Effect of Napsin A and Circulating Tumor Cells with Mesenchymal Phenotype in Lung Adenocarcinoma

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Research

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Abstract

Objective: To evaluate the clinical significance of Napsin A and circulating tumor cells（CTCs）with mesenchymal phenotype (M-CTC) in lung adenocarcinoma(LUAD).

Materials and Methods: Clinical data of 97 LUAD patients were retrieved. The CanPatrol^{TM} CTC enrichment platform was used to isolate CTCs from the peripheral blood of LUAD patients. The protein expression of Napsin A in the tumor tissues was analyzed by immunohistochemistry.

Results: 20 of the 97 patients (20.62%) were negative expression of Napsin A （Napsin A-）and 60 (61.86%) were M-CTC positive（M-CTC+). Both Napsin A expression (P=0.004) and M-CTC+ (P<0.001) showed significant correlation to lymphatic metastasis, and M-CTC+ was also significantly correlated with the tumor stage (P=0.009) but was not correlated with gender, age, smoking, tumor size and degree of differentiation. Furthermore, the Napsin A- patients had a higher positive rate of M-CTC. In addition, the recurrence-free survival (RFS; Log-rank P <0.001) and overall survival (OS; Log-rank P <0.001) of the M-CTC+ LUAD patients were significantly worse. Likewise, Napsin A- was also associated with poor RFS (Log-rank P <0.001) and OS (Log-rank P = 0.0003).

Conclusion: LUAD patients with Napsin A- have a higher frequency of M-CTC+, and the Napsin A- and M-CTC+ status portends poor prognosis.

Introduction

Lung cancer is associated with high morbidity and mortality rates worldwide and in China, and its incidence rate is increasing annually[1,2]. Non-small cell lung cancer (NSCLC) accounts for about 85% of all lung cancers, and includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma3,4, of which lung adenocarcinoma (LUAD) is the most common. Despite early detection of LUAD with low-dose spiral computed tomography (CT), patient prognosis has not improved. Therefore, there is an urgent need to identify novel prognostic markers of LUAD in order to treat patients at high risk and reduce their mortality rates.

Ashworth first proposed the concept of circulating tumor cells (CTCs)5 in 1869. CTCs are shed from the primary tumor and enter circulation, eventually seeding into distant organs and giving rise to metastatic tumors[6]. Although most CTCs are recognized and eliminated by the host immune system, a rare population of highly aggressive CTCs can evade the immune cells and cause distant metastasis[7]. There is also evidence that CTCs enter the blood circulation before metastasis[8]. CTCs can be divided into the epithelial (E-CTC), mixed (E/M-CTC) and mesenchymal (M-CTC) subtypes[9]. During epithelial-mesenchymal transition (EMT), tumor cells lose cell-to-cell contact and polarity, and undergo major cytoskeletal changes that endow them with greater mobility and invasiveness[10,11]. EMT not only increases the survival and metastatic abilities of CTCs[12] but is also crucial to chemoresistance and immune evasion[13]. Studies show that an increased frequency of CTCs with the EMT phenotype portends poor prognosis and greater aggressiveness of gastric cancer[14], colorectal cancer[15], liver cancer[16] and esophageal cancer[17].

Many techniques have been reported for the isolation and characterization of CTCs based on the surface antigen expression or physical properties of CTCs[18]. For example, the commonly used CellSearch system is the only method approved by the FDA[19]. As mentioned above, CTCs with epithelial mesenchymal transition will lose epithelial markers completely or partially[20]. And M-CTC will become more invasive, so it is necessary to identify M-CTC. Previous studies have also confirmed that CanPatrol™ has a strong ability to capture CTCs[21], and can be classified into three subgroups by RNA in situ hybridization. Compared with other techniques, CanPatrol™ can identify and classify all CTCs subpopulations, which have been widely used[22].

Napsin A is an aspartic protease primarily expressed in the lungs and kidneys[23]. It is an immunohistochemical marker of LUAD along with thyroid transcription factor-1 (TTF-1)[24], and promotes lung maturation by lysing a preform of surfactant protein B expressed in type II alveolar epithelial cells[25,26]. Napsin A also inhibits the growth of tumor cells by a mechanism independent of its catalytic activity[27], and also affects malignant transformation[28,29]. In addition, Napsin A may have function in the differentiation of epithelial cells[27]. Consistent with this, low Napsin A expression in LUAD patients is associated with poor prognosis, although the specific mechanism is not clear[30,31].

The aim of this study was to determine the prognostic value of M-CTC and Napsin A in LUAD, and their relationship. To this end, we isolated and typed CTCs from LUAD patients using the CanPatrol™ enrichment platform[32] and RNA in situ hybridization (ISH) respectively, and also evaluated Napsin A expression in tumor tissues.

Materials And Methods

Study population and design

Ninety-seven LUAD patients admitted to the First Affiliated Hospital of Guangxi Medical University (Nanning, China) from March 2014 to July 2015 were enrolled. The inclusion criteria were as follows: (i) pathologically confirmed LUAD post-surgery, (ii) underwent radical lobectomy and systemic lymph node dissection, (iii) no history of targeted therapy, radiotherapy and chemotherapy, (iv) no distant metastasis confirmed before surgery, (v) no history of other tumors, and (vi) availability of complete test results and medical records. Within three days of surgical resection, 5 ml peripheral blood was collected from the LUAD patients into anticoagulant-coated test tubes for CTCs enrichment and biochemical analysis. The ethics committee of the First Affiliated Hospital of Guangxi Medical University approved the study, and all patients provided written informed consent.

Isolation of CTCs

The CanPatrol^{TM}[33] CTC enrichment platform was used to isolate CTCs from peripheral blood samples. Briefly, the red blood cells (RBCs) were first removed with an RBC lysis buffer, and the plasma was filtered through an 8μm pore size membrane.

Tri-color RNA *in situ* hybridization (ISH) assay

RNA-ISH was performed as previously described[22] to separate the E-CTC, E/M-CTC and M-CTC. Briefly, the single cells were digested with protease (MedChemExpress, USA) and then hybridized with CD45 (leukocyte biomarker, Table S1), EpCAM, CK8/18/19 (epithelial biomarkers, Table S1), vimentin and Twist (mesenchymal biomarkers, Table S1) at 42℃ for 2 hours. The hybridization method is as described above. We used 1 ml wash buffer (0.1×SSC; Sigma, St. Louis, USA) to wash samples to remove the un-bound probes. Then we putted 0.5 fmol preamplifier and samples in 100μl preamplifier solution (1.5% sodium dodecyl sulfate, 30% horse serum from Sigma and 3mM Tris-HCl) at 42°C for 20 minutes. Then, we used 0.1×SSC to wash membrane. Then we putted it in 100μl of the amplifier solution (same composition as the preamplifier solution, pH 8) with 1 fmol amplifier. We used Alexa Fluor 647-CD45 (leukocyte), Alexa Fluor 594-CD19 (epithelial cells) and Alexa Fluor 488-Twist (mesenchymal cells) to probe cells at 42°C for 20 minutes. Finally, we used 0.1×SSC to wash the cells. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma, USA), and the cells were analyzed with fluorescence microscope (Olympus BX53, Tokyo, Japan). CTCs count of 0 was defined as negative (-), and ≥1 as positive (+).

Immunohistochemistry

Tissue specimens were fixed in 10% neutral buffered formalin, embedded with paraffin and cut into 3 mm thick sections. The tissue sections were immersed in citrate buffer (pH 6) and heated in an incubator for 20 minutes for antigen retrieval, and then incubated with a monoclonal mouse anti-human Napsin antibody (clone IP64, 1:100; Leica). The color was developed using diaminobenzidine, followed by counterstaining using hematoxylin. The sections were scored as Napsin A positive or negative based on cytoplasmic immunostaining[34].

Follow-up

All patients were followed up through telephone interviews or outpatient review until July 31, 2020. Recurrence-free survival (RFS) was defined as the time from surgery to disease recurrence or the last follow-up, and overall survival (OS) from the time from surgery to death for any reason or the last recorded follow-up visit.

Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA), and the figures were drawn using GraphPad Prism version 5.0 (GraphPad software, Inc., La Jolla, CA, USA). Logistic regression was used to estimate odds ratio (OR) and 95% CI in order to evaluate the association between clinical features, M-CTC and Napsin A. RFS and OS in patients with different CTCs phenotypes and Napsin A expression were determined by the Kaplan-Meier method. Univariate and multivariate analysis of M-CTC, Napsin A and clinical features were performed using the cox proportional regression model, and a nomogram was plotted based on the multivariate model using the rms package in R platform (R version 3.5.3, https://www.r-project.org/). $P<0.05$ was considered statistically significant.

Results

Patient characteristics

A total of 97 patients diagnosed with LUAD were enrolled from March 2014 to August 2015. There were 62 (63.9%) males and 35 females (36.1%) with median age 58 years (31–77 years). In addition, 35 patients (36.1%) had a history of smoking, and 52 (53.6%) had lymph node metastasis, and 36 (37.1%) harbored larger tumors (> 4 cm). There were 34 (35.1%), 23 (23.7%), 26 (26.8%) and 14 (14.4%) patients with stage I, II, III and IV tumors respectively. Forty-four patients (45.4%) had moderately differentiated tumors, 12 (12.4%) had well-differentiated tumors, and 41 (42.3%) had poorly differentiated tumors.

CTC counts and correlation to patient status and pathological features

The CTCs of different phenotypes are shown in Fig. 1A-C. The positive rate of CTCs was 96.91% (0 to 73) in the entire cohort, the median of CTCs was 6 and the average of CTCs values was 10.29 ± 13.43 (Table 1). The number of CTCs increased with tumor stage progression (P = 0.0340 between stage I and IV, Fig. 2A). The number and positive rate of M-CTC also increased with the stage, and were respectively 41.18%, 65.22%, 76.92% and 78.57% in stages I, II, III and IV (P = 0.0133 between stage I and II, P = 0.0015 between stage I and III, P = 0.0026 between stage I and IV, Fig. 2B). However, the frequencies of E/M-CTC and E-CTC were not affected by LUAD progression. In addition, M-CTC showed significant correlation with lymphatic metastasis (P < 0.001, OR = 5.100, 95% CI = 2.094–12.426, Table 2) and stage (P = 0.009, OR = 3.326, 95% CI = 1.344–8.227, Table 2).

Table 1
Positive expression rate of CTCs in each NSCLC stage n(%)

Stating	Numbers	CTCs	E/M-CTCs	E-CTCs	M-CTCs	Median CTCs	CTCs average	CTCs range
I	34	32(94.12)	27(79.41)	20(58.82)	14(41.18)	4.00	7.21	0–39
II	23	22(95.65)	17(73.91)	16(69.57)	15(65.22)	6.00	11.70	0–68
III	26	26(100.00)	23(88.46)	8(30.77)	20(76.92)	7.50	10.23	1–49
IV	14	14(100.00)	12(85.71)	8(57.14)	11(78.57)	13.50	15.57	1–73
total	97	94(96.91)	79(81.44)	52(53.61)	60(61.86)	6.00	10.29	0–73

Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial-mesenchymal transition phenotype; E-CTC, CTCs with epithelial phenotype; E/M-CTC, CTCs with mixed phenotypes; OR ,risk ratio; CI ,confidence interval.

Table 2
Association between clinical parameters and M-CTC

Group		M-CTC positive		OR(95%CI)	*P*-Value
	n	n	%		
Gender					
Female	35	20	57.14	1.364 (0.584–3.184)	0.473
Male	62	40	64.52		
Age					
≤65	72	47	65.28	0.578(0.229–1.450)	0.242
>65	25	13	52.00		
Smoking					
No	62	36	58.06	1.576(0.858–3.776)	0.308
Yes	35	24	68.57		
Lymphatic metastasis					
N-	45	19	42.22	5.100(2.094–12.426)	**<0.001**
N+	52	41	78.85		
Tumor Size, cm					
≤ 4	61	35	57.38	1.688(0.706–4.038)	0.239
>4	36	25	69.44		
Stage					
I + II	57	29	50.88	3.326(1.344–8.227)	**0.009**
II + IV	40	31	75.50		
Differentiated degree					
Moderately + Well	56	32	57.14	1.615(0.694–3.758)	0.266
Poorly	41	28	68.29		
Note: Bold values indicate statistically significant values.					
Abbreviations: CTC, circulating tumor cell; M-CTC, CTCs with epithelial-mesenchymal transition phenotype; OR ,risk ratio; CI ,confidence interval.					

Napsin A expression and correlation with patient status and pathological features

Twenty patients (20.62%) did not express Napsin A in the tumor tissues (Fig. 3). As shown in Table 3, Napsin A expression correlated significantly to lymphatic metastasis (P = 0.004, OR = 0.147, 95%CI = 0.040–0.543)

but not with gender, age, tumor size, stage, differentiated degree and smoking. In addition, the Napsin A- patients had a greater frequency of M-CTC compared to the Napsin A + patients (Fig. 4). The positive rate of M-CTC was also higher in the Napsin A- versus Napsin A + patients (P = 0.010, OR = 0.133, 95%CI = 0.028–0.620), whereas that of E/M-CTC and E-CTC were not significantly different between the two groups (Table 4).

Table 3
Association between clinical parameters and Napsin A

Group		Napsin A positive		OR(95%CI)	*P*-Value
	n	n	%		
Gender					
Female	35	28	80.00	0.942(0.337–2.638)	0.910
Male	62	49	79.03		
Age					
≤65	72	55	76.39	2.267(0.604–8.512)	0.225
>65	25	22	88.00		
Smoking					
Yes	62	51	82.26	0.623(0.229–1.693)	0.354
No	35	26	74.29		
Lymphatic metastasis					
N-	45	35	77.78	0.147(0.040–0.543)	**0.004**
N+	52	42	80.77		
Tumor Size, cm					
≤ 4	61	48	78.69	1.122(0.401–3.137)	0.826
>4	36	29	80.56		
Stage					
I + II	57	47	82.46	0.638(0.237–1.716_	0.374
II + IV	40	30	75.00		
Differentiated degree					
Moderately + Well	56	42	75.00	1.944(0.676–5.592)	0.217
Poorly	41	35	85.37		
Note: Bold values indicate statistically significant values.					

Table 4
Association between CTCs and Napsin A

	Napsin A			
Group	Positive(n)	Negative(n)	OR(95%CI)	*P*-Value
E/M-cells				
(+)	61	18	0.487(0.095–2.489)	0.487
(-)	16	2		
M-Cells				
(+)	42	18	0.133(0.028–0.620)	**0.010**
(-)	35	2		
E-CTC				
(+)	40	12	0.712(0.247–2.054)	0.530
(-)	37	8		
Note: Bold values indicate statistically significant values.				
Abbreviation: CTC, circulating tumor cell; M-CTC, CTCs with epithelial-mesenchymal transition phenotype; E-CTC, CTCs with epithelial phenotype; E/M-CTC, CTCs with epithelial/mesenchymal phenotype; OR ,risk ratio; CI ,confidence interval.				

Prognostic Significance Of M-ctcs And Napsin A In Luad

All patients were followed up for at least 60 months, during which 73 (75.26%) patients relapsed and 59 (60.82%) died.
The M-CTC + patients had worse RFS (P < 0.0001, Fig. 5A) and OS (P < 0.0001, Fig. 5B) compared to the M-CTC- patients. Likewise, the Napsin A- patients also showed worse RFS (P < 0.0001, Fig. 6A) and OS (P = 0.0003, Fig. 6B) compared to the Napsin + group. The patients are divided into the M-CTC+/Napsin A- (18/97, 18.56%), M-CTC-/Napsin A- (2/97, 2.06%), M-CTC+/Napsin A+ (42/97, 43.3%) and M-CTC-/Napsin A+ (35/97, 36.08%) subgroups, of which M-CTC+/Napsin A- patients had the worst RFS (P < 0.0001, Fig. 7A) and OS (P < 0.0001, Fig. 7B).

Univariate analysis (Table 5) showed that gender (P = 0.032, HR = 1.697. 95% CI = 1.034–2.785), smoking (P = 0.035, HR = 1.660, 95% CI = 1.036–2.659), M-CTC (P□0.001, HR = 2.866, 95%CI = 1.722–4.771), lymph node metastasis (P < 0.001, HR = 3.377, 95%CI = 2.054–5.553), tumor size (P = 0.007, HR = 1.904, 95%CI = 1.192–3.042), stage (P = 0.007, HR = 1.905, 95%CI = 1.196–3.035), Napsin A (P□0.001, HR = 0.321, 95%CI = 0.186–0.555) and degree of differentiation (P = 0.010, HR = 1.850, 95%CI = 1.162–2.946) was significantly correlated to RFS. OS was influenced by gender (P = 0.041, HR = 1.803. 95% CI = 1.026–3.171), smoking (P = 0.004, HR = 2.146, 95% CI = 1.282–3.591), M-CTC (P < 0.001, HR = 3.289, 95%CI = 1.798–6.014), lymph node metastasis (P < 0.001, HR = 2.681, 95%CI = 1.557–4.620), tumor size (P = 0.002, HR = 2.253, 95%CI = 1.347–

3.769), stage (P < 0.001, HR = 2.530, 95%CI = 1.510–4.238), Napsin A (P < 0.001, HR = 0.364, 95%CI = 0.206–0.643) and degree of differentiation (P = 0.004, HR = 2.115, 95%CI = 1.262–3.545). According to the multivariate analysis (Table 6), We found that the RFS of patients with M-CTC positive was shorter than that of patients with M-CTC negative, and the difference was statistically significant (P = 0.009, HR = 2.105, 95%CI = 1.206–3.676), and the RFS of patients with negative expression of Napsin A was shorter than that of patients with positive, and the difference was statistically significant (P = 0.032, HR = 0.507, 95%CI = 0.272–0.943). In addition, M-CTC-positive patients had shorter overall survival than negative patients (P = 0.010, HR = 2.319, 95%CI = 1.218–4.418), and patients with negative expression of Napsin A had worse overall survival (P = 0.046, HR = 0.504, 95%CI = 0.257–0.988). Therefore, the results of these studies indicate that positive expression of M-CTC and negative expression of Napsin A can indicate poor prognosis in patients with LUAD, and can be used as A potential marker for diagnosis, identification and prognosis evaluation of lung adenocarcinoma. Based on the multivariate Cox proportional hazard regression model, we established a nomogram on the OS and RFS of LUAD (Fig. 8A-B).

Table 5
Univariate analysis for recurrence-free survival and overall survival

Variable	Level	RFS		OS	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Gender	female/male	1.697(1.034–2.785)	**0.032**	1.803(1.026–3.171)	**0.041**
Age	≤ 65/>65	1.078(0.639–1.818)	0.779	1.215(0.684–2.158)	0.506
Smoking	Yes/No	1.660(1.036–2.659)	**0.035**	2.146(1.282–3.591)	**0.004**
M-CTC	Yes/Not	2.866(1.722–4.771)	**<0.001**	3.289(1.798–6.014)	**<0.001**
Lymphatic metastasis	N0/N+	3.377(2.054–5.553)	**<0.001**	2.681(1.557–4.620)	**<0.001**
Tumor Size, cm	≤ 4/>4	1.904(1.192–3.042)	**0.007**	2.253(1.347–3.769)	**0.002**
Stage	I + II/III + IV	1.905(1.196–3.035)	**0.007**	2.530(1.510–4.238)	**<0.001**
Napsin A	Negative/Positive	0.321(0.186–0.555)	**<0.001**	0.364(0.206–0.643)	**<0.001**
Differentiated degree	Moderately + Well / Poorly	1.850(1.162–2.946)	**0.010**	2.115(1.262–3.545)	**0.004**

Note: Bold values indicate statistically significant values.

Abbreviation: CTC, circulating tumor cell; M-CTC, CTCs with epithelial-mesenchymal transition phenotype; HR, hazard ratio; CI ,confidence interval ; RFS, Recurrence-free survival; OS, Overall survival.

Table 6
Multivariate analysis for recurrence-free survival and overall survival

Variable	RFS		OS	
	HR * (95% CI)	*P*-value*	HR* (95% CI)	*P*-value*
M-CTC				
(+)	2.105(1.206–3.676)	**0.009**	2.319(1.218–4.418)	**0.010**
(-)				
Napsin A				
Positive	0.507(0.272–0.943)	**0.032**	0.504(0.257–0.988)	**0.046**
Negative				

Notes: *HR and P-value for Cox proportional hazard regression model. adjustment by Gender, Smoking,Lymphatic metastasis□Tumor Size□Stage□Differentiated degree. Bold values indicate statistically significant values.

Abbreviation:M-CTC, CTCs with epithelial-mesenchymal transition phenotype; HR, hazard ratio; CI ,confidence interval ; RFS, Recurrence-free survival; OS, Overall surviva.

Discussion

The occurrence, development and evolution of lung cancer is a multi-gene, multi-step and complex biological process, but its mechanism is not clear. Napsin A is A new member of the aspartic acid protease family. Napsin A is A single chain protein molecule with A relative molecular weight of about 45 000, consisting of 420 amino acid residues,which is encoded by NSPSA gene located on human chromosome 19ql3.3g[35]. Napsin A is expressed in normal type II alveolar epithelial cells and plays an important role in maintaining normal lung morphology and physiological function. Lee et al. had shown that the high expression of Napsin A suggested a better prognosis, and the lack of expression might be related to the increase of tumor invasiveness[36]. However, the role of Napsin A in the occurrence and development of lung cancer and its mechanism is not very clear, which needs further research.

To my knowledge, this study is the first to report the relationship between Napsin A and M-CTC and their relationship with prognosis in patients with lung adenocarcinoma. We revealed that patients with negative Napsin A and positive M-CTC had poor prognosis. There was a significant correlation between M-CTC and lung cancer stage, and the differences between stage I and stage II, III and IV were statistically significant.

CTCs were crucial to tumor invasiveness and metastasis, therefore, they were associated with patient prognosis. In this study, we found that the numbers of CTCs and M-CTC were increased with tumor stage progression, and the M-CTC positive rate was significantly higher in patients with lymphatic metastasis and advanced tumor stage.

The difference of M-CTC between patients with and without lymph node metastasis was statistically significant (P□0.001), which was related to EMT's changes in tumor cell characteristics and the microenvironment of tumor growth, and the high expression of lytic enzymes involved in the degradation and destruction of extracellular matrix and basement membrane[12]. This changes led to the loss of the expression of the connecting molecules between cells, and the increase of the migration ability of tumor cells, making tumors more prone to metastasis, which was consistent with the results of numerous studies[37]. Sharma et al. Had also reported that the presence of CTC in peripheral blood was a prerequisite for distant metastasis of tumor and might be a key link in the formation of metastasis[38]. M-CTC was more aggressive and more likely to form new tumor lesions at a distance[39].

Consistent with this, LUAD patients harboring M-CTC had poor RFS and OS, and M-CTC positivity was identified as an independent prognostic factor. EMT was characterized by the loss of the epithelial markers such as E-cadherin and acquisition of mesenchymal markers including waveform, vimentin and Twist[40–42], which abolished cellular adhesion and enhanced their ability to penetrate into the extracellular matrix and invade the adjacent tissues[12]. Hence, EMT was crucial to cancer cell proliferation, invasion and metastasis in cancer cells[43,44]. EMT was initiated by the up-regulation of Twist1, which increased tumor cell migration and invasiveness[45–47]. As a result, we estimated that part of CTCs entering the peripheral circulation could be apoptotic through the body's immune recognition and the action of natural killer cells, and only a very few CTCs could escape immune surveillance and survive, and form tiny tumor thrombits through migration, mutual aggregation and adhesion, and evolve into metastases under specific effects, thus affecting the prognosis of patients. In addition, M-CTC was not related to other clinical characteristics of patients, such as age, gender, tumor size, smoking, and degree of differentiation, which might be related to individual tumor differences or the small sample size of this study. We expect that more conclusions with clinical significance can be drawn from larger sample size.

The numbers and positive rate of M-CTC were higher in the Napsin A negative patients, and the M-CTC+/Napsin A- patients had the worst prognosis. This is consistent with the previous finding that Napsin A overexpression inhibits proliferation and EMT of the epidermal growth factor receptor tyrosine kinase inhibitor (EGFE-TKI) resistant cells, induce apoptosis, and sensitizes them to gefitinib[48]. In addition, Napsin A also blocked the G0/G1 transition in an in vitro model of EMT, and inhibited focal adhesion kinase (FAK), which is a critical regulator of cellular adhesion, motility, metastasis and survival[49].

There were several limitations in our study that ought to be considered. The sample size was small since the patients were from a single center. Therefore, our findings will have to be validated with further multi-center prospective studies. Second, the mechanism through which Napsin A affects EMT was not studied, and will have to be determined by functional assays. Nevertheless, we established a correlation between high M-CTC count and lack of Napsin A in LUAD for the first time.

Conclusion

LUAD patients lacking Napsin A have a higher frequency of M-CTC and are more prone to EMT. The Napsin A- and M-CTC + profile are associated with poor prognosis, and a reliable early diagnostic marker.

Abbreviations

CTCs
circulating tumor cells; M-CTC:circulating tumor cells with mesenchymal phenotype;LUAD:lung adenocarcinoma. Napsin A-:negative expression of Napsin A;M-CTC+:M-CTC positive;NSCLC:Non-small cell lung cancer;CT:computed tomography;EMT:epithelial-mesenchymal transition;TTF-1:thyroid transcription factor-1;RBCs:red blood cells; ISH:in situ hybridization;RFS:Recurrence-free survival;OS:overall survival;OR:odds ratio;EGFE-TKI:epidermal growth factor receptor tyrosine kinase inhibitor;FAK:focal adhesion kinase.

Declarations

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Not applicable.

Authors' contributions

ZH designed and supervised the study. HW conducted the experiment,collected and analyzed the data and drafted the manuscript.All authors read and approved the final manuscript.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available

from the corresponding author on reasonable request.

Ethics approval and consent to participate

The experimental procedures were approved by the Ethic Committee of

the First Affiliated Hospital of Guangxi Medical University, and a written informed consent was provided by each participant.

Consent for publication

Written informed consent for publication was obtained from each participant.

Competing interests

The authors declare that they have no competing interest.

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Figures

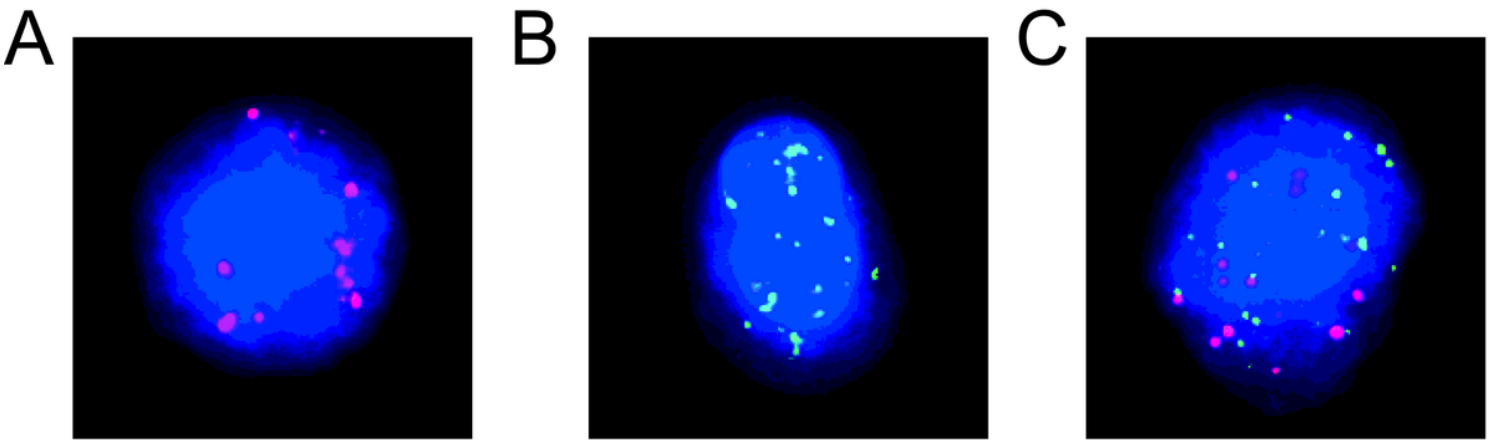

Figure 1

CTCs isolated from LUAD patients are stained with EpCAM and CK8/18/19 (red fluorescence) and Vimentin and Twist (green fluorescence) to distinguish their phenotypes. (A) E-CTC; (B) M-CTC; (C) E/M-CTC. Magnification – 100x.

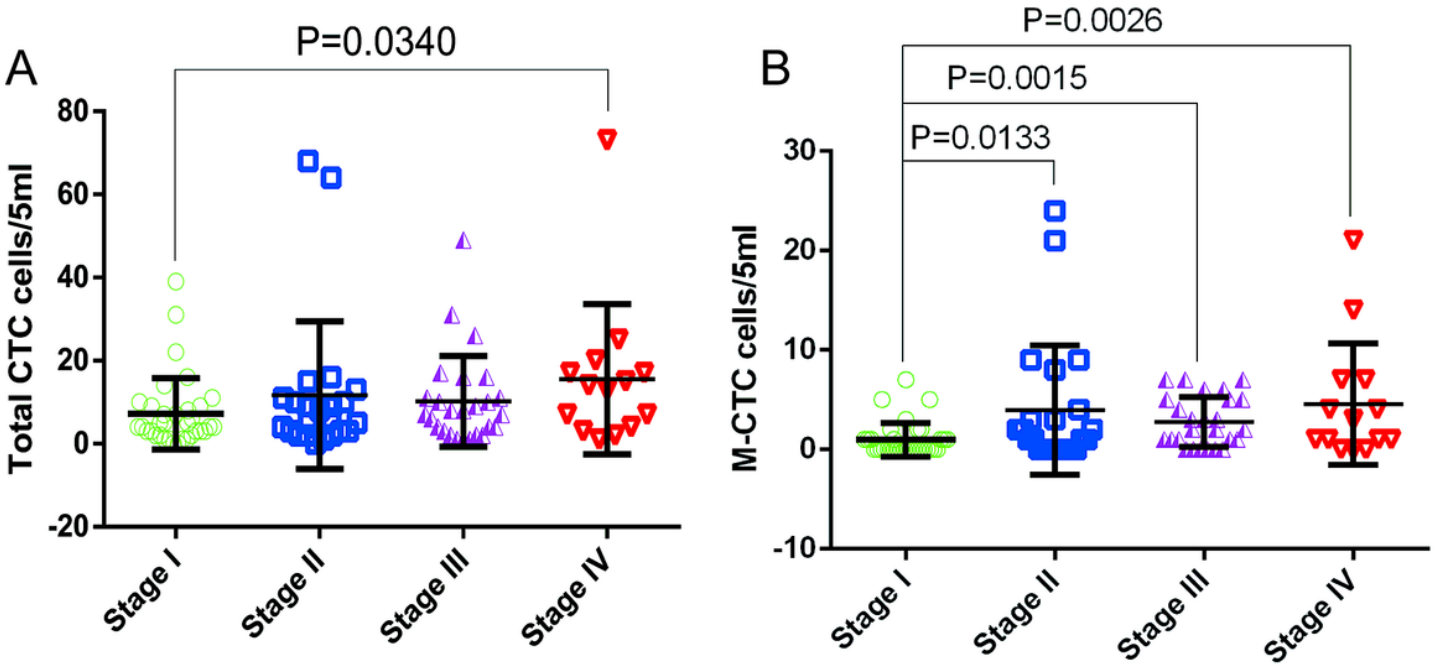

Figure 2

Distribution of CTC and M-CTC counts in LUAD patients according to tumor stage. (A) Total CTCs; (B) M-CTC.

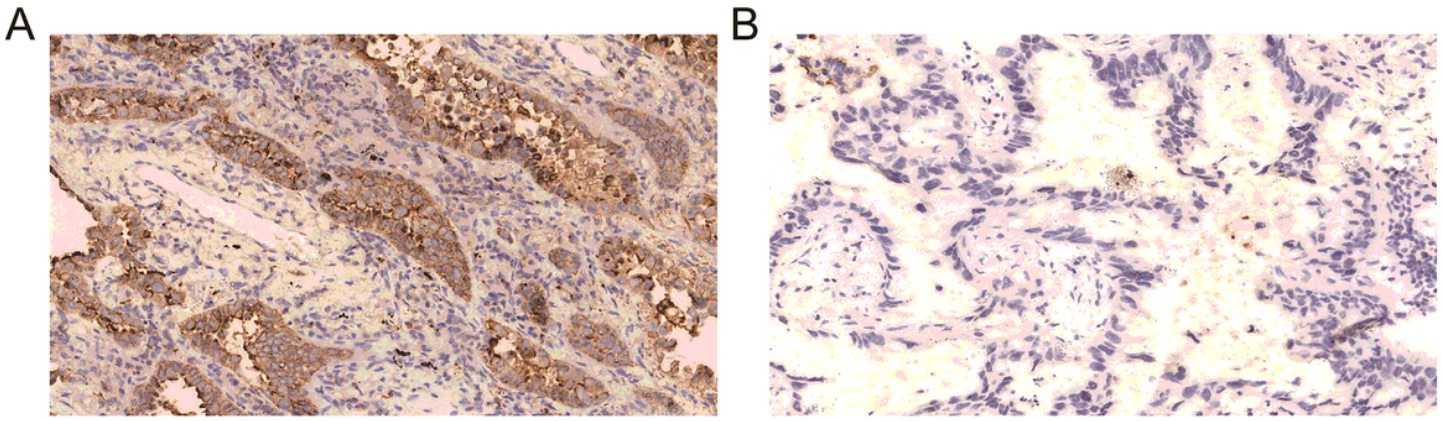

Figure 3

Staining of Napsin A on LUAD tissue samples, Napsin A -positive staining was identified as the presence of brownish-yellow granules in the nucleus: (A) Napsin A-positive;(B) Napsin A-negative.

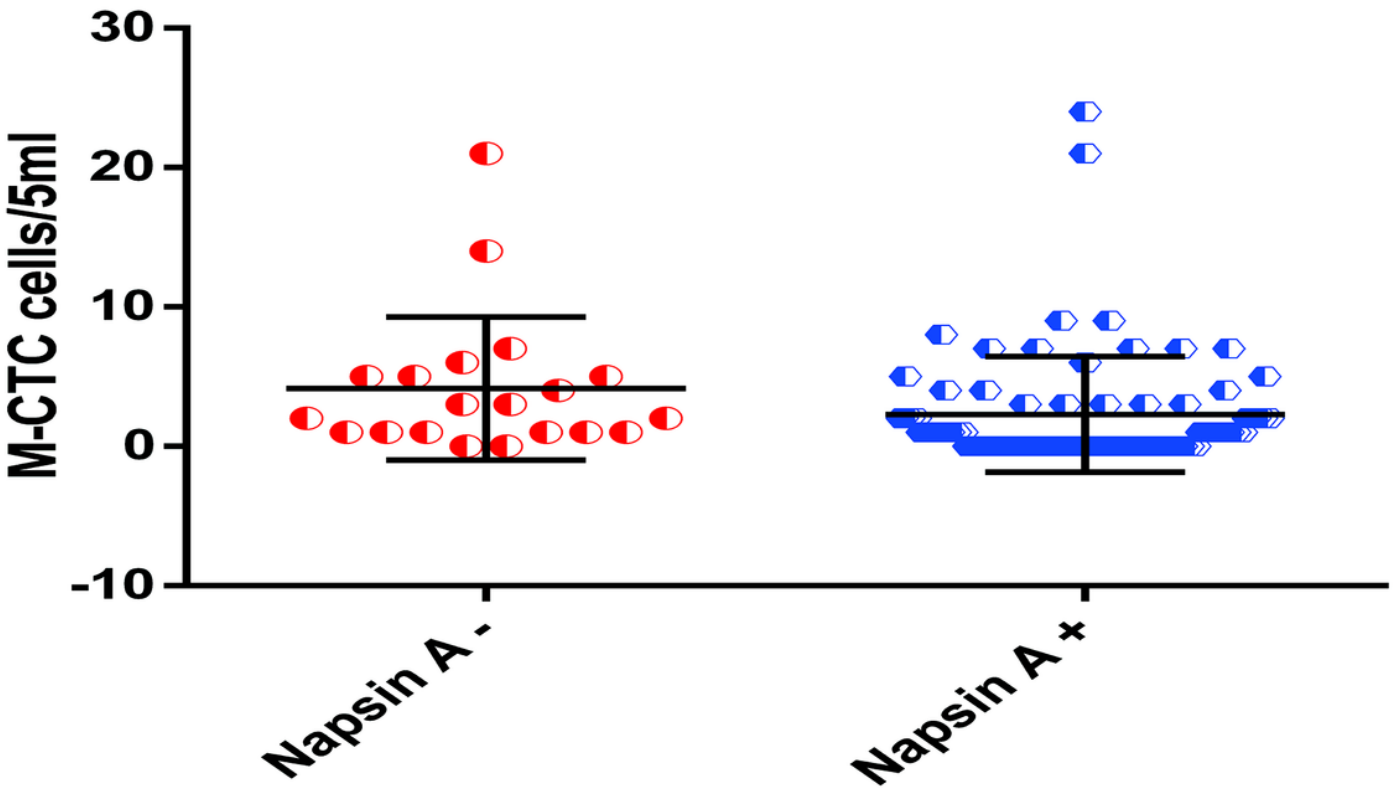

Figure 4

M-CTC in Napsin A+ and Napsin A- patients.

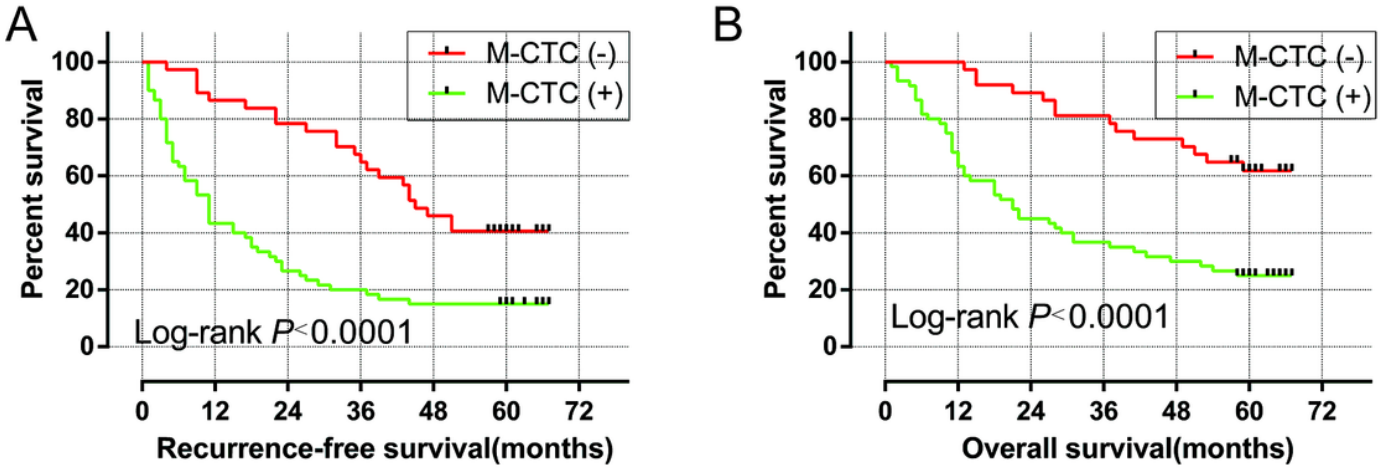

Figure 5

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

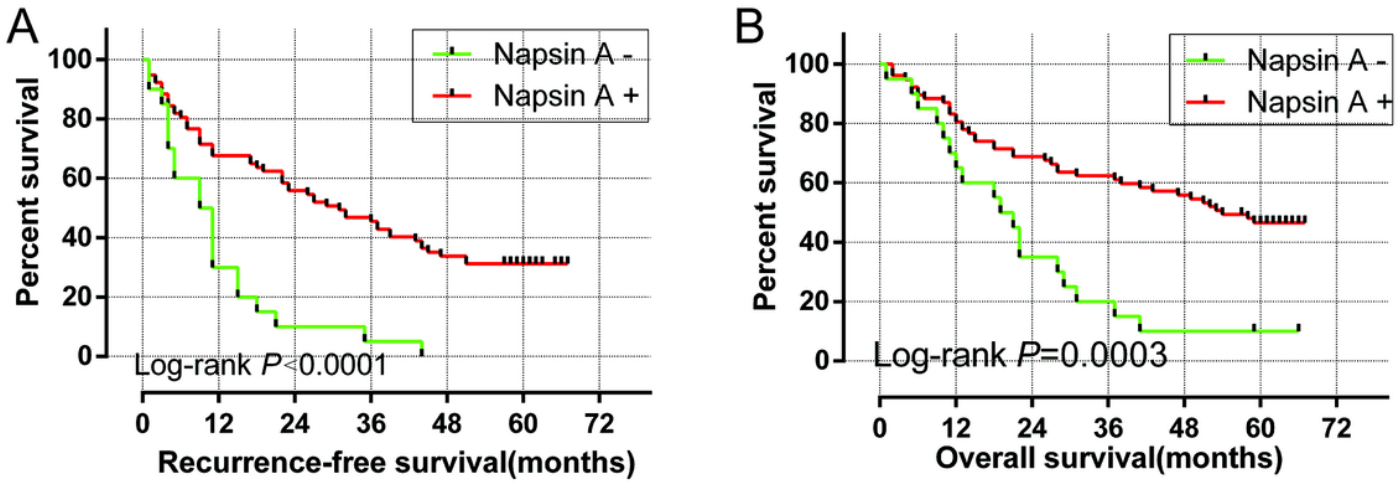

Figure 6

Kaplan -Meier survival curves of patients stratified by Napsin A expression. Patients with Napsin A- had shorter (A) RFS and (B) OS compared to those with Napsin A+.

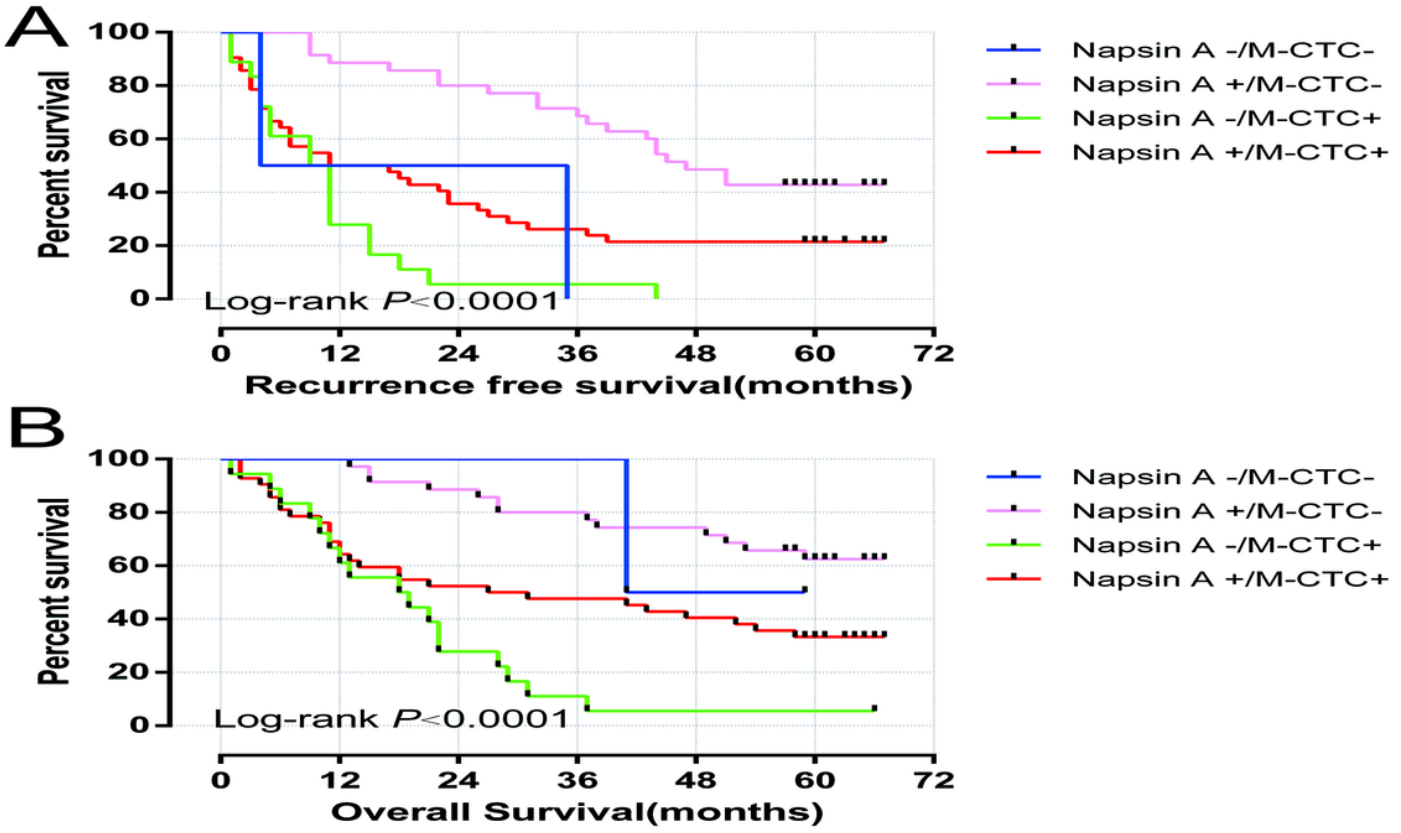

Figure 7

Kaplan-Meier survival curves of patients stratified by M-CTC and Napsin A expression. Patients with M-CTC and Napsin A- showed poor (A) RFS and (B) OS compared to those without M-CTC and Napsin A+.

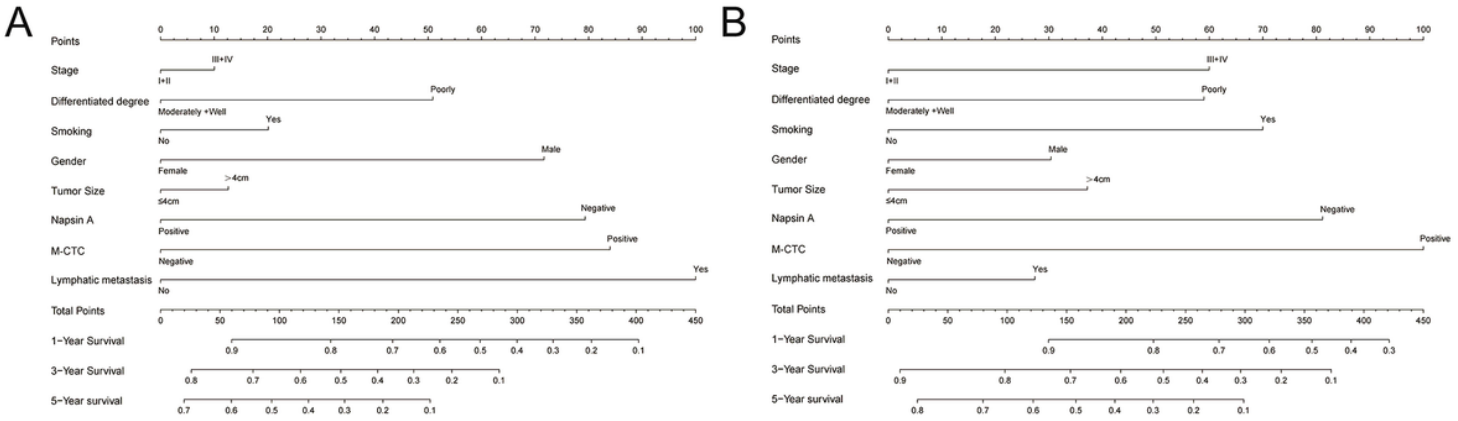

Figure 8

Nomogram module integrating smoking, gender, tumor size, lymphatic metastasis, differentiated degree, M-CTC, Napsin A 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 LUAD patients; (B) OS of LUAD patients.

Supplementary Files

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

- TableS1.docx