Review Article

Recent advances in renal cell carcinoma from a pathological point of view

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The purpose of this article is to review the recent advances in renal cell carcinoma (RCC) from a pathological point of view. Because the genetic features and morphological characteristics have become major criteria for the classification of RCC, special techniques, such as immunohistochemistry, are essential to the differential diagnosis of renal tumors. Metastasis is frequently observed among the RCC patients with curative nephrectomy, and extracellular matrix-degrading enzymes, such as matrix metalloproteinases (MMP) and heparanase, play a key role in invasion and metastasis of RCC. Snail and Slug, transcription factors of epithelial-mesenchymal transition (EMT), accelerate cancer cell invasion through downregulation of E-cadherin and up-regulation of MMP. Therapies targeted at the vascular endothelial growth factor pathway have become the standard treatment of metastatic RCC. Although they lead to tumor shrinkage mainly by inhibiting angiogenesis, they have typically been associated with drug resistance. The mechanism of the resistance remains largely unknown, but complex events including re-activation of angiogenesis, EMT and cancer stem cells, and immune escape are implicated in the refractory response to the therapy. Recent advances of the research on RCC have caused the changes of classification and therapy, and pathologists should take overall view of these as integrated pathology.

Key words: cancer stem cell, epithelial and mesenchymal transition, molecular-targeted therapy, renal cell carcinoma, resistance

INTRODUCTION

Renal cell carcinoma (RCC) represents about 90% of all malignancies of the kidney.1 Although RCC can be completely removed by surgery, it commonly recurs during follow-up. Because RCC is resistant to conventional chemotherapy, patients with metastatic RCC have poor prognoses.2 Therefore, research on its invasion and metastasis has a high priority. Matrix metalloproteinases (MMPs) and heparanase, major enzymes that degrade components of interstitial extracellular matrix (ECM) and basement membrane (BM), are involved in progression of malignant tumors. Epithelial and mesenchymal transition (EMT), a switch of polarized epithelial cells to mesenchymal phenotype, is considered as an important event during malignant tumor progression.3 EMT signal is also essential to development and maintenance of cancer stem cells (CSC).4

Newly developed therapies targeting the vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) pathways are the standard of care in metastatic RCC,5 but the treatment has typically been associated with the acquisition of resistance. Therefore, investigation of the RCC treated with these therapies is important, and resistance to anti-angiogenic therapy is recently reported to be associated with an immunosuppressive tumor microenvironment in metastatic RCC.6 The combination of anti-angiogenic therapy and immunotherapy targeting programmed death 1 (PD-1) or programmed death-ligand 1 (PD-L1) has been proposed as a potential new therapeutic approach for patients with metastatic RCC.7

Most recently, the definition of RCC subtypes was extensively changed, and genetic features, as well as morphological characteristics, have become major criteria for classification.1 The subtype of RCC is a crucial index for the therapeutic options, including molecular-targeted therapy. In this review,
we briefly introduce the diagnostic challenge of renal tumors according to the recent classification, and describe the recent progress in RCC research on the clinicopathological aspects. We also examine experimental studies focusing on invasion and metastasis, EMT and CSC, and molecular-targeted therapy and resistance mainly based on our studies.

**Diagnostic challenge of renal tumors according to the recent classification**

Renal tumors were previously classified according to the morphological features such as cytological appearance and architecture of tumor cells, but the recent classification is defined on the basis of the molecular genetic characteristics in addition to morphological and clinical features. Although histological examination using hematoxylin and eosin-stained sections allows us to make a diagnosis in the majority of cases, there is some overlap between several entities so that special techniques, such as immunohistochemistry, are necessary for the correct diagnosis. For example, clear cell RCC is defined as a malignant neoplasm composed of cells with clear or eosinophilic cytoplasm within a delicate vascular network, and most solid renal tumors with clear cytoplasm are clear cell RCC. However, there are rare cases of RCC associated with Xp11 translocations/transcription factor E3 (TFE3) gene fusions (Xp11 RCC), which show morphological features similar to clear cell RCC (Fig. 1a). Xp11 RCC predominantly affects children and young adults, and lymph node metastasis is

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**Figure 1** Representative histopathological and immunohistological features of renal tumors with (a) clear cytoplasm and (b) eosinophilic cytoplasm. (a) Clear cell renal cell carcinoma (RCC) shows diffuse and strong staining for carbonic anhydrase 9 (CA9) and negative staining for transcription factor E3 (TFE3), whereas RCC associated with Xp11 translocations / TFE3 fusions (Xp11 RCC) is positive for TFE3 and negative for CA9. (b) Morphological features of renal tumor with eosinophilic cytoplasm are similar, but the differential diagnosis can be made by using immunostaining with appropriate antibodies. CK7, cytokeratin 7; HMB45, human melanoma black 45. Bars, 50 μm.
frequently observed. Therefore, clinical information is important for diagnosis of these tumors. In the case of clear cell renal tumors of the child or young adult patient, immunostaining using antibodies to carbonic anhydrase 9 (CA9; a marker of clear cell RCC) or TFE3 (a marker of Xp11 RCC) is recommended for a differential diagnosis (Fig. 1a). CA9 immunostaining is usually negative or patchy/focal in most Xp11 RCC, whereas clear cell RCC shows strong and diffuse staining.1

Previously, the term “granular cell RCC” was used for RCC with eosinophilic cytoplasm. However, renal neoplasm other than clear cell RCC also shows this morphology (Fig. 1b). In renal tumors with eosinophilic or granular cytoplasm, the candidates for differential diagnosis include clear cell RCC, chromophobe RCC (eosinophilic variant), oncocytoma, and epithelioid angiomyolipoma (eAML). Diffuse CA9 staining can confirm the diagnosis of clear cell RCC, and CD117 and cytokeratin 7 staining is helpful for the diagnosis of chromophobe RCC and oncocytoma. Because eAML may be misdiagnosed as high-grade RCC, immunostaining of human melanoma black 45 (HMB45), Melan-A and epithelial markers should be performed in eosinophilic or granular renal tumors without characteristic features of clear cell RCC (Fig. 1b).

There are several therapeutic options for patients with metastatic RCC, and the histological subtype of RCC affects treatment options.1 Therefore, in renal tumors with unusual histology and/or clinical course, immunohistochemistry should be employed to render correct diagnosis.

Pathological prognostic factors of RCC

The prognosis of RCC patients is affected by several factors, such as histological subtype, tumor–node–metastasis (TNM) stage, nuclear grade, lymphovascular invasion, sarcomatoid component, and necrosis.1 Generally, patients with clear cell RCC have poor prognosis compared to those with non-clear cell RCC.6 Tumor stage, as defined in the TNM classification, is the most important determinant of outcome,1 and nuclear grade also represents the major prognostic variable especially in clear cell RCC. Fuhrman nuclear grading of RCC is most widely used.1 In our study on 338 patients with pT1a RCC, the recurrence rate was significantly higher in RCC patients with Fuhrman nuclear grade 3 or 4 compared to those with Fuhrman nuclear grade 1 or 2, while no significant correlation was found between tumor recurrence and other clinicopathological parameters.9 Therefore, nuclear grade may be a factor for predicting tumor recurrence and metastasis after surgery in pT1a RCC.

The Fuhrman grading system defines the grade 1–3 tumors of nuclear features, and the grade 4 tumor is characterized by the presence of nuclear pleomorphism. However, criteria for nucleolar prominence and nuclear pleomorphism are poorly defined in this system, and there is no indication regarding the relative importance of each feature. The recently updated World Health Organization (WHO) classification recommends the use of the WHO/International Society of Urological Pathology (ISUP) grading system instead of other grading systems including the Fuhrman grading system.1 According to the WHO/ISUP grading system, grade 1–3 tumors are based on nucleolar prominence. Grade 4 is defined by the presence of the pronounced nuclear pleomorphism, tumor giant cells, rhabdoid and/or sarcomatoid differentiation. This grading system is applicable to clear cell RCC and papillary RCC, but should not be used for chromophobe RCC because of its innate nuclear atypia.

A sarcomatoid component can occur in all histological subtypes of RCC and indicates an aggressive character of RCCs, showing positive association with poor prognosis in the patients with clear cell, papillary or chromophobe RCC. This is the case even in the patients with grade 4 clear cell RCC.10

Tumor necrosis is commonly observed in RCC. The presence of tumor necrosis is known to be a poor prognostic factor for the patients with clear cell RCC,1 although the patients with papillary RCC show no such association. Therefore, evaluation of the ratio of necrosis at pathological diagnosis is important for clear cell RCC, and tumor necrosis accounting for >10 % of the total tumor volume is considered to be a less favorable outcome.1

Degradation of ECM and BM by MMPs and their role in RCC invasion and metastasis

Invasion and metastasis are characteristic features of malignant tumors and a key impediment to cancer therapy. Cancer metastasis is a multistep process by which cancer cells disseminate from primary tumor to distant organs. Because ECM and BM functions as the tissue barrier, their degradation is a fundamental aspect of cancer cells and an essential event in tumor proliferation, invasion and metastasis.11 MMPs are a family of zinc-dependent endopeptidases and have been regarded as major critical molecules assisting tumor cells during invasion and metastasis, because they can digest all the components of BM and ECM such as collagens, proteoglycans, laminin, fibronectin, and vitronectin.12 In fact, MMP-2, MMP-9, MMP-11, MMP-14, and MMP16 are up-regulated in clear cell RCC.13 Among them, MMP-9 can degrade type IV collagen, a major structural component of BM, and increase the bioavailability of pro-angiogenic factors such as VEGF and transforming growth factor-β.12 The expression level of MMP-9 is related to the pathological stage, histological grade and poor progression-free and overall survivals.13,14

Proteoglycans are complex molecules consisting of coreproteins and various glycosaminoglycans chains, and classified to four major classes mainly based on localization: intracellular, cell surface, pericellular, and extracellular proteoglycans.15 Heparan sulfate proteoglycans (HSPGs) contain heparan sulfate (HS) chains covalently attached to coreproteins, and most
of HSPGs such as syndecans, glypicans, and perlecan belong to cell surface and pericellular proteoglycans. Among them, perlecan functions as a barrier of the BM and protects type IV collagen, a major BM component, from proteolytic attack (Fig. 2a). Heparanase, HS-degrading enzyme, is overexpressed in many malignant tumors with correlations with malignant phenotype. As for RCC, heparanase mRNA and protein are predominantly expressed in clear cell RCC compared to non-clear cell RCC, and its expression level is correlated with pathological tumor stage and distant metastasis, progression-free and overall survivals. In vitro analysis has revealed that inhibiting heparanase mRNA expression by small interference RNA effectively suppresses invasion of RCC cells. Because heparanase accelerates invasion and metastasis of cancer cells by degrading HS chains of HSPGs (Fig. 2a), oligosaccharide-based compounds that inhibit heparanase activity have been developed recently, aimed primarily at halting tumor growth, metastasis, and angiogenesis.

Bone metastasis of RCC and its molecular mechanism and therapy

Recent studies on metastases of the RCC patients showed that 13% of the patients develop metastases even after curative nephrectomy and bone metastasis comprises 30% of whole metastases. Metastatic bone lesions in RCC are typically osteolytic, and the patients suffer from severe bone pain and other complications, which finally lead to patient death. Histologically, accelerated bone remodeling is observed in the bone metastasis of RCC, and the ‘vicious cycle’ hypothesis about how cancer cells induce changes in bone microenvironment to drive bone destruction and tumor growth has been proposed. In brief, cancer cells stimulate osteoblasts by producing growth factors, such as parathyroid hormone-related protein, resulting in increased production of receptor activator of nuclear factor κB ligand (RANKL). RANKL binds to its receptor RANK on the osteoclastic precursor cells, and promotes...
osteoclastogenesis, leading to enhanced bone resorption. Growth factors released by osteoclastic bone resorption accelerate tumor growth. In both primary clear cell RCC and metastatic bone tissues, the expression of RANKL and RANK is upregulated, and the immunohistochemical expression is higher in high-grade clear cell RCC than in low-grade clear cell RCC. Actually, up-regulation of RANKL and RANK is a worse predictor of bone metastasis and a poor overall survival. Our experimental studies also showed that RANKL triggers the migration of RCC cell lines, and the effect is inhibited by administration of OPG, a decoy receptor for RANKL. According to these data, we propose the hypothesis that the RANK/RANKL system contributes not only to the establishment of bone metastasis by activation of osteoclastogenesis but also to distant metastasis by stimulation of carcinoma cell migration (Fig. 2b).

Zoledronic acid, a member of bisphosphonates containing one of the biochemical pyrophosphate analogues that are highly effective inhibitors of osteoclast-mediated bone resorption, has been approved for use in preventing bone pain, hypercalcemia, pathological fractures and spinal cord compression in patients with clear cell RCC. Denosumab, a human monoclonal antibody that inhibits the RANKL/RANK interaction by specific binding to RANKL is under a clinical trial. The clinical phase III trial comparing denosumab with zoledronic acid in bone metastasis of the RCC patients has demonstrated that denosumab is non-inferior to zoledronic acid, and that time to progression and overall survival is similar between the two groups.

Role of EMT in invasion and metastasis of RCC

During cancer progression, some tumor cells from the primary lesion may reactivate an embryonic program known as EMT, which is regarded as a fundamental event during embryogenesis. Cancer cells obtain mesenchymal phenotype that may contribute to invasion and metastasis through EMT, and they have the ability to cross endothelial barriers and enter blood and lymphatic circulations. The presence of a sarcomatoid component is associated with death from papillary and chromophobe RCCs as well as clear cell RCC. Transformation from conventional RCC into sarcomatoid carcinoma represents EMT, and thus EMT is considered to be an essential process for progression of both clear and non-clear cell RCC (Fig. 3a). Adhesion protein E-cadherin plays a central role in the process of epithelial morphogenesis, and its expression is downregulated during progression of malignant epithelial tumors. A hallmark of EMT is the loss of E-cadherin, and several EMT regulators have been identified as E-cadherin repressors.
Among them, Snail is a key regulator of EMT, since it induces complete EMT by repressing the E-cadherin expression and acquiring invasive and tumorigenic properties (Fig. 3b). Snail contributes to EMT in RCC cell lines. Snail expression is substantially over-expressed in high-grade clear cell RCC tissues and sarcomatoid carcinoma, showing a worse prognostic factor for patients with RCC. Our experimental studies also showed that suppression of Snail expression in RCC cell lines results in decreased expression of vimentin, MMP2, and MMP9, and up-regulation of E-cadherin expression together with inhibition of the cell invasion through Matrigel in vitro (Fig. 3b). Accordingly, these clinicopathological and experimental data indicate that Snail is a key regulator of EMT and provides RCC cells malignant phenotype by not only inducing loss of cell–cell adhesion but also promoting invasion of BM.

Exposure to environmental pollutants, including cigarette smoke, is involved in the carcinogenesis of malignant tumors, and smoking is considered to be one of the risk factors for RCC. Environmental pollutants share the same receptor, aryl hydrocarbon receptor (AhR), and AhR is a major mediator of carcinogenesis caused by environmental pollutants. Treatment of RCC cell lines with AhR ligands, indirubin and 2,3,7,8-tetachlorodibenzo-p-dioxin, activate AhR signaling pathway and up-regulate expression of Slug. Slug accelerates the invasion activity of RCC cells through up-regulation of MMPs and down-regulation of E-cadherin (Fig. 3c). These data suggest that the AhR signal pathway plays a pivotal role in the progression of RCC through inducing EMT, and may be one of the molecular targets for treatment of RCC.

Association of EMT and expression of CD44 in RCC

The concept of CSC was initially proposed in leukemia and myeloma cells based on the data that only a small percentage of tumor cells proliferate extensively and form colonies.
Since then, accumulating lines of evidence have supported the idea that cancers are diseases driven by a subpopulation of self-renewing CSC in both hematopoietic and solid tumors.4 CSC are able to differentiate, self-renew, acquire drug resistance, anchor independently and migrate.5 Recently, the association of CSC with EMT in cancer has been established, as these two fields were similar for contributing to tumor recurrence, metastasis and drug resistance.4 Many studies have used CD133- and CD44-positivity to isolate cells with CSC-like characters from solid tumors including the kidney, lung, brain, breast, liver colon, prostate and pancreatic cancers.4

Tumor necrosis factor-α (TNF-α), an important mediator for the inflammatory responses in cancers, is involved in EMT and expression of CD44 (Fig. 4a).14 Immunohistochemically, TNF-α and CD44 are localized mainly to cancer cells of high-grade clear cell RCC, and their expression levels are associated with primary tumor stage and prognosis. In addition, the expression of TNF-α and CD44 in RCC tissues is closely associated. TNF-α enhances invasion of RCC and TNF-α expression together with suppression of E-cadherin expression and up-regulation of MMP9 and CD44 in RCC cells (Fig. 4a). Thus, TNF-α may play a central role in progression of RCC by inducing EMT and promoting CD44 expression in an autocrine manner.

**Molecular-targeted therapy and resistance in RCC**

In most clear cell RCC, von-Hippel Lindau (VHL), a crucial tumor suppression gene, is inactivated. VHL protein is a component of an E3 ubiquitin-ligase complex that degrades hypoxia-inducible factor (HIF).39 In VHL-inactivated RCC, HIF is accumulated because of a defective VHL protein and causes up-regulation of many genes, such as VEGF, platelet-derived growth factor (PDGF), and transforming growth factor-α (TGF-α), all of which play roles in tumor angiogenesis and progression.5 Based on these findings, VEGF receptor tyrosine kinase inhibitors (VEGFR-TKI), including sunitinib and sorafenib, have been introduced for treatment of advanced clear cell RCC, and have shown some therapeutic effects which are superior to the cytokine-mediated approaches.40 However, resistance to the therapy is commonly observed in patients treated with sunitinib, and thus elucidation of the mechanism of resistance is an important issue. Several molecular mechanisms for the resistance are suggested as below.

(i) **Re-activation of angiogenesis**

After treatment with sunitinib, the intra-tumoral microvessel density is decreased (Fig. 4b), but the expression of several...
angiogenesis regulators is increased in the tumor tissues.41 Therefore, activation of angiogenic switch, which leads to either up-regulation of VEGF or alternative angiogenic factors, may occur in the sunitinib resistant phase.

(ii) CSC and EMT

Blocking of VEGF receptor signal by sunitinib decreases the vascularity, and may lead to focal hypoxia in the RCC. Our experimental studies have described that hypoxic treatment of RCC cells up-regulates TNF-α expression,14 and that TNF-α induces EMT and expression of CD44 (Fig. 5a). Residual carcinoma cells in the sunitinib-treated RCC tissues have shown enhanced expression of CD44 (Fig. 4b).14 Non-responder for sunitinib treatment in the patients with metastatic clear cell RCC are known to exhibit higher blood levels of TNF-α.42 Based on these findings, we think that sunitinib treatment evokes the tissue microenvironment which is suitable for EMT and survival of CSC.42

(iii) mTOR

mTOR is an important component of the phosphoinositide 3-kinase/Akt signaling pathway that mediates cell growth and proliferation.43 The mTOR signaling pathway is dysregulated in many cancers including RCC, and activation of this pathway is known to correlate with aggressive behavior and poor prognosis of RCC patients.43,44 mTOR inhibition plays an important role in the targeted treatment of various cancers including RCC, and temsirolimus and everolimus are approved as therapeutic options for the treatment of RCC. Although sunitinib is used for many patients with metastatic clear cell RCC, clinical practice guidelines recommend temsirolimus for the treatment of the RCC patients of any histology (clear and non-clear) with poor prognostic factors.45 Temsirolimus is also standard care of therapy for patients with RCC, which has progressed after VEGFR-TKI therapy.46 Since no consensus has been reached as to which of the mTOR pathway-related molecules predicts responsiveness to mTOR inhibitors,57 there is an increasing demand for predicting biomarkers to select patients that are likely to benefit from the treatment. Recent study has shown that the expression levels of phospho-mTOR may be a potential biomarker for efficacy of everolimus in patients with metastatic RCC.48 However, further investigations are necessary to determine which patients are appropriate for the administration of the mTOR inhibitor. Pathologists can immunohistochemically analyze the expression of the mTOR pathway-related molecules in the cancer tissue specimens. Thus, the immunohistochemistry of the mTOR pathway-related molecules will become a companion diagnostics of RCC in the future.

(iv) Tumor immune escape mechanism

Most patients with advanced RCC received immunotherapy with interferon-α (IFN-α) or interleukin-2 (IL-2) as standard therapy.49 Although the response rates of IFN-α or IL-2 are low (14–16 %), complete response is observed in about 5 % of the patients. These have suggested that immunotherapies may be a promising remedy for the patients with advanced RCC. Thus, new immunotherapies has recently been introduced by using PD-1, an immune check point receptor, and its ligand PD-L1 to treat the RCC patients. PD-1 is expressed on acti-
vated T-cells, natural killer cells and B-cells, and PD-L1 is expressed by many normal tissues, immune cells and solid tumor cells. PD-1 binds to PD-L1 and inhibits the activity of anti-tumor T-cell immunity (Fig. 5b). Because PD-L1 expression is associated with tumor hypoxia, and anti-angiogenic therapy induces higher infiltration of CD4+ FOXP3+ regulatory T-cells targeting VEGF signal may result in an increase in the therapeutic resistance. Accordingly, blocking antibodies targeting PD-1 or PD-L1 are expected to have promising anti-tumor effects for such patients with refractory metastatic RCC.

CONCLUSION
Recent advances in molecular biology of malignant tumors have contributed to re-classification of RCC and development of molecular-targeted therapy. Newly introduced therapy has brought some effect mainly on the prolonged survival of the patients with metastatic RCC. However, resistance to the therapy is an emerging problem and most patients die after all. Identification of the clinically available molecular biomarkers is crucially important to predict which molecular targeted therapy is more effective than the others in each patient with RCC (Fig. 6). Elucidation of the mechanism for the resistance is another critical issue. To address these problems, approaches using "integrated pathology", in which diagnostic pathology and experimental pathology are integrated together, will be necessary. On the other hand, in the daily pathological diagnosis, morphological characteristics observed from hematoxylin and eosin-stained sections of RCCs should be reinforced by the information obtained by immunohistochemistry and/or other special techniques for detection of the expression and function of the molecules critical for the subtype and targeted therapy.

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