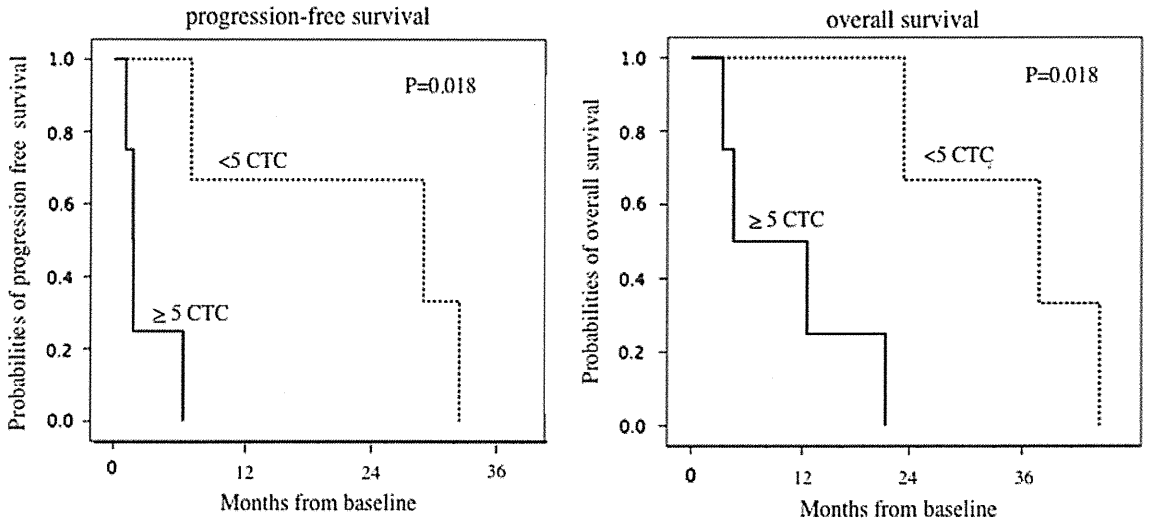

Fig. 5 Kaplan–Meier estimates of probabilities of progression-free survival and overall survival for those with positive CTCs at both pre- and post-treatment and those positive with CTCs changed to negative at evaluation period. Patients who had positive CTCs during treatment had worse PFS than patients whose CTCs level changed to negative during treatment. Patients who had positive CTCs during treatment had worse OS than patients whose CTCs level decreased below the cutoff during treatment

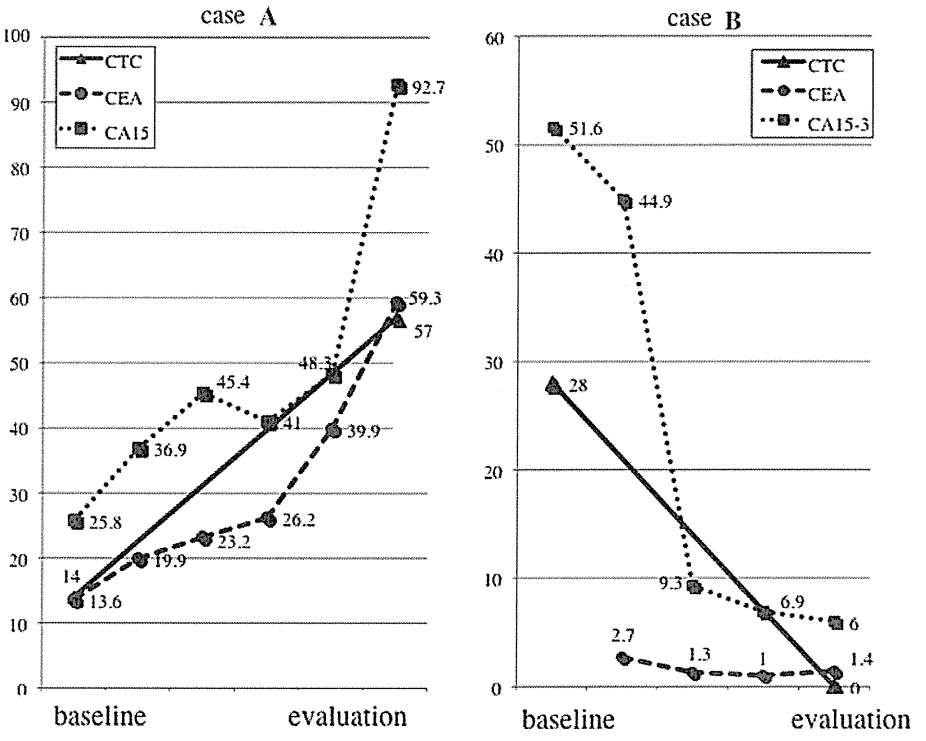

Fig. 6 *Case A* Both CTCs and tumor markers increased in this PD patient. *Case B* CTCs disappeared and tumor markers showed a tendency to decrease in this PR patient

reproducible, enabling the enumeration and characterization of CTCs [5]. It appears to provide reasonable prognostic and predictive utility in metastatic breast cancer. Commonly, clinical features including the time to first recurrence, metastatic sites, number of lesions, and tumor burden are used as prognostic factors, whereas hormone receptor status and HER2 status are usually used as predictive factors. In addition to these tools, Cristofanilli et al. described in their use of a cutoff level of CTC defined as 5 per 7.5 mL of blood [3]. The presence of more than 5 CTCs before initiation of treatment was associated with a short PFS and OS, and predicted a poorer outcome than for patients who have less than 5 CTCs detected. Similarly, the CTC level at first follow-up also predicted the treatment efficacy, PFS, and OS [3, 4]. In short, more than 5 CTCs after the initiation of treatments predicted no therapeutic

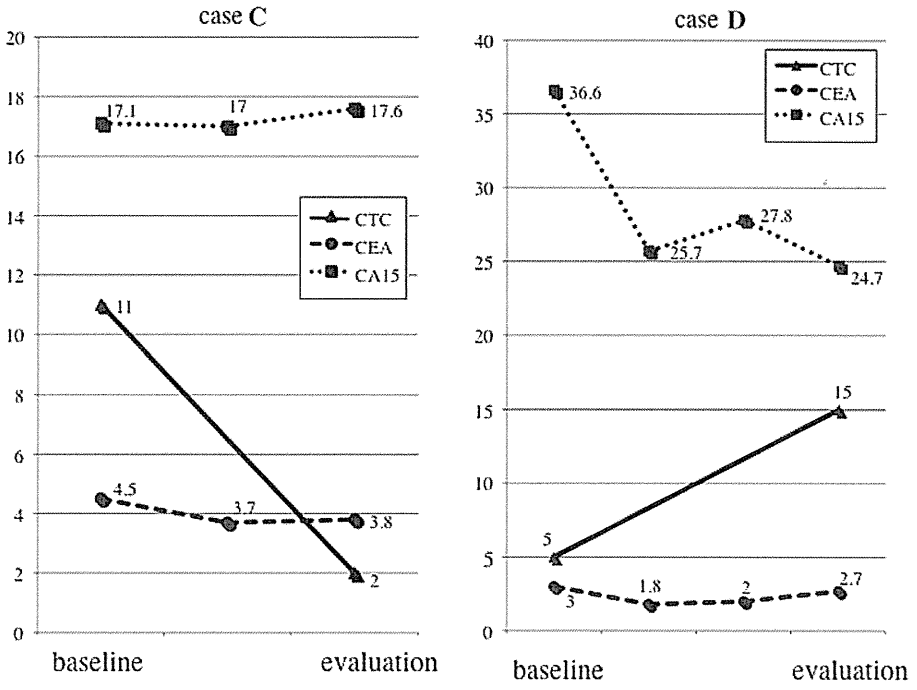

Fig. 7 *Case C* A patient whose metastatic sites showed a tendency to shrink, tumor markers did not change. On the other hand, her CTC level decreased; therefore CTC alteration was suitable to evaluate efficacy. *Case D* In spite of decreased tumor markers, the CTC level increased. Four months later, her metastatic sites progressed

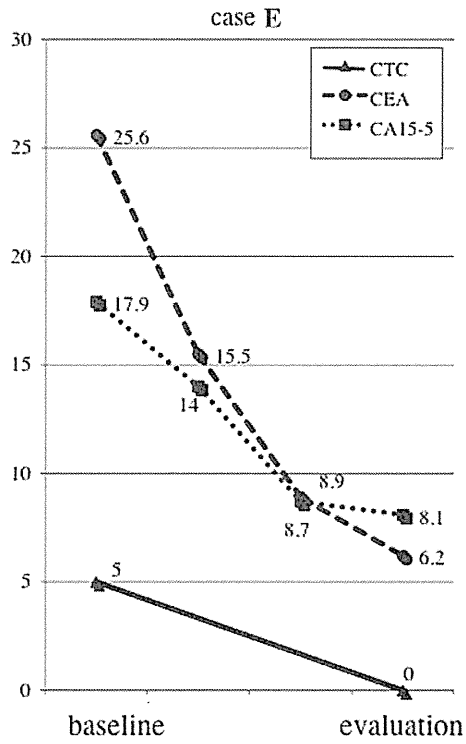

Fig. 8 *Case E* Both CTCs and tumor markers decreased in this patient with long SD who had skeletal metastasis only

benefit from the treatments. Furthermore, Hayes et al. [7]. reported that continuous detection of more than 5 CTCs at any time during therapy accurately indicated subsequent treatment failure and mortality. Although the present small study could not demonstrate that patients who initially had 5 or more 5 CTCs were associated with a worse prognosis, these data suggest that the CTC status after the treatment may be a prognostic marker. In addition, CTCs were useful to estimate treatment efficacy as a predictive marker. These results suggest that following up the number of CTCs may contribute to predict the efficacy of the treatment like tumor markers.

A subsequent report showed that CTC enumeration might provide an earlier, more reproducible indication of disease status than imaging examination. In particular, the prognosis of radiologically responding patients (stable disease and partial response) was divided into good and unfavorable prognosis groups according to the number of CTCs (5 or more, less than 5). Similarly, radiologically non-responding patients (progressive disease) were also divided into these two groups depending on the number of CTCs [8]. Furthermore, in this report, obvious radiological disease progression patients with more than 5 CTCs demonstrated a significantly worse prognosis than the patients whose CTCs level was fewer than 5.

Commonly, for monitoring patients with metastatic disease during therapy, some tumor markers can be used in conjunction with diagnostic imaging and physical examination. CA15-3 and CEA are often used in monitoring a patient's condition. However, during the first few weeks of new treatment, these serous tumor marker levels sometimes rise temporarily, and we experience difficulties in evaluating the therapeutic response [6]. As mentioned above, although alterations of tumor markers sometimes did not reflect disease condition, CTC alteration corresponded to each disease condition [9]. In our study and previous

reports, CTCs may be usable as a kind of tumor marker or surrogate marker to avoid unnecessary toxic therapy and help us develop better therapeutic strategies for our patients. After a few cycles or treatment, patients with elevated CTCs would be assigned to either the same treatment until clinical disease progression or switched to the next chemotherapeutic agent(s).

On the other hand, it is often difficult to judge treatment efficacy in the absence of measurable disease, especially with patients who have only skeletal metastasis. Usually, skeletal metastatic sites of breast cancer contain both osteolytic and osteoblastic changes. If treatment has been effective, the cancer nests sometimes reveal osteoplastic change. It is difficult to judge by radiography whether osteoblastic change is due to disease progression or to treatment efficacy. Therefore, in addition to other tumor markers, CTCs might be a useful predictor of treatment efficacy, especially with the patients whose lesions are difficult to assess.

According to the American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer, the measurement of CTCs should not be used to diagnose breast cancer or to influence any treatment decision in patients with breast cancer [10]. Similarly, the use of the recently FDA-cleared test for CTC (CellSearch Assay) in patients with metastatic breast cancer cannot be recommended until further validation confirms the clinical value of this test. Therefore, a prospective trial should be held with metastatic breast cancer patients.

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Review Article

Biomarkers to predict response to sunitinib therapy and prognosis in metastatic renal cell cancer

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Sunitinib is an orally-administered, multitargeted tyrosine kinase inhibitor. The main targets are vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2, VEGFR-3, platelet-derived growth factor receptor (PDGFR)-α, and PDGFR-β. Among therapeutic targeting agents, it is the best available in the USA for patients with metastatic renal cell cancer (RCC). Well-constructed clinical trials have led to the worldwide approval of various agents for RCC. However, in clinical practice, it remains difficult to determine the best treatment strategy with these agents. Therefore, the identification of biomarkers to predict response and side-effects and to select optimal dosages is urgently needed. Potential mechanisms of action and resistance need to be understood in order to make accurate predictions. This article briefly reviews candidate biomarkers of sunitinib therapy in terms of clinical variables, genetic factors, and circulating proteins and endothelial cells. Although further validation and implementation is necessary, if validated, biomarkers will help measure the therapeutic response in individual patients and establish treatment strategies for metastatic RCC. (*Cancer Sci* 2011; 102: 1949–1957)

Renal cell cancer (RCC) is the most lethal urologic malignancy, and its incidence is currently rising.[1] Radical nephrectomy remains the standard and only curative therapy for patients with localized RCC. However, at initial diagnosis, one-third of RCC patients exhibit visceral metastasis, and up to half of remaining patients eventually develop distant metastases.[2,3] For a long time, the only effective therapeutic and preventive agents for distant metastases and local recurrence have been interferon (IFN) and interleukin (IL)-2, although these agents only achieve a response rate of 15%.[2,3] Major recent breakthroughs have broadened our knowledge of the genetics and transduction pathways involved in various malignancies, including RCC.[4] This greater understanding of the molecular biology of RCC has led to the identification of the vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and mammalian target of rapamycin (mTOR) signaling pathways as rational targets for anticancer therapy for metastatic RCC.[4] Currently, two major subgroups of molecular-targeted agents are available in clinical practice: angiogenesis inhibitors, which include sorafenib (Nexavar; Bayer, West Haven, CT, USA), sunitinib (Sutent; Pfizer, New York, NY, USA), bevacizumab (Avastin; Genentech/Roche, Basle, Switzerland), pazopanib (Votrient; GlaxoSmithKline, Brentford, UK), and axitinib (AG-013736; Pfizer, Philadelphia, PA, USA);[5–9] and two specific inhibitors of the mTOR kinase, temsirolimus (Torisel; Pfizer) and everolimus (Afinitor; Novartis, Basel, Switzerland).[10,11] The RCC growth signals and the rationale behind these molecular-targeted agents are shown in Figure 1.

Among these targeted agents, sunitinib is an orally-administered, multitargeted tyrosine kinase inhibitor (TKI). The main targets of sunitinib are VEGF receptor (VEGFR)-1, VEGFR-2, VEGFR-3, PDGF receptor (PDGFR)-α, and PDGFR-β.[12] Sunitinib is the most readily-available therapeutic targeting agent in the USA (Research from the Synovate Healthcare US Tandem Oncology Monitor 2007–2010) for patients with RCC. A randomized, multicenter, phase-III trial enrolled 750 patients with previously-untreated metastatic RCC to receive either repeated 6-week cycles of sunitinib (at a dose of 50 mg, given orally once daily for 4 weeks, followed by 2 weeks without treatment) or IFN-α (at a dose of 9 million units, given subcutaneously three times/week). This trial demonstrated the superiority of sunitinib over IFN in the objective response rate (ORR; 31% *vs* 6%), progression-free survival (PFS; 11 *vs* 5 months), and overall survival (OS; 26.4 *vs* 21.8 months) and its acceptable safety profile.[6,13] These results and those of the clinical trials for other targeting agents indicate an improved prognosis for patients with RCC in the era of targeted therapy.

Currently, various agents, including molecular-targeted agents and immunotherapeutic agents, are treatments of choice in clinical practice. Risk algorithms, molecular diagnostics, pharmacogenomics, and pharmacodynamics are important tools to improve treatment outcomes. One of the treatment goals is personalized medicine, which offers the right treatment for the right patient at the right time.[14] Therefore, the identification of biomarkers to predict response and side-effects, and to select optimal dosages, is urgently needed. This article provides a brief overview of the genetic changes of RCC and introduces sunitinib biomarkers in terms of clinical variables, genetic factors, and circulating proteins and endothelial cells.

Renal Cell Cancer and the Hypoxia-inducible, Factor-mediated Pathway

Renal cell cancer is the most frequently-occurring solid lesion within the kidney.[1] Among RCC, clear-cell RCC (75%), alternatively called "conventional RCC", is the most common, followed by papillary RCC (10%), chromophobe RCC (5%), and renal oncocytoma (5%). In addition, the World Health Organization system includes rare, newly-recognized cancers defined by genetic factors, such as Xp11.2 translocation-associated renal cancer.[15–17]

The most important molecular disorder in RCC involves the von Hippel-Lindau (VHL) tumor suppressor gene, which is responsible for clear-cell RCC. The protein product of the *VHL* gene, which is located on chromosome 3p25, prevents angiogenesis and suppresses tumors.[15] Inactivating the phosphorylated VHL protein activates hypoxia-inducible factor (HIF) and the induction of VEGF in clear-cell RCC. In addition, mesenchymal–epithelial transition factor (MET) and fumarate hydratase (FH) are the genes responsible for types 1 and 2 papillary RCC,

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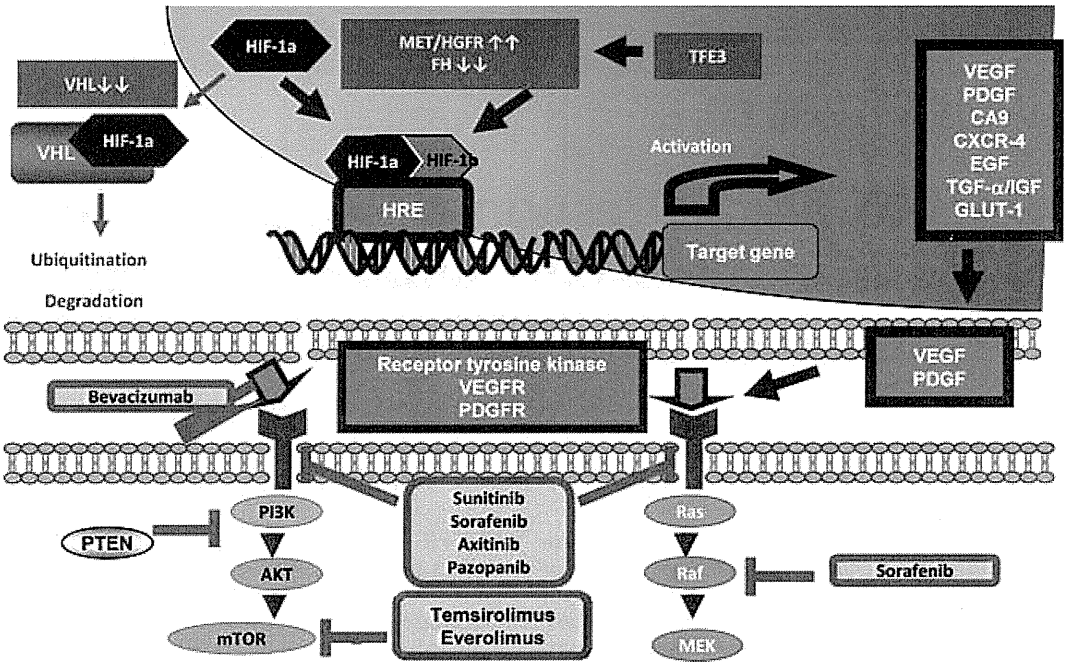

Fig. 1. Genetic alterations and growth signals in renal cell cancer and various molecular-targeted agents. CA9, carbonic anhydrase 9; CXCR-4, C-X-C chemokine receptor type 4; EGF, epidermal growth factor; FH, fumarate hydratase; GLUT1, glucose transporter 1; HGFR, hepatocyte growth factor receptor; HIF, hypoxia inducible factor; HRE, hypoxia response element; IGF-1, insulin-like growth factor 1; MET, mesenchymal–epithelial transition factor; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PI3K, phosphatidyl-inositol; TFE3, transcription factor E3; TGF-α, transforming growth factor alpha; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau tumor suppressor gene product, 3-kinases.

respectively.[18,19] Mesenchymal–epithelial transition factor, which is a proto-oncogene, encodes a tyrosine kinase membrane receptor, and the activation of MET can indirectly promote angiogenesis and tumor growth through the overexpression of VEGF.[20,21] Fumarate hydratase is an enzyme in the mitochondrial tricarboxylic acid (TCA) cycle. The loss of FH leads to pseudohypoxia through the overexpression of HIF, resulting in an increase in downstream targets, including VEGF.[20,22] Therefore, the activation of MET and the loss of FH lead to angiogenesis.[4,15] Moreover, transcription factor E3 (TFE3) and transcription factor EB (TFEB), members of the microphthalmia transcription factor/TFE, are highly expressed in the nucleus as a result of chromosomal translocations, and are responsible for the development of juvenile renal cancer.[17] They also induce the HIF-mediated angiogenesis signaling cascade. The gene products responsible for RCC are indicated in Figure 1.

Biomarkers to Predict the Response to Sunitinib Therapy

Clinical factors. In the cytokine era, prognostic factors that could predict outcome in patients with metastatic RCC treated with IFN-α as initial systemic therapy were defined by Motzer at the Memorial Sloan Kettering Cancer Center (MSKCC).[23] The MSKCC group extracted five variable risk factors for short survival: low Karnofsky performance status (PS), high lactate dehydrogenase (LDH), low hemoglobin (Hb), high corrected serum calcium (Ca), and time from the initial RCC diagnosis to the start of IFN-α therapy of <1 year (Table 1).[23] Each patient was assigned to one of three risk groups: those with no risk factor (favorable risk), those with one or two risk factors (intermediate risk), and those with three or more risk factors (poor risk).[23] The median time to death was 30, 14, and 5 months in the favorable, intermediate, and poor-risk groups, respectively.[23] These five risk criteria are now widely used and are known as the Motzer score or the MSKCC score.

Later, the same group analyzed the prognostic factors of previously-treated RCC patients who had received new agents as salvage therapy in clinical trials at the MSKCC. The median survival time for the 251 patients was 10.2 months, and the pretreatment features associated with a poor prognosis extracted by multivariate analysis were low Karnofsky PS; low Hb level; and high corrected Ca level.[24]

In the molecular-targeted therapy era, several studies have investigated clinical prognostic factors, and the findings are summarized in Table 1. Heng *et al.*[25] first reported the results from a large, multicenter study of 645 patients with anti-VEGF, therapy-naïve metastatic RCC. The study included three groups of patients: 396, 200, and 49 patients, respectively, treated with sunitinib, sorafenib, and bevacizumab, respectively; 560 patients (94%) had clear-cell RCC, while the remaining 35 patients (6%) were diagnosed with non-clear-cell RCC.[25] Four of the five adverse prognostic factors according to the MSKCC score (low Hb, high corrected Ca level, low Karnofsky PS, and time from diagnosis to treatment of <1 year) emerged as independent predictors of poor OS.[25] In addition, neutrophils greater than the upper limit of normal (ULN) range, and platelets greater than the ULN, emerged as independent adverse prognostic factors.[25]

Choueiri *et al.*[26] retrospectively identified the clinical factors associated with outcome in patients with clear-cell RCC receiving anti-VEGF agents, the majority of whom (84%) received either sorafenib or sunitinib. In total, 120 patients with metastatic clear-cell RCC were studied, and a prognostic model was constructed using PFS as an end-point.[26] In this study, all patients had undergone prior nephrectomy, and 45 patients (37%) received anti-VEGF treatment as first-line therapy, while 75 patients (63%) had previously received non anti-VEGF therapies.[26] The interval between diagnosis and anti-VEGF therapy, high corrected Ca level, poor PS, and increased platelet and neutrophil counts were identified as independent prognostic factors for poor PFS (Table 1).

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Table 1. Clinical variables correlated with better/worse survival in patients with metastatic renal cell cancer

Investigators/ references	*n*	Agent	Setting	End-point	Hb <LLN	Corrected Ca >ULN	Poor PS	From Dx to Tx <1 year	LDH >ULN	Other prognostic factors
Motzer *et al.*[23]	670	Interferon	First line	OS	Significant	Significant	Significant	Significant	Significant	
Motzer *et al.*[24]	251	Various agents	Salvage	OS	Significant	Significant	Significant	NS	NS	
Heng *et al.*[25]	645	TKI	First line	OS	Significant	Significant	Significant	Significant	NS	Neutrophil count >ULN, Platelet count >ULN
Choueiri *et al.*[26]	120	TKI	Both	PFS	NS	Significant	Significant	Significant	NS	Neutrophil count >4500/uL, Platelet count >300 000/uL
Patil *et al.*[27]	375	Sunitinib	First line	OS	Significant	Significant	Significant	Significant	Significant	Bone metastasis
Patil *et al.*[27]	375	Interferon	First line	OS	Significant	Significant	NS	Significant	Significant	Neutrophil counts, bone metastasis, lymph node metastasis
Bamias *et al.*[28]	109	Sunitinib	Both	OS	NS	NS	Significant	Significant	NS	Multiple metastatic sites
Yuasa *et al.*[29]	63	Sunitinib	Both	OS	Significant	NS	NS	NS	Significant	No history of nephrectomy, brain metastasis

Both, both first line and second line therapies; Ca, calcium; Dx, diagnosis; Hb, hemoglobin; LLN, lower limit of normal range; NS, not significant; OS, overall survival; PFS, progression-free survival; PS, performance status; TKI, tyrosine kinase inhibitor; Tx, therapy; ULN, upper limit of normal range.

Patil *et al.* reported the prognostic factors for PFS and OS, with sunitinib or IFN-α as first-line systemic therapy for patients with clear-cell metastatic RCC in a randomized, multicenter, phase-III trial, as described earlier.[6,12,27] For sunitinib, a PFS multivariate analysis identified five independent predictors, including high-serum LDH level, the presence of multiple metastatic sites, no prior nephrectomy, Eastern Cooperative Oncology Group (ECOG) PS, and baseline platelet count. The OS correlated with high LDH level, corrected Ca level, the time from diagnosis to treatment, Hb level, ECOG PS, and the presence of bone metastasis (Table 1). The authors concluded that the MSKCC model was applicable to targeted therapy.[27]

A multi-institutional, retrospective analysis of patients with metastatic RCC treated with sunitinib in six Greek oncology units was reported.[28] In this study, 109 patients were included, of whom 100 (91%) had clear-cell RCC and 17 (15%) had been treated with IFN-α, while 86 (79%) had undergone nephrectomy.[28] The time from diagnosis to the start of sunitinib of <1 year, multiple metastatic sites ($P = 0.003$), and poor PS were independently correlated with OS (Table 1).[28]

Finally, in our retrospective study of 63 native Japanese patients, all five MSKCC risk factors (ECOG PS >1, low Hb levels, high corrected Ca levels, high LDH levels, and the time from diagnosis to initial systemic therapy of <1 year) were associated with poorer OS by univariate analysis.[29] A multivariate analysis using the Cox proportional hazard model demonstrated that low Hb and elevated LDH were independently associated with poorer OS among MSKCC scores. Brain metastasis and no history of nephrectomy were also associated with poorer OS (Table 1).[29]

It is important to consider that the number of patients was different between these clinical studies, and that some studies, including our own, might be influenced by low statistical power due to relatively small sample sizes. Nevertheless, the MSKCC prognostic factors are still valid for predicting survival in metastatic RCC in the era of targeted therapy. These results indicate that the MSKCC scores are associated with the behavior of the disease, rather than with specific forms of therapy. In addition to the factors included in the MSKCC score, the number of neutrophils, the platelet count, and the number and/or location of metastatic lesions might be independent prognostic factors in patients treated with molecular-targeted agents (Table 1).

Genetic factors affecting pharmacokinetics and pharmacodynamics. The clinical efficacy of sunitinib depends on the systemic exposure of the targeted organ to the active compounds. A recent meta-analysis of pharmacokinetic data in 443 patients treated with sunitinib showed that higher plasma levels of sunitinib and of its active metabolite SU12662 were associated with prolonged PFS and OS.[30] Orally-administered sunitinib is absorbed by the intestinal mucosa and metabolized in the liver. The primary metabolite, N-de-ethylated metabolite SU12662, reaches plasma concentrations that are similar to the parent compound sunitinib and has biochemically-equivalent activity to sunitinib, but its half-life is prolonged.[13,30] The efflux transporters and the cytochrome P450 3A (CYP3A) family play a role in the absorption and metabolism of the drug.[13,30] The active metabolite and the parent compound are multitargeted TKI that inhibit PDGFR-α and PDGFR-β; VEGFR-1, VEGFR-2, and VEGFR-3; stem cell factor receptor c-KIT; Fms-like tyrosine kinase 3 receptor (Flt-3); and the glial cell line-derived neutrophic factor receptor.[13] The efflux transporters also regulate the cytoplasmic concentration of these agents.[13] Therefore, the efficacy of sunitinib can be influenced by multiple genes encoding efflux transporters, metabolizing enzymes, and targeted tyrosine kinases. Figure 2 describes the processes and enzymes/proteins involved in sunitinib activity, and the genes relevant to sunitinib response and/or toxicity are

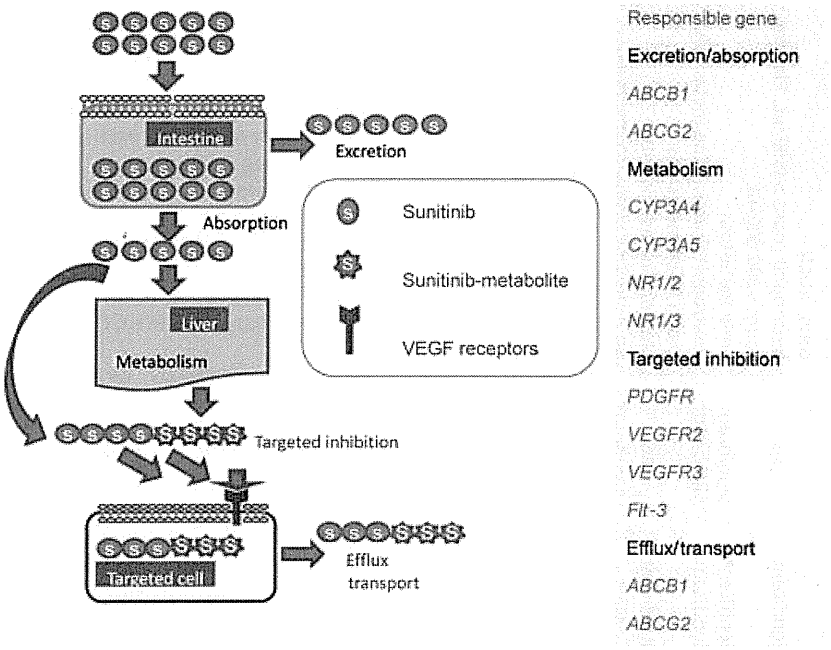

Fig. 2. Genetic prognostic factors in metastatic renal cell cancer patients treated with sunitinib. Orally-administered sunitinib is absorbed from the intestinal mucosa and metabolized in the liver to an active metabolite. Efflux transporters and the cytochrome P450 (CYP)3A family contribute to sunitinib absorption and metabolism. Metabolized active form and the parent compound inhibit platelet-derived growth factor receptor (PDGFR)-α and PDGFR-β, and vascular endothelial growth factor receptor (VEGFR)-1, VEGFR-2 and VEGFR-3. Therefore, pharmacokinetic and pharmacodynamic variables influence the efficacy of sunitinib. ABCB1, ATP-binding cassette transporter protein member B1; ABCG2, ATP-binding cassette transporter protein member G2; CYP3A4, cytochrome P450 3A4; CYP3A5, cytochrome P450 3A5; Flt-3, Fms-like tyrosine kinase receptor-3; NR1I2, nuclear receptor subfamily 1, group I, member 2; NR1I3, nuclear receptor subfamily 1, group I, member 3; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

Table 2. Genes relevant to sunitinib response and/or toxicity

Factors	Genotype	rs no.	Description	References
ABCB1	TTT haplotype*	rs1045642, rs1128503, rs2032582	Increased risk of hand–foot syndrome	32
ABCB1	TCG haplotype*	rs1045642, rs1128503, rs2032582	Improved progression-free survival	33
ABCG2	421C>A	rs2231142	High concentration, increased risk of adverse events, such as hypertension and facial acne	39
ABCG2	TT haplotype†	rs2622604	Any toxicity >grade 2	32
CYP3A5	6986 A>G	rs776746	Improved progression-free survival	33
Flt-3	738 C>T	rs1933437	Increased risk of leukopenia and thrombocytopenia	42
NR1I3	Absence of a CAT haplotype‡	rs2307424, rs2307418, rs4073054	Improved progression-free survival	33
NR1I3	Absence of a CAG haplotype‡	rs2307424, rs2307418, rs4073054	Increased risk of leukopenia	32
PDGFR-α	Homozygous of GCGT§	rs35597368, rs1800810, rs1800813, rs1800812	Decreased overall survival	33
VEGFR-2	1718 A>T	rs1870377	Improved overall survival	33
VEGFR-2	1191 T>C	rs2305948	Any toxicity >grade 2	32

Description of haplotypes: **ABCB1 3435C/T, 1236C/T,* and *2677G/T*; †*ABCG2 15622C/T* and *1143C/T*; ‡*NR1I3 5719C/T, 7738A/C,* and *7837T/G*; §*PDGFR-α 1580T/C –1171C/G -735G/A* and *-573G/T*. ABC, ATP-binding cassette; CYP3A5, cytochrome P450 3A5; Flt-3, Fms-like tyrosine kinase receptor-3; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

summarized in Table 2. To achieve personalized medicine, the complete understanding of sunitinib pharmacogenomics and the molecular profile of each individual patient are necessary.[14]

Absorption, excretion, and efflux of sunitinib. Upon oral administration, sunitinib is absorbed from the gastrointestinal tract in a process regulated by efflux transporters.[30] The ATP-binding cassette (ABC) transporter proteins, particularly multidrug resistance-1//ABC member B1 (ABCB1), formerly known as P-glycoprotein, multidrug resistance-associated protein-1/ABCC1, and breast cancer resistance protein/ABCG2, formerly known as mitoxantrone-resistant protein, mediate absorption and/or excretion through the intestinal wall and regulate the efflux of a wide variety of anticancer drugs from target cells (Fig. 2). These transporter proteins might be involved in the efficacy of sunitinib and in the resistance to sunitinib.

The T genotype in *ABCB1 3435C/T*, which is associated with higher exposure to drugs transported by ABCB1 via decreased mRNA stability, and the consequent decreased expression of *ABCB1* transporter,[31] might be a key factor in ABCB1-mediated sunitinib transport. A multicenter pharmacogenetic

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association study revealed that the *ABCB1 TTT* haplotype in *3435C/T*, *1236C/T*, and *2677G/T* was related to hand–foot syndrome (HFS).[32] In addition, van der Veldt reported that the presence of a *TCG* copy in the same *ABCB1* haplotype was a significant predictor of improved PFS.[33] Interestingly, in a report describing accelerated CYP3A4-mediated drug metabolism in *Abcb1* knockout mice, Schuetz *et al.*[34]suggested that decreased ABCB1 expression activates enzymes involved in drug absorption or disposition.

Several studies reported higher sunitinib affinity for ABCG2 than ABCB1.[35,36] Shukla *et al.*[35] demonstrated that sunitinib stimulates ATP hydrolysis by both transporters in a concentration-dependent manner, and that the affinity for ABCG2 (IC_{50}: 1.33 μM) is higher than that for ABCB1 (IC_{50}: 14.2 μM). Kawahara *et al.* analyzed the kinetics of sunitinib inhibition on ABCG2- and ABCB1-mediated transport. The authors showed that sunitinib acts as a competitive inhibitor of the transporter function of ABCG2 and ABCB1, and that sunitinib has higher affinity for ABCG2 than ABCB1.[36] A previous genetic analysis revealed that, among single nucleotide polymorphism (SNP) in the *ABCG2* gene, *ABCG2 421C/A* is the most common mutant allele in the Japanese population and in other Asian populations (>30%), and that it is associated with low ABCG2 protein expression. The authors also showed that this variable is rare in African Americans (<5%) and Caucasians (<10%).[37,38] This finding might explain the higher incidence of hematological adverse events in Asian patients. Indeed, a report suggested that the homozygous variant of *ABCG2 421C/A* might be involved in the elevated exposure to sunitinib and severe toxicity observed in a patient with RCC.[39] In addition, recent pharmacogenetic analyses revealed that two *ABCG2* gene polymorphisms (*–15622C/T* and *1143C/T*) were strongly associated with sunitinib-induced toxicity in patients.[32] Thus, *ABCG2* genetic variants might lead to increased systemic exposure to sunitinib, resulting in dose-limiting toxicities.

Metabolism. Sunitinib is metabolized in the liver, primarily by the CYP3A4 enzyme. No functional polymorphisms of *CYP3A4* have been identified. The CYP3A5 enzyme metabolizes several TKI, including erlotinib, gefitinib, and imatinib, and might be a key determinant in the interindividual differences observed in CYP3A-mediated drug metabolism.[40] In addition, the expression of CYP3A4 and CYP3A5 is regulated by the ligand-activated nuclear receptors NR1I2 (nuclear receptor subfamily 1, group I, member 2 or pregnane X receptor [PXR]) and NR1I3 (nuclear receptor subfamily 1, group I, member 3 or constitutive androstane receptor [CAR]).[41]

The *CYP3A5* gene, which is 83% homologous to *CYP3A4*, has a functional polymorphism, *6986A/G*, in intron3. The variant G allele creates a cryptic acceptor splice site and transcribes variant mRNA with an excess 131-bp fragment between exons 3 and 4.[31] The CYP3A5 protein translated from the variant mRNA is truncated at a premature stop codon, resulting in a reduced amount of complete CYP3A5 protein. van der Veldt *et al.*[33] reported that the A allele of *6986A/G* in the *CYP3A5* gene, which creates the *CYP3A5* expressor phenotype, is a predictive factor for prolonged PFS. The prolonged PFS observed in patients with the expressor phenotype might be caused by greater metabolism of sunitinib, and thereby increased levels of the active metabolite, which has a longer half-life than the parent compound.[33]

In addition, a relationship has been reported between the absence of the *CAG* haplotype in the *NR1I3* gene, which encodes the CAR, and an increased risk for leukopenia.[32] Nuclear receptor NR1I3 plays an important role in the regulation of multiple drug detoxification genes, such as *CYP3A4*.[41] Another study also extracted the polymorphic variants of *NR1I2* and *NR1I3*, identifying them as predictive factors for PFS and OS in sunitinib-treated metastatic RCC patients.[33]

Pharmacodynamics and targeted inhibition. Besides pharmacokinetic factors, pharmacodynamic factors might affect the efficacy and toxicity of sunitinib. In RCC, the major therapeutic effect of sunitinib is thought to be the inhibition of the VEGFR on tumor-associated endothelium, leading to reduced tumor angiogenesis.[13,30] In addition, the inhibition of the PDGFR might increase the anti-angiogenic effects of sunitinib by targeting pericytes, which protect endothelial cells from apoptosis.[13,30]

The presence of the A allele of the *1718T/A VEGFR-2* polymorphism and the presence of two *GCGT* copies of the *1580T/C*, *–1171C/G*, *–735G/A*, and *–573G/T* polymorphisms in PDGFR-α are associated with decreased OS and prolonged OS, respectively.[33] However, these polymorphisms are not significantly associated with prolonged PFS. These findings suggest that polymorphisms in *VEGFR-2* and *PDGFR-α* might be associated with the nature of the disease, and might therefore be prognostic instead of predictive. The presence of the T allele of the *VEGFR-2 1191C/T* polymorphism is related to the development of any toxicity higher than grade 2, including fatigue, thrombocytopenia, and hypertension.[32] Polymorphisms are also predictive for the development of coronary heart disease due to the lower binding efficiency of VEGF to the polymorphic VEGFR-2.[33] The polymorphic receptor might therefore be involved in sunitinib-induced cardiac toxicity and the development of hypertension.

In addition, the association between the Flt-3 *738T/C* polymorphism and a reduced risk of leukopenia was reported.[32] The protective effect of the Flt-3 *738C* allele against sunitinib-induced thrombocytopenia was confirmed by van Erp *et al.*[42] Therefore, the Flt-3 *738C/T* polymorphism plays a role in the variability of sunitinib-induced bone marrow toxicity.

Circulating biomarkers. Considering the pharmacological effect and biological activity of sunitinib, one should consider measuring the plasma levels of the soluble ligands (including the VEGF family and placental growth factor; PlGF), the specific ligands of VEGFR-1, and the soluble form of the receptors, which include soluble VEGFR (sVEGFR)-2 and sVEGFR-3. If the systemic exposure to sunitinib is a key factor in the predictive value, the less competitive ligands, which include VEGF and PDGF, might be associated with greater efficacy of sunitinib. Indeed, the basal levels and the fold changes of these specific ligands and their receptors have been reported as potential biomarkers of sunitinib.

The mechanisms underlying sunitinib-induced alterations in the levels of these growth factors and of their soluble receptors have not yet been elucidated. Sunitinib-induced angiogenesis inhibition might increase VEGF and PlGF as a positive feedback (Fig. 3). However, combining sunitinib with the soluble forms of the receptors might cause precipitation or degradation and decrease their levels. Rini *et al.* reported that the plasma levels of VEGF-A and PlGF in patients treated with sunitinib increased significantly after 28 days by 2.8-fold (range: 0.4- to 13.6-fold) and 3.9-fold (range: 0.8- to 20.4-fold) over baseline, respectively, whereas the mean sVEGFR-3 levels decreased by 37.6%.[43] DePrimo *et al.*[44] also reported that at the end of cycle 1 (day 28), VEGF and PlGF levels increased greater than threefold (relative to baseline) in 24 of 54 (44%) and 22 of 55 (40%) cases, respectively ($P < 0.001$). In contrast, sVEGFR-2 levels decreased by at least 20% in all patients, resulting in a 30% decrease in 50 of 55 (91%) patients ($P < 0.001$) during cycle 1, while sVEGFR-3 levels decreased ≥30% in 48 of 55 cases (87%) and ≥20% in all but two cases.[44] These levels tended to return to near baseline after 2 weeks of treatment, indicating that these effects were dependent on drug exposure. In addition to the soluble proteins, bone marrow-derived circulating endothelial progenitors (CEP) and circulating endothelial cells (CEC) increase when angiogenesis is required. Gruenwald

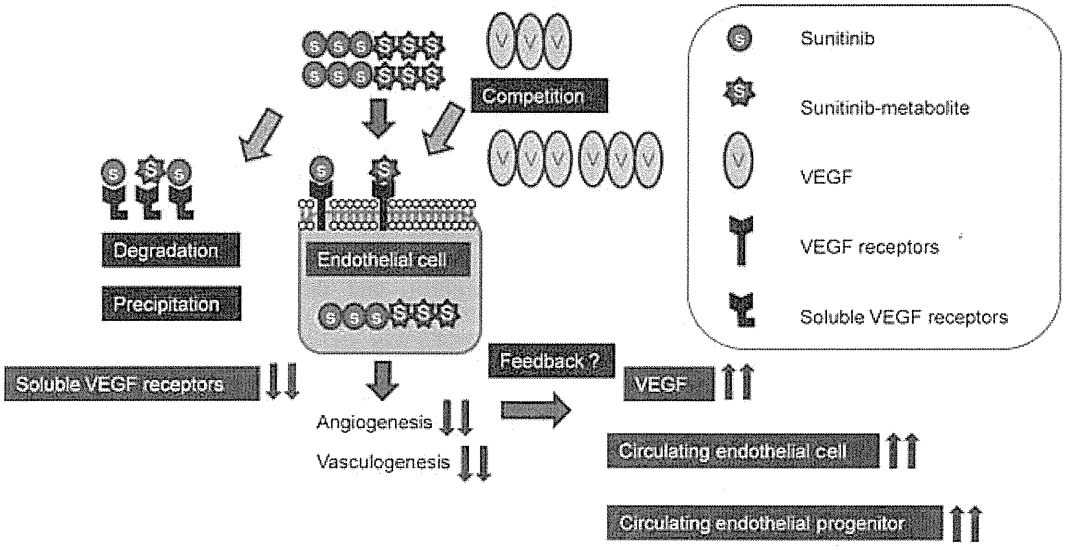

Fig. 3. Circulating soluble proteins and endothelial cells as prognostic factors in metastatic renal cell cancer patients treated with sunitinib. Mechanisms underlying sunitinib-induced alterations in the levels of these growth factors and of their soluble receptors have not yet been elucidated. Sunitinib-induced angiogenesis inhibition might increase vascular endothelial growth factor (VEGF) and circulating endothelial cells and progenitor cells as a positive feedback. In contrast, combining sunitinib with the soluble forms of the receptors might cause precipitation or degradation, and decrease their levels. VEGF, vascular endothelial growth factor.

et al. reported that CEC values in metastatic RCC patients are significantly higher than in healthy individuals (mean value: 49 ± 44 CEC/mL *vs* 8 ± 8 CEC/mL, $P = 0.0001$).[45] In this study, during the first course of sunitinib, the CEC of the patients increased from 49 ± 44 CEC/mL at baseline to 84 ± 59 CEC/mL after 14 days ($P = 0.0331$) and 89 ± 63 CEC/mL after 28 days ($P = 0.0159$) of treatment.[45] The CEC levels declined during the subsequent treatment-off period to baseline levels and below (range: 19–58 CEC/mL).[45] Figure 3 describes circulating soluble proteins and CEC/CEP as candidate biomarkers for sunitinib efficacy.

Rini *et al.* first reported that baseline sVEGFR-3 and VEGF-C levels might be prognostic factors for PFS, as well as predictive factors for objective response. Patients with lower levels than median baseline values (sVEGFR-3, 47 000 pg/mL; VEGF-C, 722.1 pg/mL) had longer PFS than patients with greater levels than the median.[43] The lack of correlation between VEGF-A or PlGF levels and PFS could be due to the fact that the study consisted of bevacizumab-refractory patients. The predictive value of baseline serum VEGF-A was reported by other groups. Porta *et al.*[46] investigated the association between sunitinib-treatment outcome and baseline serum VEGF-A levels in 85 patients treated with sunitinib, among whom 60 had a pure clear-cell RCC, whereas 12 had a predominantly clear-cell mixed histology, and 13 had a pure non-clear-cell histology. In this study, patients with increased baseline VEGF-A had a significantly decreased PFS period (odds ratio: 2.14, 95% confidence interval [CI]: 1.324–3.459).[46] This study reported that patients with increased VEGF-A have a median PFS of 4.7 months (95% CI: 2.8–8.3), whereas patients with non-elevated VEGF-A have a median PFS of 11.2 months (95% CI: 6.5–15).[46]

The fold changes in these angiogenesis-associated proteins could also be a potential biomarker of sunitinib. DePrimo *et al.*[44] reported that significantly larger changes in VEGF, sVEGFR-2, and sVEGFR-3 levels were observed in patients exhibiting objective tumor response, compared to those exhibiting stable disease or disease progression ($P < 0.05$). In addition, total drug trough, sunitinib levels, and SU12662 levels correlated modestly with the change in mean sVEGFR-2 and sVEGFR-3 plasma levels relative to baseline by linear regression analysis.[44]

Endothelial progenitors and CEC also play an integral part in tumor angiogenesis, and might be suitable predictive and prognostic biomarkers for treatment with angiogenesis inhibitors. Gruenwald *et al.*[45] reported that in patients with PFS above the median value, CEC values increased significantly from baseline (mean value: 40 ± 41 CEC/mL to 111 ± 61, $P = 0.0109$) at day 28, whereas in patients with PFS below the median value, the increase remained insignificant (mean value: 53 ± 45 CEC/mL to 69 ± 61, $P = 0.1848$). Farace *et al.*[47] reported that although baseline CEC values were not associated with PFS or OS, baseline circulating progenitor cell values were associated with PFS ($P = 0.01$) and OS ($P = 0.006$) in patients treated with TKI. In addition, changes in circulating progenitor cell values between days 1 and 14 were also associated with PFS ($P = 0.03$).[47]

Both circulating soluble proteins and endothelial cells have recently been measured in various clinical trials as potential biomarkers of the response to anti-angiogenesis agents, and research to further assess their utility is ongoing.

Others. The known adverse effects of sunitinib include HFS, diarrhea, stomatitis, hypertension, fatigue, and hypothyroidism. If adverse effects depend on the degree of systemic exposure to sunitinib, on which clinical efficacy also depends, adverse effects might be potential predictors of sunitinib efficacy in metastatic RCC patients. Correlations have been reported between clinical response and hypertension, and hypothyroidism and HFS.[48–50] Figure 4 shows the correlation between the worst adverse effects and the best clinical response in RCC patients. Rixe *et al.* retrospectively analyzed the putative correlation between sunitinib activity and adverse effects in patients ($n = 32$) with metastatic RCC. The pattern of toxicity was compared between responders and non-responders.[48] The appearance or worsening of hypertension (grade 2 or above) was found to be the single independent predictor of improved clinical response (odds ratio: 2.33, 95% CI: 1.69–3.22, $P = 0.009$) by multivariate analysis using logistic regression. By univariate analysis, a higher response rate was observed in patients with stomatitis ($P = 0.015$), fatigue ($P = 0.019$), hypertension

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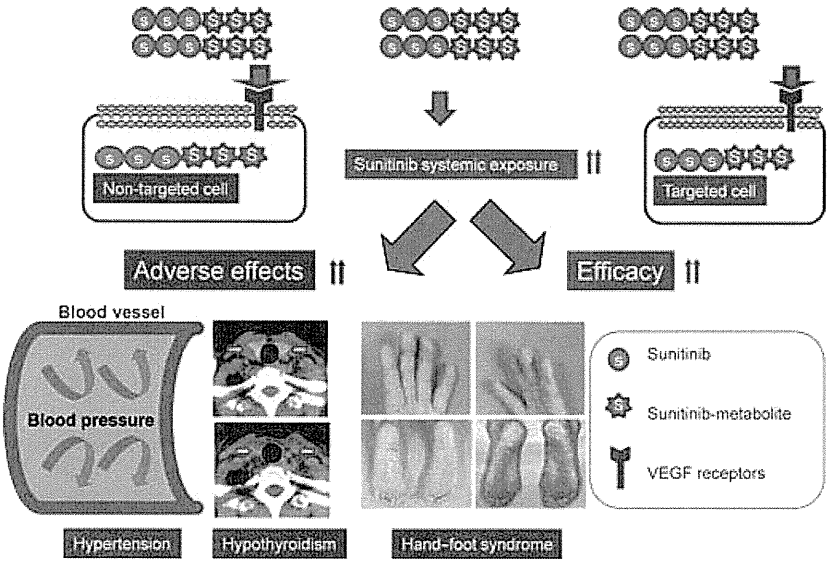

Fig. 4. Adverse effects as candidate biomarkers of favorable efficacy for sunitinib. Correlations between sunitinib efficacy and adverse effects, including hypertension, hypothyroidism, and hand–foot syndrome, were reported.

($P = 0.02$), and testicular erythema ($P = 0.04$), as well as hair depigmentation ($P = 0.042$).[48]

The possibility that the occurrence of hypothyroidism might affect the outcome of patients with metastatic RCC was prospectively investigated in consecutive patients who were to receive treatment with sunitinib or sorafenib.[49] Assessment included serum levels of thyroid-stimulating hormone (TSH), tri-iodothyronine (T3), and thyroxine (T4). Among these patients, subclinical hypothyroidism, defined as an increase in TSH above the ULN (>3.77 μM/mL), with normal T3 and T4 levels, was evident in five patients at baseline and occurred in 30 patients (36.1%) within the first 2 months after treatment initiation.[49] There was a statistically-significant correlation between the occurrence of subclinical hypothyroidism during treatment and the ORR (hypothyroid patients *vs* euthyroid patients: 28.3% *vs* 3.3%, respectively, $P < 0.001$).[49] Moreover, a multivariate analysis identified the development of subclinical hypothyroidism as an independent predictor of survival (hazard ratio: 0.31, $P = 0.014$).[49] Therefore, Schmidinger *et al.*[49] concluded that hypothyroidism might serve as a marker of favorable treatment outcome in metastatic RCC patients.

Very recently, at a meeting of the American Society of Clinical Oncology–Genitourinary, a report of a retrospective investigation of the correlation between HFS and sunitinib antitumor efficacy was presented.[50] In this study of 770 patients included in the analysis (after cycle 1, day 1), 179 (23%) developed HFS (all grades included), and 591 (77%) did not develop HFS. The median PFS (14.3 *vs* 8.3 months, $P < 0.0001$), OS (38.2 *vs* 18.9 months, $P < 0.0001$), and ORR (66.5% *vs* 31.8%, $P < 0.0001$) were significantly higher in the group with HFS than in the group without HFS.[49] Moreover, a multivariate analysis also demonstrated that treatment-emergent HFS remained a significant independent predictor of survival benefit ($P = 0.001$ and $P < 0.001$ for PFS and OS, respectively) after adjusting for other significant independent prognostic markers, including MSKCC factors.[50]

We have also found initial tumor size to be a good predictor of tumor reduction. We retrospectively analyzed 139 metastatic lesions, 16 primary lesions, 86 sunitinib-treated lesions, and 69 sorafenib-treated lesions in 54 patients with metastatic RCC.[51] A linear, moderate-to-strong association between initial tumor size and tumor size reduction rate was demonstrated (correlation coefficient: −0.441, $P < 0.001$). When these tumors were divided into two groups, according to threshold value (23.95 mm), which was determined by receiver-operating characteristic curve analysis, the smaller tumors demonstrated a significantly greater size reduction than the larger tumors by Mann–Whitney *U*-test ($P < 0.001$).[51] We believe that this simple observation constitutes useful information for physicians who treat metastatic RCC.

Biomarkers of Other Targeted Agents

In the present study, we provide a brief overview of biomarkers for other targeted agents used in the treatment of metastatic RCC. Several studies, which were introduced in this review, included patients treated with other angiogenesis inhibitors, such as sorafenib, bevacizumab, pazopanib, and axitinib,[25,26,47,49,51] and suggested that most factors might be possible universal biomarkers for these agents. Other studies investigated the association between efficacy and genetic characteristics, soluble plasma biomarkers, and clinical symptoms.

In a phase-III clinical trial of pazopanib in RCC, predictive genetic markers were explored.[52] Xu *et al.* reported that three polymorphisms in *IL8* and *HIF1A*, and five polymorphisms in *HIF1A*, *NR1I2*, and *VEGFA*, showed a nominally significant association with PFS and response rate (RR), respectively.[52] From these results, they concluded that pharmacodynamic factors might predict treatment responses to pazopanib monotherapy in patients with RCC.[52]

Plasma proteins (VEGF, soluble VEGFR-2, carbonic anhydrase IX, tissue inhibitor of metalloproteinase-1 [TIMP-1], and Ras p21) were analyzed to identify prognostic biomarkers or indicators of response to sorafenib in patients enrolled in the phase-III clinical trial Treatment Approaches in Renal Cancer Global Evaluation Trial.[53] In this study, the reciprocal changes that were also observed in VEGF and sVEGFR-2 levels following sorafenib treatment were similar to those observed with sunitinib.[53] In addition, a multivariate analysis, which included ECOG PS, the MSKCC score, and the potential biomarkers, demonstrated that the elevated plasma level of TIMP-1, which inhibits most of the matrix metalloproteinases, was an independent, poor prognostic factor.[53] Although further investigation is necessary, TIMP-1 should be

an important candidate as a potential biomarker for anti-angiogenesis therapy.[53]

Finally, some studies have suggested that hypertension is a predictive biomarker of efficacy in patients receiving targeted agents. Rini *et al.*[54] reported the final results of a phase-III trial of bevacizumab plus IFN-α versus IFN-α monotherapy in patients with metastatic RCC. In this study, patients who developed hypertension on bevacizumab plus IFN-α had a significantly improved PFS and OS versus patients without hypertension.[54] Similarly, axitinib efficacy was also reported to correlate with diastolic blood pressure. From five phase-II multicenter trials of axitinib in multiple solid tumors, including metastatic RCC, Rini *et al.* reported that the median OS (25.8 *vs* 14.9 months) and median PFS (10.2 *vs* 7.1 months) were greater in patients who developed hypertension.[55]

Conclusion

In this review, we introduced the current candidate biomarkers of sunitinib therapy. Regarding the clinical factors, the MSKCC prognostic factors seem to be valid predictors of survival in metastatic RCC, as summarized in Table 1. Host genetic factors associated with efflux transporters, metabolizing enzymes, and targeted tyrosine kinases modify the efficacy and the toxicity of sunitinib (Fig. 2). Both circulating soluble proteins and cells, which include VEGFR and their ligands, and the CEC/CEP, have been considered as potential candidate biomarkers of the response to anti-angiogenesis agents, and research to further assess their utility is ongoing (Fig. 3). Finally, we introduced severe, adverse effects as candidate biomarkers of favorable efficacy (Fig. 4). Among the targeted agents, sunitinib is an attractive clinical tool, and biomarkers of sunitinib efficacy are desirable. An important caveat is that, to date, almost all of these studies have been retrospective. Although further implementation in prospective studies is necessary, if validated, these biomarkers can be utilized to measure therapeutic response and design treatment strategies for metastatic RCC.

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Original Article

Identification of Anaplastic Lymphoma Kinase Fusions in Renal Cancer

Large-Scale Immunohistochemical Screening by the Intercalated Antibody-Enhanced Polymer Method

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BACKGROUND: Several promising molecular-targeted drugs are used for advanced renal cancers. However, complete remission is rarely achieved, because none of the drugs targets a key molecule that is specific to the cancer, or is associated with "oncogene addiction" (dependence on one or a few oncogenes for cell survival) of renal cancer. Recently, an anaplastic lymphoma kinase (ALK) fusion, vinculin-ALK, has been reported in pediatric renal cell carcinoma (RCC) cases who have a history of sickle cell trait. In this context, ALK inhibitor therapy would constitute a therapeutic advance, as has previously been demonstrated with lung cancer, inflammatory myofibroblastic tumors, and anaplastic large cell lymphomas. **METHODS:** Anti-ALK immunohistochemistry was used to screen 355 tumor tissues, using the intercalated antibody-enhanced polymer (iAEP) method. The cohort consisted of 255 clear cell RCCs, 32 papillary RCCs, 34 chromophobe RCCs, 6 collecting duct carcinomas, 10 unclassified RCCs, 6 sarcomatoid RCCs, and 12 other tumors. **RESULTS:** Two patients (36- and 53-year-old females) were positive for ALK as determined by iAEP immunohistochemistry. Using 5′-rapid amplification of complementary DNA ends, we detected *TPM3-ALK* and *EML4-ALK* in these tumors. The results of this study were confirmed by fluorescence in situ hybridization assays. The 2 ALK-positive RCCs were unclassified (mixed features of papillary, mucinous cribriform, and solid patterns with rhabdoid cells) and papillary subtype. They comprised 2.3% of non-clear cell RCCs (2 of 88) and 3.7% of non-clear cell and nonchromophobe RCCs (2 of 54). **CONCLUSIONS:** The results of this study indicate that ALK fusions also exist in adult RCC cases without uncommon backgrounds. These findings confirm the potential of ALK inhibitor therapy for selected cases of RCC. *Cancer* 2012;000:000-000. © *2012 American Cancer Society.*

KEYWORDS: anaplastic lymphoma kinase, molecular-targeted therapy, renal cell carcinoma, immunohistochemistry, intercalated antibody-enhanced polymer.

INTRODUCTION

Renal cancer is one of the major cancers. The incidence and mortality of cases are estimated at 273,518 and 116,368 in the world; 14,963 and 6957 in Japan; and 56,678 and 13,711 in the United States.[1] The 5-year survival rate of patients with localized disease is relatively good: 65% to 93% and 47% to 77% for stages 1 and 2, respectively.[2] For advanced renal cancers (34%-80% and 2%-20% 5-year survival rates in stages 3 and 4, respectively),[2] several molecular-targeted drugs have been recently approved by the US Food and Drug Administration. These drugs, which include sunitinib, sorafenib, temsirolimus, everolimus, bevacizumab, pazopanib, and axitinib, are promising. However, none of them targets a key molecule that is specific to the cancer, or is associated with "oncogene addiction" of renal cancer, namely, the dependence on one or a few oncogenes for maintenance of the malignant phenotype and cell survival.

Anaplastic lymphoma kinase (ALK) fusion is a potential vulnerability, an "Achilles' heel", of many types of human cancer, including lymphoma,[3,4] sarcoma,[5] and carcinoma.[6,7] Experimentally, lung adenocarcinomas developed in EML4-ALK (fusion of ALK with echinoderm microtubule–associated protein like 4) transgenic mice were successfully treated with an ALK inhibitor.[8] The ALK inhibitor crizotinib has recently been used in patients with lung cancer, inflammatory myofibroblastic tumors (IMTs), or anaplastic large cell lymphomas (ALCLs), which harbor various ALK fusions. The compound showed an 81% response rate in ALK-positive lung cancers defined by at least 2 diagnostic methods,[9,10] and a

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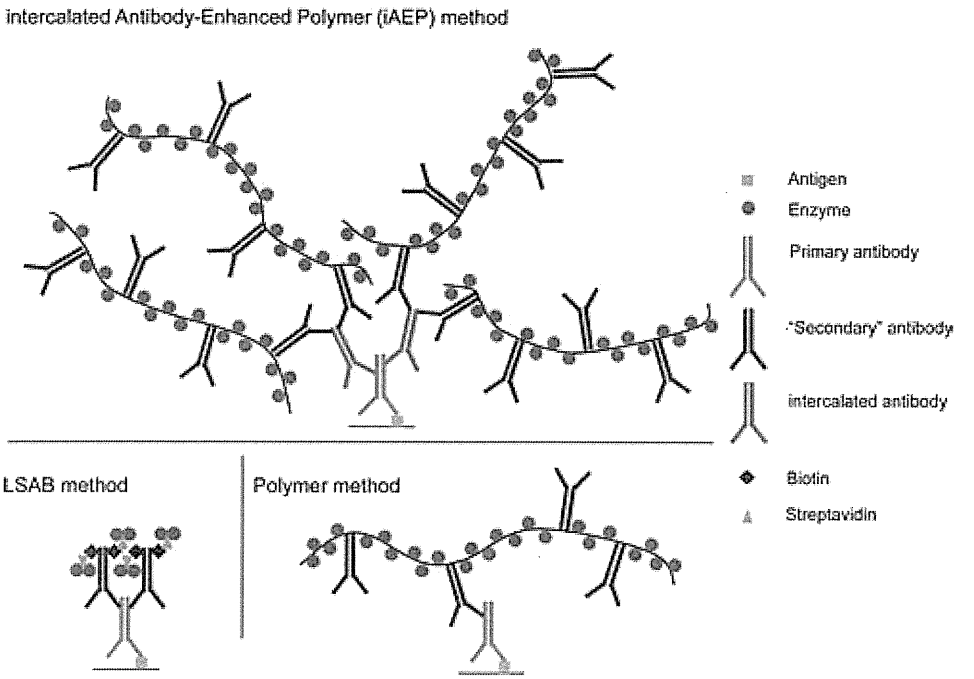

Figure 1. Schematic of intercalated antibody-enhanced polymer (iAEP) method is shown. The labeled streptavidin biotin (LSAB) and polymer methods are common conventional immunohistochemistry methods. In the iAEP method, a step of "intercalated antibody" is added between those of the primary antibody and polymer reagent. Thus, the iAEP method has an additional step compared with the polymer method, but the same number of steps as the LSAB method. There are generally 2 ways to raise the sensitivity of immunohistochemistry. The first is to raise the sensitivity of the antigen-antibody reaction, by increasing the concentration of the primary antibody, using a more sensitive antibody, antigen-retrieval technique, and so forth. The second is to raise the sensitivity of the detection system for the antigen-antibody immune complex. These 2 techniques may appear to generate the same result; however, in principle, they are totally different. The staining results are more likely to differ, especially when the antigen density is very low, such as for EML4-ALK (fusion of echinoderm microtubule-associated protein like 4 with anaplastic lymphoma kinase) or PPFIBP1-ALK (fusion of PTPRF interacting protein binding protein 1 with ALK).[13,24] In such a setting, the latter technique is more advantageous. The staining intensity depends on the density of enzyme in the antigen site. However sensitive a primary antibody is, the antigen-antibody complex cannot exceed the number of antigens. In contrast, it is easy to increase the enzyme density per antigen-antibody complex with use of the latter technique, which includes the iAEP method.

strong response in IMT for several months.[11] Two patients with ALCL who were receiving crizotinib achieved complete remission.[12] These findings indicate that ALK fusion addiction is one of the most promising targets in cancer therapy.

To ensure that such molecular-targeted therapy is effective and less toxic, accurate screening methods to detect ALK fusions are crucial. However, although immunohistochemistry has been a gold standard for the detection of ALK fusions in ALCL and IMT,[13,14] conventional anti-ALK immunohistochemistry is not sensitive enough to detect EML4-ALK, which was first described in lung cancer in 2007.[6,7] To overcome this, we developed a sensitive immunohistochemical tool, the intercalated antibody-enhanced polymer (iAEP) method (Fig. 1).[13] Combined with a conventional anti-ALK mouse monoclonal antibody 5A4, the iAEP method efficiently and consistently detected EML4-ALK in paraffin-embedded sections. In various studies on ALK-positive lung cancer, anti-ALK immunohistochemistry by iAEP or essentially equivalent methods was used to examine surgically resected specimens,[13,15-19] transbronchial lung biopsy specimens,[20] and endobronchial ultrasound-guided transbronchial needle aspiration specimens.[17,21,22] More importantly, some of the patients screened by anti-ALK iAEP immunohistochemical analysis received crizotinib therapy and showed a good response.[16,17,22] Novel ALK fusions, including v6 and v7 of EML4-ALK,[13] kinesin family member 5B (KIF5B)-ALK,[13] sequestosome 1 (SQSTM1)-ALK,[23] and PTPRF interacting protein, binding protein 1 (PPFIBP1)-ALK[24] have been identified using anti-ALK iAEP immunohistochemical analysis. Thus, anti-ALK iAEP immunohistochemistry constitutes a powerful tool for clinical and also research purposes.

The development of anti-ALK antibodies has facilitated the investigation of many types and cases of cancer, including lung cancer.[25-27] Since 1994, ALK-positive tumors have been identified exclusively in lymphoma

(ALCL and ALK-positive large B-cell lymphoma[28]) and sarcoma (IMT,[5] rhabdomyosarcoma,[26] and neuroblastoma[29]). It was not until 2007 that the presence of an ALK fusion was described in lung cancer.[6] This seems to be mainly because EML4-ALK is barely detectable by conventional anti-ALK immunohistochemistry. Considering in reverse, in cases of a tumor that is positive by anti-ALK iAEP immunohistochemistry, but negative by conventional anti-ALK immunohistochemistry, the tumor may have a novel ALK fusion partner, or express wild-type ALK at a modest level. Indeed, in "ALK-negative" IMT cases defined by conventional ALK immunohistochemistry, PPFIBP1-ALK was identified through reassessment for ALK fusions, using anti-ALK iAEP immunohistochemistry.[24] This prompted us to reevaluate other types of solid cancers for ALK fusions. Here, we describe the identification of TPM3-ALK (fusion of tropomyosin 3 and ALK) and EML4-ALK in renal cancer, by anti-ALK iAEP immunohistochemistry.

MATERIALS AND METHODS

Materials

We examined 355 renal tumor tissues from patients who had received surgery in the Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo, between 1994 and 2010. Renal tumors included 255 clear cell renal cell carcinomas (RCCs), 32 papillary RCCs, 34 chromophobe RCCs, 6 collecting duct carcinomas, 10 unclassified RCCs, 6 sarcomatoid RCCs, and 12 other tumors (4 oncocytomas, 3 angiomyolipomas, 1 solitary fibrous tumor, 2 spindle cell sarcomas, 1 desmoplastic sarcoma, and 1 anaplastic carcinoma). Surgically removed tumor specimens were routinely fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathological examination. Immunohistochemical screenings were performed using tissue microarrays. For the 2 cases positive for anti-ALK immunohistochemistry, total RNA was extracted from the corresponding snap-frozen specimen, and purified with the use of an RNeasy Mini kit (Qiagen, Tokyo, Japan). Informed consent was obtained from the patients. The study was approved by the institutional review board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 4 μm, and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 45 minutes at 102°C in antigen retrieval solution (Nichirei Bioscience, Tokyo). For conventional immunostaining, the slides were incubated at room temperature with primary antibodies: ALK (5A4), vimentin, epithelial membrane antigen (EMA), cytokeratin 7, AE1/AE3, CAM5.2, 34βE12, α-methylacyl-coenzymeA racemase (AMACR), clusters of differentiation 10 (CD10), transcription termination factor 1 (TTF1), renal cell carcinoma marker (RCC Ma), paired box 2 (PAX2), and paired box 8 (PAX8) for 30 minutes. The immune complexes were then detected with polymer reagent (Histofine Simple Stain MAX PO; Nichirei Bioscience, Tokyo, Japan). For the sensitive detection of ALK fusion proteins, the ALK Detection Kit (Nichirei Bioscience), which is based on the iAEP method, was used.

Isolation of ALK Fusions

To obtain complementary DNA (cDNA) fragments corresponding to a novel ALK fusion gene, we used a 5′ rapid amplification of cDNA ends (5′-RACE) method with the SMARTer RACE cDNA Amplification Kit (Clontech, Takara Bio Inc., Shiga, Japan). We followed the manufacturer's instructions, with a minor modification: the ALK2458R primer (5′-GTAGTTGGGGTTGTAGTC GGTCATGATGGT-3′) was used as the gene-specific reverse primer. From the deoxythymidine oligomer–primed cDNA obtained from RNA from case 1, a 385–base pair (bp) cDNA fragment containing the fusion point was specifically amplified with the primers TPM3-705F (5′-AGAGACCCGTGCTGAGTTTGCTG-3′) and ALK3078RR (5′-ATCCAGTTCGTCCTGTTCA GAGC-3′). From case 2, a 454-bp cDNA fragment containing the fusion point was specifically amplified with the primers EML4-72F (5′-GTCAGCTCTTGAGT CACGAGTT-3′) and ALK3078RR. Polymerase chain reaction (PCR) analysis of genomic DNA for TPM3-ALK in case 1 was carried out with a pair of primers flanking the putative fusion point: TPM3-705F (5′-AGA GACCCGTGCTGAGTTTGCTG-3′) and Fusion-RT-AS (5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′). For genomic PCR analysis of EML4-ALK in case 2, we used primers EML4-107F (5′-ATGAAATCACTGTGCTAA AGGCGGCT-3′) and Fusion-RT-AS (5′-TCTTGCCA GCAAAGCAGTAGTTGG-3′).

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) analysis of gene fusion was carried out with DNA probes for ALK, TPM3, EML4, and transcription factor E3 (TFE3). Unstained sections (4 μm thick) were subjected to hybridization with an ALK-split probe set (Dako, Tokyo, Japan), TFE3-split probe set (Kreatech, Amsterdam, The Netherlands), or bacterial artificial chromosome (BAC) clone-derived

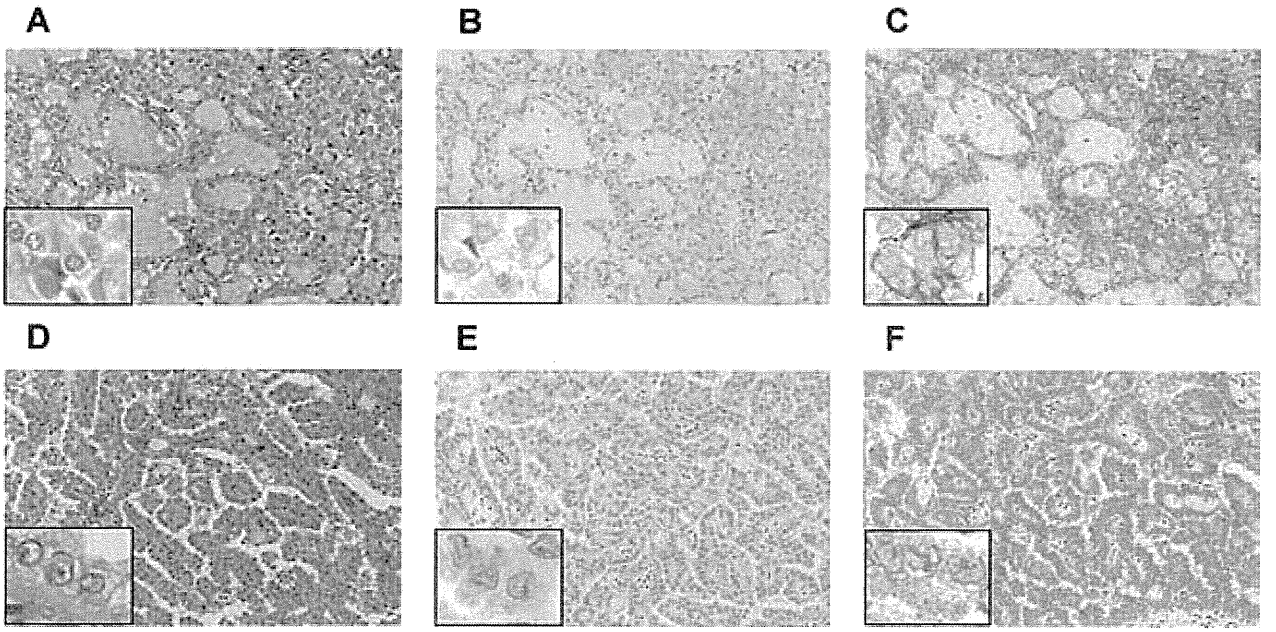

Figure 2. Histopathology of anaplastic lymphoma kinase (ALK)-positive renal cancer. Cuboidal tumor cells showed papillary, tubular, or cribriform growth patterns. The tumor cells had eosinophilic cytoplasm and round to ovoid nuclei. (A) The glandular structures possessed abundant mucin. (D) The tumor comprised a papillary structure of cuboidal or low columnar cells, with eosinophilic cytoplasm and small uniform round to oval nuclei (A,D hematoxylin and eosin stain). The tumor cells were (B) weakly positive and (E) indeterminate for ALK with conventional anti-ALK immunohistochemistry. (C,F) All of the tumor cells were clearly positive for ALK when the iAEP method was used. The staining pattern was diffuse cytoplasmic, with (C) membranous or (F) fine granular accentuation. Figures were taken using the corresponding whole sections (× 10 objective for low power view. × 40 objective for inset). Case 1 (A-C); Case 2 (D-F).

probes for ALK (RP11-984I21, RP11-62B19, RP11-701P18), TPM3 (RP11-809B24), and EML4 (RP11-996L7). Hybridized slides were then stained with 4′,6-diamidino-2-phenylindole and examined using a fluorescence microscope BX51 (Olympus, Tokyo, Japan).

Mutation Analyses for MET

A 1007-bp cDNA fragment containing the MET kinase domain was amplified using the primers MET-3186F (5′-GTCCATTACTGCAAAATACTGTCC-3′) and MET-4193R (5′-CACCTCATCATCAGCGTTATC-3′). The PCR product was sequenced after subcloning.

RESULTS

Identification of ALK Fusions in RCC Samples

Sections of tissue microarray were immunostained for ALK by the iAEP method, resulting in the detection of 2 positive cases (case 1, Fig. 2A-C; case 2, Fig. 2D-F). The positive results were also confirmed using corresponding whole histopathological sections, in which all of the tumor cells stained for ALK as other ALK-positive cancers usually do. We carried out 5′-RACE assays to determine whether these cases expressed ALK fusion or full-length ALK (mutated or unmutated). We isolated a cDNA fragment containing the exon 8 of *TPM3* fused in-frame to the exon 20 of *ALK* (Fig. 3A) in case 1, and the exon 2 of *EML4* fused to the exon 20 of *ALK* in case 2 (Fig. 3B). This EML4-ALK is called variant 5 (E2;A20) in lung cancer.[30] Reverse transcription PCR (RT-PCR) assays designed for the *TPM3-ALK* or E2;A20 successfully amplified cDNAs containing the fusion points (Fig. 3C,D). To confirm the genomic rearrangement, we performed FISH assays (Fig. 4) and genomic PCR (data not shown) for each fusion. All our results were consistent with the presence of t(1;2)(p21;p23)/*TPM3-ALK* in case 1, or inv(2)(p21p23)/E2;A20 in case 2. No other cases were positive for ALK by iAEP immunohistochemistry. All 355 cases were further examined by ALK-split FISH assay. In 12 of the cases, FISH was unsuccessful and not evaluable. In the other cases, the results were identical to those obtained by anti-ALK iAEP immunohistochemistry.

Case Presentation

Case 1

The patient was a 36-year-old woman who had a complaint suggestive of pyelonephritis. Magnetic resonance imaging and computed tomography showed a mass (4.0 cm × 4.0 cm × 3.5 cm) in the left kidney. No metastatic lesions or lymph node enlargements were identified. The patient had no past medical history of malignancy.

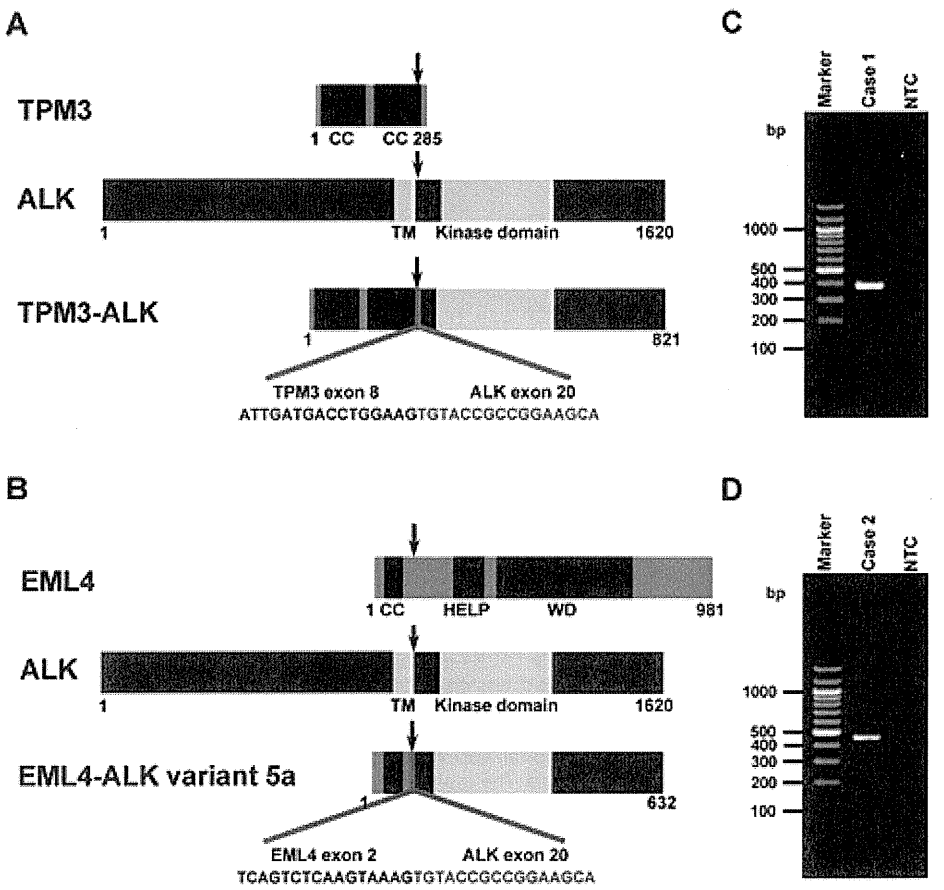

Figure 3. Identification of anaplastic lymphoma kinase (ALK) fusions. Tropomyosin 3 (TPM3) harbors 2 coiled-coil domains. (A) Case 1. A chromosome translocation generates a fusion protein in which the 2 coiled-coil domains of TPM3 and the intracellular region of ALK (containing the tyrosine kinase domain) are conserved. (B) Nucleotide sequencing of the polymerase chain reaction (PCR) products in case 2 revealed that exon 2 of echinoderm microtubule-associated protein like 4 (EML4), comprising a coiled-coil domain, was fused to exon 20 of ALK, generating the variant 5 complementary DNA (cDNA). In TPM3 and EML4 fusions, the region containing the coiled-coil domain is fused to the kinase domain of ALK. Numbers indicate amino acid positions of each protein. Arrow indicates the chromosomal breakpoint. The cDNA fragments of 385 base pairs (bp) and 454 bp were obtained by reverse transcription PCR, corresponding to (C) *TPM3-ALK* and (D) *EML4-ALK* variant 5, respectively. The left lane ("Marker") contains DNA size standards (100-bp ladder). CC indicates coiled-coil domain; HELP, hydrophobic echinoderm microtubule-associated protein; NTC, no-template control; TM, transmembrane domain; WD, WD repeats.

She underwent a translumbar left-radical nephrectomy and is currently alive and well without evidence of disease at 2 years of follow-up.

Case 2

A 53-year-old woman was found incidentally to have microscopic hematuria by medical check-up. Ultrasonography and magnetic resonance imaging showed a change in the left kidney, but the diagnosis was indefinite at that time. One year later, adenocarcinoma cells were detected by urinary cytology, and computed tomography revealed an isodence left renal mass (2.5 cm × 2.5 cm × 2.3 cm). The patient underwent a translumbar left-radical nephrectomy. She is currently alive and well at 7 years after surgery.

The patients had no episodes or family history indicative of sickle cell trait. To the best of our knowledge, there is no reported case of (genetically) Japanese individuals with sickle cell trait/disease.

Histopathological Examinations

The 2 ALK-positive renal cancers were papillary subtype and unclassified (with mixed features of papillary, mucinous cribriform, and solid patterns with rhabdoid cells). They comprised 2.3% of non–clear cell RCCs (2 of 88) and 3.7% of non–clear cell and nonchromophobe RCCs (2 of 54).

Case 1

Histologically, tumor cells were composed of papillary, tubular, or cribriform growth of cuboidal cells with

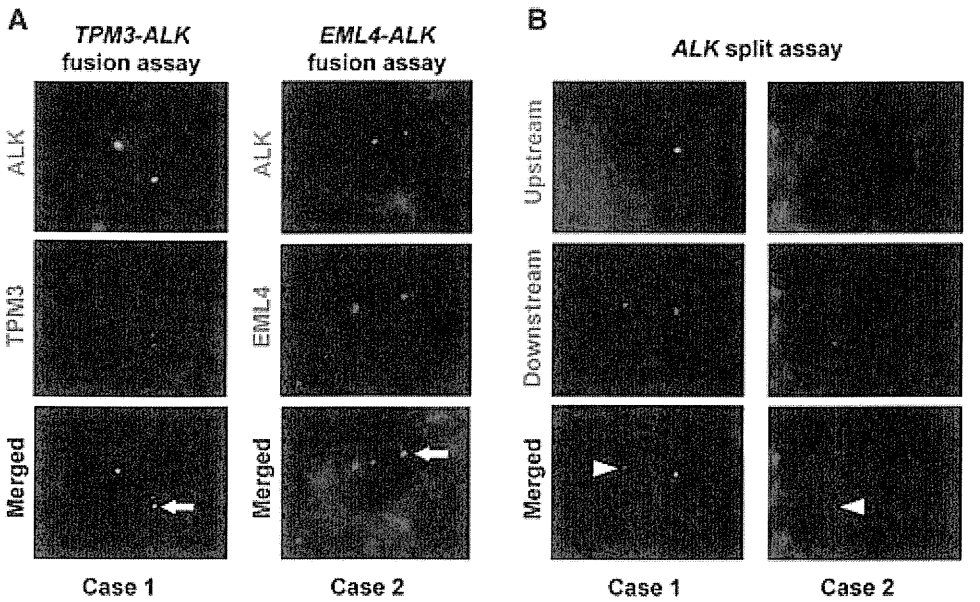

Figure 4. Fluorescence in situ hybridization analyses for *TPM3-ALK* (tropomyosin 3 fusion with anaplastic lymphoma kinase) and *EML4-ALK* (echinoderm microtubule-associated protein like 4 fusion with ALK). (A) In the *TPM3-ALK* and *EML4-ALK* fusion assays, the fusion genes are indicated by arrows. (B) The same clinical specimens as in (A) were subjected to fluorescence in situ hybridization analysis with differentially labeled probes for the upstream (green) or downstream (red) to the ALK breakpoint. In each case, the absence of 1 upstream signal indicated ALK rearrangement. Arrowhead indicates the rearranged ALK. The color of fluorescence for the bacterial artificial chromosome clones and the case numbers are indicated. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole.

eosinophilic cytoplasm. The cribriform morphology consisted of tubular structures with flattened epithelial cells, compressed by mucinous pool and inter- or intracytoplasmic vacuoles. Solid sheets of tumor cells with occasional deeply eosinophilic intracytoplasmic inclusions and eccentric nuclei, resulting in rhabdoid features, were focally identified. Nuclei were round to ovoid, and the nuclear size was basically uniform. Irregular nuclear membranes and nuclear grooves were occasionally observed. Mitotic figures were scant. The background stroma in the tumor area possessed abundant mucin. Frequent deposition of psammoma bodies and infiltration of numerous foamy macrophages were also seen. A large amount of mucinous matrix was highlighted with Alcian blue stain. These histological features resembled the mucinous cribriform pattern frequently observed in ALK-positive lung adenocarcinoma,[18,31] and also a representative case of unclassified RCC by Lopez-Beltran et al,[32] favoring a diagnosis of unclassified RCC. Immunohistochemically, neoplastic cells showed a diffuse and strong positivity for ALK (iAEP), vimentin, EMA, cytokeratin 7, AE1/AE3, cytokeratin CAM5.2, and cytokeratin 34βE12, and focally staining for PAX2, PAX8, AMACR, and CD10. TTF1 and RCC Ma were completely negative. Intracytoplasmic inclusions corresponded to aggregates of intermediate filaments of vimentin. The ALK-staining pattern appeared to be accentuated around the cell membrane of rhabdoid cells. The MIB1 (mindbomb homolog 1) labeling index was less than 1%.

Case 2

Histologically, the tumor consisted of papillary configuration of cuboidal or low columnar cells, with eosinophilic cytoplasm and small uniform round to oval nuclei. A clear cell change was focally seen. Nuclei showed a round to oval shape, and nuclear grooves were frequently observed. The size variation of nuclei was minimal, and the irregularity of the nuclear membrane was evident. Nuclear pseudoinclusions were seldom seen. Small nucleoli were occasionally identified, but mitoses were absent. The fibrovascular cores of papillary architecture contained numerous psammoma bodies and foamy macrophages. In addition, glandular lumens of tumor cells focally contained myxoid materials. These findings morphologically corresponded to papillary RCC, but did not fit to types 1 and 2 by the classification of Delahunt and Eble.[33] In contrast, the features resembled papillary RCC, type 2A, described by Yang et al.[34] Alcian blue stain highlighted a small amount of stromal-type mucin. Upon immunohistochemical analysis, neoplastic cells were diffusely and

strongly positive for ALK (iAEP), vimentin, EMA, cytokeratin 7, AE1/AE3, cytokeratin CAM5.2, cytokeratin 34βE12, and AMACR, and focally positive for PAX2 and PAX8, but negative for TTF1, CD10, and RCC Ma.

Examinations of Other Gene Aberrations

For *MET*, a cDNA fragment with the predicted size was obtained by RT-PCR in case 1. In case 2, no products were identified, indicating that the tumor of the patient did not express *MET*. No mutations were identified in case 1 by sequencing. TFE3 split signals were not observed in either of the 2 cases by FISH.

DISCUSSION

Recently, 2 independent groups have reported vinculin-ALK (VCL-ALK) in renal cancer (Table 1).[35,36] These findings broaden the spectrum of ALK fusion–positive tumors. Interestingly, the 2 patients described in the reports share several uncommon backgrounds for renal cancer: very early onset (6- and 16-year-old boys), a history of sickle cell trait, and uncommon histopathological subtypes (medullary subtype and indeterminate subtype with mixed features of medullary, chromophobe, and transitional cell subtypes). In this study, we screened 355 renal tumors, including 343 RCCs, and identified ALK fusions in 2 RCCs. Significantly, we identified ALK fusions in adult patients (36- and 53-year-old females) without sickle cell trait. This finding will provide a key to ALK inhibitor therapy for more common renal cancers.

RCC associated with *TFE3* gene fusions is already a distinctive entity in the World Health Organization classification,[37,38] and *MET* mutation has been described in 13% of sporadic papillary RCCs.[39] In the present study, we identified neither *MET* nor *TFE3* aberrations in our ALK-positive renal cancer cases. *ALK* rearrangements are recognized as almost mutually exclusive to other mutations such as *EGFR* (epidermal growth factor receptor) and *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene) in lung cancer.[6,40] All of the tumor cells in the 2 ALK-positive renal cancers observed by immunohistochemistry expressed ALK fusion protein, suggesting that all tumor cells harbor one or more *ALK* fusion genes. Therefore, as well as other ALK-positive tumors, *ALK* rearrangement in renal cancer probably occurs at a very early phase of carcinogenesis, and is likely to be a driver mutation and mutually exclusive to other driver mutations. As in the case of ALK-positive ALCL, ALK-positive renal cancer will be a distinct molecular pathological entity.

TPM3-ALK was first identified in ALCL in 1999,[41] and subsequently found in IMT in 2000.[5] Therefore, RCC is the third type of cancer that may harbor TPM3-ALK. The organ distribution of EML4-ALK is somewhat controversial. Since its discovery, EML4-ALK has been reported to be identified in lung, breast, and colon cancers. Many research groups have reported the presence of EML4-ALK in a small subset of lung adenocarcinomas (2%-10%). Interestingly, a group in the United States reported the presence of EML4-ALK in breast (5 of 209) and colorectal (2 of 83) cancers, identified by RT-PCR optimized for variants 1, 2, and 3, without showing histopathological evidence.[42] In contrast, 2 Japanese groups examined these cancers (90 breast and 96 colon cancers by RT-PCR for EML4-ALK variants 1 and 2, and 48 breast and 50 colon cancers by multiplex RT-PCR for all possible fusions), but detected no positive cases.[30,43] One possible reason for this discrepancy may be differences in ethnicity. In the present study, we showed histopathological features of the 2 ALK-positive renal cancers. In addition to morphology, the positivity of PAX2 and PAX8 and the negativity of TTF1 strongly indicated that the ALK-positive cancers of the present cases were primary RCCs, and not metastatic lesions of ALK-positive lung cancer.

The oncogenic activities of TPM3-ALK and EML4-ALK have previously been documented,[30,44] and therefore we did not demonstrate them in the present study. As in the case of other ALK-positive tumors, ALK-positive renal cancer is a promising candidate disease for ALK inhibitor therapy. In the present study, we screened surgically removable cases; the prognoses for the 2 ALK-positive patients were good, without recurrence. To realize the full potential of ALK inhibitors in renal cancers, it is important to identify the detailed clinicopathological features of ALK-positive cases, especially those of advanced or recurrent cases, by large-scale screening. For this purpose, anti-ALK immunohistochemistry can most readily be carried out as a primary screening tool. However, caution is needed; the screening immunohistochemical assay should be appropriately sensitive, because our present findings indicate that renal cancer involves EML4-ALK, which is barely detectable by conventional immunohistochemistry methods.[13,45]

Is morphology a clue to the presence of ALK fusion in renal cancers? Almost all ALK-positive lung cancers are adenocarcinomas, and more frequently show mucinous cribriform patterns and signet-ring cells than do ALK-negative adenocarcinomas.[18,31,46] ALK fusion is probably very rare in clear cell RCC, which is the most common

Table 1. ALK-Positive Renal Cancers: Present Cases and Review of Literature

Characteristic	VCL-ALK (Debelenko et al[36])	VCL-ALK (Marino-Enriquez et al[35])	TPM3-ALK (Case 1)	EML4-ALK (Case 2)
Age, y	16	6	36	53
Sex	Male	Male	Female	Female
Ethnicity	African American	African American	Japanese	Japanese
Past history	Sickle cell trait	Sickle cell trait	Tuberculosis (22 y old)	Pleomorphic adenoma (50 y old)
Karyotype	Abnormal complex karyotype	46,XY,t(2;10)(p23;q22), add(14)(p11)	Not examined	Not examined
Symptom	Right flank pain, gross hematuria	Intermittent periumbilical pain, hematuria	Pyelonephritis	Microscopic hematuria
Stage	Stage III	Stage I	Stage I	Stage I
Follow-up	9 mo, alive. No evidence of disease	21 mo, alive. No evidence of disease	2 y, alive. No evidence of disease	3 y, alive. No evidence of disease
Gross findings	6.5-cm irregularly shaped solid tumor mass with infiltrative borders centered in the right renal medulla	4.5-cm irregularly spherical mass with lobulated, fleshy light tan appearance centered in the medulla	4.0 cm × 4.0 cm × 3.5 cm irregularly shaped solid tumor with expansive borders centered in the cortex	Double cancer. A: 2.5 cm × 2.5 cm ×2.3 cm solid yellow tumor in the cortex of the left intermediate pole. B: 0.6-cm yellow mass in the cortex of the left inferior pole
Microscopic findings	Diffuse sheet-like pattern; round, oval, and polygonal tumor cells; eosinophilic cytoplasm; moderately polymorphic and vesicular nuclei	Solid growth pattern; spindle-shaped cells with large vesicular nuclei; clear coarse chromatin and abundant eosinophilic cytoplasm	Papillary, tubular, or cribriform growth of cuboidal cells with eosinophilic cytoplasm. Nuclei round to ovoid; nuclear size basically uniform	A: Papillary structure of cuboidal or low columnar cells with eosinophilic cytoplasm and small uniform round to oval nuclei. B: Clear cell
Immunohistochemistry	Positive: AE1/AE3, CAM5.2, CK7, EMA, INI1, TFE3. Negative: CD10, S100, HMB45, WT1	Positive: AE1/AE3, CAM5.2, EMA	Positive: ALK, vimentin, EMA, cytokeratin 7, AE1/AE3, CAM5.2, 34βE12, AMACR (focal), CD10 (focal), PAX2 (focal), PAX8 (focal). Negative: TTF1, RCC Ma	A: Positive: ALK, vimentin, EMA, cytokeratin 7, AE1/AE3, CAM5.2, 34βE12, AMACR, PAX2 (focal), PAX8 (focal). Negative: CD10, TTF1, RCC Ma
Diagnosis	Renal cell carcinoma, indeterminate subtype (medullary, chromophobe, transitional cell carcinoma mixed)	Renal medullary carcinoma	Renal cell carcinoma, unclassified	A: Papillary renal cell carcinoma, type 2A. B: Clear cell renal cell carcinoma

ALK indicates anaplastic lymphoma kinase; EML4, echinoderm microtubule–associated protein like 4; TPM3, tropomyosin 3; VCL, vinculin.

subtype of renal cancer; 2 previously reported cases with VCL-ALK were not clear cell RCC,[35,36] and we identified no ALK-positive cases in 255 clear cell RCCs in this study. Interestingly, case 1 showed a mucinous cribriform pattern. This may be a characteristic feature of ALK-positive carcinomas, universally applicable to carcinomas of various organs. Further study with a larger number of cases is warranted.

Molecular-targeted therapy of advanced renal cancers is starting to realize its full potential. However, complete remission is rarely achieved, because no agent targets a key molecule associated with "oncogene addiction" of renal cancer. In this context, ALK fusion constitutes a promising advance in renal cancers, as has previously been demonstrated with various other types of cancer. In the present study, we identified 2 adult cases of ALK-positive renal cancer in patients without uncommon backgrounds. Our findings confirm the potential of ALK inhibitor therapy for RCC. More detailed clinicopathological features of ALK-positive renal cancers, especially at higher clinical stages, are desirable. Hunting the "ALKoma" in various types of carcinomas, as well as in lung and kidney cancer, will provide an answer to these pathological and clinical questions.