Biochimica et Biophysica Acta 1855 (2015) 1–16

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbacan

Review

Resistance to sunitinib in renal cell carcinoma: From molecular mechanisms to predictive markers and future perspectives

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ARTICLE INFO

Article history:
Received 23 July 2014
Received in revised form 29 October 2014
Accepted 5 November 2014
Available online 11 November 2014

Keywords:
Renal cell carcinoma
Kidney cancer
Targeted therapy
Sunitinib
Drug resistance
Biological tumor markers

ABSTRACT

The introduction of agents that inhibit tumor angiogenesis by targeting vascular endothelial growth factor (VEGF) signaling has made a significant impact on the survival of patients with metastasized renal cell carcinoma (RCC). Sunitinib, a tyrosine kinase inhibitor of the VEGF receptor, has become the mainstay of treatment for these patients. Although treatment with sunitinib substantially improved patient outcome, the initial success is overshadowed by the occurrence of resistance. The mechanisms of resistance are poorly understood. Insight into the molecular mechanisms of resistance will help to better understand the biology of RCC and can ultimately aid the development of more effective therapies for patients with this infaust disease. In this review we comprehensively discuss molecular mechanisms of resistance to sunitinib and the involved biological processes, summarize potential biomarkers that predict response and resistance to treatment with sunitinib, and elaborate on future perspectives in the treatment of metastasized RCC.

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Contents

Abbreviations: (cc)RCC, (clear cell) renal cell carcinoma; FDA, Food and Drug Administration; IFN-α, interferon alpha; VHL, Von Hippel–Lindau; HIF, hypoxia inducible factor; IL, interleukin; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; TKI, tyrosine kinase inhibitor; HRE, hypoxia-responsive element; TGF-α, transforming growth factor alpha; EPO, erythropoietin; MMP, matrix metalloproteinase; EGF, epidermal growth factor; EGFR, EGF receptor; HGF, hepatocyte growth factor; HGFR/cMET, HGF receptor; SDF1, stromal-derived factor 1; CXCR4, CXC chemokine receptor 4; HAP, hypoxia-activated prodrug; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; FGFR, FGF receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PlGF, placental growth factor; Ang, angiopoietin; Tie, tunica intima endothelial kinase; DLL4, delta-like ligand 4; BMDC, bone marrow-derived cell; TAM, tumor-associated macrophage; TAF, tumor-associated fibroblast; EPC, endothelial progenitor cell; Bv8, bombina variagata peptide 8; G-CSF, granulocyte-colony stimulating factor; STAT3, signal transducer and activator of transcription 3; ALK, anaplastic lymphoma kinase; EMT, epithelial-to-mesenchymal transition; SK1, sphingosine kinase-1; S1P, sphingosine 1 phosphate; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase 3 beta; SNP, single nucleotide polymorphism; miRNA, microRNA; mTOR, mammalian target of rapamycin; ZEB, zinc finger E-box-binding homeobox; mCEC, mature circulating endothelial cell; CEP, circulating endothelial progenitor cell; TNF-α, tumor necrosis factor alpha; NGAL, neutrophil gelatinase-associated lipocalin; NEFH, neurofilament heavy polypeptide; LAD1, ladinin 1; CST6, cystatin E/M; OS, overall survival; PFS, progression-free survival; RR, relative risk; HR, hazard ratio; OR, odds ratio; 95% CI, 95% confidence interval

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http://dx.doi.org/10.1016/j.bbcan.2014.11.002
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1. Introduction

Renal cell carcinoma (RCC), which arises from the renal parenchyma, is the most common kidney cancer subtype, accounting for approximately 90% of all cases [1,2]. Of the patients diagnosed with RCC, 20–30% present with metastasized disease, and another ~30% of the patients treated for localized disease develop metastases during follow-up [3]. RCC is not a single entity, but comprises a heterogeneous group of malignancies, of which clear cell RCC (ccRCC) is the most common (75–80%) and the best studied to date. ccRCCs are highly vascularized tumors that are characterized by frequent inactivation (50–75%) of the Von Hippel–Lindau (*VHL*) gene [2,4,5]. The product of the *VHL* gene, pVHL, plays an important role in down-regulating the expression of the hypoxia inducible factor 1 (HIF1) transcription factor, which leads to decreased angiogenesis (Fig. 1). Inactivation of pVHL, e.g. by mutation, deletion or promoter CpG island methylation of the *VHL* gene, leads to accumulation of HIF1 and increased transcription of HIF1 target genes e.g. vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The frequent inactivation of *VHL* provided the rationale for the development of antiangiogenic drugs to treat ccRCC such as sunitinib, pazopanib, sorafenib, and axitinib [4]. Sunitinib is an oral multiple tyrosine kinases inhibitor (TKI), that inhibits the family of vascular endothelial growth factor receptors (VEGFR-1, VEGFR-2 and VEGFR-3), the platelet-derived growth factor

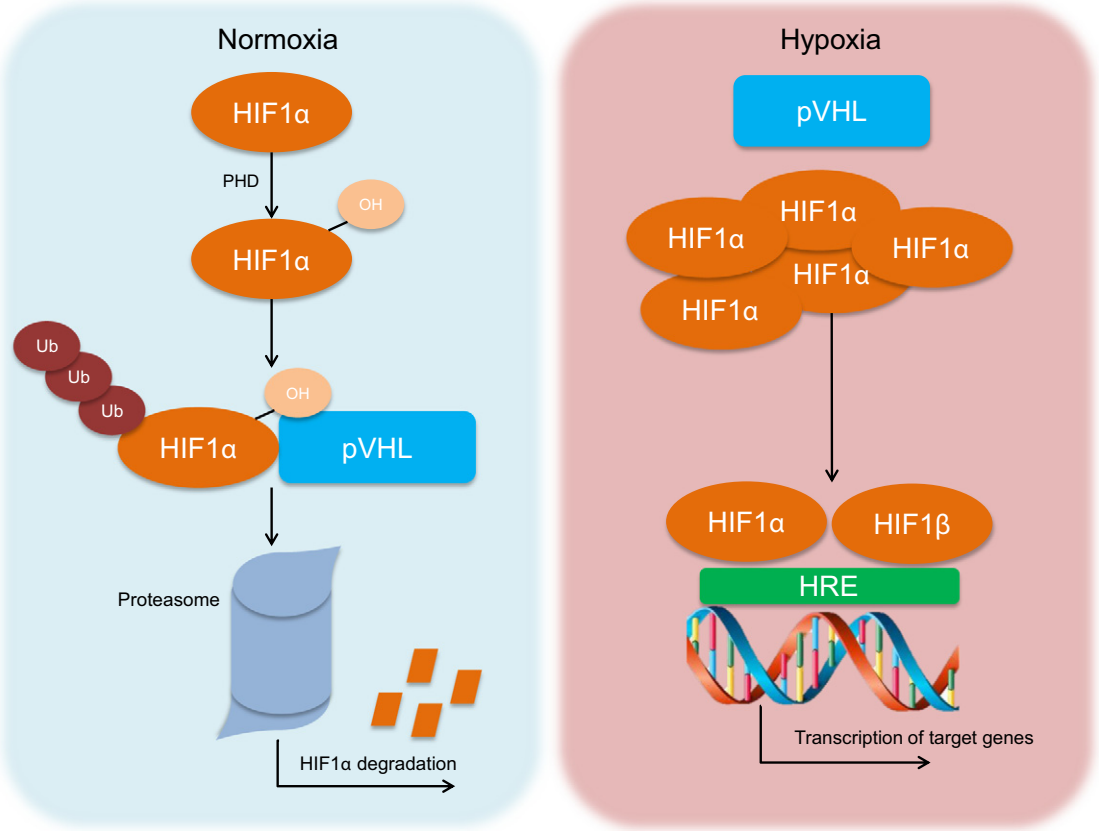

Fig. 1. VHL-signaling in the presence and absence of oxygen. Under normoxic conditions (left-hand side), the VHL protein (pVHL) binds HIF1α which makes it a target for proteasomal degradation. Under hypoxic conditions (right-hand side), or inactivation of pVHL (as in RCC), pVHL cannot bind HIF1α, which leads to the accumulation of HIF1α and binding of HIF1α to HIF1β. The so formed heterodimer translocates to the nucleus and activates the transcription of target genes such as *VEGF* and *PDGF*. Abbreviations: pVHL, VHL protein; HIF1α, hypoxia inducible factor 1α; HIF1β, hypoxia inducible factor 1β; HRE, hypoxia responsive element; PHD, prolyl hydroxylase domain; Ub, ubiquitin; OH, hydroxide.

receptors (PDGFRα and PDGFRβ), FLT3, cKIT and RET. The inhibitory effect of sunitinib on tumor angiogenesis is mainly achieved by blocking VEGFR and PDGFR, two key players in the pathogenesis of ccRCC [6,7]. In metastasized ccRCC, sunitinib treatment resulted in a significant longer progression free survival (PFS) (11 versus 5 months, $P < 0.001$) and overall survival (OS) (26.4 versus 21.8 months, $P = 0.049$) compared to treatment with interferon alpha (IFN-α) [8], and it has therefore become the mainstay of treatment.

Initial response rates to sunitinib lie between 30% and 40% [9–11]. However, disease progression usually occurs after a median of 6–15 months [12], indicating the existence of intrinsic (primary) and/or acquired (secondary) resistance. The resistance mechanisms to sunitinib can be divided in several distinct groups, namely up-regulation of proangiogenic signaling pathways, resistance mediated by the tumor microenvironment, increased tumor invasiveness and metastasis, activation of alternative signaling pathways, inadequate target inhibition, and resistance mediated by the action of microRNAs (Fig. 2). More recently, it was shown that pazopanib has similar efficacy as sunitinib, but with a favorable safety and toxicity profile [13,14]. As a result, sunitinib and pazopanib are used interchangeably in clinical practice. It is likely that the potential resistance mechanisms that are identified for sunitinib may also account for pazopanib, since they have the same molecular targets. However, to the best of our knowledge, no literature on resistance to pazopanib has yet been published. Therefore, the focus of this review will be on sunitinib resistance. We will discuss data from preclinical and clinical studies on mechanisms of sunitinib resistance, and summarize the current knowledge on potential predictive markers that can help to select patients that are likely to benefit from treatment with sunitinib. Finally, we will discuss future perspectives on how to optimize the treatment of patients with metastasized RCC.

2. Resistance mechanisms to sunitinib in RCC

2.1. Up-regulation of proangiogenic pathways

An established cause for the development of resistance to VEGF-targeted therapy is tumor hypoxia. Tumors exceeding a volume of 1 mm^3 usually contain regions of hypoxia [15]. It is well known that tumor hypoxia is associated with increased invasiveness and metastasis, and poorer patient survival [15,16]. Targeting tumor angiogenesis, e.g. by blocking VEGF signaling, leads to increased hypoxia in the tumor which in turn leads to necrosis and decrease of tumor burden.

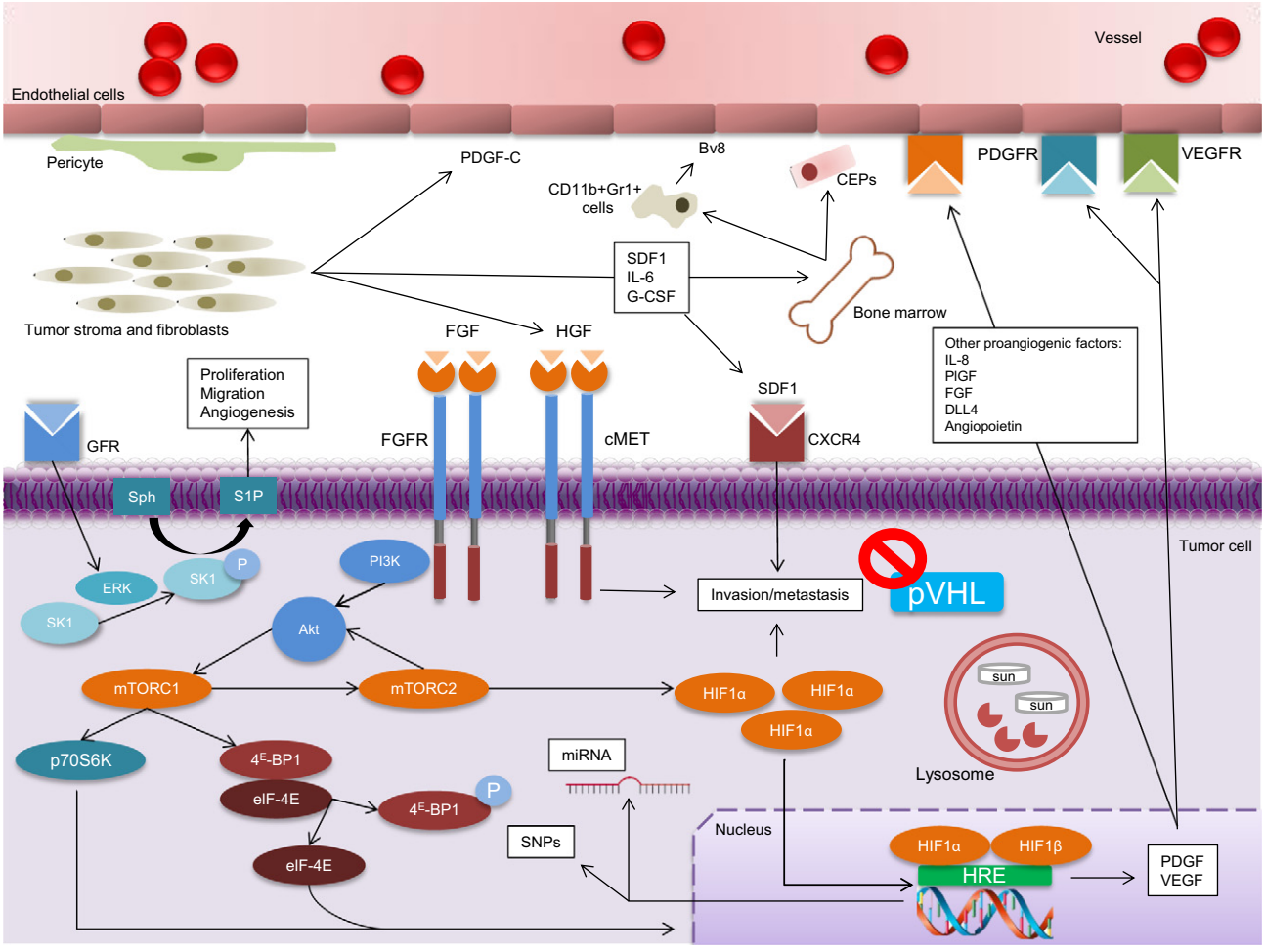

Fig. 2. Summary of potential mechanisms of resistance to sunitinib in RCC. Possible resistance mechanisms to sunitinib in RCC may include up-regulation of proangiogenic factors (e.g. IL-8, PlGF, FGF, DLL4, Ang2), increased invasive and metastatic potential of the tumor, resistance mediated by the tumor microenvironment through the recruitment of bone marrow-derived cells (CD11b + Gr1 + cells and CEPs) and through secretion of HGF and PDGF-C, activation of alternative signaling pathways, lysosomal sequestering of sunitinib, SNPs in pharmacokinetic and pharmacodynamic genes, and resistance mediated by the action of miRNAs. Abbreviations: FGF(R), fibroblast growth factor (receptor); PDGF(R), platelet-derived growth factor (receptor); VEGF(R), vascular endothelial growth factor (receptor); HGF, hepatocyte growth factor; PlGF, placental growth factor; GFR, growth factor receptor; CEPs, circulating endothelial progenitor cells; SDF1, stromal-derived factor 1; IL-6, interleukin-6; G-CSF, granulocyte-colony stimulating factor; CXCR4, CXC chemokine receptor 4; IL-8, interleukin-8; DLL4, delta-like ligand 4; miRNA, microRNA; SNPs, single nucleotide polymorphisms; HIF1α/β, hypoxia inducible factor 1alpha/beta; HRE, hypoxia responsive element; PI3K, phosphoinositide 3-kinase; p70S6K, p70S6 kinase; 4E-BP1, 4E binding protein-1; eIF-4E, eukaryotic initiation factor-4 subunit E; sun, sunitinib; SK1, sphingosine kinase-1; ERK, extracellular-signal-regulated kinase; Sph, sphingosine; S1P, sphingosine-1 phosphate.

However, hypoxia also leads to elevated expression of the HIF1 transcription factor [17] (Fig. 1). Under normoxic conditions, HIF1α is hydroxylated at two proline residues, a reaction that is oxygen-dependent and catalyzed by members of the 'prolyl hydroxylase domain' (PHD or EGLN) family. After hydroxylation, pVHL can bind and ubiquitinate HIF1α, which makes it target for proteosomal degradation [17–19]. Under hypoxic conditions, however, hydroxylation of the proline residues is prevented whereby pVHL cannot bind to HIF1α. This ensures accumulation of HIF1α and translocation to the nucleus, where it forms a heterodimer with HIF1β, which then binds to specific DNA sequences in promoter regions of target genes, called the 'hypoxia responsive elements' (HRE). Binding of the heterodimer with the HREs leads to the transcription of a large number of genes involved in numerous cellular processes, such as proliferation, angiogenesis, survival, apoptosis, glucose metabolism and pH regulation [16–19]. HRE containing genes include *VEGF*, *PDGFβ*, transforming growth factor α (*TGF-α*), erythropoietin (*EPO*), matrix metalloproteinase 1 (*MMP-1*), epidermal growth factor receptor (*EGFR*), hepatocyte growth factor receptor (*HGFR*/*cMET*), cyclin D1, stromal cell-derived factor 1 (*SDF1*) and its receptor CXC chemokine receptor 4 (*CXCR4*) [18].

Hypoxia-driven up-regulation of HIF1α thus leads to increased expression of proangiogenic factors [20,21] which could possibly cause resistance to VEGF-targeted therapy. Several hypoxia-targeted therapies have been developed, of which the hypoxia-activated prodrugs (HAPs) are the most promising [22]. The prodrug TH-302 is an example of a HAP which is metabolized to its active form, bromo-isophosphoramide mustard (Br-IPM), only under extreme hypoxic conditions. Br-IPM alkylates DNA, causing DNA to crosslink whereby replication and proliferation is suppressed. Br-IPM can also diffuse in the surrounding (normoxic) tissue, causing a so called 'bystander effect' in which non-hypoxic tumor cells are also eradicated [23]. The anti-tumor activity of TH-302, alone or in combination with other agents, has been established in several preclinical [22,24–26] and clinical studies [27,28]. Ongoing clinical studies include a phase I trial of TH-302 in combination with sunitinib in patients with advanced RCC, gastrointestinal stromal tumors and pancreatic neuroendocrine tumors (ClinicalTrials.gov Identifier NCT01381822), and sorafenib in combination with TH-302 in advanced RCC and hepatocellular carcinoma (ClinicalTrials.gov Identifier NCT01497444).

2.1.1. Fibroblast growth factor (FGF)

The first evidence that fibroblast growth factor (FGF) could be involved in mediating resistance to VEGF-targeted therapies came from a study on tumor angiogenesis in a model of pancreatic islet carcinogenesis in immunodeficient mice [20]. Inhibition of VEGFR-2 led to decreased angiogenesis, histologically confirmed by a decrease in microvessel density in these tumors. However, after 4 weeks of treatment resistance occurred, with regrowth of the tumor and reactivation of angiogenesis, despite persistent dephosphorylation of VEGFR-2. This resistance was associated with up-regulation of several proangiogenic factors, such as VEGF-A, several members of the FGF family, ephrin-A1 and angiopoietin-1. Inhibition of FGF signaling, using an FGF-trap, led to a significant decrease of tumor burden and angiogenesis [20]. Patients with recurrent glioblastoma multiforma treated with an oral TKI of VEGF receptors (AZD2171) also showed elevated circulating levels of basic FGF (bFGF) when resistance occurred [21]. Welti and colleagues showed that FGF2 can directly stimulate endothelial cell proliferation and the formation of endothelial tubules in the presence of sunitinib [29]. Furthermore, inhibition of FGF2 signaling by the small molecule PD173074 abrogated these effects of FGF2. Interestingly, analysis of 74 tumor samples from patients with metastatic RCC demonstrated that a large proportion of these tumors strongly express FGF2 [29]. The observation that up-regulation of FGF could mediate resistance to sunitinib and other VEGF-targeted therapies, led to the development of agents that inhibit FGF signaling. Very recently, it was shown in a phase III trial comparing dovitinib (an oral multi-TKI of FGFR, VEGFR and PDGFR) with sorafenib in a third-line setting, that targeting both VEGF and FGF signaling did not prove to be more effective than targeting VEGF signaling alone, since no differences in PFS (dovitinib group 3.7 months and sorafenib group 3.6 months, HR 0.86, 95% CI 0.72–1.04) or OS (dovitinib group 11.1 months and sorafenib group 11.0 months, HR 0.96, 95% CI 0.75–1.22) were observed between the two treatment groups [30]. It can be argued that FGF signaling may not be a major driver of resistance to VEGF-targeted therapy and that other alternative proangiogenic pathways (e.g. IL-8) are more important in this respect. FGF(R) could therefore be an inappropriate pharmaceutical target [31]. However, it is questionable whether sorafenib should be the comparator in this third line treatment setting, as sorafinib, based on its registration study, only showed clinical benefit after previous treatment with cytokines [32].

2.1.2. Interleukin 8 (IL-8)

Huang and colleagues identified IL-8 as a potential proangiogenic factor that mediates resistance to sunitinib in RCC. They observed higher microvessel density in sunitinib-resistant tumors compared to sunitinib-sensitive tumors in RCC xenograft models, suggesting an escape from antiangiogenesis [33]. Screening of the plasma of mice bearing sunitinib-resistant tumors revealed a significant higher plasma level of IL-8 compared to mice bearing sunitinib-sensitive tumors. Treatment of resistant tumors with an IL-8 neutralizing antibody resensitized these tumors to treatment with sunitinib. Also in human sunitinib-resistant ccRCC, higher expression of IL-8 was found compared to sunitinib-sensitive human ccRCC [33]. The role of IL-8 up-regulation was also shown in colon cancer xenografts [34] and in rhabdomyosarcoma [35]. Up-regulation of IL-8 appears to be mediated through a HIF1α-independent manner, since it was not affected by knock-down of HIF1α [35]. Inhibition of the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathway, however, was effective in inhibiting the expression of IL-8, demonstrating that IL-8 expression is regulated by NF-κB [35]. IL-8 promotes expression of VEGF mRNA and protein levels in endothelial cells through binding to CXCR2, which subsequently leads to VEGFR-2 activation in an autocrine manner [36]. These results suggest that hypoxia-induced up-regulation of IL-8 can stimulate VEGF expression in endothelial cells in a HIF1α-independent manner via NF-κB, and can promote activation of VEGFR-2 leading to persistent angiogenesis. Thus, targeting both VEGF signaling and IL-8 signaling, or preferably NF-κB signaling, seems necessary to achieve adequate inhibition of angiogenesis and to prevent sunitinib resistance.

2.1.3. Placental growth factor (PlGF)

Placental growth factor (PlGF) is a VEGF homologue that binds to VEGFR-1 which is expressed by tumor cells, endothelial cells, bone marrow-derived proangiogenic cells, inflammatory cells, and stromal cells (e.g. fibroblasts) [37,38]. Absence of PlGF under pathological circumstances (e.g. inflammation, ischemia and cancer) leads to reduced angiogenesis and vascular permeability [39]. It is thought that VEGFR-1 functions as a 'decoy receptor' for VEGF, whereby the activation of VEGFR-2 by VEGF is negatively regulated. The decreased activity of VEGFR-2 subsequently leads to reduced angiogenesis [37,39]. It is proposed that PlGF stimulates angiogenesis through several mechanisms (reviewed in [37]). Firstly, PlGF displaces VEGF from VEGFR-1, thereby increasing the bio-availability of VEGF to stimulate VEGFR-2. Secondly, it is thought that binding of PlGF to VEGFR-1 leads to intracellular signaling which in turn stimulates angiogenesis. A third mechanism is the transphosphorylation of VEGFR-2 by the PlGF/VEGFR-1-complex, which amplifies VEGFR-2 signaling. Furthermore, PlGF also stimulates angiogenesis by up-regulating the expression of VEGF-A, FGF2, PDGFβ, and matrix metalloproteinases (MMPs) as well as by the recruitment of bone marrow-derived angiocompetent myeloid cells which stimulate angiogenesis through secretion of proangiogenic cytokines [40]. Expression of PlGF and VEGFR-1 are up-regulated during carcinogenesis and

are associated with outcome and prognosis in several cancer types such as lung cancer [41], colorectal cancer [42], breast cancer [43], and gastric cancer [44]. In RCC, increased amounts of PlGF mRNA were found in tumor tissue but not in adjacent normal renal tissue [45]. Increased plasma levels of PlGF in patients with RCC were associated with tumor grade, total tumor vascularity (TTV) and survival [46]. Since expression of PlGF is up-regulated during VEGF-targeted treatment [20,21,47], and is capable of stimulating angiogenesis, agents targeting PlGF could possibly overcome resistance to these VEGF-targeted therapies. Indeed, αPlGF, an anti-PlGF antibody, inhibited growth and metastasis in VEGFR-inhibitor resistant xenograft models of pancreatic carcinoma, colon carcinoma and melanoma [48]. The role of PlGF in tumor angiogenesis and in mediating resistance to VEGF-targeted therapies was recently challenged by the observation that over-expression of PlGF inhibits angiogenesis and tumor growth [49,50] and that tumors that express PlGF are hypersensitive to anti-VEGF and anti-VEGFR-2 therapy [51]. Thus, the exact role of PlGF in angiogenesis remains unclear, as is the role of PlGF in mediating resistance to sunitinib. Nevertheless, preclinical experimental data so far encourage further research on this relationship.

2.1.4. Angiopoietin

Another pathway that is suggested to promote angiogenesis during VEGFR inhibition is the angiopoietin/tie (Ang/Tie) pathway. It has been shown that VEGFR-2 inhibition leads to up-regulation of members of the angiopoietin family in a model of pancreatic islet carcinogenesis [20]. In human RCC tissue, Ang2 expression is significantly increased and the expression of Ang2 in RCC cell lines is regulated by hypoxia and the *VHL* gene [52]. More importantly, at time of disease progression, Ang2 levels increased in RCC patients treated with sunitinib, suggesting a possible role for Ang2 in mediating resistance to sunitinib [53]. The angiopoietin family consists of four ligands (Ang1, Ang2, Ang3 and Ang4) that bind to their receptors Tie1 and Tie2. Of these, Ang1 and Ang2 and their interaction with the Tie2 receptor are the best studied to date. Ang1/Tie2 signaling plays an important role in the maturation and stabilization of the vasculature by recruitment of peri-endothelial cells [54]. In contrast, Ang2 functions as a natural antagonist of Ang1 and the Tie2 receptor, and is only expressed at sites of vascular remodeling with active angiogenesis, including pathologic angiogenesis as seen in tumors [55]. However, the function of Ang2 is context-dependent and varies with the presence of other proangiogenic signals. In absence of VEGF, inhibition of Ang1/Tie2 signaling by Ang2 leads to vascular regression, whereas Ang2 signaling in the presence of VEGF leads to destabilization, remodeling and sprouting of blood vessels [54,55] which in turn increases the exposure of endothelial cells to VEGF. MEDI3617, a monoclonal antibody targeting Ang2, led to inhibition of tumor growth in several tumor xenograft models, including RCC [56]. However, tumors treated with the anti-Ang2 antibody displayed a high degree of hypoxia with subsequent up-regulation of HIF1α and VEGF. Combined treatment with an anti-Ang2 antibody and VEGF-targeted therapies was shown to be more effective in inhibiting tumor growth than either of these agents alone [56,57], as was also true for a chimeric decoy receptor (DAAP; double antiangiogenic protein) that simultaneously binds VEGF-A and the angiopoietins [58]. The efficacy of inhibition of Ang2/Tie2 signaling in combination with VEGF-targeted therapy in preclinical models led to the implementation of this strategy in clinical trials, of which results have to be awaited.

2.1.5. Delta-like ligand 4 (DLL4)

More recently, the Delta-like ligand 4 (DLL4)/Notch signaling pathway has been implicated as an alternative angiogenic pathway that could contribute to the development of resistance to VEGF-targeted therapy. In the vasculature of human ccRCC the expression of DLL4 is almost nine times higher compared with the expression of DLL4 in normal kidney tissue [59], and expression of DLL4 on the endothelial cells is up-regulated by VEGF, FGF, and hypoxia through HIF1α [59,60]. The Notch signaling system consists of at least five ligands (Jagged1, Jagged2, Delta-Like (DLL)-1, DLL3, and DLL4) that bind to their receptors (Notch1–Notch4). DLL4 is an endothelium-specific membrane-bound ligand of Notch and is critical for normal vascular development [61] and is an important negative regulator of tumor angiogenesis [62,63]. Paradoxically, blockade of DLL4/Notch activity increases vessel density. However, these vessels are abnormal and poorly perfused, which leads to increased tumor hypoxia [62]. Blockade of Notch signaling up-regulates the expression of VEGFR-2, whilst activation of Notch signaling suppresses the expression of VEGFR-2 [64]. The increased vessel density that is seen in the face of DLL4/Notch signaling blockade could thus be caused by increased sensitivity for VEGF signaling through up-regulation of VEGFR-2 expression [64,65]. Indeed, inhibition of DLL4/Notch signaling led to inhibition of tumor growth in several tumor models, even in tumors resistant to anti-VEGF antibody treatment [62,64], and combination of anti-VEGF therapy with an anti-DLL4 antibody was even more effective in reducing tumor growth [64]. Phase I trials of the γ-secretase inhibitors RO4929097 and MK-0752, which prohibit the essential cleavage of the intracellular domain of the Notch receptor, provided preliminary evidence of clinical antitumor activity [66,67], and combination of RO4929097 with other agents (e.g. cediranib, temsirolimus and gemcitabine) is promising [68–70]. Currently, also the anti-DLL4 antibody MEDI0639 is evaluated in a first-in-human phase I study (ClinicalTrials.gov Identifier NCT01577745). Hence, the role of DLL4/Notch signaling in RCC and its possible role in mediating resistance to sunitinib needs to be further elucidated. Very recently, it was shown that DLL4/Notch signaling enhances the metastatic potential of RCC by aiding the degradation of the extracellular matrix through increased matrix metalloproteinase 9 (MMP-9) expression via the Notch effector Hey1 [71,72], and high expression of DLL4 in RCC tissue samples was indeed associated with the development of distant metastasis [71,72]. DLL4/Notch signaling thus seems to play an important role in processes such as angiogenesis and tumor metastasis in RCC, and the interplay between Notch signaling and VEGF signaling provides a rationale for combining VEGFR-inhibitors with inhibitors of DLL4/Notch signaling to treat RCC.

2.2. Resistance mediated by the tumor microenvironment

The tumor stroma consists of several cell types such as fibroblasts, pericytes, endothelial cells, and hematopoietic cells, among others [73]. It is known that stromal cells play important roles in tumorigenesis and angiogenesis either by directly contributing to the vasculature (e.g. endothelial and pericyte progenitors), by secretion of angiogenic factors (e.g. VEGF and MMP-9), or by crosstalk between tumor cells and stromal cells via cytokines and chemokines [73,74]. An important subpopulation in the stromal compartment is that of the bone marrow-derived cells (BMDCs). Since hypoxia (via HIF1α and its effectors SDF1 and VEGF [75,76]) leads to increased recruitment of several BMDCs that can promote angiogenesis [77], it is tempting to speculate that these BMDCs could contribute to mediating resistance to VEGF-targeted therapy. BMDCs can be divided into vascular progenitors (e.g. endothelial and pericyte progenitors) and vascular modulatory cells [77], including tumor-associated macrophages (TAMs) [78], Tie2 + monocytes [79], VEGFR1 + hemangiocytes [80,81] and CD11b + myeloid cells [82]. Two stromal cell populations, the CD11b + Gr1 + myeloid cells and the tumor-associated fibroblasts (TAFs), are of special interest since studies showed that they can preserve angiogenesis even in the presence of inhibition of VEGF signaling (Fig. 2), and are therefore discussed here.

2.2.1. Tumor-associated fibroblasts (TAFs)

TAFs contribute to tumor growth and angiogenesis in several ways. TAFs secrete various growth factors, cytokines and hormones, such as HGF, EGF, bFGF, SDF1, and IL-6, which can directly stimulate tumor

cell proliferation [83]. The secretion of SDF1 leads to the recruitment of endothelial progenitor cells (EPCs) into the tumor tissue, thereby promoting angiogenesis. SDF1 can also bind to its receptor CXCR4, which is expressed by tumor cells, hereby directly stimulating cell growth [84]. Expression of platelet-derived growth factor-C (PDGF-C) is up-regulated in TAFs isolated from tumors resistant to VEGF-targeted therapy [85] (Fig. 2). Angiogenesis induced by these PDGF-C secreting TAFs may be blocked using an anti-PDGF-C antibody. Combining anti-VEGF therapy with antibodies against PDGF-C indeed showed additional benefit in reducing tumor growth. These data suggest that increased expression of PDGF-C by TAFs could overcome inhibition of VEGF-mediated angiogenesis [74,85].

2.2.2. CD11b + Gr1 + myeloid cells

In 2004, Yang and colleagues demonstrated that CD11b + Gr1 + myeloid cells are significantly increased in tumor-bearing mice and can promote tumor growth by inducing angiogenesis [82]. This induction of angiogenesis was mediated by two mechanisms. First, it was shown that CD11b + Gr1 + myeloid cells produce high levels of MMP-9, an important regulator of tumor angiogenesis which increases the bioavailability of VEGF by releasing it from the matrix. Second, CD11b + Gr1 + myeloid cells can be directly incorporated into the tumor endothelium and can differentiate into endothelial cells [82]. A series of studies demonstrated that CD11b + Gr1 + myeloid cells that infiltrate the tumor are also able to confer resistance to VEGF-targeted treatment [86–88]. Tumors resistant to VEGF-targeted therapy showed higher gene expression of several cytokines involved in the mobilization and recruitment of CD11b + Gr1 + myeloid cells to the tumor, including granulocyte-colony stimulating factor (G-CSF). When CD11b + Gr1 + myeloid cells from resistant tumors were added to tumors sensitive for VEGF-targeted therapy, tumor growth was sustained even in the presence of VEGF-targeted treatment. In these admixture tumors, tumor growth was only inhibited by the combined treatment with an anti-VEGF and an anti-Gr1 antibody [86]. In a subsequent study it was shown that the CD11b + Gr1 + myeloid cells in the resistant tumors had a higher expression of the proangiogenic factor Bv8, which stimulates VEGF-independent angiogenesis in the tumor [87]. The expression of Bv8 was stimulated by G-CSF (but also IL-6 and SFD1), cytokines that are expressed by both tumor cells and tumor stroma. Blockade of Bv8 with a neutralizing antibody led to a decrease in mobilization and recruitment of CD11b + Gr1 + myeloid cells to the tumor and subsequently to inhibition of angiogenesis and tumor growth. Combining VEGF-targeted treatment with a neutralizing antibody against Bv8 had some additive effect [87,88]. These data provide evidence that up-regulation of Bv8 via G-CSF could function as an alternative proangiogenic pathway when VEGF signaling is inhibited. Very recently, Panka et al. [89] showed that treatment of RCC xenografts with sunitinib led to a significant increase in CD11b + Gr1 + myeloid cells in the tumor compared to untreated xenografts ($p < 0.0001$), which was mediated by SDF1. This study provides preliminary evidence that infiltration of the tumor with CD11b + Gr1 + myeloid cells and subsequent production of Bv8 may also play a role in mediating resistance to sunitinib in RCC.

2.2.3. Pericytes

Another population of stromal cells, which directly contribute to the formation of blood vessels, is the perivascular cells, also called pericytes [90,91]. Paracrine signaling between pericytes and endothelial cells is largely mediated by PDGF-BB (secreted by the endothelial cells) which binds to PDGFRβ on the pericytes. Binding of PDGF-BB to PDGFRβ leads to increased VEGF mRNA transcription in the pericyte via the MAPK and the PI3K pathway, which enhances the endothelial cell survival in a paracrine manner [92]. The fact that pericytes provide survival factors for endothelial cells is supported by the observation that withdrawal of VEGF only led to regression of blood vessels without pericyte coverage (immature vessels) in human glioblastoma multiforma and prostate carcinoma [93]. The enhanced survival of the endothelial cells by increased pericyte coverage and increased production of VEGF by the pericytes, could render the endothelial cells less responsive to inhibition of VEGF signaling [77]. Indeed, the approach to target both pericytes and endothelial cells by inhibiting both VEGF and PDGF signaling has proven to be more effective in reducing angiogenesis and tumor growth than targeting either of these pathways alone [94–96]. Since sunitinib targets both VEGFR and PDGFR, it is unlikely that increased pericyte coverage plays a role in mediating resistance to sunitinib. However, targeting PDGF could also have an adverse effect. Xian and colleagues showed that decreased pericyte coverage and dysfunction of pericytes led to destabilization of the vessel wall and subsequent escape of tumor cells into the vasculature, thus facilitating hematogenous metastasis [97]. This study shows that treatment with antiangiogenic agents should be carried out sensibly, since a very delicate balance exists between inhibiting tumor growth and promoting tumor progression. Destruction of the tumor vasculature deprives the tumor from oxygen en nutrients, whereby tumor growth is prohibited. However, destruction of the tumor vasculature also has a downside, since it leads to hypoxia and destabilization of the vessels, both factors that enhance the metastatic potential of the tumor. Therefore, more attention is paid to the concept of stabilization and normalization of the vasculature in antiangiogenic therapy [98]. Although normalization of the tumor vasculature leads to better oxygen and nutrient supply and thus accelerated tumor growth, it also leads to a more effective drug-delivery and immune-response, which counteracts these effects [98]. Although this concept sounds attractive, the implementation in clinic is challenging and requires a well-balanced treatment schedule, probably by combining several antiangiogenic agents.

2.3. Promotion of tumor invasiveness and metastasis

An increased metastatic potential is also seen after treatment with sunitinib in several tumor- and metastasis models in mice [99,100]. It is suggested that tumor invasiveness and metastasis is enhanced by increased tumor hypoxia through selection of more malignant metastatic cells that are less sensitive to VEGF-targeted therapy [101]. Furthermore, it was shown that hypoxia induces the expression of the cMET receptor [102] of which activation by HGF leads to cell proliferation, promotion of cell survival, and increased invasiveness through several signaling pathways including the PI3K/Akt pathway, Src, STAT3, and MAPK pathway [103]. Also, RCC cells that lack the VHL protein are highly invasive and exhibit extensive branching morphogenesis after treatment with HGF, which is associated with down-regulation of tissue inhibitor of metalloproteinase 2 (TIMP-2) and up-regulation of MMP-2 and MMP-9 [104]. Besides stimulating tumor cell proliferation and migration, activation of cMET by paracrine HGF also induces angiogenesis by stimulating proliferation, migration, and survival of endothelial cells, and by up-regulation of the expression of VEGF and down-regulation of thrombospondin-1 expression in the tumor cells [105]. A group led by Christensen showed that the HGF/cMET pathway plays a role in mediating resistance to sunitinib [103] (Fig. 2). Analysis of tumor protein lysates of sunitinib-resistant tumors indicated a higher concentration of HGF as compared with sunitinib-sensitive tumors; however, lack of HGF expression in tumor or endothelial cells indicated that the main source of HGF was the tumor stroma, as previously described [106]. Treatment of sunitinib-resistant tumors with a combination of sunitinib and a cMET-inhibitor significantly inhibited tumor growth [103]. Evidence suggests that increased activity of cMET promotes epithelial-to-mesenchymal transition (EMT) [107]. In tumor samples of patients treated with VEGF-targeted therapy an increase of the mesenchymal markers vimentin and CD44 was observed [107,108]. Evidence of EMT as a resistance mechanism to sunitinib in RCC has also been published [109], and could probably be due to increased cMET activity. Several preclinical studies have demonstrated that combined inhibition of cMET and VEGF signaling suppresses tumor growth, angiogenesis, tumor invasion and metastasis [103,107,108,110,111]. A phase

II trial of cabozantinib [112], an oral inhibitor of multiple tyrosine kinases including cMET, VEGFR-2, KIT, and RET, in patients with heavily pretreated metastatic RCC, demonstrated clinical activity with a high objective response rate of 28% and stable disease rate of 62%. Currently, a phase III trial comparing cabozantinib with everolimus as second-line treatment in patients who progressed on previous treatment with a VEGFR TKI is ongoing (ClinicalTrials.gov Identifier: NCT01865747). Cabozantinib has also shown to be very effective in the treatment of patients with castration-resistant prostate cancer (CRPC), in which the HGF/cMET and the VEGF signaling pathway also play critical roles in tumor development and progression [113]. As in CRPC, cabozantinib demonstrated efficacy against bone metastases in RCC, a factor that predicts poor prognosis in patients with metastasized RCC treated with VEGF-targeted therapy [114]. To date, cMET seems to be the most promising target for the treatment of therapy-resistant RCC, as demonstrated by the divers molecular processes involved in tumor progression in which the HGF/cMET plays an important role, as well as by the clinical activity of cabozantinib.

2.4. Activation of alternative signaling pathways

Although the anti-tumor efficacy of sunitinib is mainly achieved indirectly by inhibiting angiogenesis [7], sunitinib also has a direct effect on tumor cells [6,115]. Gao et al. found that both sphingosine kinase-1 (SK1) and ERK, a downstream target of SK1, were activated in sunitinib-resistant RCC cells [116] (Fig. 2). SK1 is an important regulator of sphingolipid signaling. The sphingosine-1 phosphate (S1P) concentration in cells is kept low through a balance between S1P synthesis and degradation. Especially the balance between the S1P-precursors (ceramide and sphingosine) and S1P, which is regulated by SK1, is important in determining whether a cell proliferates or undergoes apoptosis. Phosphorylation of sphingosine by SK1 leads to the production S1P, which stimulates growth and angiogenesis and prevents apoptosis. At the same time, SK1 also lowers the intracellular levels of the pro-apoptotic molecule ceramide, further preventing apoptosis [117]. Furthermore, SK1 modulates the expression and transcriptional activity of HIF1α. Activation of SK1 is stimulated under hypoxic circumstances. SK1 stabilizes the level of HIF1α through inhibition of the proteasomal degradation of HIF1α via the Akt/GSK-3β signaling pathway which leads to accumulation and enhanced transcriptional activity of HIF1α. Pharmacological inhibition and RNA silencing of SK1 prevented the accumulation of HIF1α and its transcriptional activity in several human cancer cell lines, including RCC [118]. Downstream inhibition of SK1 signaling with an anti-S1P monoclonal antibody also blocked endothelial cell migration, inhibited VEGF and bFGF induced tumor angiogenesis, inhibited S1P-induced proliferation and release of proangiogenic cytokines such as IL-6, IL-8 and VEGF in several cancer cell lines [119].

2.5. Inadequate target inhibition

2.5.1. Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are single base pair changes that achieve a population frequency of at least one percent. SNPs that could be involved in mediating resistance to sunitinib are possibly located in genes that regulate the pharmacokinetics and pharmacodynamics of this drug. Pharmacokinetic genes of special interest are the genes that encode the efflux transporters ABCB1 (P-glycoprotein; P-gp) and ABCG2. These efflux transporters are expressed on enterocytes and could influence the absorption, and thus the systemic exposure, of orally administered sunitinib [120]. The association between the polymorphism in the *ABCB1* haplotype (TCG copy) and improved patient outcome suggests that this SNP leads to decreased efflux of sunitinib and thus increased systemic exposure [120]. Sunitinib is predominantly metabolized by CYP3A4 and also partly by CYP3A5, two enzymes of the cytochrome P450 family of which the expression of the former is regulated by the ligand activated nuclear receptors NR1I2 and NR1I3. SNPs in the genes that encode these enzymes may also influence the effective concentration of sunitinib [120]. The presence of an A-allele in *CYP3A5* (6986A/G), which leads to the *CYP3A5* expressor phenotype, was associated with increased PFS [120]. This longer PFS could be caused by increased metabolism of sunitinib, which leads to an increased concentration of SU12662, the active metabolite of sunitinib. SNPs in *NR1I2* and *NR1I3* are associated with decreased PFS and/or OS [120,121] possibly due to a negative regulation of CYP3A4 expression. SNPs in pharmacodynamics factors, such as the molecular targets of sunitinib (e.g. VEGF- and PDGF receptors), could also contribute to the development of resistance to sunitinib. Multiple studies confirmed that several SNPs in these genes are indeed associated with survival of patients treated with sunitinib [120–125] (Table 1). Interestingly, the SNPs rs307826 and rs307821 in *VEGFR-3* were associated with decreased patient survival in two independent studies [121,122]. SNPs in genes involved in the pharmocodynamics and pharmacokinetics of sunitinib could thus possibly serve as predictive markers of sunitinib efficacy, however, validation in larger patient cohorts is necessary.

2.5.2. Lysosomal sequestering

Since increased drug efflux is a classic mechanism of resistance, the intracellular concentration of sunitinib in resistant and sensitive renal cell- and colon carcinoma cell lines was determined [126]. Surprisingly, the intracellular concentration of sunitinib was ten-fold higher in resistant cells compared to sensitive cells. Using fluorescence microscopy, increased sequestering of sunitinib in acidic lysosomes was revealed in resistant cells. Despite the increased intracellular concentration of sunitinib in resistant cells, levels of p-ERK and p-Akt were unaffected and similar to the levels in the parental cells. Thus, increased lysosomal sequestering presents a novel potential mechanism of resistance to sunitinib. Furthermore, lysosomal sequestering as a resistance mechanism proved to be reversible, which supports retrospective clinical data of effectiveness of sunitinib rechallenge after disease progression on prior treatment with sunitinib [127,128]. The clinical value of rechallenge with sunitinib in the third-line setting is currently prospectively investigated in a phase II trial, that includes patients with metastasized RCC who had benefit from prior treatment with sunitinib and who progressed on both sunitinib and second-line therapy with an mTOR inhibitor (or a period of more than 3 months without treatment) (ClinicalTrials.gov Identifier: NCT02071641).

2.6. Resistance mediated by the action of microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that control gene expression by controlling mRNA behavior, either by direct cleavage of the targeted mRNAs or by inhibiting translation through complementarity at their 3′ untranslated regions [17]. MiRNA profiling studies identified different patterns of miRNA expression in RCC [129,130]. Furthermore, several potential targets of the aberrantly expressed miRNAs were identified, including ccRCC oncogenes such as *mTOR*, *VHL*, *HIF1α* and *PDGFβ* [131]. One of the few studies on the effect of miRNAs in mediating resistance to sunitinib, assessed miRNA expression in peripheral blood of patients receiving sunitinib for advanced RCC [132]. Comparison of the poor response (PFS < 6 months) and the prolonged response (PFS > 18 months) groups with regard to miRNA expression, found significant differences in the expression of 28 miRNAs, which were used to build several models predicting poor and prolonged response to sunitinib. Prolonged response was related to down-regulation of miRNA-410, miRNA-1181, and miRNA 424. Poor response was associated with down-regulation of miRNA-192, miRNA-193a-3p, and miRNA-501-3p. Ontology analyses of the genes regulated by these miRNAs showed that they are involved in cancer-related pathways such as angiogenesis and apoptosis. Recently, higher expression of miRNA-942, miRNA-133a, miRNA-628-5p and miRNA-484 was seen in sunitinib-resistant tumors as compared to sunitinib-sensitive tumors from patients with metastasized RCC, with

Table 1
Association of SNPs in genes that regulate the pharmacokinetics and pharmacodynamics of sunitinib with outcome in RCC.

Association of SNPs and sunitinib outcome in RCC					
Study	Outcome	SNP	HR (95% CI)	P-value	Ref.
van der Veldt et al. N = 136	PFS	A-allele in *CYP3A5* 6986A/G	0.266 (0.079–0.892)	0.032	[120]
		Presence of CAT-copy in the *NR1I3* haplotype	1.758 (1.108–2.790)	0.017	
		TCG copy in the *ABCB1* haplotype	0.522 (0.287–0.950)	0.033	
	OS	A-allele in *VEGFR-2* 1718 T/A	2.907 (1.224–6.903)	0.016	
Garcia-Donas et al. N = 89	PFS	*VEGFR-3* rs307826	3.57 (1.75–7.30)	0.0079	[122]
		VEGFR-3 rs307821	3.31 (1.64–6.68)	0.014	
Beuselinck et al. N = 88	PFS	*FGFR-2* rs2981582	2.669 (1.094–6.511)	0.031	[121]
		NR1I2 rs2276707	2.978 (1.012–8.761)	0.047	
	OS	*NR1I3* rs2307424	1.913 (1.006–3.636)	0.048	
		VEGFR-3 rs307826	2.223 (1.187–4.163)	0.013	
	PFS and OS	*ABCB1* rs1128503	NA	0.027 and 0.025 resp.	
		NR1I3 rs4073054	NA	0.025 and 0.035 resp.	
		VEGFR-3 rs307821	NA	0.032 and 0.011 resp.	
Beuselinck et al. N = 91	RR	CC-variant in *VEGFR-1* rs9582036	NA	0.028	[123]
	PFS	CC-variant in *VEGFR-1* rs9582036	NA	0.06	
		AA-variant in *VEGFR-1* rs9554320	NA	0.005	
	OS	CC-variant in *VEGFR-1* rs9582036	NA	0.008	
		AA-variant in *VEGFR-1* rs9554320	NA	0.067	
Scartozzi et al.N = 84	PFS	*VEGF-A* rs833061	0.71 (NA)	0.0197	[124]
		VEGF-A rs2010963	0.19 (NA)	0.0201	
		VEGFR-3 rs6877011	0.35 (NA)	<0.0001	
	OS	*VEGF-A* rs833061	0.69 (NA)	0.0011	
		VEGFR-3 rs6877011	0.39 (NA)	P < 0.0001	
Kim et al. N = 63	OS	*VEGF* rs3025039 plus *VEGFR-2* rs2305948	3.18 (1.12–8.99)	0.03	[125]

Abbreviations: SNP, single nucleotide polymorphism; PFS, progression free survival; OS, overall survival; RR, response rate; HR, hazard ratio; NR1I3 and NR1I2, nuclear receptor 1I3 and 1I2; VEGFR-1, -2, -3, vascular endothelial growth factor 1, 2, and 3; VEGF(A), vascular endothelial growth factor (A); FGF, fibroblast growth factor receptor.

up-regulation of miRNA-942 being the most accurate predictor of sunitinib efficacy [133] (Table 2). High expression of each of these four miRNAs was significantly associated with decreased time to progression (TTP) and OS. Overexpression of miRNA-942 in a metastatic RCC cell line led to increased secretion of MMP-9 and VEGF and led, via a paracrine loop, to promotion of migration of endothelial cells and sunitinib resistance. More specifically, Berkers et al. [134] found a miRNA that is down-regulated in sunitinib-resistant metastatic ccRCC (Table 2). Compared to good responders (PFS ≥ 12 months), miRNA-141 was significantly down-regulated in tumors from poor responders (PFS < 6 months) to sunitinib. Further, miRNA-141 was associated with EMT [134]. MiRNA-141 inhibits the activity of the transcriptional repressors ZEB1 and ZEB2, factors that are known to be implicated in EMT, tumor metastasis, angiogenesis induction and chemotherapy resistance. Thus, expression of certain miRNAs can possibly be used as predictive markers that also provide a clue on the underlying resistance mechanism since they modulate important processes such as angiogenesis and EMT (Table 2).

3. Potential predictive biomarkers of response and resistance to sunitinib

As described above, a subgroup of RCC patients is intrinsically resistant to, or develops acquired resistance upon, treatment with sunitinib. (Bio)markers accurately identifying these patients will help managing this disease more optimally.

3.1. Circulating biomarkers

3.1.1. Circulating endothelial cells

Two distinct groups of circulating endothelial cells can be distinguished: mature circulating endothelial cells (mCECs) and circulating endothelial progenitors (CEPs). mCECs are derived from the blood vessel wall and are released into the circulation upon vessel damage but also during normal angiogenesis [135]. CEPs originate from the bone marrow and differentiate into mature endothelial cells, whereby they directly contribute to neovascularization through vasculogenesis [136]. An elevation of CECs (mCECs and CEPs) has been observed in patients with several different cancer types as compared to healthy patients [137,138]. Increased levels of CECs were observed in a preclinical model of RCC [139], but also in patients with RCC [137,140]. Especially the fact that mobilization of CEPs is regulated by the HIF1-regulated factors VEGF and SDF1, points to a possible role of these CECs in RCC [140]. Since mCECs are released into the circulation following vessel damage, they could represent a marker for efficacy of antiangiogenic drugs, such as sunitinib [141]. Furthermore, CEPs express VEGFR-2 on their cell surface which is also a target of many angiogenesis inhibitors [142]. Based on these observations, it can be reasoned that treatment with antiangiogenic drugs would lead to increased levels of mCECs and decreased levels of CEPs by inhibition of their mobilization [141]. Indeed, the level of CECs in patients with metastasized RCC was increased during treatment with sunitinib, which was associated with longer PFS when compared to patients with a decreased number of CECs [143,144] (Table 2). High level of CEPs at baseline was associated with higher risk of progression (HR 2.50, P = 0.010) and poorer prognosis (HR 3.30, P = 0.006) compared to patients with a low baseline level of CEPs [145]. These data show that an increase in circulating endothelial cells could reflect vascular damage and thus efficacy of angiogenesis inhibitors and could possibly be used as a marker to predict response to treatment with sunitinib (Table 2).

3.1.2. Circulating cytokines and proangiogenic factors, including VEGFRs

In order to identify possible markers that predict sunitinib activity, Perez-Gracia et al. used an extreme phenotype selection approach in combination with a high-throughput human cytokine array [146]. Baseline levels of TNF-α and MMP-9 were significantly higher in patients who progressed than in patients with no progression, and high levels of both MMP-9 and TNF-α at baseline predicted reduced TTP,

Table 2
Possible biomarkers that predict response to treatment with sunitinib in patients with RCC.

Predictive biomarkers for sunitinib efficacy in RCC									
Type of biomarker	Study	No. of patients	Marker	Association with outcome	HR (95% CI)	RR (95% CI)	OR (95% CI)	P-value	Ref.
Circulating biomarkers									
▪ *Circulating endothelial cells*	Vroling et al.	21	CECs	Increased number of CECs after 14 days was associated with longer PFS	–	–	–	0.034	[143]
	Gruenwald et al.	13	CECs	In patients with PFS above the median (249 days) mean CEC values increased significantly from baseline 40 ± 41 CEC/ml to 111 ± 61 at day 28.In patients with PFS below the median, the increase from 53 ± 45 to 69 ± 61 CEC/ml remained insignificant.	–	–	–	0.0109	[144]
	Farace et al.	55	CEPs	CEPs levels at baseline >2% were associated with shorter PFS and OS	2,5 (NA) and 3.3 (NA), resp.	–	–	0.01 and 0.006, resp.	[145]
▪ *Cytokines and proangiogenic factors*	Perez-Gracia et al.	21	TNF-α	TNF-α baseline levels above the median (50 pg/ml) predict risk of reduced TTP	2.63 (0.986–7.058)	–	–	0.053	[146]
			MMP-9	MMP-9 baseline levels above the upper tercile (4062 ng/ml) predict risk of reduced TTP	3.05 (1.09–8.51)	–	–	0.033	
			TNF-α and MMP-9	High baseline levels of TNF-α and MMP-9 predict risk of reduced TTP	10.323 (1.028–103.64)	–	–	<0.047	
	DePrimo et al.	63	VEGF	Significantly larger changes (increase) in VEGF were observed in patients with objective tumor response to sunitinib compared with those exhibiting stable disease or disease progression	–	–	–	<0.05	[148]
	Porta et al.	84	VEGF	Low serum baseline VEGF level was independently associated with increased PFS	–	1.96 (1.47–2.45)	–	–	[150]
			NGAL	Low serum baseline NGAL levels were independently associated with increased PFS	–	1.91 (1.39–2.42)	–	–	
	Rini et al.	57	VEGF-C	Patients with baseline VEGF-C level below the median baseline value (722.1 pg/ml) had longer PFS	0.3662 (0.1567–0.6000)	–	–	0.0006	[149]
	Harmon et al.	63	IL-8	Low serum baseline IL-8 level was independently associated with increased OS	1.110 (1.022–1.20)	–	–	0.013	[147]
▪ *Circulating VEGFRs*	DePrimo et al.	63	sVEGFR-2 and sVEGFR-3	Significantly larger changes (decrease) in sVEGFR-2 and sVEGFR-3 were observed in patients with objective tumor response to sunitinib compared with those exhibiting stable disease or disease progression	–	–	–	<0.05	[148]
	Rini et al.	59	sVEGFR-3	Patients with baseline sVEGFR-3 level below the median baseline value (47,000 pg/mL)	0.4457 (0.2121–0.7753)	–	–	0.006	[149]

(continued on next page)

Table 2 *(continued)*

Predictive biomarkers for sunitinib efficacy in RCC									
Type of biomarker	Study	No. of patients	Marker	Association with outcome	HR (95% CI)	RR (95% CI)	OR (95% CI)	P-value	Ref.
				had longer PFS					
	Harmon et al.	63	sVEGFR-3	Low serum baseline sVEGFR-3 level was independently associated with increased OS	1.064 (1.004–1.13)	–	–	0.037	[147]
Genetic biomarkers									
▪ *SNPs*	See Table 1.								
Epigenetic biomarkers									
▪ *miRNAs*	Berkers et al.	20	miRNA-520 g	Significantly up-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.036	[134]
			miRNA-155	Significantly up-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.040	
			miRNA-526b	Significantly up-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.0067	
			miRNA-144	Significantly down-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.016	
			miRNA-141	Significantly down-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.0098	
			miRNA-376b	Significantly down-regulated in non-responders to sunitinib (PFS < 6 months) compared to responders (PFS > 12 months)	–	–	–	0.032	
	Prior et al.	20	miRNA-942	Significantly higher expression in sunitinib-sensitive patients (TTP > 22 months or PR > 50%) compared to sunitinib-resistant patients (TTP ≤ 5 months)	–	–	–	0.0074	[133]
			miRNA-942	High expression was significantly associated with TTP and OS	–	–	–	0.0030 and 0.0009, resp.	
			miRNA-628-5p	High expression was significantly associated with TTP and OS	–	–	–	0.0270 and 0.0161, resp.	
			miRNA-133a	High expression was significantly associated with TTP and OS	–	–	–	0.0153 and 0.0092, resp.	
			miRNA-484	High expression was significantly associated with TTP and OS	–	–	–	0.0001 and 0.0036, resp.	
▪ *DNA methylation*	Dubrowinskaja et al.	18	*NEFH*	Low methylation of *NEFH* was associated with increased OS	–	–	–	0.028	[165]
	Peters et al.	18	*CST6*	Low methylation of *CST6* was associated with increased PFS and OS	4.1 (1.3–12.6) and 4.1 (1.3–13.4), resp.	–	–	0.009 and 0.011, resp.	[166]
			LAD1	Low methylation of *LAD1* was associated with increased PFS and OS	6.4 (1.6–26.0) and 2.9 (1.0–8.6)	–	–	0.004 and 0.043, resp.	

Abbreviations: HR, hazard ratio; RR, relative risk; OR, odds ratio; 95% CI, 95% confidence interval; PFS, progression free survival; OS, overall survival; TTP, time-to-progression; ref, reference; NA, not available; CECs, circulating endothelial cells; CEPs, circulating endothelial progenitors; TNF-α, tumor necrosis factor-alpha; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor; NGAL, neutrophil gelatinase-associated lipocalin; IL-8, interleukin-8; sVEGFR, soluble vascular endothelial growth factor receptor; miRNA, microRNA; SNP, single nucleotide polymorphism; NEFH, Neurofilament Heavy polypeptide; CST6, cystatin E/M; LAD1, Ladinin 1.

independent of other factors (HR 10.323, 95% CI 1.028–103.64, P = 0.047) [146] (Table 2). In a phase II trial of sunitinib, baseline IL-8 was a significant predictor of overall survival in the sunitinib arm (HR 1.110, 95% CI 1.022–1.20, P = 0.013) [147] (Table 2). Measuring the circulating soluble ligands of VEGFRs (e.g. the VEGF family and PlGF) as possible markers for sunitinib efficacy has been performed in several studies. Levels of VEGF and PlGF significantly increased during treatment with sunitinib, and significantly larger changes in VEGF levels were observed in patients with objective tumor response compared to patients with stable disease or disease progression [148] (Table 2). In another phase II trial, mean plasma levels of VEGF-A and PlGF also significantly increased whereas VEGF-C levels decreased during treatment

with sunitinib [149], of which the latter was associated with higher objective response rate and longer PFS (HR 0.3662, 95% CI 0.1567–0.6000, P = 0.0006) (Table 2). Besides high baseline levels of VEGF, Porta et al. also found that high baseline levels of neutrophil gelatinase-associated lipocalin (NGAL) were associated with significant shorter PFS than normal baseline levels (RR 1.96, 95% CI 1.47–2.45 and RR 1.91, 95% CI 1.39–2.42, respectively) [150] (Table 2). In several of the above discussed studies, soluble VEGFRs (sVEGFR) have also been investigated as potential predictive markers for sunitinib efficacy (Table 2). In some of these studies, levels of sVEGFR-2 and/or sVEGFR-3 decreased during treatment with sunitinib, and greater decrease of one or two of these circulating receptors was associated with better tumor response or PFS [148,149]. Moreover, low baseline levels of sVEGFR-3 were identified as a significant, independent predictor of overall survival (HR 1.064, 95% CI 1.004–1.13, P = 0.037) [147]. Instead of measuring soluble VEGFRs in the plasma, the expression of ten molecular markers was determined in radical nephrectomy specimens from patients with metastatic RCC treated with sunitinib [151]. PFS in patients with strong expression of VEGFR-2 was significantly longer than in patients with weak expression of VEGFR-2.

3.2. Genetic and epigenetic biomarkers

3.2.1. Inactivation of the VHL gene

Although inactivation of the *VHL* gene, either by mutation, deletion or promoter CpG island hypermethylation, is a frequent event in the carcinogenesis of both sporadic and hereditary RCC, no consistent correlation between *VHL* inactivation and patient prognosis has been demonstrated [152–155]. The possibility that inactivation of *VHL* could serve as a predictive marker for efficacy of VEGF-targeted treatment has also been studied. Rini et al. determined the activation status of the *VHL* gene in tumor samples of 43 patients with metastatic RCC treated with either sunitinib (56%), axitinib (25%) or bevacizumab + interferon alpha (19%) [156]. They found mutations in *VHL* in 25 patients and promoter methylation in one patient, and these patients had an objective response rate of 48% compared to 35% in patients with no *VHL* mutation or methylation. A trend towards increased TTP was seen in patients with *VHL* methylation, frameshift mutations and truncating mutations compared to patients without these features (13.3 months versus 7.4 months, P = 0.06). No association between *VHL* status and OS was observed (P = 0.97) [156]. Besides higher response rates in patients with *VHL* inactivation compared to those with wild-type *VHL* (41% versus 31%, P = 0.034), patients with loss of function mutations (frameshift, nonsense, splice and in-frame deletions/insertions) had an even higher response than patients with wild-type *VHL* (52% versus 31%, P = 0.04) [157]. However, also in this study no association between *VHL* status and survival in patients with metastatic RCC treated with VEGF-targeted therapy was found.

3.2.2. DNA methylation

A variety of methylation markers that predict prognosis independent from clinicopathological factors, such as tumor size, grade, stage and presence of metastasis, in RCC have been reported [158–163]. However, identification of methylation biomarkers that predict response to therapy in RCC is still in its infancy. In a preclinical study, methylation of the promoter regions of the *VEGFR-1* (*FLT1*) and *VEGFR-2* (*KDR*) genes and the influence on TKI (sunitinib and PTK/ZK) efficacy was studied [164]. Treatment of cancer cell lines without *VEGFR*-methylation with sunitinib or PTK/ZK led to inhibition of proliferation. In contrast, proliferation in cell lines with *VEGFR*-hypermethylation was not significantly inhibited after treatment with either one of these agents. Combined treatment of the hypermethylated cells with the demethylating agent 5-aza-2′-deoxycytidine (DAC) and PTK/ZK had a synergistic effect and led to increased inhibition of proliferation compared to hypermethylated cells treated with DAC or PTK/ZK monotherapy [164]. Efficacy of treatment with sunitinib could thus be dependent on the promoter methylation status of the VEGF receptor genes, and could represent a possible predictive biomarker, although it cannot be excluded that other mechanisms of action of DAC are responsible for the observed effects. Therefore, the presence of *Flt1* and *KDR* promoter methylation and the relevance of this in predicting response to sunitinib in RCC has to be further elucidated in clinical studies. Recently, promoter hypermethylation of Neurofilament Heavy polypeptide *(NEFH)* was not only reported as an independent predictor of PFS in patients with RCC, but is also as a predictor of response to VEGF-targeted therapy [165]. Patients with high *NEFH* promoter methylation had an OS of 9.8 months versus 29.8 months for patients with low methylation (P = 0.028) (Table 2). In another study by the same group, promoter hypermethylation of cystatin E/M *(CST6)* and Ladinin 1 *(LAD1)* was associated with PFS and OS of patients with metastatic RCC undergoing VEGF-targeted therapy [166]. Patients with low methylation of *CST6* had a PFS of 11.4 months and OS of 22.9 months, compared to 2.0 months and 3.4 months in patients with high methylation, respectively (P = 0.009 and P = 0.011, respectively). Low methylation of *LAD1* was associated with a PFS of 11.4 months and OS of 16.4 months, whereas PFS in patients with high methylation was 2.0 months and OS 3.4 months (P = 0.004 and P = 0.043, respectively) (Table 2). However, the number of patients in these studies was small, so further validation of *NEFH*, *CST6* and *LAD1* promoter methylation as predictive biomarkers for VEGF-targeted therapy response is needed.

4. Conclusions and future perspectives

Although different mechanisms of resistance have been identified such as up-regulation of proangiogenic pathways, recruitment of BMDCs, promotion of tumor invasiveness and metastasis, and activation of alternative signaling pathways, one factor seems to play a crucial role in all these processes: tumor hypoxia. Targeting the tumor vasculature with VEGF-targeted agents renders the tumor cells hypoxic with subsequent accumulation of HIF1, which in turn leads to up-regulation of proangiogenic factors that stimulate angiogenesis, up-regulation of cMET which is involved in increased tumor invasiveness and EMT, and up-regulation of SDF1 which is implicated in the recruitment of proangiogenic BMDCs. Furthermore, tumor hypoxia also leads to the selection of more malignant tumor cells that have increased invasive potential. Another danger of destroying the tumor vasculature with sunitinib, is the facilitation of hematogenous metastasis by destabilization of the vessel wall, and in the case of sunitinib especially through decreased pericyte coverage of the tumor vessels. Targeting hypoxia with for instance HAPs or decreasing tumor hypoxia by trying to normalize the tumor vasculature with antiangiogenic therapy, could therefore be attractive treatment options. Preventing the accumulation of HIF1 with an mTOR-inhibitor could also be useful in this respect. However, the combination of everolimus with sunitinib was associated with significant toxicities [167]. Some of the findings from preclinical studies have fostered the development of agents that target the resistance mechanisms, and consequently led to the implementation of these agents in clinical trials. However, dovitinib, that targets VEGFR, PDGFR and FGFR, showed no clinical benefit over sorafenib in a third-line setting [30], which might indicate that FGF is not the main driver of sunitinib resistance, or that other factors play an equally important role. This would imply that, in order to overcome sunitinib resistance, combinational therapies that target multiple proangiogenic factors is needed. However, this is almost impossible due to the development of severe toxicities. To date, only cabozantinib, that targets cMET, showed clinical results that hold promise for the treatment of sunitinib-resistant RCC. Due to the lack of durable responses with VEGF-targeted therapy, renewed attention is also paid to the use of immunotherapy in the treatment of metastatic RCC. In this respect, the interaction of programmed death 1 (PD1) with its ligand PD-L1, a negative regulator of T-cell activation, seems to be an interesting target since it is shown that renal tumors that express PD-L1 are more aggressive [168], and that

infiltration of RCC with PD1-positive immune cells is associated with poor outcome [169]. Targeting the PD1/PD-L1 interaction with monoclonal antibodies has proven to be successful and even led to durable antitumor responses in RCC in several phase I/II clinical trials [170–174]. The results of a phase I clinical trial of the anti-PD-1 antibody nivolumab in heavily pre-treated patients with metastatic RCC were very promising [174] and currently a phase III trial of nivolumab is conducted (ClinicalTrials.gov Identifier: NCT01668784).

Another emerging concept is the fact that resistance to sunitinib seems to be transient and reversible [109,126], as was also demonstrated by the effectiveness of sunitinib rechallenge in the clinic [127,128]. This implies that tumors can be VEGF driven again after a period 'off-sunitinib treatment' instead of being driven by other proangiogenic factors/pathways [31], which could also explain why combined treatment targeting both the alternative proangiogenic pathway and the VEGF pathway is usually more effective than targeting the alternative proangiogenic pathway alone. Although this concept seems promising, it has yet to be prospectively determined whether retreatment with sunitinib is of meaningful clinical benefit.

The results of individual trials with targeted therapies underscore the importance of determining the clinical relevance of the identified resistance mechanisms and the need for a personalized approach in the treatment of metastasized RCC. Up until now, choice of treatment for patients with metastasized ccRCC is based on clinical factors such as performance status and several laboratory variables, as used in the Memorial Sloan Kettering Cancer Center (MSKCC) criteria [175] and the modified Heng criteria [176]. However, there are no validated biomarkers yet that can predict response to therapy in patients with RCC. Of special interest are markers that predict response to sunitinib, since this is the most widely used first-line agent in the treatment of RCC. These predictive markers will enable us to select patients that will benefit from treatment with sunitinib, and prevent overtreatment and serious side effects experienced by a large group of patients. Despite great efforts in this field, no baseline predictive biomarkers for sunitinib efficacy have been validated for the use in clinic so far. A lot of biomarker discovery studies are conducted in only a small number of patients, and validation in large patient cohorts and in prospective studies is necessary before these potential predictive markers can find their way to the clinic. Furthermore, focus should also be on the combination of several markers in a panel, to improve predictive capacity. Validation of the resistance pathways in humans can not only help to develop agents that target these pathways, but can possibly also identify new predictive markers.

In conclusion, more insight into the molecular mechanisms underlying sunitinib resistance is needed to improve and optimize the treatment of patients with metastasized RCC. Moreover, the identification of predictive markers of response and resistance to sunitinib can help to determine the suitable treatment algorithm for each individual patient and greatly improve the prognosis of patients with metastasized RCC.

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