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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 perfundate 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.
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. |
|---|---|---|---|---|
| n | 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.

* Control group has been published in reference 10.

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.
After washing the filter with 0.9% NaCl, the residue was collected, followed by standard paraffin embedding of the solidified agarose in 2% liquid agarose (LE, analytical grade, Promega, Madison WI) to detect vessel basement membrane, collagen IV (Abcam, Cambridge, United Kingdom) to detect endothelial cells, and carbonic anhydrase IX (M75, Dr. Oosterwijk) to detect tumor cells metastasized in the histopathology of RCCs. In our series, two renal tumors were of the micronodular phenotype. We included these tumors in the analyses to investigate if the effects of sorafenib on these tumors were minimal compared to the effects on the non-micronodular tumors.

**Statistical Analysis**

Relationships between neoadjuvant treatment and occurrence of metastasis, blood vessel wall changes, carbonic anhydrase-IX expression, and microbiologic invasion and inflammation were determined 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 ccRCCs. In our series, two renal tumors were of the nonclear cell type. A tumor type analysis was performed independently by two investigators (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.

**Table 2. Clinical Parameters.**

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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.

**Immunohistochemistry**

Sorafenib and Circulating Tumor Cells in RCC Kats-Ugurlu et al. 223

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.
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 Perfusate**

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 perfusate (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).
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
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 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 wound-healing complications. It was recently described that discontinuation of sunitinib treatment in patients with ccRCC during which theoretically morphologic changes may occur. This 3-day period is inevitable to prevent 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.

References
