Am J Transl Res 2020;12(6):2916-2928
www.ajtr.org /ISSN:1943-8141/AJTR0107961

Original Article

Clinical significance of Ki67 and circulating tumor cells with an epithelial-mesenchymal transition phenotype in non-small cell lung cancer

Huajian Peng*, Xiang Tan*, Yongyong Wang, Lei Dai, Guanbiao Liang, Jianji Guo, Mingwu Chen

*Department of Thoracic Surgery, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, People's Republic of China. *Equal contributors and co-first authors.*

Received January 16, 2020; Accepted June 1, 2020; Epub June 15, 2020; Published June 30, 2020

Abstract: Circulating tumor cells (CTCs) are a heterogeneous population of tumor cells with distinct clinical and biological properties. The aim of the present study was to evaluate the relationship between CTCs with the epithelial-mesenchymal transition phenotype (CTC EMT) and the proliferative marker Ki67, and their prognostic value in non-small cell lung cancer (NSCLC). CTCs were isolated from the peripheral blood of 84 NSCLC patients using the CanPatrol™ CTC enrichment method, and the expression of Ki67 in tumor tissues were detected by immunohistochemistry. Almost two-thirds (61/84) of the patients were positive for CTC EMT, and 55 (65.4%) patients had high in-situ expression of Ki67 (≥ 14%) in the tumor tissues. CTC EMT was not significantly associated with tumor size and differentiation, age, gender and histological type, but correlated with lymphatic metastasis, tumor stage and Ki67 overexpression. Furthermore, the CTC EMT+ NSCLC patients had a significantly lower recurrence-free survival (RFS) and overall survival (OS) compared to the negative patients. Similarly, Ki67 levels ≥ 14% were associated with a significantly lower RFS and OS. In conclusion, CTC EMT is significantly related to Ki67 expression, and is a risk factor of NSCLC.

Keywords: Circulating tumor cell, epithelial-mesenchymal, Ki67, non-small-cell lung cancer, survival

Introduction

Lung cancer is the most commonly diagnosed malignancy worldwide and in China, and the leading cause of cancer-related deaths [1, 2]. Non-small cell lung cancer (NSCLC), including squamous cell carcinomas, adenocarcinomas and large cell carcinomas, account for about 85% of all lung cancers [3, 4]. Most lung cancer patients are diagnosed in the advanced stages which precludes surgery. Although novel diagnostic and treatment methods have improved the prognosis of lung cancer patients, reliable biomarkers for early diagnosis need to be explored in order to improve prognosis and treatment outcomes.

Circulating tumor cells (CTCs) are shed from the primary tumor into the circulation and drive cancer metastasis [5]. Studies show that CTCs enter the bloodstream long before metastasis, and may even be released before primary tumor formation [6]. Although most CTCs can be cleared by the host immune system, some with high invasiveness may escape immune surveillance and cause tumor metastasis or recurrence [7]. CTCs are classified into the epithelial, epithelial-mesenchymal transition (EMT) and mixed phenotypes [8], and EMT frequently occurs in the CTCs during tumor progression [9, 10]. The transition of epithelial cells to mesenchymal cells is a key process accompanying normal embryonic development, as well as pathological conditions like tissue fibrosis, tumorigenesis and cancer progression [11, 12]. It is driven by gene expression changes resulting in enhanced mobility and greater invasiveness [13, 14], which are conducive to tumor recurrence and distant metastasis. At the molecular level, EMT is characterized by increased expression of interstitial markers like vimentin and twist [12, 15]. The latter is a key transcription involved in embryonic development [16], organ growth and development,

Table 1. CD45, CK19, and Twist capture probe sequences

	Sequences (5'-3')
CD45	TCGCAATTCTTATGCGACTC
	TGTCATGGAGACAGTCATGT
	GTATTTCCAGCTTCAACTTC
	CCATCAATATAGCTGGCATT
	TTGTGCAGCAATGTATTTCC
	TACTTGAACCATCAGGCATC
CK19	AAGTCATCTGCAGCCAGACG
	CTGTTCCGTCTCAAACTTGG
	TTCTTCTTCAGGTAGGCCAG
	CTCAGCGTACTGATTTCCTC
	CTGTAGGAAGTCATGGCGAG
	AAGTCATCTGCAGCCAGACG
Twist	ACAATGACATCTAGGTCTCC
	CTGGTAGAGGAAGTCGATGT
	CAACTGTTCAGACTTCTATC
	CCTCTTGAGAATGCATGCAT
	TTTCAGTGGCTGATTGGCAC
	TTACCATGGGTCCTCAATAA

tumorigenesis, cell proliferation and differentiation, and is overexpressed in multiple tumors like prostate cancer [17], gastric cancer [18], breast cancer [19] and early resected NSCLC [20]. Recent studies show that CTCs drive progression of gastric cancer [21], hepatocellular carcinoma [22], colorectal cancer [23], prostate cancer [24], and NSCLC [25]. However, the prognostic value of the CTCs with EMT phenotype (CTC EMT) in NSCLC has not yet been evaluated so far.

The proliferation-related protein Ki-67 [26] is expressed in all phases of mitosis but not the G_0 phase [27], and associated with tumor proliferation, invasion, metastasis and prognosis [28, 29]. Ki67 overexpression in cancer cells significantly increases their proliferation, invasiveness and migration [30, 31], making it a suitable biomarker of cancer progression.

The aim of this study was to explore the relationship between CTC EMT and Ki67 in NSCLC, and their respective prognostic values. To this end, we isolated and typed CTCs from NSCLC patients using the advanced CanPatrol™ CTC enrichment method [32] and in situ hybridization, and also analyzed Ki67 expression in the tumor tissues.

Materials and methods

Study population and design

Eighty-four NSCLC patients were enrolled between March 2014 and July 2014 at the First Affiliated Hospital of Guangxi Medical University (Nanning, China). The inclusion criteria were as follows: (i) pathologically confirmed NSCLC, (ii) radical lobectomy and systemic lymph node dissection, (iii) no history of radiotherapy or chemotherapy, (iv) no distant metastasis before surgery, (v) lack of any other tumors, and (vi) availability of complete medical records. Five milliliter peripheral blood was collected from patients within three days after surgery into anticoagulant-coated tubes for CTCs isolation or biochemical assays. The study was approved by the ethical committee of the First Affiliated Hospital of Guangxi Medical College, and all patients provided informed consent.

Isolation of CTCs

The CanPatrol™ CTC enrichment method was used for isolating the CTCs. Briefly, the erythrocytes were first removed using a red blood cell lysis buffer (0.1 mM EDTA, 10 mM $KHCO_3$ and 154 mMNH_4Cl in deionized water), and the remaining cells were resuspended in 4% formaldehyde/PBS for 5 minutes. The cell suspension was then transferred to a filter tube (SurExam, Guangzhou, China) fitted with an 8 μm diameter pore filter (Billerica micropores, USA), and the vacuum pump (Auto Science, Tianjin, China) was set to 0.08 mpa. The E-Z96 vacuum manifold (Omega, Norcross, USA) and plate valve (SurExam, Guangzhou, China) was then switched on for filtering.

Tri-color RNA in situ hybridization (ISH) assay

RNA-ISH was performed as previously described [10] to separate the epithelial, EMT and mixed CTCs. Briefly, the single cells were digested with protease and then hybridized with CD19 and Twist-specific probes (**Table 1**) at 42°C for 2 hours. After washing thrice with 1 ml wash buffer (0.1×SSC; Sigma, St. Louis, USA) to remove the un-bound probes, the samples were incubated with 0.5 fmol preamplifier in 100 μl preamplifier solution (1.5% sodium dodecyl sulfate, 30% horse serum from Sigma and 3 mM Tris-HCl) at 42°C for 20 minutes. The membranes were washed three times with 1 ml

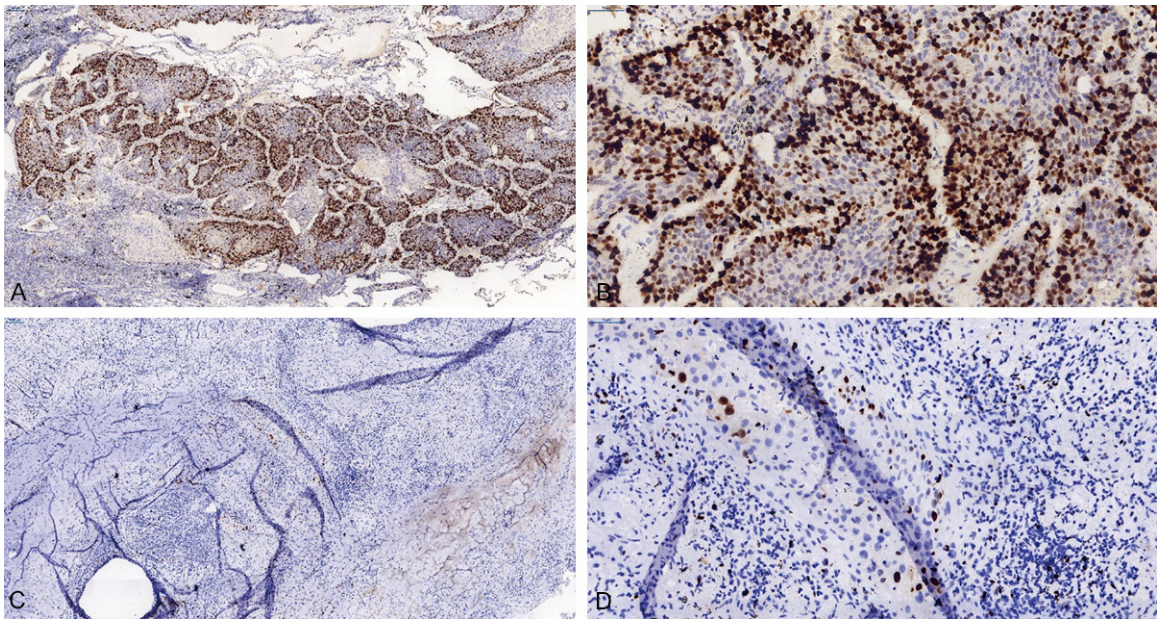

Figure 1. Staining of Ki67 on NSCLC tissue samples, Ki67-positive staining was identified as the presence of brownish-yellow granules in the nucleus. A, B. Ki67 ≥ 14%; C, D. Ki67 < 14%.

wash buffer, and incubated with 1 fmol amplifier in 100 μl of the amplifier solution (same composition as the preamplifier solution, pH 8). The cells were then probed with Alexa Fluor 647-CD45 (leukocyte), Alexa Fluor 594-CD19 (epithelial cells) and Alexa Fluor 488-Twist (mesenchymal cells) at 42°C for 20 minutes. After a final wash with 0.1×SSC, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Olympus BX53, Tokyo, Japan). CTC count of 0 was defined as negative (-), and ≥ 1 as positive (+).

Immunohistochemistry

Tumor tissue sections were deparaffinized, hydrated, rinsed thrice with PBS, and immersed in 3% hydrogen peroxide to quench the endogenous peroxidases. After rinsing thrice with PBS, the sections were incubated with the primary anti-Ki67 antibody provided in the EliVision^{TM}Plus kit for 60 min at room temperature. The slides were washed thrice with PBS, incubated with polymer enhancer (reagent A) for 20 min at room temperature, rinsed again, and incubated with the enzyme-labeled anti-mouse polymer (reagent B) at room temperature for 30 min. Following a final rinse with PBS, the sections were stained with freshly prepared DAB, rinsed with tap water, counterstained with hematoxylin, and differentiated using 0.1% hydrochloric acid. The slides were rinsed again with tap water and PBS, dehydrated through an ethanol gradient, and mounted with neutral resin coverslips. Ki67-positive staining was identified as the presence of brownish-yellow granules in the nucleus, and the samples were graded as Ki67^{lo} or Ki67^{hi} when < 14% and ≥ 14% of the tumor cells respectively stained positive (**Figure 1**) [33]. The samples were graded independently by two pathologists.

Follow-up

All patients were followed up through outpatient review or telephone interviews till July 30, 2019. Recrudesce-free survival (RFS) was defined as the date from surgery to disease recurrence or the last follow-up. Overall survival (OS) was defined as the time from surgery to death for any reason or the last recorded follow-up visit.

Statistical analysis

All statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA) and the figures were prepared using GraphPad Prism version 5.0 (GraphPad software, Inc., La Jolla, CA, USA). The nomogram was plotted using the rms package in R plat-

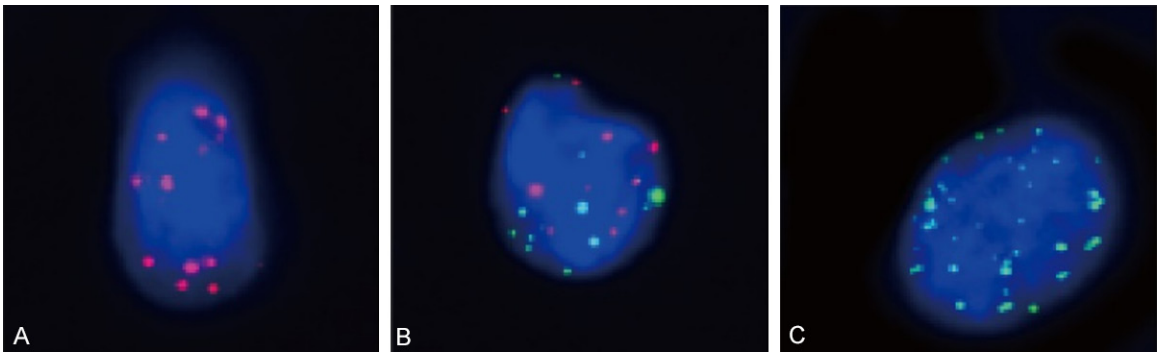

Figure 2. CTCs isolated from NSCLC patients are stained with CK19 (red fluorescence) and Twist (green fluorescence) to distinguish their phenotypes. A. Epithelial cells; B. CTC EMT; C. Mixed cells. Magnification - 100×.

Table 2. Positive expression rate of CTCs in each NSCLC stage n (%)

Stating	Numbers	CTCs	mixed cells	epithelial cells	CTC EMT	Median CTCs	CTCs average	CTCs range
I	30	29 (96.7)	25 (83.3)	17 (57.7)	11 (36.7)	5.00	7.73	0-54
II	20	19 (95.0)	15 (75.0)	15 (75.0)	13 (65.0)	5.00	11.85	0-68
III	25	23 (92.0)	22 (88.0)	8 (32.0)	17 (68.0)	8.00	9.44	0-43
IV	9	9 (100.0)	7 (77.8)	4 (11.1)	8 (88.9)	9.00	9.67	1-21
total	84	80 (95.2)	67 (82.1)	44 (52.4)	61 (72.6)	5.00	9.43	0-68

Abbreviations: CTC, circulating tumor cell.

form (R version 3.5.3, https://www.r-project.org/). Logistic regression was used to estimate odds ratio (OR) and 95% CI in order to evaluate the association between clinical features, CTC EMT and Ki67. Kaplan-Meier survival curves were used to determine RFS and OS in different CTC phenotypes. Univariate and multivariate analysis of CTC EMT, Ki67 and clinical features were performed using the cox proportional regression model. Nomogram was established based on all independent prognostic factors identified by multivariate analysis. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

There were 52 (61.9%) males and 32 (38.1%) females, and the median age of the patients was 59 years (33-79 years). In addition, 30 (35.7%) patients had a history of smoking, whereas 54 (64.3%) were non-smokers. Lymph node metastasis was detected in 38 (45.2%) patients, and 32 patients (38.1%) had large (> 4 cm) tumors. Furthermore, 30 (35.7%), 20 (23.8%), 25 (29.8%) and 9 (10.7%) patients had stage I, II, III and IV tumors respectively. Poorly differentiated tumors were seen in 33 patients (39.3%), while 45 (53.6%) and 6 (7.1%) patients respectively harbored moderately and highly differentiated tumors. Histologically, 24 patients (28.6%) had squamous cell carcinoma and 60 (71.4%) had adenocarcinoma.

CTC count and its association with patient status and pathological features

The CTCs of the different phenotypes isolated from the NSCLC patients are shown in **Figure 2A-C**. The average positive rate of CTCs was 95.9%, and the CTC counts ranged from 0-68 (**Table 2**; **Figure 3A**), with median and average CTCs 5 and 9.42 respectively. The positive rate of CTCs was the lowest in stage III (92%, **Table 2**), and that of epithelial CTCs was highest in stage II (75%, **Table 2**), indicating that the total and epithelial CTC numbers were not associated with the TNM stage. In addition, the positive rates of CTC EMT were 36.7%, 65%, 68% and 88.9% respectively in the stage I, II, III and IV patients (**Table 2**), with significant differences between stage I and stage III ($P = 0.0151$, **Figure 3B**), as well as between stages I and IV ($P = 0.002$, **Figure 3B**). Therefore, NSCLC progression is likely associated with an increase in CTCs undergoing EMT. The relationship between CTC EMT and clinical parameters are

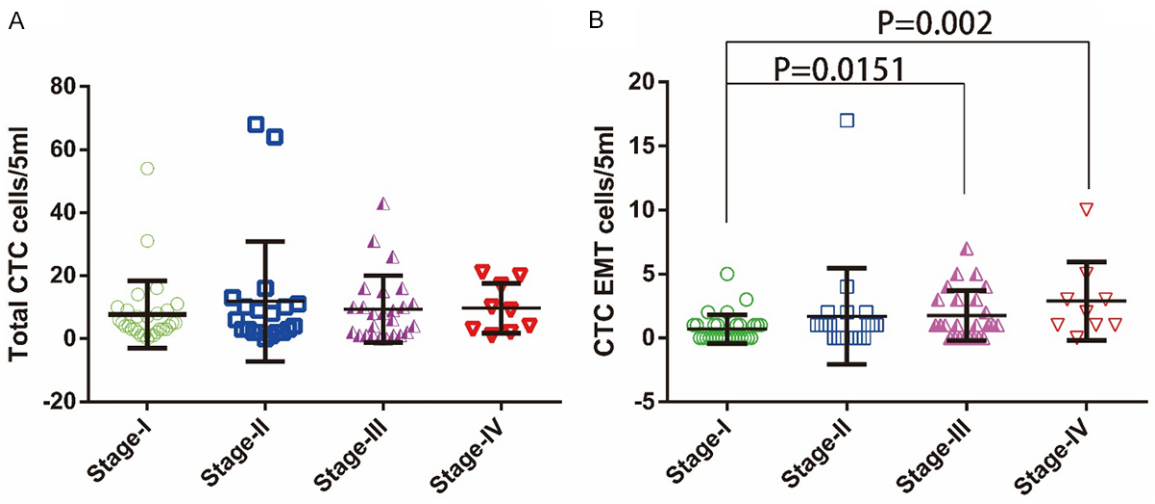

Figure 3. Distribution of CTC count and CTC EMT in NSCLC patients according to tumor stage. (A) CTC counts among the tumor stages (B) CTC EMT counts among the tumor stages (I vs. III, P = 0.0151; I vs. IV, P = 0.002).

Table 3. Association between clinical parameters and CTC EMT

Group	n	CTC EMT positive		OR (95% CI)	*P*-Value
		n	%		
Gender					
Male	52	32	61.5	0.646 (0.264-1.585)	0.341
Female	32	17	53.1		
Age					
≤ 65	60	36	60.0	0.788 (0.303-2.047)	0.625
> 65	24	13	54.2		
Smoking					
Yes	30	20	66.7	1.724 (0.681-4.364)	0.250
No	54	29	53.7		
Lymphatic metastasis					
N-	38	16	42.1	3.490 (1.406-8.664)	**0.007**
N+	46	33	71.7		
Tumor Size, cm					
≤ 4	52	27	50.0	2.556 (0.995-6.562)	0.051
> 4	32	23	71.9		
Stage					
I+II	50	24	48.0	3.009 (1.173-7.723)	**0.022**
II+IV	34	25	73.5		
Differentiated degree					
Poorly	33	19	57.6	1.053 (0.443-2.557)	0.910
Moderately+Well	51	30	58.8		
Pathology					
Squamous cell carcinoma	24	17	70.8	0.471 (0.170-1.300)	0.146
Adenocarcinoma	60	32	53.3		

Note: Bold values indicate statistically significant values. Abbreviations: CTC, circulating tumor cell; CTC EMT, CTCs with epithelial-mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval.

shown in **Table 3**. Patients with stage III/IV tumors had significantly higher numbers of CTC EMT compared to those with stage I/II tumors (P = 0.022). Patients with lymphatic metastasis

Table 4. Association between clinical parameters and Ki67

Group	n	Ki67 positive		OR (95% CI)	*P*-Value
		n	%		
Gender					
Male	52	38	73.1	2.612 (1.028-6.634)	**0.044**
Female	32	17	53.1		
Age					
≤ 65	60	40	66.7	1.200 (0.448-3.215)	0.717
> 65	24	15	62.5		
Smoking					
Yes	30	29	96.7	31.231 (3.966-245.944)	**0.001**
No	54	26	48.1		
Lymphatic metastasis					
N-	38	18	47.4	4.568 (1.736-12.020)	**0.002**
N+	46	37	80.4		
Tumor Size, cm					
≤ 4	52	27	51.9	6.481 (1.991-21.103)	**0.002**
> 4	32	28	87.5		
Stage					
I+II	50	28	56.0	3.031 (1.113-8.250)	**0.030**
II+IV	34	27	79.4		
Differentiated degree					
Moderately+Well	51	28	54.9	3.696 (1.303-10.484)	**0.014**
Poorly	33	27	81.8		
Pathology					
Squamous cell carcinoma	24	19	79.2	2.533 (0.833-7.705)	0.101
Adenocarcinoma	60	36	60.0		

Note: Bold values indicate statistically significant values. Abbreviation: CTC, circulating tumor cell; CTC EMT, CTCs with epithelial-mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval.

also had increased CTC EMT compared to those without metastasis (P = 0.007). In contrast, tumor size (P = 0.051) and differentiation status (P = 0.91), gender (P = 0.604) and smoking habits (P = 0.25) were not significantly associated with CTC EMT.

Ki67 index and its association with patient status and pathological features

Fifty-five patients (65.5%) were $Ki67^{hi}$ according to the in-situ tumor expression levels (**Table 4**). Ki67 expression was significantly associated with the tumor stage (P = 0.03), lymph node metastasis (P = 0.002), tumor size (P = 0.002), tumor differentiation (P = 0.014) and gender (P = 0.044), but not with smoking habits (P = 0.250). In addition, neither CTC EMT positive rate nor Ki67 overexpression was significantly associated with age or tumor histological type (P > 0.05, **Table 4**). The relationship between Ki67 and CTCs phenotypes were also examined (**Table 5**). While the positive rates of total, epithelial and mixed CTCs were not significantly associated with Ki67 expression (P > 0.05), that of CTC EMT was significantly higher in the $Ki67^{hi}$ compared to the $Ki67^{lo}$ groups (70.91%, 39/55 vs 34.48%, 10/29; P = 0.002).

Prognostic significance of CTC EMT and Ki67 in NSCLC

Univariate analysis (**Table 6**) showed that CTC EMT positive rate (log-rank P < 0.001, HR = 2.743. 95% CI = 1.612-4.665), lymphatic metastasis (log-rank P < 0.001, HR = 2.525, 95% CI = 1.499-4.253), tumor size (log-rank P = 0.00, HR = 2.381, 95% CI = 1.435-3.949), smoking (log-rank P = 0.008, HR = 2.008, 95% CI = 1.203-3.352), tumor stage (log-rank P = 0.001, HR = 2.275, 95% CI = 1.376-3.761), Ki67 expression (log-rank P = 0.001, HR =

Table 5. Association between CTCs and Ki67

Group	Ki67		OR (95% CI)	*P*-Value
	High expression	Low expression		
CTCs				
(+)	52	28	1.615 (0.160-16.263)	0.684
(-)	3	1		
Mix cells				
(+)	46	23	0.750 (0.238-2.364)	0.623
(-)	9	3		
Epithelial Cells				
(+)	26	18	1.825 (0.729-4.571)	0.199
(-)	29	11		
CTC EMT				
(+)	39	10	0.216 (0.083-0.565)	**0.002**
(-)	16	19		

Note: Bold values indicate statistically significant values. Abbreviation: CTC, circulating tumor cell; CTC EMT, CTCs with epithelial-mesenchymal transition phenotype; OR, risk ratio; CI, confidence interval.

Table 6. Univariate analysis for recurrence-free survival and overall survival

Variable	Level	RFS		OS	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Gender	Women/men	1.738 (1.020-2.961)	**0.042**	1.711 (0.946-3.095)	0.076
Age	≤ 65/> 65	0.969 (0.555-1.692)	0.912	1.302 (0.720-2.352)	0.382
Smoking	Yes/No	2.008 (1.203-3.352)	**0.008**	2.712 (1.559-4.719)	**< 0.001**
CTC EMT	Yes/Not	2.743 (1.612-4.665)	**< 0.001**	2.236 (1.246-4.014)	**0.007**
Lymphatic metastasis	N0/N+	2.525 (1.499-4.253)	**< 0.001**	2.304 (1.293-4.106)	**0.005**
Tumor Size, cm	≤ 4/> 4	2.381 (1.435-3.949)	**0.001**	2.325 (1.335-4.050)	**0.003**
Stage	I+II/III+IV	2.275 (1.376-3.761)	**0.001**	3.084 (1.765-5.390)	**< 0.001**
Ki67	< 14/≥ 14	2.776 (1.558-4.947)	**0.001**	2.699 (1.406-5.180)	**0.003**
Differentiated degree	Moderately+Well/Poorly	1.915 (1.161-3.160)	**0.011**	1.901 (1.094-3.305)	**0.023**
Pathology	Squamous cell carcinoma/Adenocarcinoma	1.406 (0.813-2.434)	0.223	1.818 (1.022-3.236)	**0.042**

Note: Bold values indicate statistically significant values. Abbreviation: CTC, circulating tumor cell; CTC EMT, CTCs with epithelial-mesenchymal transition phenotype; HR, hazard ratio; CI, confidence interval; RFS, Recurrence-free survival; OS, Overall survival.

2.776, 95% CI = 1.558-4.947), gender (log-rank P = 0.042, HR = 1.738, 95% CI = 1.020-2.961) and degree of tumor differentiation (log-rank P = 0.011, HR = 1.915, 95% CI = 1.161-3.160) were significantly associated with RFS. In addition, the OS was also influenced by CTC EMT (log-rank P = 0.007, HR = 2.236, 95% CI = 1.246-4.014), lymphatic metastasis (log-rank P = 0.005, HR = 2.304, 95% CI = 1.293-4.106), tumor size (log-rank P = 0.003, HR = 2.325, 95% CI = 1.335-4.050), smoking (log-rank P < 0.001, HR = 2.712, 95% CI = 1.559-4.719), tumor staging (log-rank P < 0.001, HR = 3.084, 95% CI = 1.765-5.390), Ki67 levels (log-rank P = 0.003, HR = 2.699, 95% CI = 1.406-5.180), pathological stage (log-rank P = 0.042, HR = 1.818, 95% CI = 1.022-3.236), and degree of tumor differentiation (log-rank P = 0.023, HR = 1.901, 95% CI = 1.094-3.305). However, multivariate analysis (**Table 7**) showed that only the CTC EMT were an independent factor of RFS (log-rank P < 0.001, HR = 2.696, 95% CI = 1.554-4.677) and OS (log-rank P = 0.032, HR = 1.940, 95% CI = 1.060-3.550), indicating that CTC EMT is a prognostic indicator of NSCLC. A nomogram of NSCLC RFS and OS was next established based on the multivariable Cox proportional hazard regression model (**Figure 4A**, **4B**), which indicated that while CTC EMT contributed the most to RFS (**Figure 4A**), its prognostic impact on OS ranked after that of tumor stage and differentiated degree (**Figure 4B**). Taken together, CTC EMT plays a key role in NSCLC progression.

Table 7. Multivariate analysis for recurrence-free survival and overall survival

Variable	RFS		OS	
	HR* (95% CI)	*P*-value*	HR* (95% CI)	*P*-value*
CTC EMT				
(-)	1		1	
(+)	2.696 (1.554-4.677)	**< 0.001**	1.940 (1.060-3.550)	**0.032**
Ki67				
< 14%	1		1	
≥ 14%	1.653(0.827-3.305)	0.155	1.276 (0.574-2.837)	0.550

Notes: *HR and *P*-value for Cox proportional hazard regression model. adjustment by Smoking, Lymphatic metastasis, Tumor Size, Stage, Differentiated degree, Pathology. Bold values indicate statistically significant values. Abbreviation: CTC EMT, CTCs with epithelial-mesenchymal transition phenotype; HR, hazard ratio; CI, confidence interval; RFS, Recurrence-free survival; OS, Overall survival.

During the 60 months of follow-up, 63 patients (75.3%) experienced recurrence, and 51 patients (60.7%) died. The $Ki67^{hi}$ group showed poor RFS (**Figure 5A**, P < 0.001) and OS (**Figure 5B**, P = 0.002) compared to patients with low Ki67 expression. The 5-year RFS rates of the $Ki67^{hi}$ and $Ki67^{lo}$ patients were 44.8% and 14.5% respectively, and the 5-year OS rates were 58.6% and 29.1%. In addition, the patients lacking CTC EMT had significantly longer RFS (**Figure 6A**, P < 0.001) and OS (**Figure 6B**, P = 0.005) compared to the CTC EMT+ patients. The respective 5-year RFS rates were 60% and 14.3%, and the 5-year OS rates were 51.4% and 30.6%. Based on the CTC EMT phenotype (positive or negative) and Ki67 expression levels (≤ 14% or ≥ 14%), the patients were stratified into the $Ki67^{hi}$/CTC EMT+ (39/84, 46.4%), $Ki67^{hi}$/CTC EMT- (16/84, 19%), $Ki67^{lo}$/CTC EMT+ (10/84, 11.9%) and $Ki67^{lo}$/CTC EMT- (19/84, 22.6%) subgroups. As shown in **Figure 7A**, **7B**, the $Ki67^{hi}$/CTC EMT+ patients had significantly worse RFS and OS compared to the other groups (RFS, log-rank P < 0.0001; OS, log-rank P = 0.0006). Taken together, a high frequency of CTC EMT indicates poor prognosis of NSCLC patients, and timely intervention can improve the chances of survival.

Discussion

Since the discovery of CTCs in the peripheral blood of cancer patients, considerable efforts were made to detect and isolate these cells in order to monitor tumor progression and treatment outcomes. However, the clinical application of CTCs was limited by the low purification rates, sensitivity and enrichment of the isolation methods. CanPatrol™ is a second-generation CTC enrichment method based on immuno-capture and nanofiltration. Its high recovery rate and lack of dependence on surface antigens makes it highly suitable for CTCs research and clinical applications. In the current study, we isolated CTCs from the peripheral blood of NSCLC patients using CanPatrol™. The positive rate of CTCs was already high in patients with early stage tumors, indicating that tumor cells entered the circulation early during progression. Furthermore, while the epithelial and mixed CTCs had similar frequencies across the different tumor stages, that of CTC EMT was significantly higher in the advanced versus early stage tumor-bearing patients, as well as in patients with lymphatic metastasis compared to those without. Therefore, the proportion of CTCs undergoing EMT increase during the progression of NSCLC and likely promote distant metastasis, which can affect the prognosis of NSCLC patients.

EMT is accompanied by the degradation of extracellular matrix and basement membrane [9] and loss of junction proteins between cells, which increase tumor cell migration and allows them to metastasize [9, 34]. Consistent with the poor prognostic outcomes of metastasis, the CTC EMT+ patients had worse RFS and OS compared to the CTC EMT- patients, and presence of these CTCs was identified an independent risk factor of NSCLC. Furthermore, CTC EMT was not related to patient age, tumor size, smoking history, tumor type and degree of differentiation, which is consistent with a previous report [34]. Ki67 is a prognostic biomarker in multiple cancers, and its expression was positively correlated with the male sex, lymphatic metastasis, larger tumors (≥ 4 cm), advanced stages (stage III+IV), smoking, and poor tumor differentiation in the NSCLC cohort. Furthermore, the $Ki67^{hi}$ patients had worse RFS and OS, indicating a prognostic role of Ki67 as well in NSCLC. Consistent with previous findings on colorectal cancer [35], there

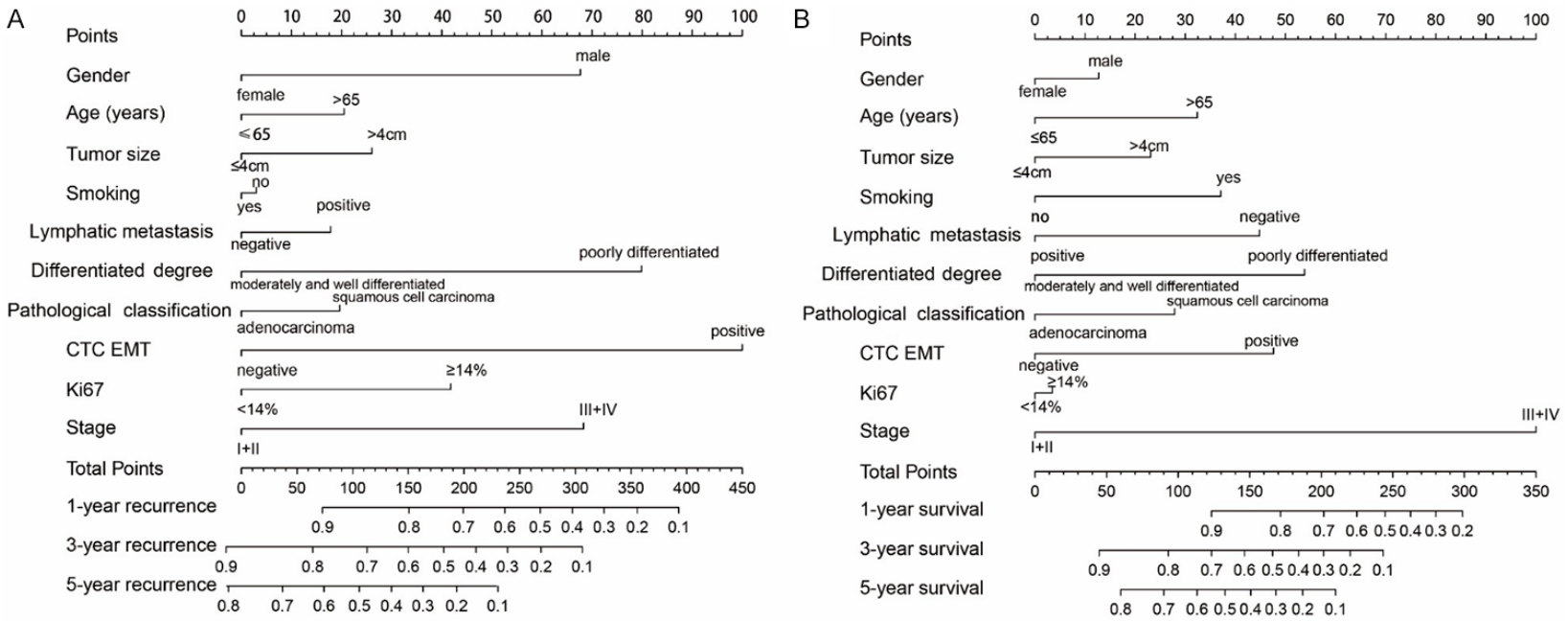

Figure 4. Nomogram module integrating gender, age, tumor size, smoking, lymphatic metastasis, differentiated degree, pathological classification, CTC EMT, Ki67 and stage. The points identified on the top scale for each independent covariate were added to determine the estimated overall survival and the probability of 1-, 3- and 5-year recurrence or survival; (A) RFS of NSCLC patients; (B) OS of NSCLC patients.

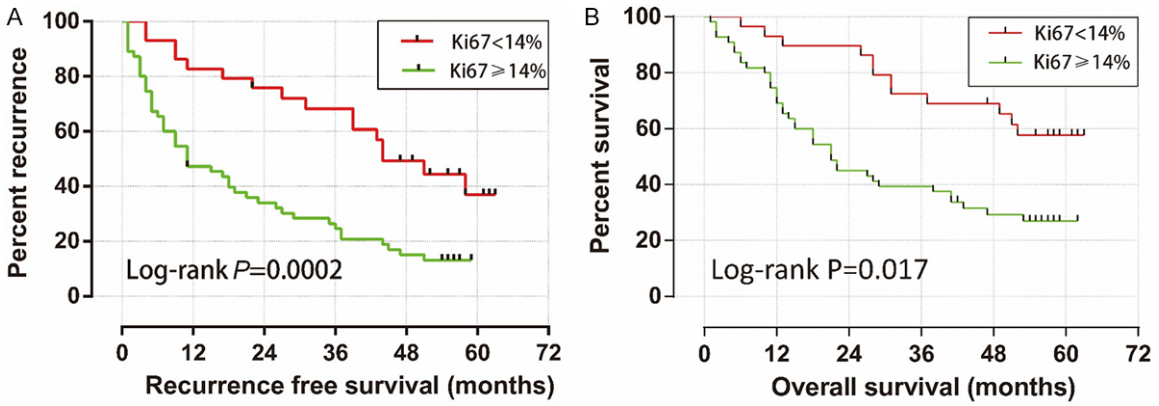

Figure 5. Kaplan-Meier survival curves of patients stratified by Ki67 expression. Patients with Ki67 ≥ 14% had shorter (A) RFS and (B) OS compared to those with Ki67 ≥ 14%.

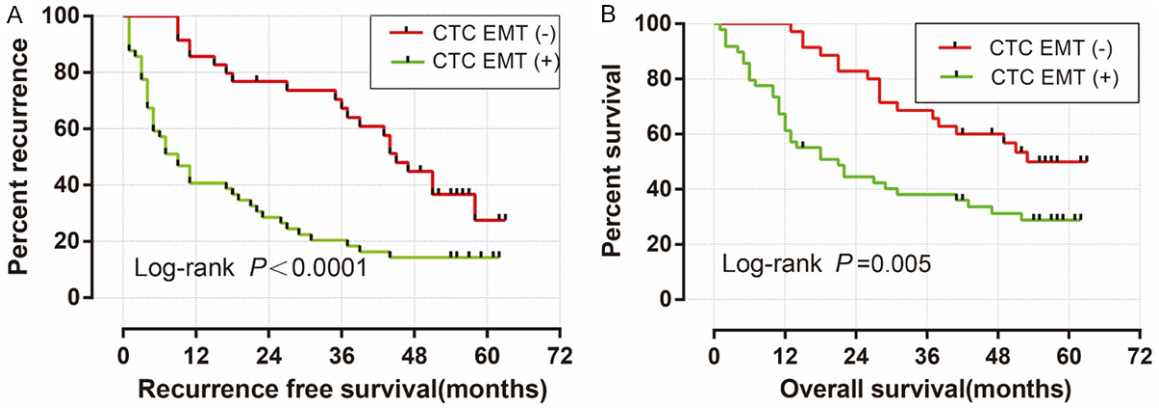

Figure 6. Kaplan-Meier survival curves of patients stratified by CTC EMT. Patients with CTC EMT had shorter (A) RFS and (B) OS compared to patients lacking CTC EMT.

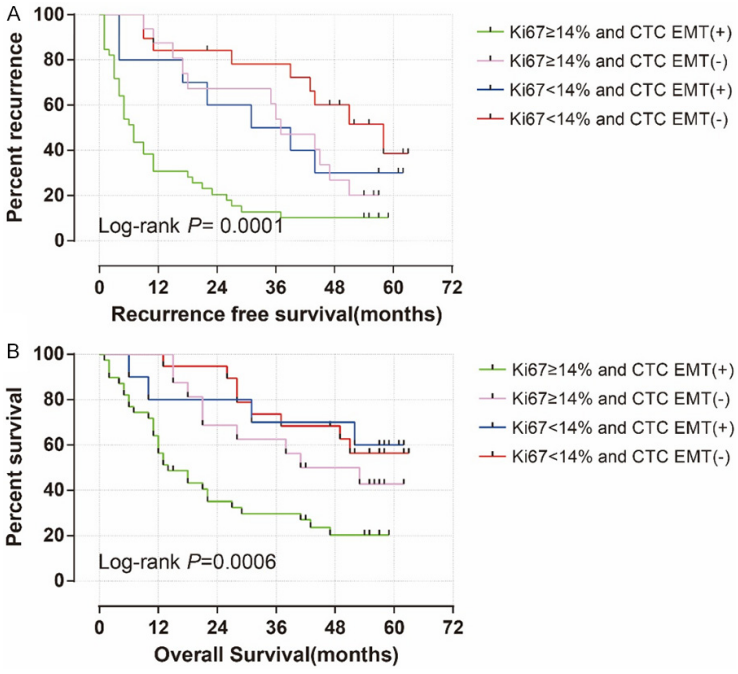

Figure 7. Kaplan-Meier survival curves of patients stratified by CTC EMT and Ki67 expression. Patients with CTC EMT and Ki67 ≥ 14% showed poor (A) RFS and (B) OS compared to those without CTC EMT and Ki67 < 14%.

was no significant correlation between Ki67 expression and CTCs in the NSCLC patients. However, Ki67 overexpression correlated with increased CTC EMT, and the Ki67^{hi}/CTC EMT+ patients had significantly worse prognosis compared to the other groups stratified on the basis of these markers. A previous study showed that the increased Twist levels during EMT of murine mammary epithelial cells pro-

tected them against apoptosis [36]. It is possible therefore that the proliferative effects of Ki67 synergizes with the EMT of NSCLC CTCs to further promote their migration, which translates to increased metastasis and recurrence.

Our study has certain limitations that ought to be addressed. First, the sample size was small and derived from a single center, which prevented subgroup analysis. Furthermore, we did not elucidate the mechanistic basis of the relationship between CTC EMT and Ki67 overexpression. However, we show for the first time that the EMT of CTCs is related to NSCLC prognosis and the proliferation of tumor cells. Early detection of CTC EMT is predictive of poor prognosis, and timely treatment can improve the prognosis and survival of NSCLC patients.

Conclusion

High in situ expression of Ki67 in NSCLC tissues and increased CTC EMT are positively correlated with poor prognosis. The CTC EMT and Ki67 levels can stratify NSCLC patients into four prognostic subgroups. Although the prognostic value of Ki67 needs to be verified, CTC EMT is a reliable prognostic biomarker and its early detection and reduce the mortality in NSCLC patients.

Acknowledgements

The authors thank Prof. Nuo Yang and Prof. Huafu Zhou from the Department of Thoracic Surgery, The First Affiliated Hospital of Guangxi Medical University, for their contributions in manuscript revision. This work was supported in part by the National Natural Science Foundation of China (81660387).

Disclosure of conflict of interest

None.

Address correspondence to: Mingwu Chen, Department of Thoracic Surgery, The First Affiliated Hospital of Guangxi Medical University, Shuang Yong Road 6, Nanning 530021, Guangxi Zhuang Autonomous Region, People's Republic of China. Tel: +86-0771-5356708; Fax: +86-0771-5350031; E-mail: chen535@126.com

References

[1] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68: 394-424.

[3] Matthews MJ, Mackay B and Lukeman J. The pathology of non-small cell carcinoma of the lung. Semin Oncol 1983; 10: 34-55.

[4] Spiro SG, Gould MK and Colice GL; American College of Chest Physicians. Initial evaluation of the patient with lung cancer: symptoms, signs, laboratory tests, and paraneoplastic syndromes: ACCP evidenced-based clinical practice guidelines (2nd edition). Chest 2007; 132: 149S-160S.

[5] Pantel K and Alix-Panabieres C. Circulating tumour cells in cancer patients: challenges and perspectives. Trends Mol Med 2010; 16: 398-406.

[6] Togo S, Katagiri N, Namba Y, Tulafu M, Nagahama K, Kadoya K, Takamochi K, Oh S, Suzuki K, Sakurai F, Mizuguchi H, Urata Y and Takahashi K. Sensitive detection of viable circulating tumor cells using a novel conditionally telomerase-selective replicating adenovirus in non-small cell lung cancer patients. Oncotarget 2017; 8: 34884-34895.

[7] Wu XL, Tu Q, Faure G, Gallet P, Kohler C and Bittencourt Mde C. Diagnostic and prognostic value of circulating tumor cells in head and neck squamous cell carcinoma: a systematic review and meta-analysis. Sci Rep 2016; 6: 20210.

[8] Tsongalis GJ. Branched DNA technology in molecular diagnostics. Am J Clin Pathol 2006; 126: 448-453.

[9] Hollier BG, Evans K and Mani SA. The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies. J Mammary Gland Biol Neoplasia 2009; 14: 29-43.

[10] Wu S, Liu S, Liu Z, Huang J, Pu X, Li J, Yang D, Deng H, Yang N and Xu J. Classification of circulating tumor cells by epithelial-mesenchymal transition markers. PLoS One 2015; 10: e0123976.

[11] Kalluri R and Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest 2003; 112: 1776-1784.

[12] Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002; 2: 442-454.

[13] Arrazubi V, Mata E, Antelo ML, Tarifa A, Herrera J, Zazpe C, Teijeira L, Viudez A, Suarez J, Hernandez I and Vera R. Circulating tumor cells in patients undergoing resection of colorectal

cancer liver metastases. clinical utility for long-term outcome: a prospective trial. Ann Surg Oncol 2019; 26: 2805-2811.
[14] Gheldof A and Berx G. Cadherins and epithelial-to-mesenchymal transition. Prog Mol Biol Transl Sci 2013; 116: 317-336.
[15] Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. J Clin Invest 2009; 119: 1417-1419.
[16] Yu W, Kamara H and Svoboda KK. The role of twist during palate development. Dev Dyn 2008; 237: 2716-2725.
[17] Cho KH, Choi MJ, Jeong KJ, Kim JJ, Hwang MH, Shin SC, Park CG and Lee HY. A ROS/STAT3/HIF-1alpha signaling cascade mediates EGF-induced TWIST1 expression and prostate cancer cell invasion. Prostate 2014; 74: 528-536.
[18] Zhang H, Gong J, Kong D and Liu HY. Anti-proliferation effects of Twist gene silencing in gastric cancer SGC7901 cells. World J Gastroenterol 2015; 21: 2926-2936.
[19] Paterlini-Brechot P and Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. Cancer Lett 2007; 253: 180-204.
[20] Jiang W, Pang XG, Wang Q, Shen YX, Chen XK and Xi JJ. Prognostic role of Twist, Slug, and Foxc2 expression in stage I non-small-cell lung cancer after curative resection. Clin Lung Cancer 2012; 13: 280-287.
[21] Cheng B, Tong G, Wu X, Cai W, Li Z, Tong Z, He L, Yu S and Wang S. Enumeration and characterization of circulating tumor cells and its application in advanced gastric cancer. Onco Targets Ther 2019; 12: 7887-7896.
[22] Ye X, Li G, Han C, Han Q, Shang L, Su H, Han B, Gong Y, Lu G and Peng T. Circulating tumor cells as a potential biomarker for postoperative clinical outcome in HBV-related hepatocellular carcinoma. Cancer Manag Res 2018; 10: 5639-5647.
[23] Nanduri LK, Hissa B, Weitz J, Scholch S and Bork U. The prognostic role of circulating tumor cells in colorectal cancer. Expert Rev Anticancer Ther 2019; 19: 1077-1088.
[24] de Kruijff IE, Sieuwerts AM, Onstenk W, Kraan J, Smid M, Van MN, van der Vlugt-Daane M, Hoop EO, Mathijssen RHJ, Lolkema MP, de Wit R, Hamberg P, Meulenbeld HJ, Beeker A, Creemers GJ, Martens JWM and Sleijfer S. Circulating tumor cell enumeration and characterization in metastatic castration-resistant prostate cancer patients treated with cabazitaxel. Cancers (Basel) 2019; 11: 1212.
[25] Das M, Riess JW, Frankel P, Schwartz E, Bennis R, Hsieh HB, Liu X, Ly JC, Zhou L, Nieva JJ, Wakelee HA and Bruce RH. ERCC1 expression in circulating tumor cells (CTCs) using a novel detection platform correlates with progression-free survival (PFS) in patients with metastatic non-small-cell lung cancer (NSCLC) receiving platinum chemotherapy. Lung Cancer 2012; 77: 421-426.
[26] Coates PJ, Hobbs RC, Crocker J, Rowlands DC, Murray P, Quinlan R and Hall PA. Identification of the antigen recognized by the monoclonal antibody BU31 as lamins A and C. J Pathol 1996; 178: 21-29.
[27] Scholzen T and Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000; 182: 311-322.
[28] Xie S, Liu Y, Qiao X, Hua RX, Wang K, Shan XF and Cai ZG. What is the prognostic significance of Ki-67 positivity in oral squamous cell carcinoma? J Cancer 2016; 7: 758-767.
[29] Klapper W, Hoster E, Determann O, Oschlies I, van der Laak J, Berger F, Bernd HW, Cabecadas J, Campo E, Cogliatti S, Hansmann ML, Kluin PM, Kodet R, Krivolapov YA, Loddenkemper C, Stein H, Moller P, Barth TE, Muller-Hermelink K, Rosenwald A, Ott G, Pileri S, Ralfkiaer E, Rymkiewicz G, van Krieken JH, Wacker HH, Unterhalt M, Hiddemann W and Dreyling M; European MCL Network. Ki-67 as a prognostic marker in mantle cell lymphoma-consensus guidelines of the pathology panel of the European MCL Network. J Hematop 2009; 2: 103-111.
[30] Mitchell KG, Parra ER, Nelson DB, Zhang J, Wistuba II, Fujimoto J, Roth JA and Antonoff MB; MD Anderson Lung Cancer Immune Microenvironment Working Group. Tumor cellular proliferation is associated with enhanced immune checkpoint expression in stage I non-small cell lung cancer. J Thorac Cardiovasc Surg 2019; 158: 911-919.
[31] Martin B, Paesmans M, Mascaux C, Berghmans T, Lothaire P, Meert AP, Lafitte JJ and Sculier JP. Ki-67 expression and patients survival in lung cancer: systematic review of the literature with meta-analysis. Br J Cancer 2004; 91: 2018-2025.
[32] Wu S, Liu Z, Liu S, Lin L, Yang W and Xu J. Enrichment and enumeration of circulating tumor cells by efficient depletion of leukocyte fractions. Clin Chem Lab Med 2014; 52: 243-251.
[33] Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B and Senn HJ; Panel members. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen international expert consensus on the primary therapy of early breast cancer 2011. Ann Oncol 2011; 22: 1736-1747.
[34] Mego M, Karaba M, Minarik G, Benca J, Silvia J, Sedlackova T, Manasova D, Kalavska K, Pindak D, Cristofanilli M, Reuben JM and Mardiak J. Circulating tumor cells with epithelial-to-mesenchymal transition phenotypes associat-

ed with inferior outcomes in primary breast cancer. Anticancer Res 2019; 39: 1829-1837.
[35] Yang Y, Li J, Jin L, Wang D, Zhang J, Wang J, Zhao X, Wu G, Yao H and Zhang Z. Independent correlation between Ki67 index and circulating tumor cells in the diagnosis of colorectal cancer. Anticancer Res 2017; 37: 4693-4700.
[36] Robson EJ, Khaled WT, Abell K and Watson CJ. Epithelial-to-mesenchymal transition confers resistance to apoptosis in three murine mammary epithelial cell lines. Differentiation 2006; 74: 254-264.