: 21911936]
2. Bair SM, Choueiri TK, Moslehi J. Cardiovascular complications associated with novel angiogenesis inhibitors: emerging evidence and evolving perspectives. Trends Cardiovasc Med. 2013 5;23(4):104–13. [PubMed: 23290365]
3. Boursiquot BC, Zabor EC, Glezerman IG, Jaimes EA. Hypertension and VEGF (Vascular Endothelial Growth Factor) Receptor Tyrosine Kinase Inhibition: Effects on Renal Function. Hypertension. 2017 7 24 pii: HYPERTENSIONAHA.117.09275.
4. Broxterman HJ, Georgopapadakou NH. Anticancer therapeutics: “Addictive” targets, multitargeted drugs, new drug combinations. Drug Resist Updat. 2005 8; 8(4):183–197. [PubMed: 16154800]
5. Donoviel DB, Freed DD, Vogel H, et al. Proteinuria and perinatal lethality in mice lackingNEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol. 2001; 21(14):4829–4836. [PubMed: 11416156]
6. Eremina V, Quaggin SE. Biology of anti-angiogenic therapy-induced thrombotic microangiopathy. Seminars in nephrology. 2010; 30(6):582–590. [PubMed: 21146123]
7. Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest. 2003;111(5):707–716. [PubMed: 12618525]
8. Eskens FA, Verweij J. The clinical toxicity profile of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor (VEGFR) targeting angiogenesis inhibitors a review. Eur J Cancer. 2006; 42(18):3127–3139. [PubMed: 17098419]
9. Garovic VD, Wagner SJ, Petrovic LM, et al. Glomerular expression of nephrin and synaptopodin, but not podocin, is decreased in kidney sections from women with preeclampsia. Nephrol Dial Transplant. 2007; 22(4):1136–1143. [PubMed: 17255128]

10. Gu JW, Manning RD Jr, Young E, Shparago M, Sartin B, Bailey AP. Vascular endothelial growth factor receptor inhibitor enhances dietary salt induced hypertension in Sprague-Dawley rats. Am J Physiol Regul Integr Comp Physiol. 2009;297(1):R142–R148. [PubMed: 19420288]

11. Hamnvik OP, Choueiri TK, Turchin A, McKay RR, Goyal L, Davis M, Kaymakcalan MD, Williams JS. Clinical risk factors for the development of hypertension in patients treated with inhibitors of the VEGF signaling pathway. Cancer. 2015 1 15;121(2):311–9. [PubMed: 25236375]

12. Hayman SR, Leung N, Grande JP, Garovic VD. VEGF inhibition, hypertension, and renal toxicity. Curr Oncol Rep. 2012 8;14(4):285–94. [PubMed: 22544560]

13. Hye Khan MA, Kolb L, Skibba M, Hartmann M, Blöcher R, Proschak E, Imig JD. A novel dual PPAR-γ agonist/sEH inhibitor treats diabetic complications in a rat model of type 2 diabetes. Diabetologia. 2018 10;61(10):2235–2246. [PubMed: 30032428]

14. Jászai J, Schmidt MHH. Trends and Challenges in Tumor Anti-Angiogenic Therapies. Cells. 2019;8(9):1102.

15. Kitamoto Y, Tokunaga H, Miyamoto K, Tomita K. VEGF is an essential molecule for glomerular structuring. Nephrol Dial Transplant. 2002; 17(Suppl 9):25–27.

16. Lankhorst S, Baelde HJ, Claesen-van Groningen MC, Smedts FM, Danser AH, van den Meiracker AH. Effect of high salt diet on blood pressure and renal damage during vascular endothelial growth factor inhibition with sunitinib. Nephrol Dial Transplant. 2016;31(6):914–21. [PubMed: 26681729]

17. Liu F, Zhang S, Xu R, Gao S, Yin J. Melatonin Attenuates Endothelial-to-Mesenchymal Transition of Glomerular Endothelial Cells via Regulating miR-497/ROCK in Diabetic Nephropathy. Kidney Blood Press Res. 2018;43(5):1425–1436. [PubMed: 30212830]

18. Ma Z, Zhu L, Liu Y, Wang Z, Yang Y, Chen L, Lu Q. Lovastatin Alleviates Endothelial-to-Mesenchymal Transition in Glomeruli via Suppression of Oxidative Stress and TGF-B1 Signaling. Front Pharmacol. 2017 Jul 18:8:473.

19. Machnik A, Dahlmann A, Kopp C, Goss J, Wagner H, van Rooijen N, Eckardt KU, Müller DN, Park JK, Luft FC, Kerjaschki D, Titze J. Mononuclear phagocyte system depletion blocks interstitial toxicity-responsive enhancer binding protein/vascular endothelial growth factor C expression and induces salt-sensitive hypertension in rats. Hypertension. 2010;55(3):755–61. [PubMed: 20142563]

20. Mantia CM, McDermott DF. Vascular endothelial growth factor and programmed death-1 pathway inhibitors in renal cell carcinoma. Cancer. 2019;125(23):4148–4157. [PubMed: 31532565]

21. Nagasawa T, Hye Khan MA, Imig JD. Captopril attenuates hypertension and renal injury induced by the vascular endothelial growth factor inhibitor sorafenib. Clin Exp Pharmacol Physiol. 2012 5;39(5):454–61. [PubMed: 22443474]

22. Ollero M, Sahali D. Inhibition of the VEGF signaling pathway and glomerular disorders. Nephrol Dial Transplant. 2015 Sep;30(9):1449–55.

23. Piera-Velazquez S, Li Z, Jimenez SA. Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. Am J Pathol. 2011;179:1074–80. [PubMed: 21763673]

24. Shankland SJ. The podocyte’s response to injury: role in proteinuria and glomerulosclerosis. Kidney Int. 2006 6:69(12):2131–47 [PubMed: 16688120]

25. Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, Kalluri R. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem. 2003;278(15):12605–12608. [PubMed: 12538598]

26. van den Meiracker AH, Danser AH. Mechanisms of Hypertension and Renal Injury During Vascular Endothelial Growth Factor Signaling Inhibition. Hypertension. 2016;68(1):17–23. [PubMed: 27185750]
27. Wu S, Chen JJ, Kudelka A, Lu J, Zhu X. Incidence and risk of hypertension with sorafenib in patients with cancer: a systematic review and meta-analysis. Lancet Oncol. 2008 2;9(2):117–23. [PubMed: 18221915]

28. Zhao L, Zhao J, Wang X, Chen Z, Peng K, Lu X, Meng L, Liu G, Guan G, Wang F. Serum response factor induces endothelial-mesenchymal transition in glomerular endothelial cells to aggravate proteinuria in diabetic nephropathy. Physiol Genomics. 2016 10 1;48(10):711–718. [PubMed: 27565710]
**Figure 1:**
A schematic depicting the experimental protocol used in the study.
Figure 2:
Systolic blood pressure in different experimental groups (A). Proteinuria expressed as the ratio of urinary protein to creatinine excretion in different experimental groups (B). *P vs. Normal Control-Vehicle. All data are expressed as mean ±SEM, N=6.
Figure 3:
Glomerular injury index in different experimental groups (A). A representative photomicrograph depicting glomerular injury in normal control-vehicle and rats administered sorafenib (B). Kidney cortical expression of several glomerular proteins in different experimental groups (C-F). *P vs. Normal Control-Vehicle. All data are expressed as mean +/-SEM, N=6.
Figure 4:
Kidney cortical expression of an endothelial cell marker, WT-1 (A), and several mesenchymal markers (Col III, α-SMA, FSP-1 and vimentin) in different experimental groups (B-E). *P vs. Normal Control-Vehicle. All data are expressed as mean ± SEM, N=6.