

Research Progress on Circulating Tumor DNA in Renal Cell Carcinoma

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Abstract

Renal cell carcinoma (RCC) is one of the most common tumors in urinary system, and its incidence ranks 7th and 8th in male and female in the United States with a continuous upward trend in last 5 years. In China, RCC also shows a significant growth trend. Because the early symptoms are not obvious, many patients are diagnosed at an advanced stage of the disease and often have a poor prognosis. Therefore, early diagnosis and treatment are particularly important for RCC control. With the advent of the era of innovation in imaging modalities, even early detection of RCC in patients is not possible. However, with the steady increase in the value of liquid biopsy and the emergence of Next-generation sequencing technology, the research on tumor genomics continues to advance, and sequencing combined with liquid biopsy is applied in solid tumors. With this merging, circulating tumor DNA (ctDNA) detection is becoming more and more mature, providing a new tool to resolve this problem. Circulating tumor DNA (ctDNA) is a tumor-derived fragment of DNA in blood or body fluids. It can reflect the information of the entire tumor genome and is easy to obtain. ctDNA has important clinical application in tumor diagnosis, prognosis, and monitoring of disease recurrence, therapeutic effect and chemotherapy resistance. Recent studies show that ctDNA also have clinical value in RCCS as circulating tumor DNA detection may serve as a biomarker for early diagnosis, and monitoring disease course. This article reviews the application of ctDNA in the clinical diagnosis and treatment of RCCS.

Key words Circulating tumor DNA, gene mutation, methylation, renal cell carcinoma, clinical research

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Introduction

Renal cell carcinoma (RCC) is a malignant tumor of the urinary system and is the seventh most common tumor worldwide, accounting for 2.4% of adult malignant tumors in the world, and its incidence is increasing year by year [1, 2]. In China, RCC also shows an obvious trend of growth. At present, the diagnosis of RCC mainly relies on imaging and pathological examination. However, these methods have obvious shortcomings such as imaging examination can detect space-occupying lesions of the kidney, it is not enough to determine the histological type of lesions; Histopathological examination is invasive and requires strict medical indications. In addition, imaging and pathology tests are not of great value in assessing the risk of metastasis and disease progression, or in monitoring the therapeutic efficacy of tumors. Unlike other solid tumors, currently no clinically applicable biomarkers have been found in RCCS, which is not conducive to the screening of high-risk groups and early detection of the disease. Due to the lack of clinical symptoms in early RCCS, 30% of patients are diagnosed with an advanced stage of the disease. The prognosis for patients with advanced RCC is generally poor, with an overall 5-year survival rate of only 8% [3-5]. It is reported that about 15% of patients with initial diagnosis of RCC have developed distant metastases, and another 10% to 20% of patients with localized RCC still develop metastatic renal carcinoma (mRCC) after treatment [2, 6]. In addition, more than 30% of RCCS will relapse or metastasize after undergoing radical nephrectomy [3]. The prognosis of mRCC is poor, the 5-year survival rate is about 10%, and the median survival time is only 10 months [4, 5]. Therefore, early diagnosis and detection of micrometastases are very important. In addition, there is still no precise targeted drug for patients with mRCC [7-14]. At the same time, the traditional imaging monitoring methods have poor real-time performance and poor sensitivity. In the last few years, treatment strategies for patients with advanced or metastatic renal cell carcinoma (mRCC) have developed rapidly. For example, immunotherapy [programmed death-1, PD-1] antibody opdivo and anti-cytotoxic T lymphocyte-associated protein 4 antibody ipilimumab [15], protease inhibitor [mammalian target of rapamycin, mTOR] inhibitor everolimus [16] and tyrosine kinase inhibitors (Sunitinib, sorafenib, pazopanib, and asitinib) [17] all benefit significantly in patients with advanced RCC or mRCC. However, these therapies still require certain immunological or molecular indications, and drug tolerance remains a key issue limiting efficacy. In this context, there is an urgent need for reliable and non-invasive biomarkers for RCCS to enable early disease diagnosis, guide targeted drug selection, monitor therapeutic effects, and detect and evaluate drug tolerance so that treatment regimens can be adjusted for higher efficacy.

Cell-free DNA (cfDNA) refers to nucleation that can be detected in almost all body fluids, including blood. The short DNA fragments are involved in various physiological and pathological processes such as immunity, coagulation, aging, carcinogenesis, etc. In cancer patients, a portion of the cfDNA in the plasma originates from the tumor, called circulating tumor DNA (ctDNA), and may have the same mutations and genetic alterations as the primary tumor. However, ctDNA detection technique provides a new opportunity to solve the above problem. ctDNA is a fragment of DNA in the blood or body fluids derived from tumor cells, it is mainly derived from apoptosis, necrosis and extracellular vesicle secretion of tumor cells [18-34]. It is a characteristic tumor biomarker. By analyzing the characteristics of ctDNA, a wide range of information about the tumor can be obtained. The core advantage of ctDNA detection is that it reflects the information of the entire tumor genome [35, 36], and it is real-time and easy to obtain. This technique avoids clinical biopsy puncture damage

and the internal heterogeneity of the tumor, the tumor molecular typing is more accurate. The genomic information obtained from patient's guide the course of comprehensive treatment may provide a basis for adjusting clinical treatment plans. Furthermore, patients can be monitored noninvasively to detect prospective tumor progression. Thus, the application value of ctDNA in the fight against cancer has received great attention in recent years, but the application of ctDNA in the clinical diagnosis and treatment of RCC is still in the early stage of exploration. Further study of its clinical value will benefit patients at all stages of RCC and help to provide personalized treatment for mRCC. This review introduces the clinical research progress of ctDNA in the diagnosis of RCC, prognosis assessment, disease recurrence monitoring, therapeutic effect monitoring and acquired drug resistance.

ctDNA content and fragmentation

The sequence information, the content and fragment size of ctDNA in peripheral blood of patients with RCC have certain clinical application value. Due to the extremely low content of ctDNA, the detection requires non-normal sensitive methods. Currently, the commonly used methods for ctDNA detection include: digital PCR (dPCR), digital PCR-beaming, next generation sequencing, (NGS) and amplification system (amplification refractory mutation system, ARMS) [37]. Studies have found that the content of ctDNA in peripheral blood of mRCC patients correlate with tumor load. ctDNA was detected in peripheral blood of 53% of patients with mRCC, and the tumor volume of these patients was significantly larger than that of patients without ctDNA detection [38]. Wan et al. [39] further confirmed that the level of plasma ctDNA was correlated with the recurrence of RCC, and patients with high plasma ctDNA level before nephrectomy had a significantly higher tumor recurrence rate than patients with low plasma ctDNA level. The level of ctDNA also plays an important role in therapeutic monitoring. Feng et al. [40] quantitatively detected the plasma ctDNA level of 18 mRCC patients treated with sorafenib at different time points, and found that the plasma ctDNA level negatively correlate with the prognosis during treatment. Yamamoto et al. [41] conducted a study and found that the plasma median ctDNA level in RCC patients was significantly higher than that in healthy control group. In addition, the plasma ctDNA level increased with the increase of tumor TNM stage and Fuhrman grade. The level of ctDNA in patients with lymphatic infiltration was also significantly higher than that in patients without infiltration. Plasma ctDNA levels were significantly higher even in patients with early stage (cT1aN0M0) RCC compared to healthy controls. The research team also revealed that the fragment level of ctDNA is also of clinical application value, that is, the length of plasma ctDNA fragment in RCC patients is significantly shorter than that in healthy controls, and the size of ctDNA fragment is negatively correlated with progression-free survival in RCC patients [41]. Moreover, in patients with higher Fuhrman grading and positive lymphatic infiltration, the mean length of ctDNA was shorter [41, 42]. All of these findings suggest that the content and fragmentation level of ctDNA are potentially important for the diagnosis, disease staging and grading, and prognostic judgment of patients with RCC. **Figure 1** suggests that samples for liquid biopsy of RCC using blood samples comprising ctDNA, and isolation, detection, and characterization of ctDNA and the associated clinical value. **Table 1** did a summary on tumor-guided analysis of plasma for ctDNA detection.

Mutation of ctDNA

The mutation map of RCC patients is the basis of its molecular diagnosis. The research team of Pal and others interpreted the

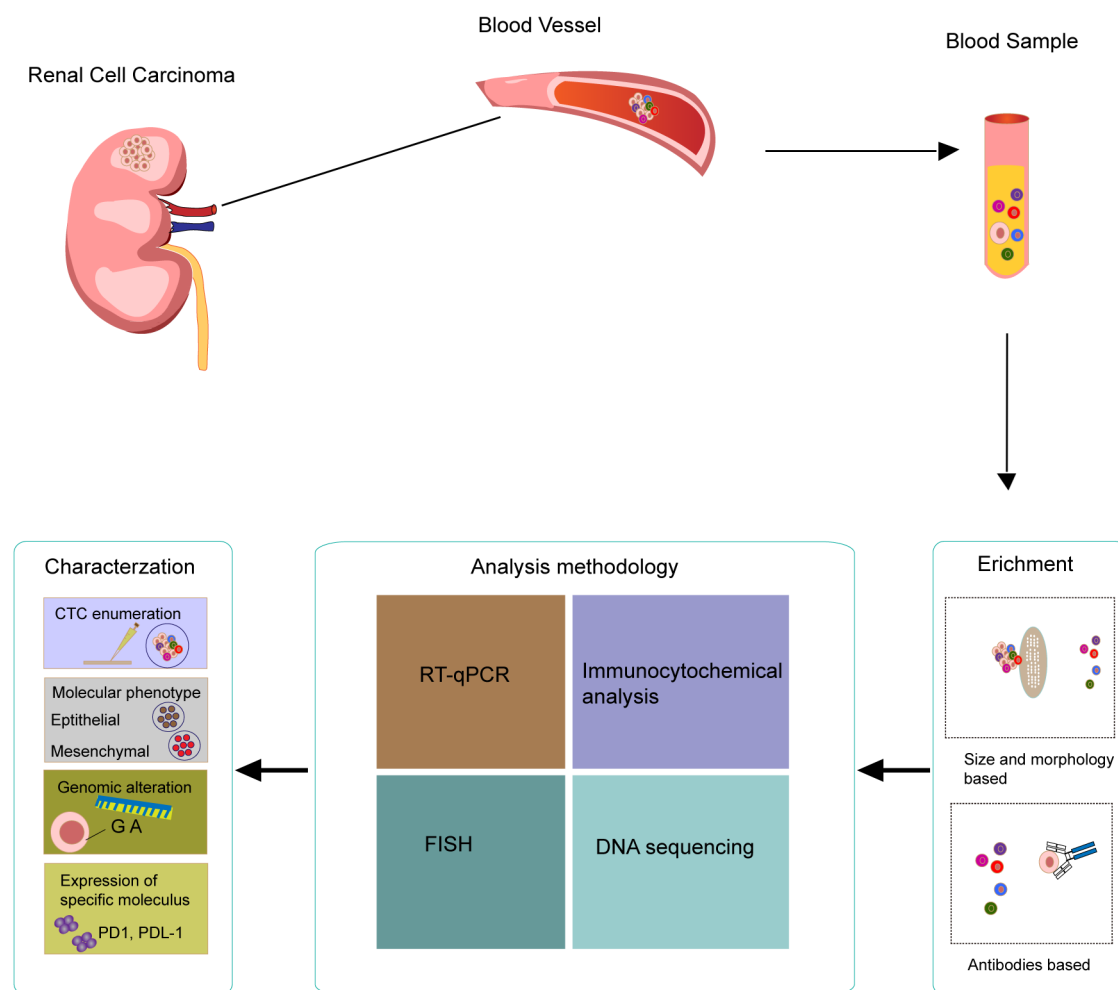


Figure 1. Samples for liquid biopsy of RCC using blood samples comprising circulating tumor DNA (ctDNA), and isolation, detection, and characterization of ctDNA and the associated clinical value.

mutation information of ctDNA of renal cancer for the first time [43]. The research team conducted mutation detection on ctDNA of 220 mRCC patients (Guardant360), 78.6% of 220 patients were found to have at least one mutation. Among them, the five major factors with high mutation frequency were TP53 (35%), VHL (23%), EGFR (17%), NF1 (16%) and ARID1A (12%). Although the study identified genes with a higher frequency of abrupt changes in ctDNA, it was limited by the fact that patients choose mRCC rather than RCC. ctDNA is derived from genomic DNA, and the number of base mutations carried by ctDNA is directly related to the mutation load of a patient's tumor cells. Detection of ctDNA mutation spectrum can help predict whether a patient will benefit from immunological checkpoint inhibitor therapy [44]. Opdivo, a monoclonal antibody against PD-1, has recently been approved for the treatment of malignant tumors associated with mismatch repair defects [45], for which mRCC is an ideal candidate. Immune checkpoint inhibitors play an extremely important role in the treatment of mRCC, and an increasing number of related drugs are expected to be approved for the treatment of mRCC in the near

future. In addition, the combination of multiple immunological checkpoint inhibitors is expected to be the first-line therapy for mRCC [45-47]. The number of base mutations in ctDNA can predict the response of patients with RCC to immunotherapy, which further demonstrates the clinical application value of ctDNA mutation detection. In addition, the mutational characteristics of RCCS are also closely related to the therapeutic response and efficacy. It has been found that ctDNA with methoprenetolerant (MET) resistant mutations can be detected in the plasma of RCC mice, the level of which is correlated with the volume of the primary tumor and changes with treatment of cabotininib [48]. rs9582036 mutation in the vascular endothelial growth factor receptor 1 (VEGFR1) gene was predictive of Sunitinib. The effect of therapy is expected to become an important predictive biomarker [49]. Pal et al. [43, 50] tested the ctDNA mutant profile of 220 patients with mRCC and classified it to assess whether there were significant differences in the reactivity of patients with different ctDNA mutations to targeted therapy. It was found that the increased mutation level of p53 gene in ctDNA was associated

Table 1. Tumor-guided analysis of plasma.

Study	Stage	Methods	ctDNA detection rate (%)	Findings
Maia MC 2017 [38]	mRCC	CLIA-certified, College of American Pathology-accredited comprehensive plasma assay; The enriched digital sequence libraries were analyzed using the HiSeq2500 Sequencing System (Illumina) with an average coverage depth of 15,000 x.	53	The content of ctDNA in peripheral blood of mRCC patients is correlated with tumor load, and the tumor volume of these patients is significantly larger than that of patients without ctDNA detected.
Wan J 2013 [39]	Various	Quantitative real-time PCR was performed on an ABI Prism® 7900HT. Each 10-μl reaction consisted of 1XSYBR® GreenER™ quantitative PCR SuperMix, 1 μl DNA sample, and 0.2 μmol/l forward and reverse primers (ACTB: forward GCTATCCCTGTACGCCTCTG; reverse AGGAAGGAAGGCTGGAGAG; size of PCR product is 384 bp).	Not Applicable	The level of plasma ctDNA is associated with the recurrence of RCC, and the recurrence rate of tumor in patients with high plasma ctDNA level before nephrectomy is significantly higher than that in patients with low plasma ctDNA level.
Feng G 2013[40]	mRCC	Quantitative real-time PCR was performed on an ABI Prism 7900 HT.	Not Applicable	Plasma ctDNA levels were negatively correlated with prognosis during treatment.
Yamamoto Y 2018 [41]	various	Quantitative real-time PCR analysis was performed using a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) to detect levels of plasma ctDNA.	63	The median plasma ctDNA level in RCC patients was significantly higher than that in healthy control group, and the plasma ctDNA level increased with the increase of tumor TNM stage and Fuhrman grade. The ctDNA level in patients with lymphatic vessel infiltration was also significantly higher than that in patients without invasion. Compared with healthy controls, plasma ctDNA levels are significantly higher even in patients with early (cT1aN0M0) RCC.
Yamamoto 2019 [42]	Various	Target capture and further library preparation processes were carried out according to the manufacturer's instructions for the Agilent SureSelectXT Target Enrichment System (Agilent Technologies) with minor modification.	30	Plasma ctDNA fragment length was significantly shorter in RCC patients than in healthy controls, and ctDNA fragment size was inversely associated with progression-free survival in RCC patients, with shorter mean ctDNA length in patients with higher Fuhrman grade and positive lymphatic infiltration.
Khagi Y 2017 [44]	Various	Sequencing was performed by a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory, Guardant Health, Inc. (http://www.guardanthealth.com).	91	ctDNA is derived from genomic DNA, and the number of base mutations carried by ctDNA is directly related to the mutation load of a patient's tumor cells. Detecting the mutation profile of CTDNA can help predict whether a patient can benefit from immunological checkpoint inhibitor therapy.

Table 1. Tumor-guided analysis of plasma (Continued).

Study	Stage	Methods	ctDNA detection rate (%)	Findings
Pal SK 2017 2018 [43, 50]	Various	Enriched digital sequence libraries are subsequently analyzed using the HiSeq2500 Sequencing System (Illumina, San Diego, CA, USA), achieving an average raw coverage depth of 15 000 (minimum: 2000, average Q-score: 20).	78.6	The ctDNA mutant profile of 220 patients with mRCC and classified it to assess whether there were significant differences in the reactivity of patients with different ctDNA mutations to targeted therapy. The increased mutation level of p53 gene in ctDNA was associated with resistance to Sunitinib, pazopanib, bevacizumab and other targeted drugs.
Lu H 2016 [57]	Various	DNA was quantified using Quant-iT™ PicoGreen® dsDNA Reagent (ThermoFisher, Invitrogen, Darmstadt, Germany) in Greiner 384 wellplates (Sigma-Aldrich, Taufkirchen, Germany) on the microplate reader Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany).	Not Applicable	The plasma cfDNA and cfmtDNA concentrations of 145 non-metastatic RCC patients and 84 mRCC patients, and established a diagnostic model of RCC by combining the concentration of nuclear genome and mitochondrial genome (AUC up to 0.84).
Skrypkina I 2016 [26]	Various	The methylation status of the different genes was determined qualitatively by the methylation-specific polymerase chain reaction (MS-PCR).	LRRC3B (74.1%), APC (51.9%), FHIT (55.6%), and RASSF1 (62.9%) genes	Ras association domain family 1A could be detected in the plasma DNA of RCC patients (ras association domain family 1A, RASSF1A), fragile histidine triad (FHIT) and adenomatous polyposis coli (APC) methylation having great value in the diagnosis of RCC.
Jung M 2019 [58]	Various	Quantification of SHOX2 DNA methylation in ccDNA and tissues from the UKB cohort was conducted using a methylation-specific quantitative PCR (qMSP).	Not Applicable	The methylation level of short stature homeobox protein 2 (SHOX2) in RCC tissue and plasma samples and evaluated its clinical significance. It was found that patients with RCC who are still at high risk of death after nephrectomy can be identified by testing SHOX2 methylation levels in ctDNA before treatment, and may benefit from adjuvant therapy or early palliative care through early intervention.
Costa VL 2011 [60]	Various	Chemiluminescence detection, image acquisition and analysis were performed using an Applied Biosystems Chemiluminescence Detection Kit (P/N 4342142) and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (P/N 4338036) following the manufacturer's protocol.	67	PCDH17 methylation in urine samples from RCC patients has the potential to be a biomarker for urinary tumors, including bladder cancer, RCC, and prostate cancer.

Table 1. Tumor-guided analysis of plasma (Continued).

Study	Stage	Methods	ctDNA detection rate (%)	Findings
Xin J 2016 [61]	Various	Following PCR amplification, pyrosequencing was performed using a PyroMark Gold Q96 SQA Reagents kit (Qiagen, Inc.) and a PSQ96 HS DNA analyzing system (Qiagen, Inc.) according to the manufacturer's protocol.	89	The level of TCF21 in the urine of RCC patients positively correlate with its level in tumor tissue, and has certain diagnostic value for RCC.
Santoni M 2018 [62]	Various	The genomic DNA of patients was extracted from blood, using the DNA Blood Mini kit according to the manufacturer's instructions. Genotyping of polymorphisms was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Amplifications and analysis were carried out on the 7300 Real-Time PCR System (Applied Biosystems), using the SDS software v1.4.0 for allelic discrimination (Applied Biosystems).	Not Applicable	The genotype of autophagy genes in peripheral blood to evaluate its correlation with the risk and prognosis of renal clear cell carcinoma.

with resistance to Sunitinib, pazopanib, bevacizumab and other targeted drugs. Drug resistance caused by tumor heterogeneity is the root cause that limits the therapeutic effect of tumors, and RCC is no exception [51]. In tumor tissues, even if only a very small number of cancer cells carry drug-resistant mutations, these cells may survive in the course of treatment and then form drug-resistant tumor tissues [52]. These tumor cells may be missed in the initial pathological biopsy due to the small number or spatial distribution, etc. ctDNA can provide a more representative "full view" of the genetic diversity of the tumor, so as to make up for this deficiency to some extent, which is of great significance for guiding the treatment decision of RCC.

ctDNA methylation

In addition to gene mutation, DNA methylation is also an important index to establish a diagnostic model. ctDNA methylation markers have become a topic of interest in the clinical treatment of cancer, not only because the methylation change is an early and independent event of tumor occurrence, but also because the methylation pattern of ctDNA in peripheral blood, which has a high degree of tumor specificity. The peripheral blood Sept 9 methylation detection kit developed by Epigenomics has been approved by the US Food and Drug Administration for the screening and early diagnosis of colorectal cancer, and has important clinical application value in tumor recurrence monitoring, prognostic judgment, and therapeutic monitoring [53, 54]. ctDNA methylation has emerged as a promising epigenetic marker in the diagnosis and prognosis assessment of a variety of tumors, including RCCS. Numerous studies have shown that DNA methylation is involved in the occurrence and development of RCCS and can reflect patient responsiveness to treatment [55, 56]. DNA methylation usually occurs in the early stages of cancer, making DNA methylation biomarkers ideal targets for early detection. By detecting CpG island methylation in cell-free DNA (cfDNA) in plasma of 35 RCC patients, Hauser and other research teams found that the methylation level of RCC patients was higher than that of normal control group [25]. This index has the potential to be used as a biological indicator for the diagnosis of RCC. Later, Lu et al. [57] analyzed the plasma cfDNA and cfmtDNA concentrations of 145 non-metastatic RCC patients and 84 mRCC patients, and established a diagnostic model of RCC by combining the concentration of nuclear genome and mitochondrial genome (AUC up to 0.84). Skrypkina et al. [26] found that Ras association domain family 1A could be detected in the plasma DNA of RCC patients (ras association domain family 1A, RASSF1A), fragile histidine triad (FHIT) and adenomatous polyposis coli (APC) methylation having great value in the diagnosis of RCC. In addition, DNA methylation is often associated with drug resistance and sensitivity to therapeutic agents. Thus, methylation biomarkers are useful both to stratify patients for effectiveness before drug therapy and to monitor disease progression during treatment. Jung et al. [58] examined the methylation level of short stature homeobox protein 2 (SHOX2) in RCC tissue and plasma samples and evaluated its clinical significance. It was found that patients with RCC who are still at high risk of death after nephrectomy can be identified by testing SHOX2 methylation levels in ctDNA before treatment, and may benefit from adjuvant therapy or early palliative care through early intervention. Another study found that serum protocadherin-17 precursor (PCDH17) methylation is a common event in RCCS and can indicate poor prognosis and may be a biomarker for prognosis in postoperative RCCS [59]. In addition to blood samples, PCDH17 methylation in urine samples from RCC patients has the potential to be a biomarker for urinary tumors, including bladder cancer, RCC, and prostate cancer [60]. Another methylation biomarker identified in urine samples of RCC

patients is transcription factor 21 (TCF21). The level of this gene in the urine of RCC patients positively correlate with its level in tumor tissue, and has certain diagnostic value for RCC [61]. The study of Costa et al. [60] further confirmed this conclusion. In fact, at the histological level, the methylation levels of many genes are closely related to the prognosis of patients with kidney cancer, but whether the methylation levels of these genes in plasma are also related to the prognosis of patients with kidney cancer remains to be further studied. Theoretically, the methylation levels of specific genes in tumor tissue should be consistent with or correlated with the levels in plasma or urine [61], and the clinical value of methylation levels of these genes in peripheral blood or urine in disease prognosis needs further attention.

Others

Polymorphism

Autophagy plays an important regulatory role in the occurrence and progression of RCC. Santoni et al. [62] analyzed the genotype of autophagy genes in peripheral blood to evaluate its correlation with the risk and prognosis of renal clear cell carcinoma. The autophagy-related genes selected included ATG4A, ATG4B, ATG4C, ATG5, ATG16L1, ATG16L2 and IRGM in autophagy-related gene (ATG). Single nucleotide polymorphism (SNP) of these genes was analyzed in 40 mRCC patients treated with pazopanib, and the incidence of ATG16L2-rs10751215 was significantly reduced in mRCC patients compared with the general population. ATG4A-rs7880351, ATG4C-rs6670694, and ATG5-rs490010 were associated with disease-progression-free survival in pazopanib treated RCC patients.

Mitochondria

Lu et al. [57] investigated the application potential of genomic and mitochondrial derived ctDNA fragments in the diagnosis and prognosis of RCC patients. They extracted ctDNA from 40 healthy controls and 229 patients with RCC (145 RCC and 84 mRCC) for analysis. Two mitochondrial fragments (65 bp and 175 bp) were found to be able to effectively distinguish healthy controls from patients with RCC and mRCC. And one genomic ctDNA fragment (306 bp) was found to be able to distinguish between healthy controls and patients with RCC. These results suggest that genomic and mitochondrial ctDNA fragments may contribute to the early diagnosis of mRCC, which may have important applications in adjuvant therapy of RCC.

Problems and Prospects

Peripheral blood ctDNA of patients with RCC has its own characteristics. Compared with other solid tumors (such as pancreatic cancer, ovarian cancer, colorectal cancer, bladder cancer, etc.), the ctDNA level of RCC patients is relatively low [63, 64]. The level of ctDNA in patients with RCC correlate with disease progression, and patients with high tumor load tend to have higher levels of ctDNA [63]. However, for patients with early RCC, more sensitive detection methods are needed. As an important branch of liquid biopsy, ctDNA testing has the potential to transform the landscape of cancer treatment. However, the widespread use of this new method still faces a number of challenges, including improvement in the sensitivity and specificity of ctDNA testing in peripheral blood of patients with early RCC; to develop uniform and standardized procedures to make test results comparable between different laboratories; and to reduce the cost of testing so that the test is suitable for early cancer screening. As the gold standard of tumor diagnosis, histopathologic examination

still plays an irreplaceable role. With the deepening of the research, ctDNA test in RCC diagnosis, prognosis assessment, disease recurrence monitoring, treatment effect monitoring and acquired drug resistance and other aspects has received increasing attention. As a non-invasive detection method, ctDNA detection can be easily tolerated by patients, and can meet the clinical requirements for real-time and continuous sampling. Therefore, ctDNA test may become an indispensable supplement to tissue biopsy, and its clinical application value is expected.

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Ethical policy

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Approval from institutional ethical committee was taken.

Availability of data and materials

All data generated or analysed during this study are included in this publication.

Author contributions

XZ wrote the manuscript draft and prepared the figures and tables. MD and KZ edited and approved the final manuscript.

Competing interests

All authors declare no competing interests.

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