Clinical Significance of Circulating Tumor Cells and Ki-67 in Renal Cell Carcinoma

Jinbo Song
Zhengzhou University
Zhe Yu
Zhengzhou University
Bingqi Dong
Zhengzhou University
Mingkai Zhu
Zhengzhou University
Xiaofeng Guo
Zhengzhou University
Yongkang Ma
Zhengzhou University
Shiming Zhao
Zhengzhou University
Tiejun Yang (tiejunyang@126.com)
Department of Urology Surgery, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, China https://orcid.org/0000-0002-9635-1707

Research

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Abstract

Background: Renal cell carcinoma (RCC) is a common maligant tumor of the genitourinary system. We aimed to analyze the potential value of the metastasis-related biomarker, circulating tumor cells (CTCs) and proliferative marker Ki-67 in diagnosis of RCC.

Methods: Data from 24 laparoscopic radical nephrectomy (RN) and 17 laparoscopic partial nephrectomy (PN) were collected in 2018. The number and positive rate of CTCs and circulating tumor microemboli (CTM) was obtained at three different time points in peripheral blood just before, immediately and one week after surgery. Ki-67 protein expression was evaluated in RCC tissue by immunohistochemistry.

Results: No association between the number and positive rate of perioprative CTC and clinicopathological features was found, except for the statistically significant association between the preoprative CTC counts and tumor size. The CTC counts gradually decreased during the perioperative period, and at one week after surgery was significantly lower than that before surgery. Higher Ki-67 expression was significantly correlated positively with preoprative CTC counts. In addition, a higher Ki-67 express has been found in high CTCs group (CTCs $\geq$ 5).

Conclusion: Our results suggest that surgical nephrectomy is a*s*sociated with a decrease in CTC counts of RCC patients. CTCs can act as a potential biomarker for the diagnosis and prognostic of RCC. Careful and sufficient long-term follow-up is needed for patients with high preoprative CTC counts.

Introduction

Renal cell carcinoma (RCC) is one of the most prevalent urological tumors. Its incidence rate is about 2% to 3% still increasing over the years.[1] It is a serious threat to the health and life of patients. Because of the poor response to radiotherapy and chemotherapy, surgery (radical nephrectomym and partial nephrectomy) is the first treatment option for RCC.[2] However, postoperative recurrence and metastas is still occur in up to 20% to 40% of RCC patients.[2, 3] Transcirculatory metastasis is the most important pathway for the formation of renal cell carcinoma metastases foci. Circulating tumor cells (CTCs) are defined as tumor cells shedding from the primary tumor site or metastases into the peripheral blood and also called circulating tumor microemboli (CTM) when they present as a cluster of cells. They may attach to and grow in distant organs and have long been considered as a marker of tumor invasiveness.[4, 5]

There are Several methods for the precise diagnosis of RCC. Except based on pathological diagnosis, various molecular biomarkers are available to enable the early detection of RCC or evaluation the progression and prognosis.[6-8] Among these molecules, Ki67 has been considered to be an effective diagnostic marker for a variety of cancer, including RCC.[9-12] Previous studys have indicated that tumor proliferation rate is an important prognostic indicator with high tumor spread rates leading to worse patient outcomes.[13] Ki-67 contributes to enhanced proliferative activity of intrinsic cell populations in malignant tumors which is a nuclear protein that is associated with ribosomal RNA synthesis and may be

necessary for proliferating cells.[14] Therefore, Ki-67 has been considered as a biomarker of RCC which could be used in routine clinical practice.[11, 12, 15, 16] In view of its clinical significance, the Ki67 has been widely applied as a diagnostic approach for assessing a number of human malignancies.[17, 18]

The aim of the present study was to detect the changes of CTC and CTM counts and positive rate in peripheral blood of patients with RCC during perioperative period, and to explore their correlation with clinical and pathological features. Especially, we analyzed the correlation between CTCs and Ki-67 index and discussed the prognostic value of CTCs in RCC.

Materials And Methods

Clinical specimens

A total of 50 patients with kidney tumor who had received resection at The Afliated Cancer Hospital of Zheng zhou University between January 2018 and December 2018 were enrolled in the study. The inclusion criteria were (1) definitive pathological diagnosis of primary RCC; (2) received surgical resection, defined as complete removal of the macroscopic tumor; (3) margin-negative R0 resection; (4) no prior anticancer treatment; and (5) aged between 18 and 80 years. The exclusion criteria included (1) incomplete clinical evaluations; (2) history of any urological surgery; (3) receiving any treatments in preoperative, benign final pathology or upper tract urothelial cell carcinoma. (4) having other active or preexisting malignancies. Finally, 41 patients was included. All surgical procedures were performed by the same surgeon in this department. This study has been approved by the Ethical Committee of The Affliated Cancer Hospital of Zhengzhou University, and all patients provided written informed consent.

Immunohistochemical (IHC) staining of Ki-67

The stained slides were examined by two independent pathologists who were totally blinded to the clinical data. For Ki-67 antibody, cells labelled by the antibody displayed a nuclear staining pattern. The score was assigned according to the average of the extent of immuno-expression (0%–100% percentage of cells staining).

Collection and Detection of CTCs

5ml peripheral blood was collected before, immediately and one week after surgery. The samples were stored in anticoagulant vessels with ethylene diamine tetraacetic acid (EDTA), then mixed up and down slowly for 8 times. After collection, the specimens were stored at room temperature and processed within 2h. Otherwise, they were stored in a refrigerator at 4 ° C for no more than 24 hours. Samples were reheated at room temperature for at least 30min before process. CTCs/CTM were analyzed using the CTC-BIOPSY system (Youzhiyou, Wuhan, China),[19] as previously described, a semi-automatic CTCs detection system based on the membrane filtration separation technology (Isolating by size of epithelial tumor cells, ISET).[19, 20] Briefly, 5 ml specimens were diluted to 8 ml using buffer containing 0.2% formaldehyde, and then transferred to the filtration membrane with pore diameter of 8μm. The sample was separated semi-

automatically by the instrument, and then the cells remaining on the filter membrane were distinguished by Diff-Quik staining. CTCs / CTM were defined based by morphological standard:[21] (1) nucleocytoplasmic ratio> 0.8; nucleoli is abnormally large; (2) cell nucleus diameter> 18 mm; nucleus is irregularly shaped; (3) nuclear membrane is thickened, wrinkled, non-uniform stained, and chromatin shifted laterally; (4) CTM: There are 3 or more CTCs aggregates. All candidate CTCs/CTM were blindly reviewed and identifed independently by three senior cytopathologists. The staining results are shown in Fig 1.

Statistical analysis

All statistical analyses were performed using the Graphpad Prism 7.0 (GraphPad Software, California, USA) and SPSS 22.0 statistic software (SPSS Chicago, USA). The results were presented as mean ± standard deviation (M±SD). The Student's t test and one-way analysis of variance (ANOVA) with Tukey's test were used for continuous variables, and chi-square test or Fisher exact test for categorical variables. As for non-normal distribution samples, Wilcoxon two sample test were used. Differences were considered statistically significant when the P value was <0.05.

Results

Patient characteristics

Among the 41 patients finally included, there were 22 males and 19 females. The age ranged from 20 to 76 years (53.0±12.1), and 34% of patients were older than 60 years. Pathological type: 37 cases of clear cell carcinoma, 4 cases of non-clear cell carcinoma (papillary cell carcinoma 3 cases and 1 case of chromophobe cell carcinoma); surgical method: 17 cases reveived partial nephrectomy and 24 cases reveived radical nephrectomy. The baseline characteristics of the patients are shown in Table 1.

Preoperative intraoperative and postoperative positive rate of CTCs/CTM and CTC/CTM counts

The positive rate of CTCs in peripheral blood of RCC patients before, immediately and one week after surgery were 82.9%, 85.4% and 73.2%, respectively. The positive rate of CTCs one week after surgery was lower than that before surgery, but the difference was not statistically significant (P > 0.05); the CTC counts were 11.56±12.92/5ml 9.29±11.79/5ml 4.12±5.71/5ml, respectively. The counts of CTC at one week after surgery was significantly lower than that before surgery (P = 0.001) (Fig 2A). The detection rate of perioperative CTM in RCC patients was low. There was no statistically significant difference between perioperative CTM counts or positive rate (Fig 2B). Thus, we did not perform the analysis on whether the change of perioperative CTM affects diagnosis and prognosis. Compared with preoperative CTC counts, CTC counts decreased in 26 (63.4%) patients, increased in 11 (26.8%) patients, and were unchanged in 4 (9.8%) patients immediately after surgery (Fig 2C); CTC counts decreased in 30 (73.2%) patients, increased in 9 (21.9%) patients, and were unchanged in 2 (4.9%) patients one week after surgery (Fig 2D).

CTC counts and its association with clinical and pathological features of RCC patients

The correlation of CTC counts with clinicopathological features of patients before, immediately and one week after surgery were analyzed. As shown in Table 2, There were 17 cases with tumor mass 5 cm or more in diameter and 24 cases with tumor diameter less than 5cm, a significant correlation was observed between the preoperative CTC counts (17.12±16.94 vs. 7.13±7.67) and tumor diameter (P=0.018). Accordingly, linear regression analysis demonstrated that significant positive correlations were found between preoperative CTC counts and tumor diameter (P = 0.020; Fig 3D). But there was no statistical difference immediately after surgery (P = 0.438; Fig 3E) and one week after surgery (P = 0.342; Fig 3F). Futhermore, the correlation analysis showed that there was no correlation between the distribution of CTC-positive patients or CTC counts and clinical TNM stage before, immediately and one week after surgery (Table 2, Supplementary table 1 and Supplementary table 2).

As shown in Table 3, the patients were divided into 24 laparoscopic radical nephrectomy (RN) and 17 laparoscopic partial nephrectomy (PN) according to the sugrical approaches. The changes of perioperative CTC counts were compared between the two groups, and the difference had no statistical significance (P > 0.05); However, the positive rate of peripheral blood CTCs in the RN group before surgery was significantly higher than that in the PN group (P = 0.014).

Then, using a preoperative CTCs of 5 as the cutoff value, 41 RCC patients were divided into high CTCs group (24 cases with CTCs ≥ 5) and low CTCs group (17 cases with CTCs < 5). There was no statistically significant difference in the change of CTCs between two groups immediately after surgery(P = 0.885; Table 4). However, In the high CTCs group, CTC counts decreased in 21 (87.5%) patients one week after operation. Compared with low CTCs group (52.9%), the difference was statistically significant (P = 0.014; Table 4).

Correlation between CTC counts and Ki-67 expression of patients with RCC

According to the results of IHC staining, the patients were divided into three Ki67 index groups, including 26 cases with indices less than 10%, 8 cases with indices between 10 and 20%, and 7 cases with 21% or more. Tumors with Ki67 index of 10% or more were considered to be highly proliferative. Based on this criterion, 15 out of the 41 patients were showed highly proliferative potential. As shown in Tables 5-7. The results suggested that the proportion of RCC patients with high CTC levels (CTCs ≥ 5) was higher in the high proliferation group than in the low proliferation group before surgery and one week after surgery. Meanwhile, the positive rate of preoperative CTCs in the high proliferation group was significantly higher than that in the low proliferation group (P=0.035). However, the difference between them was not statistically significant immediately after surgery. Linear regression analysis demonstrated that significant positive correlations were found between preoperative CTC counts and Ki-67 index (P < 0.001; Fig 3A). However, no significant correlations were observed between the intra- and postoperative CTC counts and Ki-67 index (Fig 3B and 3C).

Discussion

The detection of CTCs provides a new powerful tool to evaluate tumor load and invasiveness. In recent years, more and more studies have been conducted on CTCs of RCC, which play an important role in the detection of early recurrence and metastasis.[22] Our results showed that preoperative CTC counts was higher in the tumor size ≥ 5cm group and reduced by surguical treatment. Besides, preoperative CTC counts was correlated to proliferation marker Ki-67.

At present, there are few studies on CTCs in renal cell carcinoma. Here, we determined the change of perioperative CTC counts and positive rate, and evaluated whether they were correlated with clinicopathological features. The statistical analyses results demonstrated that only tumor diameter affected preoperative CTC counts. Our findings are not identical to previous studies which found that the TNM-Staging are related to the positive rate and level of CTCs.[23, 24] One reason could be included that most patients were diagnosised with early clinical TNM Stage.

Surgical resection is the best treatment for local renal cell carcinoma, The results of this study showed that the positive rate of CTCs in RCC patients slightly increased immediately after surgery but rapidly decreased one week after surgery, however, the difference was not statistically significant. The mean level of CTCs gradually decreased during perioperative period. Some studies have shown that in the surgical treatment of non-small cell lung cancer and prostate cancer CTCs may fall off due to invasive operation, leading to blood-derived spillover of cancer cells.[25, 26] Zhang et al [21] showed that the levels of CTC in breast cancer patients was higher on the 3rd day after surgery than that before surgery, but decreased significantly on the 7th day after surgery.[27] Invasive operation might result in a transiently increase in CTCs due to squeeze the tumor. Overall, our findings are consistent with the previous studies, complete removal of the tumor will reduce CTC counts after surgery.

RN and PN are the main surgical approaches for renal cell carcinoma. According to the literature, different surgical methods may affect periopretive CTC counts. Haga et al[28] compared four surgical approaches, laparoscopic RN, laparoscopic PN, open RN, and open PN. They found open RN resulted in a significantly more postoperative CTC counts than in the laparoscopic RN group or in the open or the laparoscopic PN group. Our data suggested that the RN group showed a tendency to have higher positive rate of CTCs and more preoperative CTC counts than PN group. *The reason may be that* patients had greater tumor diameter and TNM stage, which resulted in a higher preoperative CTC counts and positive rate in the RN group. However, the perioperative change in positive rate of CTCs or CTC counts did not differ significantly among the surgical methods. The laparoscopic surgery was used for all patients in this study.
Fine manipulation could be more possible in laparoscopic kidney surgery than in open surgery. Thus, laparoscopic kidney surgery might be preferable from preventing blood-derived spillover of cancer cells.

Does preoperative CTC counts affect the change of CTCs after surgery? At present, there is no uniform definition of CTCs positivity.[29] Some studies have reported that CTCs ≥ 5 is an independent risk factor for recurrence and metastasis of breast cancer and non-small cell lung cancer.[27, 30] Using a CTCs of 5 as the cutoff value, the present study demonstated that, in the high CTCs group, more patients showed CTC

counts decreased at one week after surgery compared to low CTCs group. To some extent, the high CTCs group may receive extra benefit from surgical treatment.

Ki67 is a nuclear antigen that is present in almost all human malignancies. A growing body of research on lymphomas,[31] bladder cancer,[32] colorectal cancer[33] and gastric cancer,[34] have shown that overexpression of Ki-67 is associated with tumor cell growth, biological aggressiveness and the prognosis of these malignancies. Moreover, In RCC labeling indexes of Ki67 was found to be positively associated with advanced tumour stage and grade, and provide an additional prognostic indication of biological aggressiveness.[35, 36] Tollefson et al[37] reported that patients with high Ki67 expression were 68% more likely to die from RCC. Then, we analyzed the correlation between CTC counts and Ki-67 index and evaluate the prognostic value of CTCs in RCC. Our results indicated that a higher Ki-67 expression was significantly correlated positively with the absolute number of preoprative CTCs using the linear regression analyses. In addition, A higher Ki-67 express has been found in high CTCs group (CTCs ≥ 5). Therefore, CTCs≥5 may be an prognostic indicator of renal cell carcinoma. This finding was consistent with these reports that demonstrated in breast cancer and non-small cell lung cancer.[27, 30]

The methods of detecting CTCs should be noted for this study. The CellSearch system was approved by the FDA as a CTCs detection platform, which analyzes CTCs by detecting epithelial cellular adhesion molecule (EpCAM) expression in individual tumors. However, the expression of EpCAM in RCC is not high,[38] so this method has a low detection rate of CTCs in RCC.[39] In this study, a semi-automatic CTCs detection system based on ISET technology, namely the CTC-BIOPSY device, was used to analyze CTCs. Compared with the CellSearch system, ISET has a higher detection rate for CTCs of RCC and more advantages in detecting renal cancer CTCs with low expression of EpCAM.[23] In recent studies, markers of G250 antigen[40, 41] or CA9 combined with CD147[42] showed good prospects in the detection of CTCs in RCC patients.

A few limitations of the this study need to be considered. First, in order to execute the inclusion and exclusion criteria in strict rotation, the number of cases enrolled in this study was small. Second, although preoperative CTC counts was correlated to proliferative marker Ki-67 in the current study, the prognostic and predictive impact of CTCs in the perioperative period would need more than several years of observation because the follow-up period is still too short. Future studies with longer postoperative follow-up is necessary to assess the clinical significance of perioperative CTCs detection in the diagnosis and treatment of RCC.

Conclusion

our results showed the effect of surgical nephrectomy on CTCs in patients with RCC. Our findings supported surgical treatment was the direct reason for the decrease of CTC counts in RCC patients. Our results also showed a high association between CTC counts and the proliferation marker Ki-67 further confirming the potential of the CTCs as a diagnosis and prognostic biomarker of RCC. It is necessary to detect CTCs of RCC patients during perioperative period, especially for the tumor size ≥5cm. Careful and

sufficient long-term follow-up is needed for patients with high preoprative CTC counts (CTCs≥5). In the future study, using of the combination of the CTC counts and the Ki67 index or other biomarkers might provide better diagnostic accuracy and precision of RCC.

Declarations

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Author information

Jinbo Song and Zhe Yu contributed to this work equally as co-first authors.

Affiliation

Department of Urology Surgery, The Affiliated Cancer Hospital of Zhengzhou University, Henan Cancer Hospital, Zhengzhou, China

Jinbo Song, Zhe Yu, Bingqi Dong , Mingkai Zhu, Xiaofeng Guo, Yongkang Ma, Shiming Zhao&Tiejun Yang

Contributions

Tiejun Yang designed the study. Jinbo Song and Zhe Yu drafted the manuscript. Bingqi Dong and Mingkai Zhu were responsible for the collection and analysis of the experimental data. Xiaofeng Guo, Yongkang Ma, Shiming Zhao, and Zhe Yu revised the manuscript critically for important intellectual content. The authors read and approved the final manuscript.

Corresponding author

Correspondence to Tiejun Yang.

Ethics declarations

This study has been approved by the Ethical Committee of The Affliated Cancer

Hospital of Zhengzhou University. All patients provided written informed consent.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no conflict of interests.

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Tables

Table 1 The baseline characteristics of 41 RCC patients

Parameter	No. of patients	%
Sex		
Male	22	53.7
Female	19	42.3
Age		
≤60 Years	27	65.9
>60 Years	14	34.1
Tumor location		
Upper	8	19.5
Middle	16	39.0
Lower	17	41.5
Surgical Methods		
Partial nephrectomy	17	41.5
Radical nephrectomy	24	58.5
Pathological type		
Clear cell carcinoma	37	90.2
Non-clear cell carcinoma	4	9.8
Fuhrman grade		
G1	14	34.1
G2	18	43.9
G3	6	14.6
G4	3	7.3

Table 2 The correlation of CTC counts with clinicopathological features of patients before surgery

Clinicopathological features	Cases (n=41)	CTC counts (M±SD)	*P*-value	CTCs, n (%)		*P*-value
				Negative(n=7)	positive(n=34)	
Tumor size			0.018*			0.512
<5 cm	24	7.13±7.67		4(16.7)	20(83.3)	
≥5 cm	17	17.12±16.94		3(17.6)	14(82.4)	
T-Staging			0.621			0.433
T1-2	36	12.06±14.08		7(19.4)	29(80.6)	
T3-4	5	9.50±6.37		0(0.0)	5(100.0)	
N-Staging			0.089			0.614
N0	38	11.92±13.34		7(18.4)	31(71.6)	
N1	3	7.00±3.00		0(0.0)	3(100.0)	
M-Staging			0.354			0.517
M0	37	11.70±13.38		7(18.9)	30(71.1)	
M1	4	10.25±8.66		0(0.0)	4(100.0)	
AJCC Staging			0.943			0.202
I	29	11.75±14.36		7(24.1)	22(75.9)	
II	4	14.25±13.52		0(0.0)	4(100.0)	
III	4	8.75±4.27		0(0.0)	4(100.0)	
IV	4	10.25±8.66		0(0.0)	4(100.0)	

AJCC: American Joint Committe on cancer

Table 3 The counts and positive rate of CTC in different sugrical approaches

Groups	Before surgery		Immediately after surgery		One week after surgery	
	Positive (%)	CTC counts	Positive (%)	CTC counts	Positive (%)	CTC counts
PN （n=17）	11 (64.71)	10.29±9.67	12 (70.59)	10.18±11.32	13 (76.47)	5.00±5.18
RN （n=24）	23 (95.83)	12.46±14.34	23 (95.83)	8.79±7.76	17 (70.83)	3.50±4.22
P-value	0.014*	0.603	0.066	0.728	0.965	0.415

Table 4 The number and change of patients in different CTCs groups during perioperative period

Before surgery	CTCs<5 (n=17) (%)		CTCs≥5 (n=24) (%)		*P*-value
	Decreased	No decreased	Decreased	No decreased	
Immediately after surgery	11 (64.7)	6 (35.3)	15 (62.5)	9 (37.5)	0.885
One week after surgery	9 (52.9)	8 (47.1)	21 (87.5)	3 (12.5)	0.014*

Table 5 The correlation between CTC counts and Ki67 index before surgery

		CTC counts, n		*P*-value	CTCs, n (%)		*P*-value
		<5 (n=17)	≥5 (n=24)		Negative(n=7)	positive(n=34)	
Ki67 index	<10 (n=26)	15	11	0.012*	7(26.9)	19(73.1)	0.114
	10-20 (n=8)	2	6		0(0.0)	8(100.0)	
	21-40 (n=7)	0	7		0(0.0)	7(100.0)	
	Low proliferation (n=26)	15	11	0.005*	7(26.9)	19(73.1)	0.035*
	High proliferation (n=15)	2	13		0(0.0)	15(100.0)	

Table 6 The correlation between CTC counts and Ki67 index immediately after surgery

		CTC counts, n		*P*-value	CTCs, n (%)		*P*-value
		<5 (n=18)	≥5 (n=23)		Negative(n=6)	positive(n=35)	
Ki67 index	<10 (n=26)	14	12	0.288	5(19.2)	21(80.8)	0.583
	10-20 (n=8)	2	6		0(0.0)	8(100.0)	
	21-40 (n=7)	2	5		1(14.3)	6(85.7)	
	Low proliferation (n=26)	14	12	0.091	5(19.2)	21(80.8)	0.388
	High proliferation (n=15)	4	11		1(6.67)	14(93.3)	

Table 7 The correlation between CTC counts and Ki67 index one week after surgery

		CTC counts, n		*P*-value	CTCs, n (%)		*P*-value
		<5 (n=28)	≥5 (n=13)		Negative(n=11)	positive(n=30)	
Ki67 index	<10 (n=26)	21	5	0.028*	7(26.9)	19(73.1)	0.612
	10-20 (n=8)	5	3		3(37.5)	5(62.5)	
	21-40 (n=7)	2	5		1(14.3)	6(85.7)	
	Low proliferation (n=26)	21	5	0.038*	7(26.9)	19(73.1)	0.719
	High proliferation (n=15)	7	8		4(26.7)	11(73.3)	

Figures

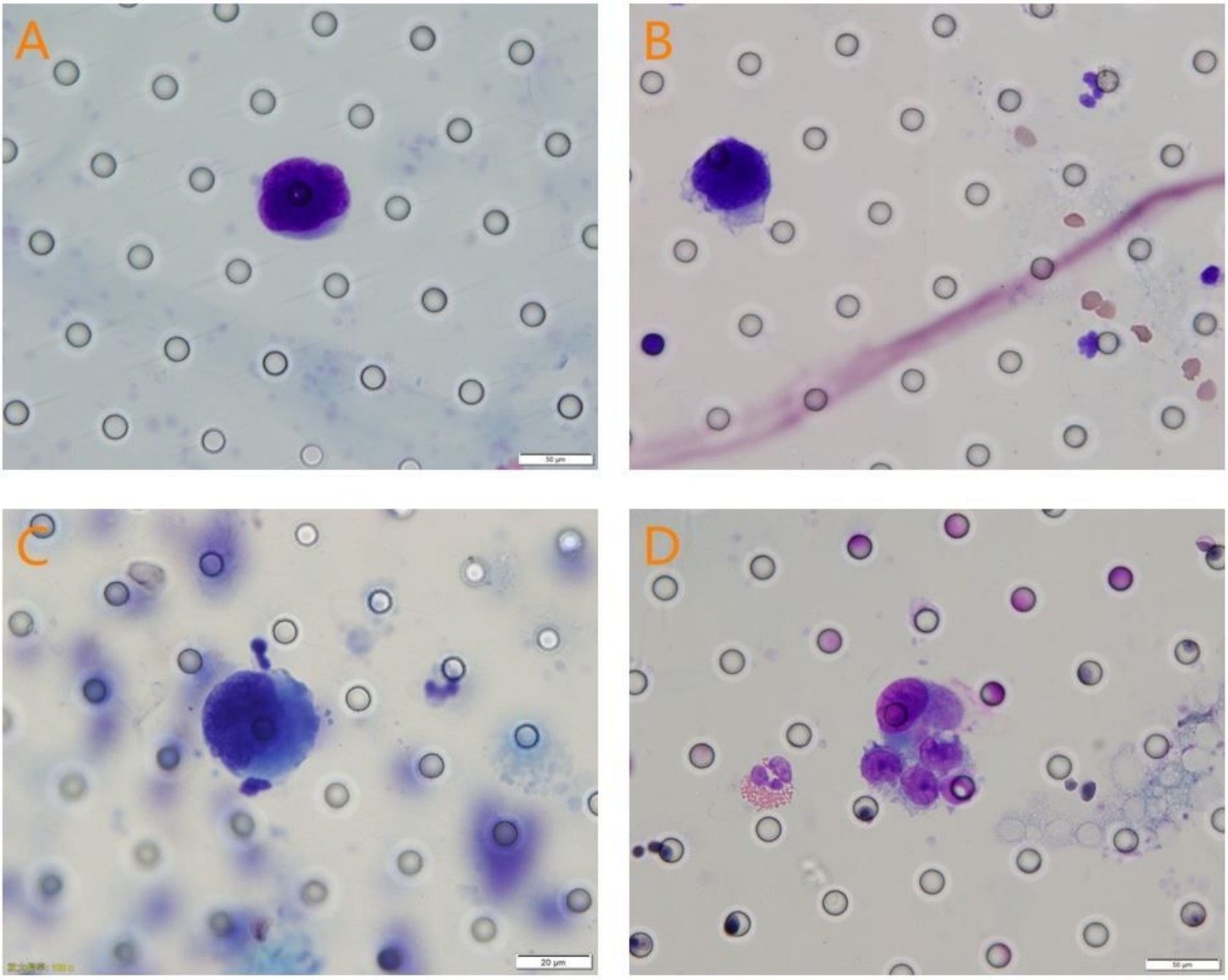
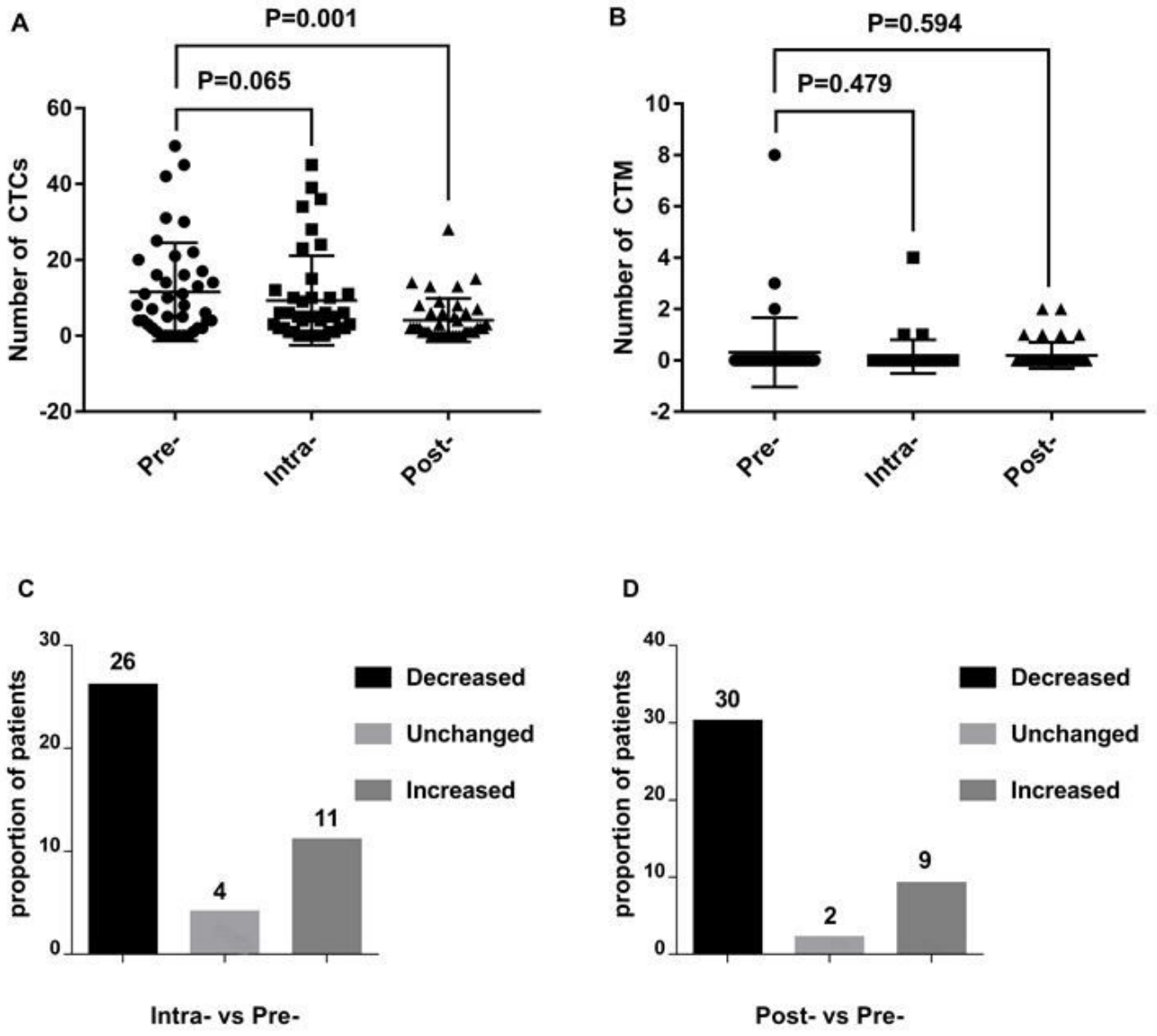


Figure 1

Cell morphology analysis of CTCs / CTM detected in the peripheral blood of RCC patients: A-D was separated by ISET method, and the cells of RCC patients were stained by Diff-Quik showing cell malignant characteristics. CTCs (A-C) : (1) nucleocytoplasmic ratio> 0.8; abnormally large nucleoli; (2) cell nucleus diameter> 18 mm; irregular nucleus shape; (3) thickened jagged and wrinkled nuclear membrane, uneven staining, and lateral shift of chromatin. CTM (D) : Presence of accumulation of tumor cells (≥ 3).



Pre: preoperation (before surgery); Intra: intraoperation (immediately after surgery); Post: postopperation (one week after surgery).

Figure 2

A: Peripheral blood CTC counts in RCC patients before surgery, immediately after surgery and one week after surgery; B: Before surgery, immediately after surgery and one week after surgery peripheral blood CTM counts in RCC patients; C: The incidence of increased, decreased or no change in CTC counts in the same RCC patients immediately after surgery compared to preoperative; D: The incidence of increased, decreased or no change in the counts of CTCs in the same RCC patients one week after surgery compared to preoperative [Pre: preoperation (before surgery); Intra: intraoperation (immediately after surgery); Post: postopperation (one week after surgery).]

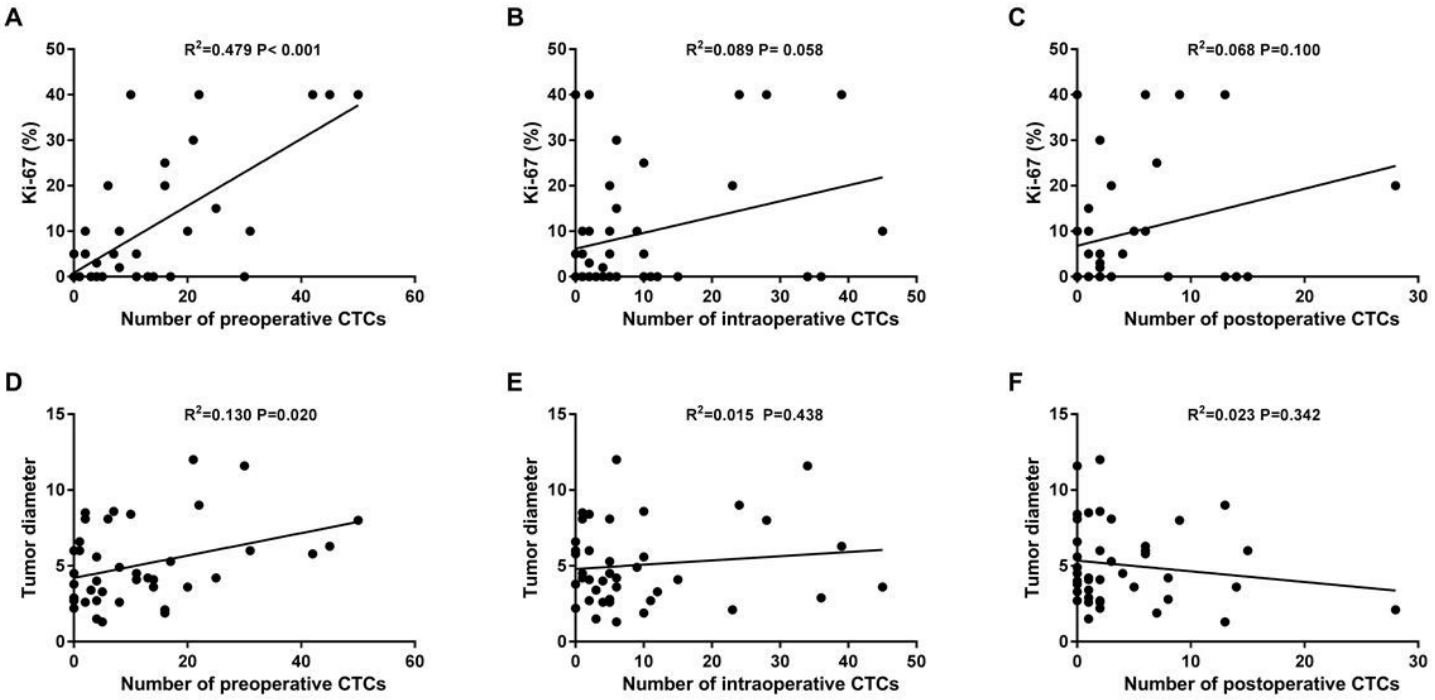


Figure 3

Correlation between number of CTCs and Ki-67and between number of CTCs and tumor diameter. A :Correlation between Ki-67 and number of CTCs before surgery. B: Correlation between Ki-67 and number of CTCs immediately after surgery (number of intraoperative CTCs). C: Correlation between Ki-67 and number of CTCs one week after surgery.D: Correlation between tumor diameter and number of CTCs before surgery. E: Correlation between tumor diameter and number of CTCs immediately after surgery (number of intraoperative CTCs). F: Correlation between tumor diameter and number of CTCs one week after surgery

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.doc
- Supplementarytable2.doc