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Neoadjuvant Sorafenib Treatment of Clear Cell Renal Cell Carcinoma and Release of Circulating Tumor Fragments¹

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Abstract

BACKGROUND: Clear cell renal cell carcinoma (ccRCC) is characterized by high constitutive vascular endothelial growth factor A (VEGF-A) production that induces a specific vascular phenotype. We previously reported that this phenotype may allow shedding of multicellular tumor fragments into the circulation, possibly contributing to the development of metastasis. Disruption of this phenotype through inhibition of VEGF signaling may therefore result in reduced shedding of tumor fragments and improved prognosis. To test this hypothesis, we investigated the effect of neoadjuvant sorafenib treatment on tumor cluster shedding. **PATIENTS AND METHODS:** Patients with renal cancer ($n = 10$, of which 8 have ccRCC) received sorafenib for 4 weeks before tumor nephrectomy. The resection specimens were perfused, and the perfusate was examined for the presence of tumor clusters. Effects of the treatment on the tumor morphology and overall survival were investigated (follow-up of 2 years) and compared with a carefully matched control group. **RESULTS:** Neoadjuvant sorafenib treatment induced extensive ischemic tumor necrosis and, as expected, destroyed the characteristic ccRCC vascular phenotype. In contrast to the expectation, vital groups of tumor cells with high proliferation indices were detected in postsurgical renal venous outflow in 75% of the cases. Overall survival of patients receiving neoadjuvant treatment was reduced compared to a control group, matched with regard to prognostic parameters. **CONCLUSIONS:** These results suggest that neoadjuvant sorafenib therapy for ccRCC does not prevent shedding of tumor fragments. Although this is a nonrandomized study with a small patient group, our results suggest that neoadjuvant treatment may worsen survival through as yet undefined mechanisms.

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Introduction

Although the molecular and cellular mechanisms that are involved in metastatic spread of tumors are gradually being unraveled, much is still poorly understood. Because metastatic disease is the leading cause of death of patients with cancer, deciphering these mechanisms is of extreme importance. The currently most popular paradigm is the seed and soil hypothesis, put forward by Paget already in 1889 [1]. This hypothesis states that solitary tumor cells that are shed from a tumor (the seeds) can develop into clinically relevant lesions only in

Abbreviations: ccRCC, clear cell renal cell carcinoma; VEGF, vascular endothelial growth factor

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compatible tissues (the soil) where interactions with the specific tissue microenvironment allow tumor outgrowth. Epithelial-to-mesenchymal transition is nowadays seen as a key developmental program that needs to be activated during cancer invasion and metastasis [2]. The mesenchymal phenotype allows cells to migrate and spread through surrounding extracellular matrix in a protease-dependent manner before eventually invading into blood vessels and entering the circulation [3,4].

We and others have proposed an alternative mechanism of metastasis that is independent of epithelial-to-mesenchymal transition and does not require proteolytic degradation of the vessel wall [5–7]. When investigating effects of constitutive overexpression of vascular endothelial growth factor A (VEGF-A) on tumor histology and biology, we found that this factor induced a highly metastatic phenotype in melanoma xenografts. This phenotype could be attributed to VEGF-A–induced development of a so-called micronodular phenotype, which develops by budding of clusters of tumor cells into dilated tumor vasculature. During this budding process, the clusters become gradually covered with vessel wall elements as they enter the blood vessel. We demonstrated that these clusters may eventually enter the circulation and are ultimately caught in the lung vasculature where they may grow out to pulmonary metastases [5].

In approximately 80% of clear cell renal cell carcinomas (ccRCCs), the *Von Hippel-Lindau* (VHL) gene is mutated resulting in nonfunctional *Von Hippel Lindau* protein (pVHL) [8]. As normal pVHL is involved in the controlled degradation of hypoxia-inducible factor 1 α , pVHL mutations result in accumulation of this transcription factor and constitutive expression of its target gene *VEGF-A*, also under normoxic conditions [9]. The majority of VEGF-A–expressing ccRCCs present with a micronodular phenotype [10]. We previously reported that ccRCC tumor cell clusters are often located in the lumen of vessels from where they can enter the circulation [11]. We observed a correlation between the occurrence of these clusters in renal venous outflow and the presence of pulmonary metastases suggesting that shedding of tumor cell clusters into the circulation results in subsequent capture in the lung capillary bed, followed by outgrowth.

The well-recognized importance of VEGF and PDGF signaling pathways in ccRCC has provided a rationale for treating patients with metastatic and progressive disease with tyrosine kinase inhibitors, among which is sorafenib, a small-molecule inhibitor of VEGF receptor and platelet derived growth factor receptor tyrosine kinases and RAF kinase [12–14]. Although these therapies are indicated for disseminated disease, patients with locally extensive RCC have also been treated in phase II trials in a neoadjuvant setting with the aim to reduce tumor size and downstage the tumors, facilitating complete resection [15–17]. Limited histopathologic data show extensive

necrosis and fibrosis in the primary tumor on neoadjuvant treatment, although a risk of fulminant angiogenic outgrowth during drug holidays has been observed [18]. The destruction of the micronodular phenotype of the tumor, which is a direct result of anti-VEGF treatment, may also impinge on the metastatic potential of these tumors, as has been suggested in previous preclinical studies [5].

Here, we investigated the effects of neoadjuvant sorafenib treatment on the histopathology of the primary tumor, on shedding of tumor cell clusters into the circulation, and on survival.

Materials and Methods

Patients

The study described here was a side study to Trial No. NCT08602862 in which molecular imaging is performed on patients with renal cell cancer after neoadjuvant sorafenib treatment. Part of the data from the trial have been published before [19,20]. All patients included in this study signed informed consent. Ten patients were included in a period of 2 years. Eligible for sorafenib treatment, followed by nephrectomy, were patients with RCC with a Karnofsky score >70% who did not receive prior chemotherapy, immunotherapy, or radiation therapy. The decision to participate in the study was not further influenced by clinical performance as Motzer scores were calculated only at poststudy evaluation. Patient characteristics and clinicopathologic parameters have been published before, and an updated summary is given in Table 1, with more detailed information on individual cases in Table 2 [19]. TNM staging was performed according to the TNM classification, seventh edition. Tumors were classified as ccRCC ($n = 8$), chromophobe RCC ($n = 1$), and papillary RCC ($n = 1$). All tumors were pT3a, pT3b, or pT4 and G2 to G4. Seven patients in the treatment group were in retrospect suspect for having metastatic disease. Patients were treated for 4 weeks with sorafenib (400 mg twice a day), and nephrectomy was performed 3 days after the last administration.

For survival analyses, we carefully composed a control group of 12 patients with ccRCC from our previous study who underwent tumor nephrectomy without any neoadjuvant treatment [11]. This group was matched to the treatment group with respect to TNM staging. All patients in this control group had confirmed metastatic disease at first presentation.

Perfusion of Nephrectomy Specimen and Analysis of Renal Venous Blood

Renal outflow from tumor nephrectomies was examined essentially as described [11]. In short, a balloon catheter was placed in the renal artery, and the kidney was perfused with a controlled flow of 0.9% NaCl using a peristaltic pump (Watson-Marlow, Rotterdam, The Netherlands). Perfusion was continued at a rate of 30 ml/min until

Table 1. Patient Characteristics and Occurrence of Tumor Cell Clusters in Venous Outflow in Patients with Renal Carcinoma after Neoadjuvant Sorafenib Treatment and in Untreated Patients with ccRCC.

	<i>n</i>	Median Age	Sex	Tumor Size	BVI	No. with Shed Tumor Clusters
Neoadjuvant	8 ccRCCs	58 (45-78)	70% M	9.4 cm (5-15)	7 of 8 (88%)	6 of 8 (75%)
	2 Non-ccRCCs	57 (49-64)	50% M	6.1 cm (5.5-7.7)	0 of 2 (0%)	0 of 2 (0%)
Untreated*	14 ccRCCs	61 (43-70)	71% M	10.5 cm (6-16.5)	6 of 8 (75%)	10 of 14 (72%)

BVI, blood vessel invasion.

Difference between shedding of tumor cell clusters in neoadjuvant treated and untreated tumors is not different. Note that in the treated group, seven patients in retrospect were suspect of metastatic disease at first presentation.

* Control group has been published in reference 10.

Table 2. Clinical Parameters.

Patient No.	Sex	Age	LDH (Units/l)	Hb (mM)	Leuco (10 ⁹ /l)	Neutro (%)	Thrombo (10 ⁹ /l)	Calcium (mM)	KPS (%)	Metastasized at Diagnosis	Stage and Grade	Diameter	Microvascular Invasion	Tumor Fragments	Overall Survival (months)	Systemic Treatment
Control group																
1	F	61.2	402	6.1	8.5	72	331	2.55	100	Yes	pT3b/G3	7.5	NS	No	65+	None
2	M	67.7	483	8	7.7	NA	331	2.46	90-100	Yes	pT3b/G2	6.0	+	Yes	20	INF- α and sunitinib
3	M	60.7	464	8.8	5	67	240	2.43	90-100	Yes	pT3b/G4	10.5	+	Yes	18	Sunitinib
4	F	42.7	NA	8.2	9.6	NA	403	2.61	90-100	Yes	pT2/G34	9.1	-	No	37	Sunitinib
5	M	66.6	625	6.9	5.1	NA	178	2.51	90-100	Yes	pT3b/G4	16.5	+	Yes	13	INF- α
6	M	67.5	398	6.9	8.4	77	350	2.38	90-100	Yes	pT3a/G3	12.0	NS	No	27	INF- α and RIT
7	M	44.7	347	8.9	6	NA	265	2.35	90-100	Yes	pT3b/G3	9.5	+	Yes	66	Triple, RIT, and sunitinib
8	M	61.5	361	8.8	6.7	NA	281	2.32	90-100	Yes	pT3b/G2	9.0	NS	Yes	75	RIT, sunitinib
9	M	54.6	490	10	5.4	NA	209	2.52	90-100	Yes	pT3a/G3	12.3	-	Yes	78+	None
10	F	68.1	372	9.1	10.2	NA	235	2.58	90-100	Yes	pT3a/G2	14.5	NS	Yes	77+	RIT
11	M	69.3	889	5.6	8	78	437	2.88	90	Yes	pT3b/G3	9.0	+	Yes	Lost to follow-up	None
12	M	60.5	313	7.8	5.5	68	189	2.42	100	Yes	pT3b/G3	14.0	NS	Yes	56+	Sunitinib and temsirolimus
13	F	69.8	348	8.7	7.4	62	228	2.74	100	Yes	pT3b/G3	9.0	NS	Yes	64+	Pazopanib
14	M	46.2	291	9.8	9.5	67	240	2.38	90-100	Yes	pT4/G4	8.5	+	No	85+	Sunitinib, temsirolimus, and pazopanib
Neoadjuvant sorafenib group																
15	M	58.1	523	8.9	4.8	68	213	2.53	90-100	Yes	pT3b/G3	13.0	NS	No	35	RIT
16	M	77.8	500	7.9	9	72	274	2.56	100	Yes	pT3b/G4	14.0	NS	Yes	43	None
17	F	61.8	304	6.4	8.8	56	850	2.69	90	Yes	pT3b/G2	6.5	+	No	71+	None
18	M	47.1	427	7.7	12.2	78	489	2.68	90	Yes	pT3b/G3	9.5	+	No	7	Sorafenib
19	M	45.1	532	8.3	5.6	72	261	2.33	100	Yes	pT3a/G34	6.5	+	Yes	7	Sunitinib
20	M	57.5	360	7.2	9.1	64	309	3.32	80	Yes	pT4/G4	15.0	+	NA	10	Sunitinib
21	M	50.1	333	7.2	8.1	75	320	3.38	100	Yes	pT3a/G4	5.5	-	NA	7	Sorafenib

NA, not available; NS, not scored; LDH, lactate dehydrogenase; Hb, hemoglobin; Leuco, leukocytes; Neutro, neutrophils; Thrombo, thrombocytes; Calcium, corrected calcium; KPS, Karnofsky performance score; INF- α , interferon- α ; RIT, radioimmunotherapy; Triple, interferon- α plus interleukin-2 plus 5-fluorouracil.

the perfundate was clear. Typically, 500- to 750-ml perfundate was collected. Outflow was filtered through a 75- μ m mesh nylon sieve. After washing the filter with 0.9% NaCl, the residue was collected, concentrated by centrifugation (500g for 5 minutes), and fixed in Unifix (Klinipath, Duiven, The Netherlands). Subsequently Agar-Cyto blocks were prepared by carefully resuspending the fixed residue in 2% liquid agarose (LE, analytical grade, Promega, Madison WI) followed by standard paraffin embedding of the solidified agarose blocks [21]. After this procedure, primary tumor tissue was processed for standard diagnosis and immunohistochemical staining.

Immunohistochemistry

AgarCyto blocks and corresponding primary tumors were analyzed by hematoxylin and eosin (H&E) staining and immunohistochemistry using antibodies against CD31 (Dako, Glostrup, Denmark) to detect endothelial cells, collagen IV (Abcam, Cambridge, United Kingdom) to detect vessel basement membrane, α -smooth muscle actin (Sigma, Zwijndrecht, The Netherlands) to detect pericytes, and carbonic anhydrase IX (M75, Dr. Oosterwijk) to detect tumor cells [22]. Proliferating tumor cells were detected using an antibody against Ki67 (clone sp6; Abcam). Inflammatory cells were detected using antibodies against CD2 (Neomarkers, Fremont, CA) to detect T-lymphocytes and myeloperoxidase (MPO; Neomarkers) to detect granulocytes. Antibodies were visualized by appropriate biotin-labeled secondary antibodies and avidin peroxidase (Vector Laboratories, Burlingame, CA), as previously described [23]. Primary tumors were assessed for necrosis, blood vessel wall changes, carbonic anhydrase-IX expression, micronodular phenotype, (micro)vascular invasion, and inflammation. To minimize sampling errors, at least 10 paraffin blocks taken from different areas from each tumor were analyzed. Ten untreated ccRCCs from our local pathology archive were included as controls to assess extent of necrosis. Scoring for

micronodular phenotype, based on CD31 stainings, was performed independently by two investigators (G.K.-U. and W.L.). To quantify inflammatory cells, MPO and CD2 immunoreactivity was scored independently by two pathologists (H.v.K. and G.K.-U.) as ranging from 0 (no stained cells) to 3 (high density of positive cells). In case of discordance, consensus was reached at a double-headed microscope.

Statistical Analysis

Relationships between neoadjuvant treatment and occurrence of tumor material in renal outflow were analyzed in 2 \times 2 contingency tables using Fisher exact test (two sided) in IBM SPSS Statistics version 20.0 (Chicago, IL). Median survival differences were tested in GraphPad Prism version 5.03 (Graphpad Software Inc., San Diego, CA) for significance using a Mantel-Cox test and were considered significant at $P < 0.05$.

Results

Effects of Sorafenib Treatment on Primary Tumors

We first analyzed the effects of neoadjuvant sorafenib treatment on the histopathology of RCCs. In our series, two renal tumors were of the nonclear cell type. These tumor types do not constitutively express VEGF-A and do not present with a micronodular vascular phenotype. We included these tumors in the analyses to investigate if lack of constitutive VEGF expression affects response to sorafenib. Effects of sorafenib on the morphology of these tumors were minimal (not shown). In contrast, sorafenib induced profound effects on the ccRCCs; these all showed extensive areas of necrosis, although areas of vital tumor tissue were still present (Figure 1, A and B). At macroscopic cross-sectioning, abundant liquefactive necrosis was observed more often than in historical control tumors that had been processed similarly in our department (not shown).

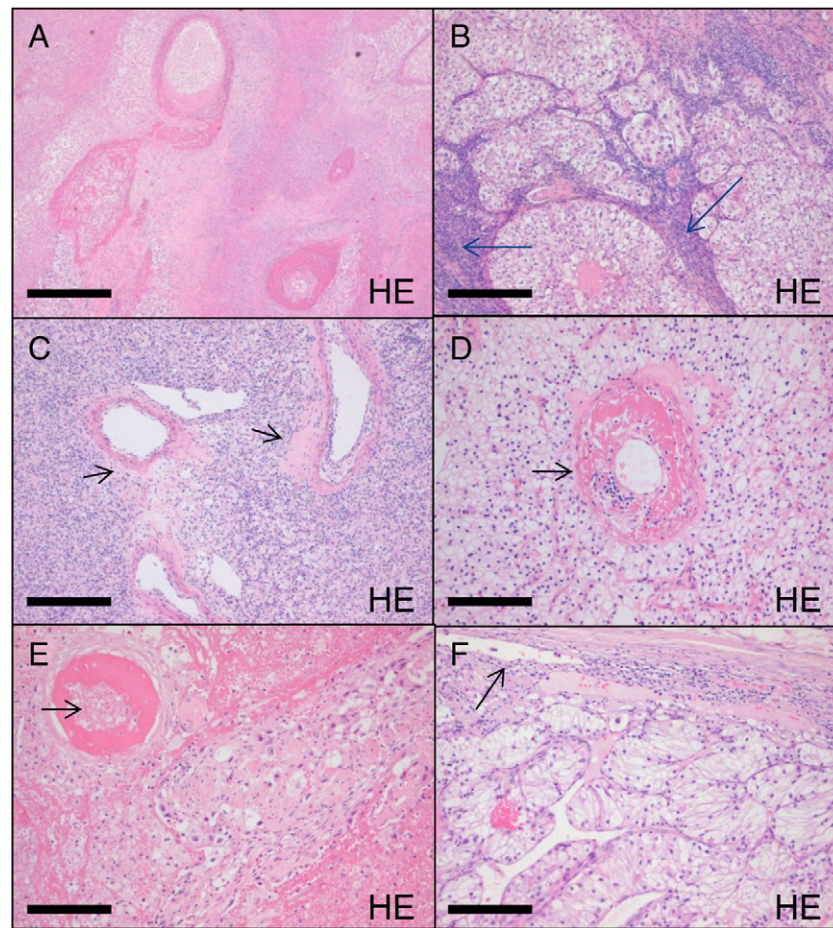


Figure 1. Histologic analysis of effects of sorafenib treatment on primary tumor. Throughout the tumor, extensive areas of necrosis are seen (H&E staining in A; note also necrotic remnants of blood vessels), whereas also areas of vital tumor can be observed, interspersed with heavily inflamed fibrovascular structures (see H&E staining in B). A spectrum of tumoral blood vessel responses to sorafenib treatment is seen, ranging from edema of the vessel wall (see arrows in C) to fibrinoid necrosis with endothelial cell fragmentation, fibrin deposition, inflammatory cells (D, arrow), and obliteration (E, arrow). For comparison, F shows a nontreated control tumor with the characteristic micronodular phenotype and undamaged blood vessels (arrow). Scale bar, 400 μm (in A); 200 μm (in B and C); 100 μm (in D–F).

Histologically, the treated ccRCCs frequently showed prominent edema and fibrinoid necrosis of the blood vessel walls (Figure 1, C and D, arrows). Vessel lining by endothelial cells was often discontinuous, with fragmentation and sometimes thrombus formation (Figure 1E, arrow). In central necrotic areas, remnants of necrotic vessels were also observed (Figure 1A). Importantly, the vascular changes induced by sorafenib were tumor specific as vessels in normal renal tissue distant from the tumor showed a normal appearance (not shown). Furthermore, the vasculature in vital tissue in control (nontreated) ccRCC showed a regular endothelial lining (Figure 1F).

In seven of eight ccRCCs, prominent blood vessel (micro)invasion was observed (Table 1). None of the treated tumors showed a micronodular phenotype (not shown), whereas this phenotype was seen in 45% of the nontreated tumors [11].

In treated tumors, intratumoral lymphocyte densities were higher than in control tumors (78% score 2 or higher *vs* 50% in control tumors; Figure 2 and Table 3). A consistent observation was that very high densities of lymphocytes were found associated with fibrovascular structures (Figures 1B, arrows, and 2, A and B) and damaged

tumor vasculature (Figure 2, C and D), whereas nontreated ccRCC showed some lymphocytic infiltrates mainly concentrated in the tumor rim (Figure 2, E and F). Granulocyte counts as determined by MPO staining were in general low (not shown).

Presence of Tumor Cell Groups in the Perfundate

In six of eight patients with ccRCC in the neoadjuvant group (75%), small groups of CA-IX–positive tumor cells (3–15 cells) could be detected in the filtered perfundate (a representative example is shown in Figure 3A; see also Table 1). The cells were sometimes loosely attached to each other and showed irregular cytoplasmic extensions. All these groups contained proliferating tumor cells (Ki67 staining in Figure 3B). Association with endothelial cells or pericytes, as demonstrated by CD31 or α -smooth muscle actin staining, was not observed in any of the collected tumor cell groups (not shown). This was in contrast to our previous study, where we found vessel basement membranes and/or endothelial cell linings in 30% of tumor cell clusters in untreated patients (see example in Figure 3C) [11]. Venous outflow material from the chromophobe and papillary RCC did not contain tumor cells or clusters (Table 1).

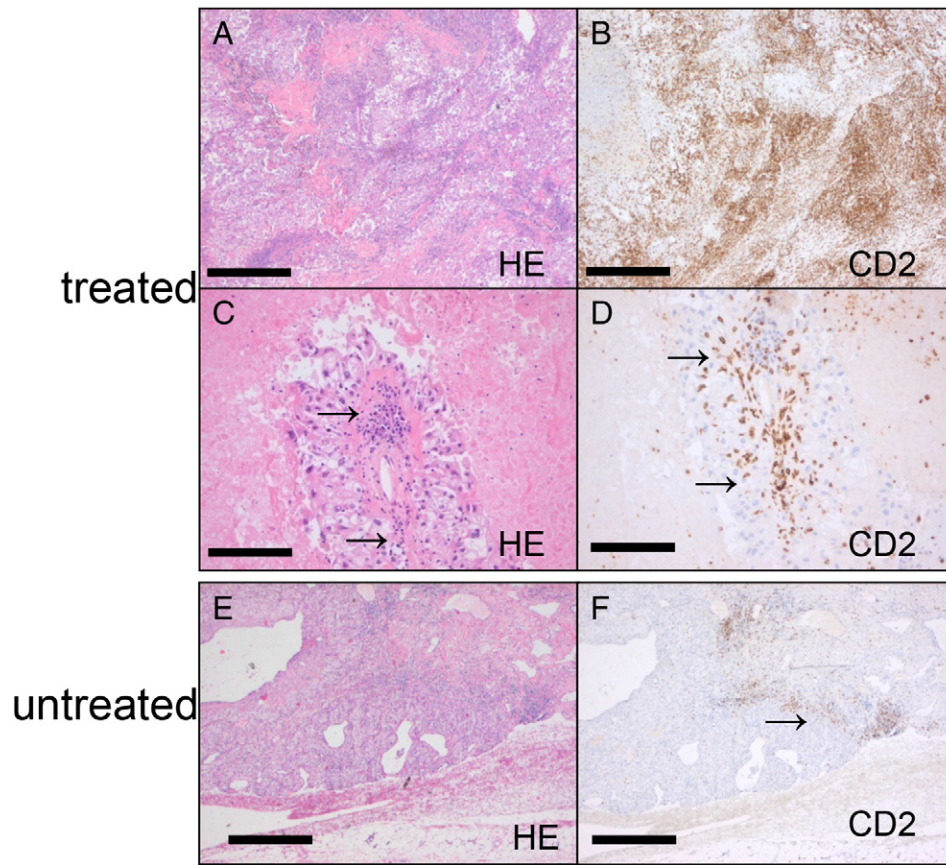


Figure 2. Inflammatory response in RCC after neoadjuvant sorafenib treatment. Shown are serial sections, stained with H&E (A) and the T-lymphocyte marker CD2 (B). Note heavy infiltration of CD2-positive cells in B and perivascular lymphocytes in C and D (arrow). E and F show H&E staining and CD2 staining, respectively, of serial sections of an untreated tumor (E). Lymphocyte infiltration is much less profound and is mainly localized in the rim of the tumor (arrow in F). Scale bar, 400 μ m (in A, B, E, and F); 100 μ m (in C and D).

Effect of Neoadjuvant Sorafenib Treatment on Survival

Because the control group was heterogeneous and to prevent bias, the control group was narrowed down to match the treated group with respect to disease stage and pathophysiological and clinical parameters, ultimately resulting in 12 control patients and 7 patients in the neoadjuvant group (see Table 2). Kaplan-Meier analysis of patients in the neoadjuvant group revealed significantly poorer overall survival than the control group (Figure 4) [11]. Even with this small group, a significantly longer median survival was found in the control group (66 months, range = 13-85 months, six patients still alive), compared to the patients in the neoadjuvant sorafenib-treated group (10 months, range = 7-71 months, one patient still alive); $P = .038$. This resulted in a hazard ratio of 4.42 (confidence interval = 1.09-16.81).

Discussion

We previously reported that in 33% of patients with ccRCC, tumor cell clusters can be detected in renal venous outflow and that the occurrence of these shed clusters correlates with the presence or metachronous development of pulmonary metastasis [11]. The presence of such tumor tissue fragments was also associated with the micronodular phenotype that is characteristically found in tumors with high VEGF-A expression [7,10]. Disruption of the VEGF-A-induced micronodular phenotype by VEGFR2 inhibition in a mouse model of human melanoma xenografts effectively inhibited pulmonary metastasis [5]. In the present study, the effects of blocking VEGF-A activity with sorafenib on shedding of tumor cell clusters from human renal cell cancers was investigated.

Sorafenib treatment of papillary and chromophobic RCC (lacking constitutive VEGF expression) was apparently ineffective: these tumors contained minimal amounts of necrosis and hypoxia and contained high vessel densities (data not shown). In contrast, treatment of the ccRCCs destroyed the characteristic micronodular phenotype and induced extensive necrosis, although large vital areas were still present in all tumors. These areas homogeneously expressed CA-IX [20], confirming the clear cell origin of these tumors. The vascular endothelium in treated ccRCCs was damaged, resulting in edema of the vessel walls, vasculitis, and fibrinoid necrosis. Although on the basis of its tyrosine kinase inhibition profile, sorafenib affects endothelial cells, we cannot completely

Table 3. Distribution of Lymphocytes Versus Granulocytes in ccRCC.

	MPO	CD2
Neoadjuvant Treated		
Score 0 or 1	92%	22%
Score >1	7%	78%
Control group		
Score 0 or 1	95%	50%
Score >1	5%	50%

Note that on sorafenib treatment, a lymphocytic infiltrate is induced.

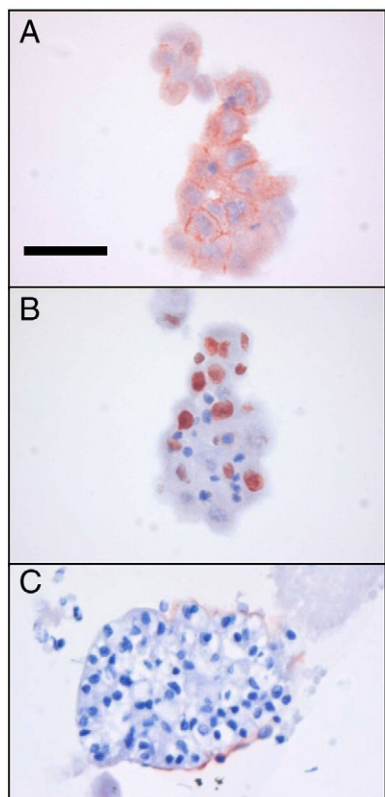


Figure 3. Characteristic example of a tumor cell group, collected after perfusion of a ccRCC nephrectomy specimen (A and B). Tumor origin of the cells is evidenced by immunohistochemical staining for the ccRCC marker CA-IX (A). Note the high proliferation index in this tumor cell cluster as evidenced by immunostaining for the proliferation marker Ki67 (B). C shows an example of a laminin-stained tumor cell cluster from the control group, illustrating that this cluster is covered by wall elements. Scale bar, 50 μ m.

exclude additional direct proapoptotic effects on tumor cells, as has been suggested before [24].

In contrast to the initial expectation of lower shedding of tumor cell groups, these were still observed in 75% of sorafenib-treated patients. This was in line with the notion that seven of eight patients had in retrospect suspected metastatic disease at the start of treatment. In the subgroup of metastasized patients in the control group, a similar percentage was seen (66%). We therefore conclude that neoadjuvant sorafenib treatment does not have impact on the

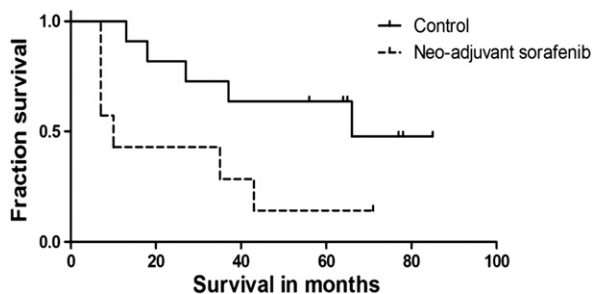


Figure 4. Kaplan-Meier survival curve shows a significant decrease in overall survival in the neoadjuvant treatment group. $P = .038$; HR = 4.4.

shedding of tumor clusters. Of importance, most of the tumor cell groups in the treatment group contained viable and even proliferating cells. These cell groups were deprived of an endothelial cell lining, in contrast to those in control patients where 30% of clusters were associated with vessel wall elements.

The mechanism of tumor cluster shedding from treated nonmicronodular tumors is not clear. All patients in whom tumor cell groups were detected in the perfundate of the postnephrectomy venous outflow presented with (micro)vascular invasion of tumor in the vital tumor areas. Shed tumor cell groups may therefore represent the vital remnants of these intravascularly located tumor fragments that are shed from a destroyed tumor vasculature. Sorafenib-induced destruction of the tumor vasculature and damaged vessel walls may also facilitate direct entry of tumor cell clusters into the circulation. Absence of vessel wall elements suggests that entrance of these cell clusters into the circulation occurs through a mechanism that is distinct from intravascular budding. Alternatively, the micronodular phenotype, which had been initially present, may have been destroyed by sorafenib by a direct toxic effect on endothelial cells, resulting in denudation of the endothelial covering of the intravascular buds. Of interest, a short course of antiangiogenic tyrosine kinases has been shown to provoke a more invasive tumor phenotype in animal models of cancer [25,26]. It remains to be established whether our observations have a physiological link to these data.

We have previously examined the effects of anti-VEGF therapies on angiogenic melanoma xenografts in mice and found a reduction in the number of metastases in the lungs. This may be explained by the fact that in that situation, the formation of a micronodular phenotype is prevented, whereas in the current clinical study, established tumors with such phenotype are subjected to treatment.

Our study suggests that neoadjuvant sorafenib treatment has a negative impact on prognosis. However, we realize that there are limitations to our study. As this was a side study to clinical trial, designed to assess the effect of sorafenib on antibody-mediated molecular imaging, the setup did not allow a randomized approach, and clinicopathologic parameters could only be determined postnephrectomy. Furthermore, the number of included patients was limited, and importantly, retrospective analysis revealed that most of the patients in the treatment group had metastatic disease already at the start of treatment. It would be of high importance to study the effects of neoadjuvant treatment in patients without evidence of metastatic disease. In addition, there were differences in treatment regime at tumor recurrence that may have impacted on survival (see Table 2). On the basis of our data, it is not possible to judge the potential predictive value of Motzer scores for response to neoadjuvant treatment. Yet, the highly significant negative effect of neoadjuvant treatment on survival (hazard ratio = 4.4) in this small patient population indicates that the use of sorafenib for neoadjuvant treatment of ccRCCs, especially in patients with metastatic disease, should be reconsidered and further studies are needed on the basis of the ongoing trials. Whether this study should be extended to larger groups of patients should be a serious matter of debate given our results [25,26]. Of note, treatment with sunitinib, a tyrosine kinase inhibitor with a similar target specificity as sorafenib, in a phase II neoadjuvant setting did not reveal a negative effect [17]. Whether this is related to intrinsic differences between sorafenib and sunitinib is unclear.

Another intrinsic limitation of our study is that the study design did not allow pretreatment tumor sampling. However, because more

than 80% of ccRCCs show VHL mutations leading to high VEGF-A levels and a micronodular phenotype, we consider it justified to assume that the absence of this phenotype in the tumors in the present study is the result of sorafenib treatment. A final potential drawback is that sorafenib treatment was stopped 3 days before tumor nephrectomy during which theoretically morphologic changes may occur. This 3-day period is inevitable to prevent wound-healing complications. It was recently described that discontinuation of sunitinib treatment in patients with ccRCC in a neoadjuvant setting may result in compensatory angiogenesis and a tumor rebound effect [27]. In the sorafenib-treated tumors of the current study, no indication for this phenomenon was found, but the time span between the end of therapy and subsequent surgery might have been too short. Neoadjuvant treatment of patients with ccRCC with the anti-VEGF antibody bevacizumab does not appear to cause compensatory angiogenesis, possibly due its circulation half-life of approximately 21 days [27]. Although it would be interesting to investigate the effects of this anti-VEGF antibody on tumor-cluster shedding, such studies are not feasible because of expected wound-healing complications during tumor nephrectomy.

A remarkable observation was the profound inflammatory response in the vital tumor areas that was significantly higher than in control tumors. How this inflammatory response contributes to tumor biology is unclear. Sorafenib has been shown to inhibit natural killer cell-mediated cytotoxicity *in vitro*, an effect that was not seen with sunitinib [28]. Conversely, it has been shown that sorafenib decreases the influx of immune-suppressing regulatory T cells (Treg) in clinical samples of ccRCC, potentially leading to an enhanced immune response and improved clinical outcome [19]. If and how these counteractive effects have contributed to reduced survival in the group of sorafenib-treated patients are not clear, and a more detailed analysis of the lymphocytic infiltrates in treated *versus* control tumors will be needed to evaluate the effects of these therapies on antitumor immunity.

In conclusion, this study strongly suggests that neoadjuvant sorafenib treatment of ccRCC destroys the characteristic VEGF-A-induced micronodular phenotype of these tumors, causes specific changes in tumor vasculature, and induces profound necrosis as well as intratumoral inflammation. In contrast to the initial expectations, neoadjuvant treatment did not reduce shedding of tumor fragments into the circulation. Furthermore, neoadjuvant sorafenib treatment of metastasized patients with ccRCC may worsen prognosis.

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