Perioperative circulating tumor cells (CTCs) and CTC-white blood cells detected by a size-based platform predict worse prognosis in renal cell cancer patients

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Research

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Abstract

Purpose To explore the clinical significance of perioperative CTCs counts, EMT-CTCs and CTC- white blood cells (WBCs) in renal cancer patients.

Materials and Methods A total of 131 patients with renal cancer who underwent operation from the Second Affiliated Hospital of Xi'an Jiaotong University were enrolled. 20 patients with benign renal diseases were used as control. 5 mL Peripheral blood was extracted from 131 patients with renal cancer before operation and about 3 months after operation. Control patients took blood at the corresponding time. CanPatrol CTC detection technique was used to enrich and identify CTCs, EMT-CTCs and CTC-WBCs simultaneously.

Results All patients enrolled were T1-3N0M0. 52 renal cancer patients received radical resection, while other 79 patients underwent nephron-sparing surgery. The positive rate of CTC, mesenchymal CTCs (MCTC) and CTC-WBC before surgery were 95.4% (125/131), 61.1% (80/131) and 11.5% (15/131), respectively. Preoperative total CTCs, mesenchymal CTCs or CTC-WBCs were poorly correlated with patients' parameters. Preoperative CTC, MCTC or CTC-WBC showed little association with progression-free survival (PFS), while post-operation total CTC (≥ 6), positive mesenchymal CTCs and positive CTC-WBC significantly correlated with early recurrence and metastasis, and remained independent indicators for worse PFS. In addition, the elevation of CTC and MCTC after operation were also correlated with unfavourable PFS.

Conclusions With the detection of CTCs in peripheral blood, better risk stratification of renal cancer patients might therefore help to identify a subset of patients that might have higher recurrent risk than the overall population of patients.

Introduction

Worldwide, renal cancer represents one of the 10 most frequently diagnosed cancers in adults, accounting for 5% in men and 3% in women of all oncological diagnoses [1]. Renal cancer is a very invasive diseases and benefits poorly from adjuvant chemotherapy or radiation therapy[2]. Surgical resection remains an effective therapy for clinical localized renal cancer, with options including radical nephrectomy and nephron-sparing surgery [3]. Importantly, 20% to 30% of patients with localized renal cancer recur or metastasize after surgical excision. The median time to relapse after surgery is 1 to 2 years, with most relapse occurring within 3 years [4]. Even in patients considered to be potentially curable by surgery, metastasis can occur in 5-10 years. It is urgent to development biomarkers for better risk stratification of renal cancer patients, which might allow identification of patients with a high risk of recurrence after nephrectomy.

Circulating tumor cells (CTCs) are cells of tumor origin circulating in the blood and believed to be vital seeds for hematogeneous tumor metastasis [5]. Evidence has shown that CTC counts has clinical relevance as a surrogate biomarker to noninvasive monitor for cancer progression and therapeutic decision-making [6-9]. In patients with early-stage hepatocellular carcinoma, higher CTC levels correlated with early relapse. In addition, the epithelial cells can disseminate from tumors and penetrate into blood vessels by epithelial mesenchymal transition (EMT)[10]. Hence, CTCs may be classified into three types: epithelial, mesenchymal (MCTC), and epithelial/mesenchymal hybrids. Increased MCTC have been reported as a predictor of disease progression in breast cancer [11]. Furthermore, apart from single CTC and CTC clusters, researchers have also isolated and characterized CTC-white blood cell (WBC) clusters. The association between neutrophils and CTCs can expand the metastasis potential of CTCs. Compared with patients with no CTC-WBC cluster or at least five CTCs, patients with the presence of CTC-WBC clusters showed the worst PFS in breast cancer [12].

So far, published CTC data for renal cancer mostly rely on small patient cohorts of different disease stages using various CTC techniques, and they show little results about correlation of CTC phenotypes and PFS in early stage of renal cancer [13-16]. In the present study, we evaluated CTCs in the peripheral blood obtained from patients with renal cancer by using filtration method for CTC capture. A tri-color mRNA in situ hybridization(ISH) assay was conducted to identify and classify CTCs. Our goal was to investigate the clinical significance of CTCs and CTC-WBC regarding PFS in operable renal cancer patients. Better risk stratification of renal cancer patients might therefore help to identify a subset of patients that might have higher recurrent risk than the overall population of patients.

Materials And Methods

We retrospectively reviewed 131 consecutive patients with renal cancer patients ($T_{1-3}N_0M0$ stage) receiving operation from January 2015 to January 2020 at the Second Affiliated Hospital of Xi'an Jiaotong University, China. This study was reviewed and approved by the review board and ethics committee of the Second Affiliated Hospital of Xi'an Jiaotong University (2018021). Informed consent was obtained from all patients participated. Peripheral blood samples or CTC analysis were obtained from all patients before surgery and about three months after surgery. In addition to the patient recruitment described above, blood specimens were also collected from 10 healthy donor.

The first patient follow-up was administered at three months after radical resection, then every 3~6 months for the first two years, and every 6–12 months thereafter. The follow-up intervals can be assessed more frequently for patients suspected of recurrence. The follow-up time ranged from 6 to 61 months. Progression free survival was defined as the time from surgery to diagnosis of local recurrence, distant metastasis or last follow-up.

The strategy of enrichment and characterization of renal cancer CTCs was described in the previously published report. 5ml Peripheral blood samples were collected before surgery and 10 days to 1 month after surgery. Samples were processed within 4 h of collection. Samples were centrifuged for 5 min at 1500 rpm, and the plasma phase was removed. CTCs were further separated by using CanPatrol CTC enrichment technique (SurExam, Guangzhou, China).

A tri-color RNA in situ hybridization (ISH) assay was used to characterize CTC and CTC-WBC cluster, including epithelial markers (EpCAM, CK8/18/19, labeled with Alexa Fluor 594), mesenchymal markers (Vimentin and Twist, labeled with Alexa Fluor 488), white blood cells marker (labeled by Alexa Fluor 750 conjugated anti-CD45), and nuclear marker (DAPI). CD45 was only expressed in leukocytes but not in tumor cells. CTCs were identified as epithelial and/or mesenchymal marker-positive DAPI+CD45-. CTC-WBC clusters were identified as one CTC with one/two-associated WBCs (representative pictures were shown in Figure 1).

The association between preoperative CTC level and CTC-WBC positivity and clinicopathological parameters were performed by the chi-square test. Progression-free survival (PFS) was defined as the time from the date of diagnosis to the date of progression (local recurrence or distant metastasis) or censored at the date of last follow-up. PFS was calculated by the Kaplan–Meier method and compared with the log-rank test. Univariate and multivariate analysis were performed using Cox's proportional hazards regression model with a forward stepwise procedure. Analyses were performed using the statistical software package IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA). All two-sided P-values less than 0.05 were considered to be significant.

Results

The study included a total of 131 renal cancer patients with TNM stage $T_{1-3}N_0M_0$. The clinicopathological features of the study cohort were summarized in Table 1, including age, sex, histology, differentiation, T stage, surgery types and renal score. We obtained blood samples from 131 patients and enriched for CTCs using Canpatrol^R technique. Based on the EMT markers, the detected CTCs could be classified into three phenotypes: epithelial, bi-phenotypic and mesenchymal (Fig. 1a). In the present study, whereas the majority of CTCs were single, we also detected CTC-WBC clusters. Three types of CTCs and CTC-WBC clusters were depicted in Fig 1. No CTC was detected in patients with benign renal disease. Baseline CTCs were detected (≥ 1/5 mL) in 125 out of 131 renal cancer patients (95.4%) with a median of 6 CTCs/5 mL blood, ranging from 0/5 mL to 53/5 mL. The positive rate of mesenchymal CTCs (MCTC) before surgery in all patients was 61.1% (80/131), ranging from 0/5 mL to 15/5 mL. CTC-WBC clusters were present in 11.5% (15/131) patients, ranging from 0/5 mL to 2/5 mL.

For better deciphering the potential application of CTCs in clinical practice, patients were divided into two parts according to the median number of total CTC counts (CTC<6 or CTC ≥6), MCTCs (negative/positive) and CTC-WBCs (negative/positive), respectively. Of the 131 patients included in the CTC analysis, 58 (44%) had CTCs<6 and 73 (56%) had CTC ≥6 at baseline. The preoperative serum CTC level showed no association with clinicopathological features, while CTC level after surgery was significantly correlated with types of surgery, P=0.007 (Table 1). Similarly, the preoperative and postoperative MCTC showed little correlation with patients' parameters. The positive rate of CTC-WBC clusters was 11.5% (15/131) before operation, and 13.0% (17/131) after operation. Similarly, no association between the existence of CTC-WBC and clinicopathological characteristics was found.

The follow-up duration of all patients was 6 to 61 months, with a median of 24 months. In total, 129 (98.5%) patients were alive and 111 (84.7%) patients were in the disease-free status at the end of follow-up. The Kaplan–Meier's survival curves revealed that patients with CTCs ≥6 after surgery had significantly poorer PFS ($P < 0.001$) than subgroup with CTC< 6, while the CTC level at baseline was found to have no significant correlation with PFS ($P = 0.459$, Fig. 2A and B). Similarly, patients with the presence of MCTCs per 5 mL blood after surgery was more likely to have unfavourable PFS compared with those without MCTCs($P = 0.002$, Fig. 2D). Likewise, the survival curves revealed that patients with/without CTC-WBC clusters before surgery exhibited no remarkable differences in PFS, while the negative of CTC-WBC after surgery was significantly associated with longer PFS ($P = 0.916$ and 0.017, respectively, Fig. 2E and F).

CTC counts after operation were available from all patients. Patients with CTCs post-operation increased when compared with CTCs pre-operation were categorized as CTC increased, the other patients were categorized as CTC decreased or no change. Similarly, these patients were also categorized according to the variations of MCTC during operation. The increase of CTC or MCTC were significantly associated with poorer PFS of renal cancer patients, P=0.006 and 0.012, respectively (Figure 3A and 3B).

In univariate analysis, clinical factors significant for survival were T stage and operation methods. CTC related univariate analyses revealed significant association between post-operative CTC/MCTC/CTC-WBC, CTC/MCTC changes and PFS (all, P<0.05) (Table 2). In multivariate Cox regression analysis, after adjusting for the clinically significant univariate factors, CTC level (HR 7.521, 95%CI 2.065-27.397, P=0.001), existence of MCTC (HR 8.232, 95%CI 1.826-37.820, P=0.006) and CTC-WBC (HR4.108, 95%CI 1.507-11.199, P=0.006) after operation remained as highly significant predictors of PFS. Patients with increased CTC (HR 2.784, 95%CI 1.001-7.743, P=0.05) or MCTC (HR 2.329, 95%CI 0.901-6.021, P=0.081) had slightly higher risk of progression compared with those in the CTC or MCTC decreased/unchanged group.

Discussion

The current study evaluated the relationship between perioperative CTC subpopulations, CTC-WBCs and PFS in operable renal cancer patients. Our findings demonstrated post-operation total CTC (CTC ≥ 6), positive mesenchymal CTCs and positive CTC-WBC significantly correlated with early recurrence or metastasis, and remained independent indicators for worse PFS.

CTCs are considered the pivotal component of the metastatic cascade and are being extensively studied only in the last decade or so [10, 17]. One well-validated clinical application of CTC is assessment of their prognostic value by CellSearch assay at pretreatment baseline in advanced diseases, which depends on the expression of epithelial marker EpCAM [6-8]. At the same time, clinical significance of CTCs in early stages cancers has also attracted much attention [18-21]. However, CTCs with low or absence of EpCAM expression may be dismissed by the dependence of CTC isolation on epithelial cell-specific technique [22]. In 2011, ANGELA GRADILONE noticed the possible mechanism underlying the low number of CTCs detected with CellSearch technique in 25 advanced renal cancer patients [23]. CanPatrolR(Surexam, Guangzhou, China) technique is a combination of membrane filtration and epithelial/ mesenchymal biomarkers-based identification, enables the isolation of CTCs with low/no epithelial markers possible[24].

So far, several published studies has focused on CTCs in advanced renal cancer patients with various techniques. Liu showed that the number of CTCs (DAPI+/CK+/CD45-) was associated with clinical stages of renal cancer. Patients in late-stage (III and IV) had 1.2-fold more CTCs than patients in early-stage (I and II) [25]. Bluemke found that the presence of CTCs were correlated with positive lymph node and synchronous metastases before surgery [13]. Few studies concerned CTCs in early stage renal cancer patients. In the present study, we firstly demonstrated total CTCs, mesenchymal CTCs and CTC-WBC before or after surgery showed little association with clinical characteristics in T_{1-3}N0M0 renal cancer patients. In non-metastasis breast cancer, the preoperative CTCs were also poorly associated with tumor size, grade or lymph node status [26].

Circulating tumor cells appear to add important reference information regarding an individual patient's risk for relapse or progression. Bluemke discovered that positive CK+ CTCs was significantly correlated with inferior overall survival for renal cancer patients [13]. Nel found that the presence and quantity of CD133-positive or N-cadherin-positive CTC was associated with poor PFS in 14 renal cancer patients [27]. Due to the relative short follow-up time and relative early stage of patients, 15.3% patients (20/131) experienced recurrence or metastasis in our study. Patients with higher CTCs, mesenchymal CTCs were more likely to get progression during follow up. In addition, total CTC (CTC≥6), positive mesenchymal CTCs were independent prognostic indicators

for poorer PFS. However, current clinical practice guidelines, including NCCN and ASCO, do not recommend routine use of CTC for therapeutic decision making in non-metastasis breast cancer patients, not to mention in early stage renal cancer patients. Future clinical trials needed to demonstrate clinical utility of CTC for decision-making and monitoring response to therapy, as well as prognostic indicators.

The success rate of blood metastasis is low since only a few of the thousands of CTCs daily released in the bloodstream finally survive and establish secondary lesions. In peripheral blood, CTCs migrate as single cells or clusters, named 'circulating tumor microemboli' (CTMs), which also comprise leukocytes, endothelial cells, and other cells held together by cell adhesion proteins [28]. In any case, it is conceivable that CTCs, leaving the protected microenvironment of the primary tumor, encounter challenge from immune surveillance in nontumor tissues. After entering blood stream, CTCs are thought be guarded by platelets from immune elimination [29, 30]. In addition, Szczerba found that in advanced breast cancer patients and breast cancer mouse models, CTCs were associated with non-malignant cells such as white blood cells (WBCs) [12]. We found that patients with the positive detection of CTC-WBC three months after surgery were characterized by inferior PFS than those without CTC-WBC. Similar to our findings, Szczerba showed that PFS in patients with positive CTC-WBC clusters were significantly shorter than patients with no CTC-WBC or patients with no less than 5 CTCs [12]. More fundamental and clinical research were needed for further elucidation of CTC-WBC in cancer metastasis.

There were still several limitations within present study, including lack of long time period follow-up research, single-center study. Subgroup analysis was needed to be conducted in future studies. Thus, further multi-center studies are still needed to validate our findings and form a more comprehensive clinical conclusion.

Conclusions

In conclusion, the current study demonstrated that CTCs subpopulation and CTC-WBC were novel and promising prognostic indicator in early stage renal cancer patients, which significantly correlated with the patients' relapse. Future multi-center studies are needed to validate our findings, and further clarify the value of integrating the indicator with current clinical strategies in improving risk stratification of early stage renal cancer patients.

Abbreviations

CTC=circulating tumor cell

WBC=white blood cell

PFS=progression-free survival

EMT= epithelial mesenchymal transition

CTM= circulating tumor microemboli

Declarations

Author Contributions: Concept and design, Z.L.W. and P.Z.; investigation and data curation, Y.Q.X., X.J.Y., H.C.L., Y.P.Z., Z.L.L., L.X., H.L.L., and Q.C.; data analysis, writing and editing of the manuscript, Z.L.W., P.Z and Y.C.; supervision, T.C.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Tables

Table 1. Relationship of perioperative total CTCs, mesenchymal CTC, CTC-WBC and clinical characteristics.

Clinical parameters	n	Total CTC				Mesenchymal CTC				CTC-WBC			
		Pre-surgery		Post-surgery		Pre-surgery		Post-surgery		Pre-surgery		Post-surgery	
		CTC<6	CTC≥6	CTC<6	CTC≥6	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Age													
<50	32	13	19	17	15	9	23	13	19	28	4	29	3
≥50	99	45	54	55	44	42	57	41	58	88	11	85	14
P			0.633		0.81		0.149		0.937		0.76		0.762
Sex													
Male	83	35	48	44	39	37	46	30	53	70	13	69	14
Female	48	23	25	28	20	14	34	24	24	46	2	45	3
P			0.523		0.555		0.081		0.121		0.051		0.107
Pathological subtypes													
Clear-cell carcinoma	113	52	61	60	53	47	66	45	68	100	13	98	15
Others	18	6	12	12	6	4	14	9	9	16	2	16	2
P			0.314		0.283		0.117		0.415		1		1
Differenciation													
Low	22	7	15	13	9	7	15	11	11	19	3	21	1
Middle	102	48	54	57	45	41	61	42	60	92	10	88	14
High	7	3	4	2	5	3	4	1	6	5	2	5	2
P			0.446		0.38		0.79		0.295		0.248		0.189
T Stage													
T1	112	52	60	63	49	43	69	45	67	99	13	97	15
T2	15	4	11	6	9	5	10	6	9	13	2	13	2
T3	4	2	2	3	1	3	1	3	1	4	0	4	0
P			0.327		0.39		0.379		0.454		0.811		1
Types of surgery													
Nephron-sparing surgery	79	35	44	51	28	27	52	34	45	68	11	68	11
radical excision	52	23	29	21	31	24	28	20	32	48	4	46	6
P			0.993		0.007		0.169		0.603		0.273		0.691
Renal score													
Low risk	28	12	16	17	11	7	21	13	15	24	4	24	4
Middle risk	72	32	40	42	30	31	41	32	40	63	9	66	6
High risk	31	14	17	13	18	13	18	9	22	29	2	24	7
P			0.983		0.243		0.232		0.283		0.576		0.126

Table 2. Univariate and multivariate Cox regression analysis for prediction of PFS

Variable	Univariate COX			Multivariate COX*		
	HR	95%CI	*P*	HR	95%CI	*P*
Preoperative CTC（CTC≥6/CTC<6）	1.398	0.569-3.438	0.465	——		
Postoperative CTC（CTC≥6/CTC<6）	7.803	2.278-26.728	0.001	7.521	2.065-27.397	0.001
CTC variation（elevated/not elevated）	3.481	1.329-9.119	0.011	2.784	1.001-7.743	0.05
Preoperative MCTC（positive/negative）	1.996	0.718-5.549	0.185	——		
Postoperative MCTC（positive/negative）	7.19	1.665-31.05	0.008	8.232	1.826-37.820	0.006
MCTC variation（elevated/not elevated）	3.006	1.218-7.42	0.017	2.329	0.901-6.021	0.081
Preoperative CTC-WBC（positive/negative）	0.925	0.212-4.029	0.917	——		
Postoperative CTC-WBC（positive/negative）	3.079	1.169-8.111	0.023	4.108	1.507-11.199	0.006

*（Adjusted by age, sex, T stage, types of surgery and tumor differention.

Figures

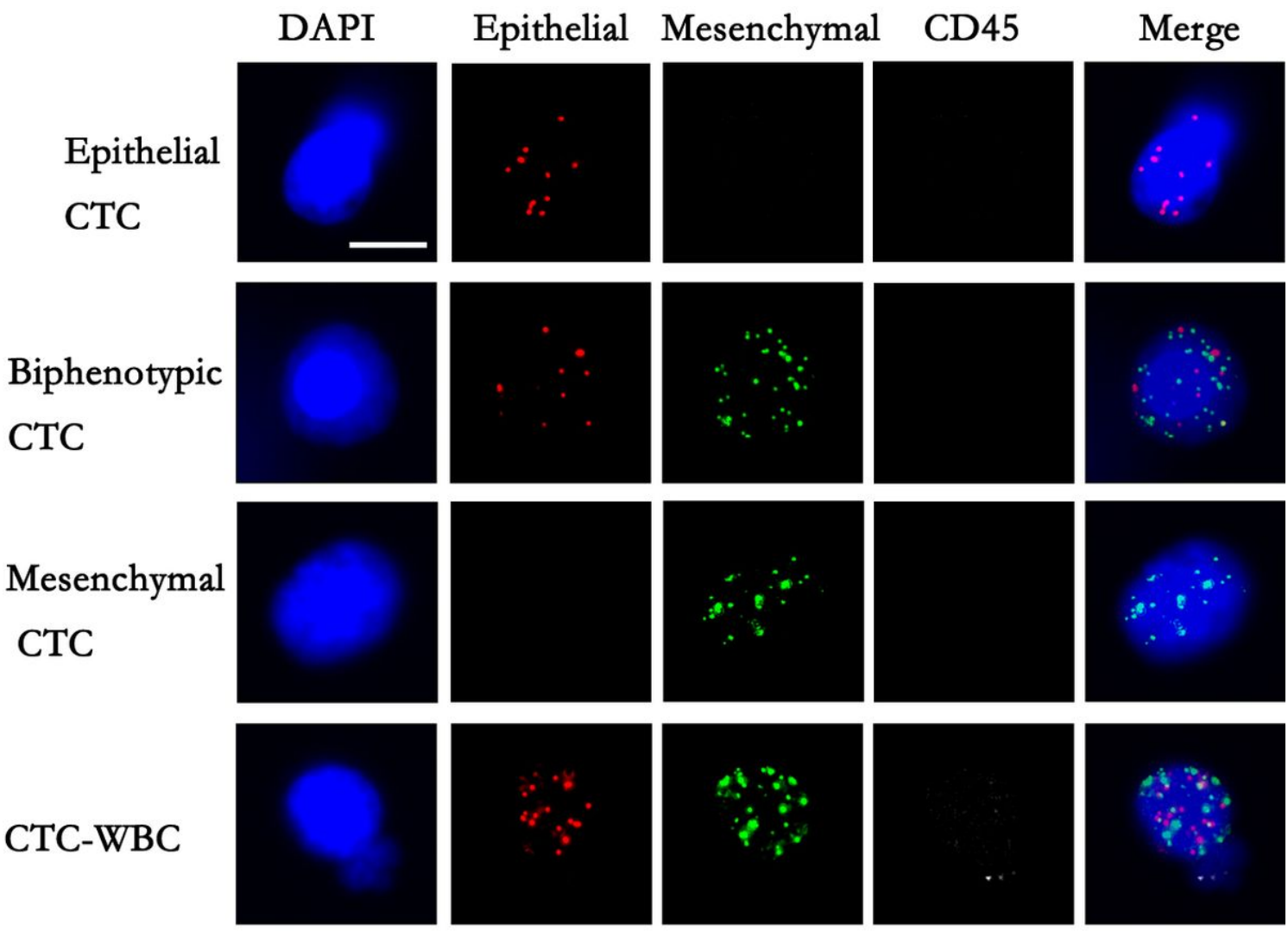

Figure 1

EMT phenotypes of CTCs and CTC-WBC detected by the RNA in situ hybridization in renal cancer patients. Fluorescence microscopy images of three types of CTCs with positive expression of epithelial markers (EpCAM and CK8/18/19, red dots), biphenotypic markers (red dots and green dots and mesenchymal markers (Vimentin and Twist, green dots). CTC-WBC was characterized as DAPI+CD45+Epithalial+and/or mesenchymal+. Scale bar = 10 μm.

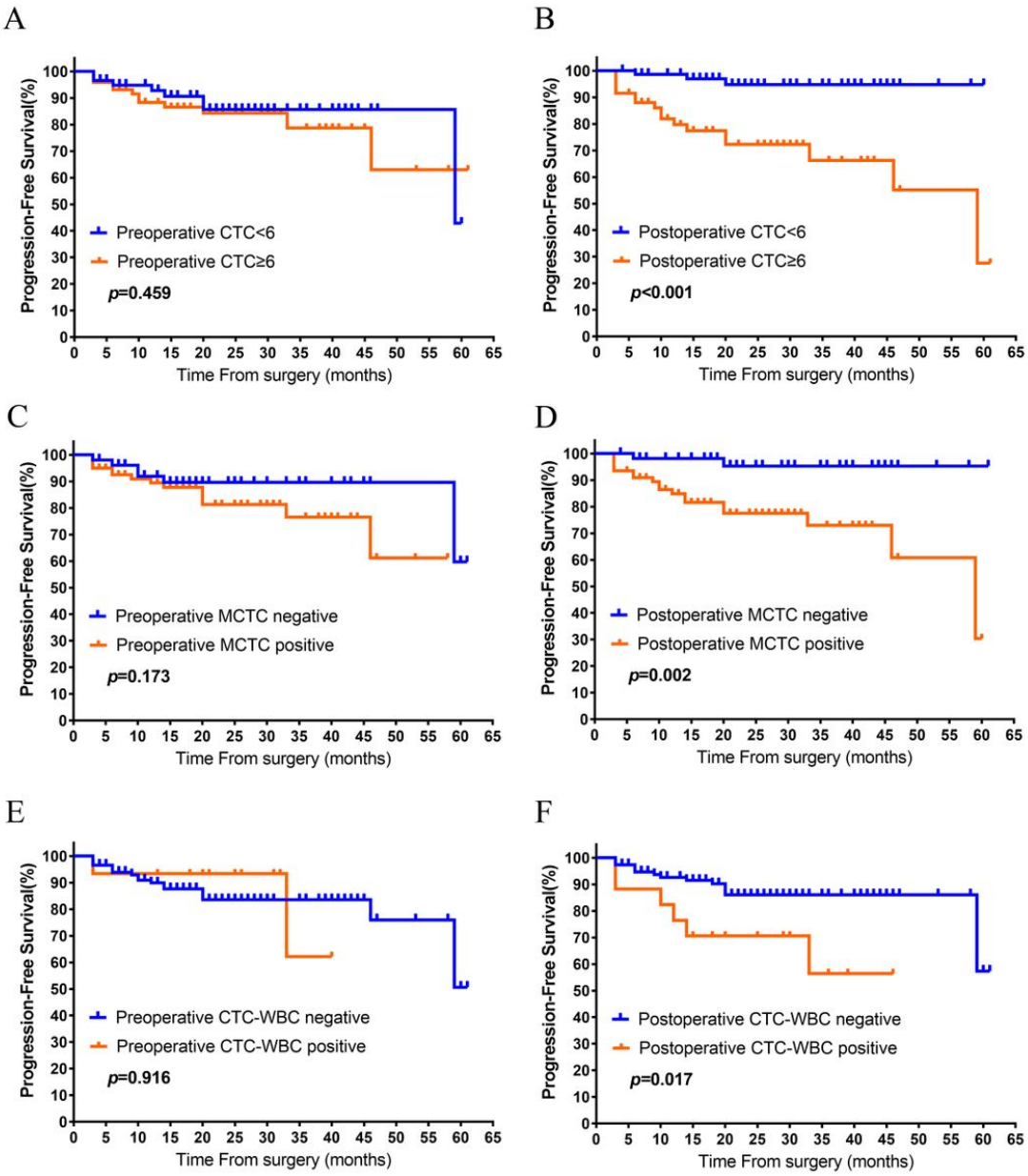

Figure 2

Kaplan-Meier curves for progression-free survival (PFS) of patients according to CTC, mesenchymal CTC (MCTC) and CTC-WBC before and after surgery. (A)Preoperative CTC and postoperative CTC (B) with PFS. (C, D) Preoperative MCTC and postoperative MCTC with PFS. (E, F) Preoperative CTC-WBC and postoperative CTC-WBC with PFS. N=131.

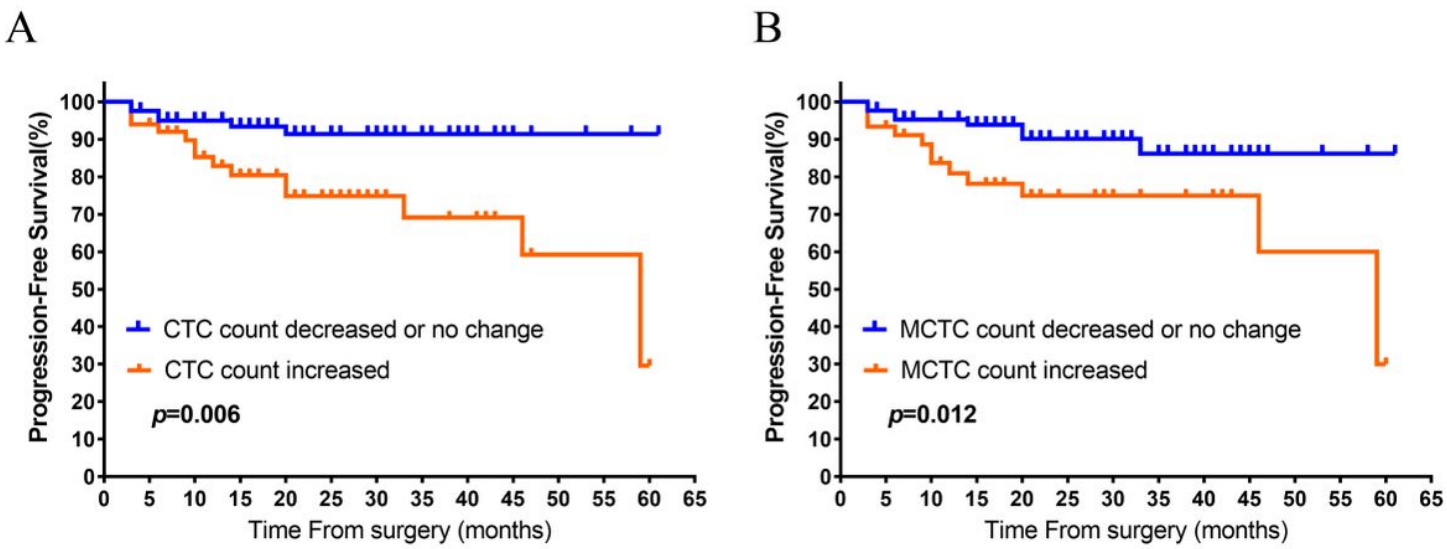

Figure 3

Kaplan-Meier curves for progression-free survival (PFS) of patients according to CTC or mesenchymal CTC (MCTC) variations before and after surgery. (A) Perioperative CTC variation and PFS, (B) perioperative MCTC variation and PFS.