Stem cells increase in numbers in peri-necrotic areas in human renal cancer

Mariana Varna1,2, Guillaume Gapihan1,2, Jean-Paul Feugeas1, Philippe Ratajczak1,2, Sophie Tan2, Irmine Ferreira1,2, Christophe Leboeuf1,2, Niclas Setterblad1, Arnaud Duval1,2, Jérôme Verine1,2,3, Stéphane Germain1,4,5,6,7, Pierre Mongiat-Artus1,2,8, Anne Janin1,2,3,8*, Guilhem Bousquet1,2,8*

1Université Paris Diderot, Sorbonne Paris Cité, Laboratoire de Pathologie, UMR-S 1165,
F-75010, Paris, France
2INSERM, U1165-Paris, F-75010, France
3AP-HP-Hôpital Saint-Louis, Service de Pathologie–Paris, F-75010, France
4Collège de France, Center for Interdisciplinary Research in Biology (CIRB), Paris, F-75005, France
5CNRS UMR 7241, Paris, F-75005, France
6INSERM U 1050, Paris, F-75005, France
7MEMOLIFE Laboratory of excellence and Paris Science Lettre
8AP-HP-Hôpital Saint-Louis, Service d’Urologie–Paris, F-75010, France

* These authors are co-senior authors

Running title: hypoxia and cancer stem-cells in renal cancer
Keywords: human renal cancer, hypoxia, stem cell, sunitinib, drug resistance

#Correspondance: Anne Janin, U1165, Université Paris7, Inserm, Hôpital Saint-Louis, 1 avenue Claude Vellefaux, Paris, F-75010, France, Email : anne.janin728@gmail.com, Tel: +(33) 1 42 49 45 70, Fax :+(33) 1 42 49 92 81
Dr Guilhem Bousquet, U1165, Université Paris7, Inserm, Hôpital Saint-Louis, 1 avenue Claude Vellefaux, Paris, F-75010, France, Email : guilhem.bousquet@sls.aphp.fr, Tel: +(33) 1 42 38 54 28, Fax :+(33) 142 49 49 22

Downloaded from clincancerres.aacrjournals.org on July 16, 2017. © 2014 American Association for Cancer Research.
hypoxia and cancer stem-cells in renal cancer

This work was supported by University-Paris-Diderot, INSERM, ANR, and Canceropole-INCa.

The authors declare that no conflict of interest exist

The manuscript contains 3311 words (excluding references), 4 figures and 2 tables
hypoxia and cancer stem-cells in renal cancer

**Statement of translational relevance**

Developing strategies to overcome resistance to sunitinib is a major challenge in human renal-cell carcinoma (RCC). Anti-angiogenic therapies act through hypoxia-induced tumor necrosis, but the mechanisms by which hypoxia induces treatment resistance in tumors remains unknown.

In primary RCC from patients with metastatic cancer, we identified CD133/CXCR4-coexpressing cells in peri-necrotic areas. We reproduced this preferential distribution in six xenograft models established from tumor samples from patients with metastatic RCC. In two models responder to sunitinib, we showed that sunitinib-induced hypoxia increased the number of peri-necrotic cancer stem-cells, and that these cells had a lower sensitivity to the drug.

The clinical relevance is high since resistance to anti-angiogenic drugs is challenging daily oncology practice. Important data is added to existing knowledge about cancer treatment with anti-angiogenic drugs because it opens a new field of pharmacological research based on the fact that an anti-angiogenic drug, initially efficient on a metastatic cancer, is also able to induce resistance to its own therapeutic effect.
Abstract

Purpose: Developing strategies to overcome resistance to sunitinib is a major challenge in human renal-cell carcinoma (RCC). We hypothesized that sunitinib-induced tumor necrosis-associated hypoxia could interact with renal cancer stem-cells in patients with metastatic RCC.

Experimental design: We studied tissue samples from seven patients with primary metastatic RCC, before and after sunitinib treatment, and from six xenograft models derived from human RCC. Two xenograft models were responders to sunitinib, the four others were non-responders. CD133/CXCR4 co-expressing cells derived from the two responder xenograft models were used for in vitro studies.

Results: In the seven primary RCC, we identified a significantly larger number of CD133/CXCR4-coexpressing cells in peri-necrotic versus peri-vascular areas. Their numbers also significantly increased after treatment, in peri-necrotic areas. We reproduced these clinical and pathological results in all six RCC xenograft models with again a preferential peri-necrotic distribution of CD133-expressing cells. Necrosis occurred at Day7 in the two responder models treated with sunitinib, while it occurred at Day21 in the untreated controls and in the four non-responder models. Strikingly, when we studied the 6 RCC xenograft models at the time necrosis, whether spontaneous or sunitinib-induced, occurred, necrosis area correlated with stem-cell number in all 120 xenografted RCCs.

When studied under experimental hypoxia, the number of CD133/CXCR4 co-expressing cells and their tumorigenic potency increased while their sensitivity to sunitinib decreased.

Conclusions: In human RCC, sunitinib was able to generate resistance to its own therapeutic effect via induced hypoxia in peri-necrotic areas where cancer stem cells were found in increased numbers.
hypoxia and cancer stem-cells in renal cancer

**Introduction**

Anti-angiogenic therapies have improved the prognosis of patients with metastatic renal-cell carcinoma (RCC), but secondary resistance constantly occurs (1-3). These innovative therapies act through hypoxia-induced tumor necrosis (4), necrosis being considered an indirect marker of oxygen depletion (5).

Preclinical data suggest a central role of hypoxia in the resistance to tyrosine-kinase-inhibitors such as sunitinib (6-7). Hypoxia induces resistance to chemotherapy in human cancer progenitors (8-9), and to targeted therapies in chronic myeloid leukemia cells (10).

Hypoxia and necrosis occur in the spontaneous evolution of cancer. Hypoxia-inducible-factor-1α (HIF1α) stimulates angiogenesis by target gene activation (11), and promotes endothelial-cell progenitor and cancer stem-cell development (6, 12-14). In tissues, where microenvironment conditions are thought to govern cancer stem-cells, little is known about the role of hypoxia.

According to consensus criteria, cancer stem-cells are able to self-renew and have tumorigenic potential (15). Different surface markers characterize them, including CD133, expressed by hematopoietic stem-cells (16), renal progenitors (17), brain (18) and colon cancer stem cells (19), and CXCR4, expressed by human hematopoietic progenitors, renal progenitors (20), and pancreatic cancer stem-cells (21-22).

Using CD133 and CXCR4 on human primary RCC biopsies and xenografted samples, we identified stem-cells around areas of spontaneous or sunitinib-induced necrosis. When these cells were sorted from xenografted human RCC before treatment, we demonstrated that hypoxia increased their tumorigenic potency, and decreased their sensitivity to sunitinib. Our results therefore strongly suggest that in human RCC sunitinib was able to generate resistance to its own therapeutic effect via induced hypoxia in peri-necrotic areas where cancer stem cells are found in increased numbers.
hypoxia and cancer stem-cells in renal cancer

**Materials and Methods**

**Patients and RCC samples**

Samples were obtained from primary tumors of seven patients with metastatic RCC, both before any medical treatment and after 3 months of sunitinib treatment (Pfizer, U.S.A.) at 50mg/day with a 4 weeks on and 2 weeks off schedule (1), sunitinib being the first line of treatment. Imaging-guided pre-treatment biopsies were similarly performed at a minimum distance of 1 cm from necrotic areas detected on computed tomography, and processed as described in Supplementary methods. Table 1 shows tumor characteristics.

In compliance with French Bioethics-law (2004-800, 06/08/2004), all patients had been informed of the research use of the part of their samples remaining after diagnosis had been established, and did not oppose it. Informed consent was obtained for each patient. The study was approved by the University Board Ethics Committee.

**Human RCC xenografts**

Six xenograft models of human RCC in nude mice were studied (see Supplementary methods). The characteristics of the 6 xenografted human RCC models are shown in Table 2. The day when tumors reached a volume of 300 mm$^3$ – *i.e.* 100% tumor volume – was considered as Day0. Then, 15 of the 35 mice xenografted with human RCC were treated by gavage with sunitinib diluted in 0.9% NaCl, at 20mg/kg/day.

At Day21, when mice treated with sunitinib were compared with untreated mice, the xenograft model was considered as “responder” to sunitinib if tumor volumes were significantly smaller (p<0.01).

**Peri-necrotic CD133-positive cell counts**

We counted CD133-positive cells on tumor samples of primary human RCCs and all RCC xenograft models (see Supplementary methods).

**Assessment of CD133, CXCR4 and HIF1α in peri-necrotic cells**
hypoxia and cancer stem-cells in renal cancer

For human primary RCCs before and after treatment with sunitinib, multiple-fluorescent immunostainings were performed (see Supplementary methods).

Assessment of necrosis area

Necrosis areas were assessed on tumor samples from all RCC xenograft models, at Day0, Day7, Day14 and Day21 (see Supplementary methods).

Cancer stem-cells from xenografted human RCC: spheres and tumorigenicity

We studied stem-cells from all RCC xenograft models. In the responder and non-responder models, we analysed the samples obtained at Day0, before any treatment. Cells obtained from dissociated sections were cultured as described in Supplementary methods, to obtain spheres. We then assessed the tumorigenicity of CD133/CXCR4 co-expressing cells isolated from these spheres (see Supplementary methods).

Experimental hypoxia, assessment of proliferation and microvessel density

We studied spheres obtained from untreated tumor samples (Day0) from the six models. These spheres were separated into two groups, maintained in a humidified chamber for seven days, one under experimental hypoxia (1% O₂) and the other under normoxia (20% O₂). Assessment of proliferation and microvessel density was performed on tumor samples of xenografts obtained after engraftment of spheres cultured under normoxic or hypoxic conditions (see Supplementary methods).

Experimental hypoxia, tumorigenicity and cancer stem-cell sensitivity to sunitinib

As described in Supplementary methods, we assessed the relative number of CD133, CXCR4, and CD133/CXCR4 expressing cells in hypoxic and normoxic spheres by flow cytometry. We then assessed tumorigenicity of normoxic and hypoxic spheres, and checked whether CD133/CXCR4 cells from normoxic and hypoxic spheres had different sensitivity to sunitinib.

Statistical analyses
Calculated using SPSS Statistics 17.0 software or the R 2.15.2 statistical software (R Foundation for Statistical Computing, Vienna, Austria) (see Supplementary methods).
Results

Stem-cells in perinecrotic areas

In the primary tumor samples of the 7 patients with metastatic RCC, before treatment and after 3 months of sunitinib, we identified large cells expressing CD133. When counted independently from their location in the tumor, there was no significant change after treatment (from 2.4% to 3.7%, p=0.1). Interestingly, CD133-expressing cells were significantly more numerous in peri-necrotic than in peri-vascular areas, in both untreated patients (4.7% versus 1.3%, p<0.01) and treated patients (8.3% versus 1.9%, p<0.01). In treated patients, CD133-expressing cells were significantly more numerous than in untreated patients but only in peri-necrotic areas (8.3% versus 4.7%, p<0.05) (Figure 1A).

In peri-necrotic areas in all patients, 84.5% (+/- 2.5%) of these cells co-expressed CD133 and CXCR4, suggesting a stem-cell phenotype. These CD133/CXCR4-coexpressing cells were significantly more numerous in treated patients compared to untreated patients (7.3% versus 3.9% p<0.05). In addition, HIF1α, a marker of hypoxia, was co-expressed in 95% of CD133/CXCR4 cells (Figure 1B).

Six xenograft models of human RCC in nude mice were studied, obtained from engraftment of RCC primary tumor samples from patients with metastatic RCC. Each of the 6 xenograft models was treated with sunitinib for 21 days. A model was considered as “responder” to sunitinib treatment if its tumor volume at Day21 was significantly smaller than the mean tumor volume among untreated mice (p<0.01). When there was no significant difference in tumor volume between treated and untreated mice, the xenograft model was considered as non-responder to sunitinib treatment. Two of the 6 xenograft models were responder to sunitinib and the 4 others were non-responder models. In the two xenograft models responder to sunitinib (HRCC1 and HRCC8), a significant increase in necrotic areas was found between Day0 and Day7 of treatment with sunitinib (5.4 ±4.1% versus 32.2 ± 10.6% of total-tumor
hypoxia and cancer stem-cells in renal cancer

surface area for pooled results of these two models, p<0.05). In contrast, it took 21 days to observe necrotic areas both in the group of untreated mice (26.3 ±9.8%), and in the group of treated mice not responding to sunitinib (24.9% ±9.4) (Figure 2B and Supplementary Figure S1).

When we studied the six xenograft models, we again demonstrated that CD133-expressing cells were significantly more numerous in peri-necrotic than in peri-vascular areas, in both treated and untreated tumors. In addition, when we compared untreated and treated mice, CD133-expressing cells were significantly more numerous in treated mice, but only in peri-necrotic areas (p<0.05) (Figure 2C and Supplementary Figure S2).

These six pre-clinical models enabled us to perform in situ analyses on whole xenografted tumors. After removal of the whole tumor and realization of five representative full sections, the percentages of necrotic areas and of CD133 positive cells were assessed in the five different sections for each tumor (Figure 2D). When we studied untreated and treated mice together, including responders and non-responders to sunitinib (n=120 mice), the necrosis ratio in tumor tissue sections was significantly related to the mean number of CD133-expressing cells (p<0.01) (Figure 2D).

When we used pimonidazole, another hypoxic marker, we showed that hypoxic living cells stained for pimonidazole were distributed around necrotic areas. This was observed in the RCC xenograft models when necrosis occurred, at Day21 for one untreated RCC xenograft model, and at Day7 under treatment for one xenograft model that was responder to sunitinib (Supplementary Figure S3)

Since tissue necrosis is directly related to hypoxia, these results strongly suggest a link between hypoxia, whether sunitinib-induced or spontaneous, and an increased number of RCC stem-cells.

**CD133/CXCR4 stem-cells had tumorigenic potential**
To determine if CD133/CXCR4 co-expressing cells were tumorigenic in vivo, we grew spheres from untreated RCC xenografts. When these spheres were cultured in normoxic conditions, confocal microscopy identified CD133/CXCR4 co-expressing cells (Figure 3A). These cells isolated from normoxic spheres induced tumor growth 3 weeks after injection of $2 \times 10^3$ cells into nude mice. This tumorigenic potential was observed with CD133/CXCR4 co-expressing cells from normoxic spheres derived both from the two sunitinib responder models (Figure 3B) and the four non-responder models (Figure 4A).

As controls, $2 \times 10^3$ cells from the CD133/CXCR4 double-negative fraction of normoxic spheres injected in nude mice did not induce any tumor growth over a period of two months (Supplementary Table). Microscope analyses and cell counts showed that tumors that developed from CD133/CXCR4 co-expressing cells reproduced the initial primary human RCC features for morphology, cell proliferation, microvessel density, and stem-cell density (Figure 3B and 4A).

**Experimental hypoxia increased stem-cell tumorigenicity**

We compared the tumorigenic potential of spheres cultured under hypoxic and normoxic conditions. After injection of CD133/CXCR4 co-expressing cells sorted from spheres, there was a significantly larger mean tumor volume after injection of hypoxic stem-cells rather than normoxic stem-cells ($p<0.05$) (Figure 3C and 4B). This difference was significant from 42 days on for the two responder models (Figure 3C) and for the four non-responder models (Figure 4B). To rule out a difference due to proliferation or angiogenesis, we counted Ki67- and CD31-expressing cells in tumor sections, and no significant difference was found in tumors grown from normoxic and hypoxic spheres (Figure 3C).

When we tested the tumorigenic potential of serial dilutions of isolated CD133/CXCR4 cells from normoxic and hypoxic spheres, a significant increase in tumor growth occurred after injection of $2 \times 10^2$ and $2 \times 10^3$ cells when hypoxic spheres were compared to normoxic spheres.
hypoxia and cancer stem-cells in renal cancer

The difference was not significant after injection of a larger number of cells ($2.10^4$ cells) (Figure 3C and 4C).

**Experimental hypoxia increased the number of stem-cells and decreased their sensitivity to sunitinib**

To assess the role of experimental hypoxia on the number of renal cancer stem-cells, spheres derived from untreated tumors of the six human RCC xenograft models were cultured under hypoxic conditions.

After 5 days of experimental hypoxia, FACS analyses showed a significantly larger number of CD133/CXCR4 cells in hypoxic spheres compared to normoxic spheres ($p<0.01$). This was found in the two responder models ($7.6\% \pm 2$ versus $2.1\% \pm 0.7$) ($p<0.01$) (Figure 3D), and the four non-responder models ($9.2\% \pm 2.2$ versus $4.6\% \pm 1.1$) ($p<0.01$) (Figure 4C).

To assess sunitinib effects on RCC stem-cells, we then analysed whether CD133/CXCR4 cells from hypoxic spheres were more sunitinib-resistant than CD133/CXCR4 cells from normoxic spheres, using an *in vitro* cytotoxicity assay. The half-maximum-inhibition-concentration (IC50) of sunitinib was higher for hypoxic than for normoxic cells for both the two responder models (Figure 3D), and the four non-responder models (Figure 4D).
Discussion

In patients with metastatic RCC who were responders to sunitinib, we identified CD133/CXCR4-coexpressing stem-cells in peri-necrotic areas, and showed that their numbers increased after sunitinib administration. To our knowledge, this has never been reported in human RCC.

We chose CD133 and CXCR4 because they are two well-known markers of stem-cells (16-20). In a series of 240 RCC, more than 20% positive cells were found for CD133-expressing cells in 22.5% of RCC, and for CXCR4-expressing cells in 60.8% of RCC (23), and high CXCR4 expression was predictive of poor response to sunitinib (24). The seven patients we studied had at least a stable disease under sunitinib treatment (for this reason they underwent nephrectomy after treatment), and when we assessed the number of CD133/CXCR4 co-expressing cells, we found a mean percentage from 4.2% to 7.7%.

To further study these stem-cells, we xenografted human RCC in nude mice, and performed a sequential study in untreated and sunitinib-treated mice. We obtained 6 models, two of which were responders to sunitinib. Using these two responder models, we were able to reproduce the clinical and pathological results found in the seven patients, thus showing that sunitinib significantly increased the number of CD133/CXCR4 co-expressing stem-cells with a preferential peri-necrotic distribution. When all xenografted human RCC, whether treated or untreated, responders or non-responders to sunitinib, were considered (i.e. 120 tumor samples), we demonstrated that the number of CD133/CXCR4 co-expressing cells was related to the extent of tissue necrosis. In a preclinical model of breast cancer cell lines (7), the number of cancer stem-cells increased under sunitinib via hypoxia. Here, we studied renal and not breast cancer, and human tumor samples and not cancer cell lines.

Regarding renal cancer stem-cells, CD105, more than CD133, has been recommended following studies using cells selected by flow cytometry (25). However, CD105 is not
hypoxia and cancer stem-cells in renal cancer

appropriate for studies in whole tumor tissue sections, because it is also expressed by tumor endothelial cells (26) and carcinoma-associated fibroblasts (27).

In our preclinical model of human RCC xenografts, the number of stem-cells, preferentially distributed in peri-necrotic areas, was related to the extent of tissue necrosis, whatever the origin of necrosis, spontaneous or treatment-induced.

*VHL*-gene mutations are found in 70% of human RCC (28), as was the case in our patients and the xenografted RCC. When present, *VHL*-gene mutations lead to HIF accumulation (29), including HIF1α, recently characterized as a tumor suppressor (30-31), and HIF2α, characterised as an oncoprotein (30). Since HIFs and necrosis are two indirect markers of oxygen depletion (5), for this study based on human cancer tissue analysis we chose to use the extent of necrosis rather than the number of HIF1α- or HIF2α-expressing cells as markers of hypoxia.

After sorting from untreated xenografted human RCC, the CXCR4/CD133 co-expressing stem-cells were able to form spheres and to induce tumors in mice, two characteristic features of cancer stem-cells (15). We showed in vitro that experimental hypoxia increased both their number and their tumorigenicity. Hypoxia can increase the number of glioblastoma (12) and breast cancer (14, 22) stem-cells, but we have demonstrated here for the first time that hypoxia can increase tumorigenic potential and resistance to sunitinib of cancer stem-cells from untreated human RCC.

The question of additional hypoxia on RCC bearing *VHL* mutation opens discussion of the relative importance of intrinsic and extrinsic hypoxia in RCC cells. It has recently been shown that clear-cell RCC can express low levels of HIF1α, particularly when chromosome 14q is deleted (30, 32). Therefore, it is possible that not all RCC cells have the same constitutive pseudo-hypoxic level. In our pre-clinical models, human xenografted RCC were treated by
hypoxia and cancer stem-cells in renal cancer

sunitinib which effects on endothelial cells induces necrosis (4), and thus hypoxia in tumor cells through an extrinsic mechanism.

In patients with metastatic RCC, resistance to anti-angiogenic treatment is a major cause of poor survival (2-3). In preclinical studies, it has been shown that resistance to sunitinib occurred because cancer cells acquired metastatic potential (33-34), and that sunitinib mainly acts by vascular effect and induction of necrosis (4). Our demonstration that hypoxia increases the number of cancer stem-cells while decreasing their sensitivity to sunitinib implies that the drug, by way of its initial effect on the tumor microvessels, is able to induce mechanisms of resistance to its own effect. This is coherent with the clinical observation showing that the benefit of sunitinib is only transitory (2).

Designing new strategies to optimize anti-angiogenic therapies needs to take into account the deleterious effect of therapy-induced hypoxia, which, as shown in this study, is responsible for the emergence of cancer stem-cells in RCC. Targeting cancer stem cells might thus be a promising future research approach to prevent acquired resistance to sunitinib. Molecules targeting endothelial cell progenitors such as TRC105, an anti-endoglin antibody, are under development in metastatic RCC (35), but the effect of therapeutic antibodies targeting CXCR4 and/or CD133 on RCC cancer stem-cells remains unknown.

The clinical relevance of our results is a major strength of this work, as resistance to anti-angiogenic drugs is challenging daily practice in oncology. In addition, this study is to our knowledge the first to be performed on human RCC samples before and after treatment, the relevant results being reproduced in xenograft models responding to sunitinib treatment, thus generalizing the conclusions obtained in the patient samples.
hypoxia and cancer stem-cells in renal cancer

Important data is added to existing knowledge about cancer treatment with anti-angiogenic drugs because it opens a new field of pharmacological research based on the fact that an anti-angiogenic drug, initially efficient on a metastatic cancer, is also able to induce resistance to its own therapeutic effect
hypoxia and cancer stem-cells in renal cancer

Acknowledgments

We thank J. Lehmann-Che for determination of the VHL-status; H.de The and M. Crisan for critical reading of the manuscript. A. Swaine reviewed English language.

Author Contributions

M. Varna, G. Bousquet, S. Germain and A. Janin conceived and designed the study. G. Bousquet enrolled patients. P. Mongiat-Artus performed the surgery. J. Verine performed histological diagnosis of human tumor samples. M. Varna, G. Bousquet, C. Leboeuf, I. Ferreira, G. Gapihan, N. Setterblad, A. Duval and A. Janin collected data and/or performed experiments for the study. M. Varna, G. Bousquet, C. Leboeuf, J-P. Feugeas and A. Janin analyzed the data. J-P. Feugeas performed the statistical analyses. G. Bousquet, M. Varna and A. Janin contributed to drafting the manuscript. All authors approved the final version of the manuscript.
hypoxia and cancer stem-cells in renal cancer

References


9. Lau CK, Yang ZF, Ho DW, Ng MN, Yeoh GC, Poon RT, et al. An Akt/hypoxia-inducible factor-1alpha/platelet-derived growth factor-BB autocrine loop mediates hypoxia-
hypoxia and cancer stem-cells in renal cancer


hypoxia and cancer stem-cells in renal cancer


hypoxia and cancer stem-cells in renal cancer


Table 1 – Primary tumor characteristics.

<table>
<thead>
<tr>
<th>Type of metastasis</th>
<th>Lung, bone</th>
<th>Lung, pancreas</th>
<th>Lung, brain</th>
<th>Lung</th>
<th>Lymph node, lung</th>
<th>Lung, brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial TNM status*</td>
<td>pT1aN1M1</td>
<td>pT4NXM1</td>
<td>pT3NXM1</td>
<td>pT3bNXM1</td>
<td>pT3aNXM1</td>
<td>pT1aNXM1</td>
</tr>
<tr>
<td>VHL status</td>
<td>mutated</td>
<td>mutated</td>
<td>Wild-type</td>
<td>mutated</td>
<td>mutated</td>
<td>mutated</td>
</tr>
<tr>
<td>Fürhman grade</td>
<td>III</td>
<td>IV</td>
<td>III</td>
<td>I</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>Tumor</td>
<td>RCC1</td>
<td>RCC2</td>
<td>RCC3</td>
<td>RCC4</td>
<td>RCC5</td>
<td>RCC6</td>
</tr>
</tbody>
</table>

*according to 2009 TNM classification [F.L. Greene, M. Gospodarowicz and C. Wittekend et al., American Joint Committee on Cancer (AJCC) staging manual (ed 7.), Springer, Philadelphia, PA (2009)]
Table 2 - Characteristics of the 6 xenografted tumor models

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Sample from</th>
<th>Führman grade</th>
<th>VHL status</th>
<th>Initial TNM* status at time of graft</th>
<th>Metastases</th>
<th>Responder to sunitinib</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-treatment biopsy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRCC1</td>
<td>+</td>
<td>III</td>
<td>mutated</td>
<td>pT1aN1M1</td>
<td>Lung, bone</td>
<td>Yes</td>
</tr>
<tr>
<td>HRCC8</td>
<td>+</td>
<td>II</td>
<td>mutated</td>
<td>pT1aNxM1</td>
<td>Lung</td>
<td>Yes</td>
</tr>
<tr>
<td>HRCC9</td>
<td>+</td>
<td>IV</td>
<td>wild-type</td>
<td>pT3bNxM1</td>
<td>Bone</td>
<td>No</td>
</tr>
<tr>
<td>HRCC10</td>
<td>+</td>
<td>IV</td>
<td>mutated</td>
<td>pT3aNxM1</td>
<td>Lung, liver</td>
<td>No</td>
</tr>
<tr>
<td>HRCC11</td>
<td>+</td>
<td>IV</td>
<td>mutated</td>
<td>pT4NxM1</td>
<td>Lung</td>
<td>No</td>
</tr>
<tr>
<td>HRCC12</td>
<td>+</td>
<td>IV</td>
<td>mutated</td>
<td>pT1bNxM1</td>
<td>Lymph node,</td>
<td>No</td>
</tr>
</tbody>
</table>

*according to 2009 TNM classification [F.L. Greene, M. Gospodarowicz and C. Wittekend et al., American Joint Committee on Cancer (AJCC) staging manual (ed 7.), Springer, Philadelphia, PA (2009)]
Figure 1: Stem-cell distribution in perinecrotic areas of primary human renal cell carcinoma

A. In primary tumors of seven patients with metastatic RCC, before treatment and after 3 months of treatment with sunitinib, areas of necrosis are surrounded by carcinoma cells expressing CD133 (small black arrows). Bar =50 µm. The number of CD133 positive cells is significantly larger in peri-necrotic than in peri-vascular areas, and when only peri-necrotic areas are considered, they are significantly larger after treatment.

B. Using combined CD133/CXCR4/HIF1α immunostaining, triple positive cells (white arrowheads) can be identified in areas surrounding necrosis (N), before treatment and after 3 months of treatment with sunitinib. Bar =50 µm. The numbers of peri-necrotic positive cells were significantly larger after treatment.

**: p<0.01, *: p<0.05

Figure 2: Stem-cell distribution in perinecrotic areas of xenografted human renal cell carcinoma

A. In the 6 models xenografted with human RCC, the follow-up of tumor growth identifies 2 models responding to sunitinib treatment (HRCC1 and HRCC8), and 4 non-responders (HRCC9 to HRCC12).

B. In RCC xenografts untreated and treated with sunitinib (n=5 mice at each time-point), necrosis occurs after seven days of treatment (Day7) in responder models while it occurs two weeks later (Day21) in untreated mice and in non-responder models. *: p<0.05, ns = non significant.

C. On xenografted RCC responding to sunitinib (HRCC1 and HRCC8), untreated (Day0) and treated with sunitinib (Day7) (n=5 mice at each time-point), we identify single cells
expressing CD133 in peri-necrotic areas. N=necrosis, bar =50µm. CD133 positive cells are significantly more numerous in peri-necrotic compared to peri-vascular areas, and in treated mice compared to untreated mice, **: p<0.01 and *: p<0.05

D. On xenografted tumor section represented here on a virtual slide, the periphery of the tumor is surrounded by a white broken line and areas of necrosis by black broken lines. For each area of necrosis, the surface is automatically calculated in µm².

When all 6 xenografted RCC are considered, at four different time-points: Day0, Day7, Day14 and Day21 (n=120 mice), the numbers of CD133-expressing cells are significantly related to the extent of necrosis (Kendall Correlation $R^2 = 0.82$, p<0.01).

**Figure 3: In the two responder models, hypoxia increased the number of stem-cells, their tumorigenicity and resistance to sunitinib**

A. Under normoxic conditions, confocal microscopy (Zeiss LSM510, Germany) shows that RCC sphere cells co-express the stem cell markers CD133 and CXCR4.

B. When grafted sub-cutaneously, CD133/CXCR4 co-expressing cells isolated by magnetic sorting from HRCC1 or HRCC8 spheres induce tumor growth in two groups of 5 nude mice.

At Day63, microscope analyses show that tumors developed from CD133/CXCR4 co-expressing cells reproduce the initial RCC features in terms of proliferation (Ki67 positive cell-count), microvessel density (CD31 positive cell-count), and stem-cell density (CD133/CXCR4 cell-count)

C. Tumor growth following sub-cutaneous injection of $2.10^3$ CD133/CXCR4 cells obtained from HRCC1 or HRCC8 spheres cultured either under hypoxic or under normoxic conditions is significantly different (permutation test, p<10$^{-5}$). At Day 49 tumor growth for hypoxic cells is larger than for normoxic cells (n=10 mice in each group). **: p<0.01.
Microscope analyses show that the differences in growth observed between xenografted tumors developed from hypoxic and normoxic spheres do not result from differences between numbers of Ki67- and CD31-positive cells. Follow-up of tumor growth after sub-cutaneous injection of serial dilutions of CD133+/CXCR4+ cells isolated from hypoxic and normoxic spheres shows that only hypoxic cells are able to induce tumor growth at the minimal dilution of $2 \times 10^2$ cells, and that they induce a significantly larger tumor growth than normoxic cells at the dilution of $2 \times 10^3$ cells (n=5 mice in each group).

*: p<0.05.

D. Flow cytometry analyses of HRCC1 and HRCC8 spheres cultured under normoxic and hypoxic conditions show significantly larger numbers of cells expressing CXCR4, and double positive CD133/CXCR4 cells in hypoxic spheres. **: p<0.01.

MTT assays show that double positive CD133/CXCR4 cells sorted from hypoxic spheres are more resistant to sunitinib than CD133/CXCR4 cells sorted from normoxic spheres in the two responder models. **: p<0.01.

**Figure 4: In the four non-responder models, hypoxia also increased tumorigenicity of stem-cells and their resistance to sunitinib**

A. When grafted sub-cutaneously, CD133/CXCR4 co-expressing cells isolated by magnetic sorting from HRCC9, HRCC10, HRCC11 or HRCC12 spheres induce tumor growth in 4 groups of 5 nude mice each.

At Day56, microscope analyses show that tumors developed from CD133/CXCR4 co-expressing cells from each of the 4 non-responder models reproduce the initial RCC features in terms of proliferation (Ki67 positive cell-count), microvessel density (CD31 positive cell-count), and stem-cell density (CD133/CXCR4 cell-count)
B. Sub-cutaneous injection of $2 \times 10^3$ CD133/CXCR4 cells from spheres cultured either under hypoxic or under normoxic conditions shows that tumor growth for each of the 4 non-responder models is significantly different (permutation test, $p<10^{-5}$). At Day 49, tumor growth for hypoxic cells is larger than for normoxic cells (n=10 mice in each group). *: $p<0.05$, **: $p<0.01$.

C. Follow-up of tumor growth after sub-cutaneous injection of serial dilutions of cells isolated from hypoxic and normoxic spheres shows that only hypoxic cells are able to induce tumor growth at the minimal dilution of $2 \times 10^2$ cells, and that they induce a significantly larger tumor growth than normoxic cells at the dilution of $2 \times 10^3$ cells (n=5 mice in each group). *: $p<0.05$.

Flow cytometry analyses of HRCC9, HRCC10, HRCC11 and HRCC12 spheres (pooled data) cultured under normoxic and hypoxic conditions show significantly larger numbers of cells expressing CXCR4, and double positive CD133/CXCR4 cells in hypoxic spheres. *: $p<0.05$; **: $p<0.01$.

D. MTT assays show that double positive CD133/CXCR4 cells sorted from hypoxic HRCC9, HRCC10, HRCC11 or HRCC12 spheres are more resistant to sunitinib than CD133/CXCR4 cells sorted from normoxic spheres sorted from the corresponding models. *: $p<0.05$. 
Figure 1

A  
Necrosis

CD133

Before treatment

After treatment

B  
In perinecrotic areas

CD133

CXCR4

HIF1α

Overlay

Before treatment

After treatment

% positive cells

CD133+ cells
CD133+/CXCR4+ cells
CD133+/CXCR4+/HIF1α cells

% positive cells

CD133+ cells
CD133+/CXCR4+ cells
CD133+/CXCR4+/HIF1α cells

% positive cells

CD133+ cells
CD133+/CXCR4+ cells
CD133+/CXCR4+/HIF1α cells

Figure 1
Xenografted human renal cell carcinoma

A

Untreated

Responders

Non responders

B

% of necrotic areas

Day0 Day7 Day14 Day21

Day0 Day7 Day14 Day21

Day0 Day7 Day14 Day21

% of necrotic areas

Day0 Day7 Day14 Day21

Day0 Day7 Day14 Day21

Day0 Day7 Day14 Day21

C

Responders

Untreated (Day0)

Treated (Day7)

% CD133 positive cells

CD133 positive cells

% CD133 positive cells

% CD133 positive cells

% vascular peri necrotic

% vascular peri necrotic

% vascular peri necrotic

% vascular peri necrotic

D

Assessment of necrotic areas

All xenografts

% of necrotic areas

% CD133 positive cells

R² = 0.83

p<0.01

1. 302956 µm²
2. 165281 µm²
3. 51865 µm²
4. 14883 µm²
5. 3368 µm²
6. 24942 µm²
7. 13366 µm²
8. 299431 µm²

Figure 2
Figure 3
Xenografted human renal cell carcinoma before treatment II-four non-responder models

Figure 4
Clinical Cancer Research

Stem cells increase in numbers in peri-necrotic areas in human renal cancer

Mariana Varna, Guillaume Gapihan, Jean-Paul FEUGEAS, et al.

Clin Cancer Res Published OnlineFirst December 11, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-0666

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/12/12/1078-0432.CCR-14-0666.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.