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Original Article

Significance of circulating tumor cells in non-small-cell lung carcinoma

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Abstract: Background: Non-small-cell lung carcinoma (NSCLC) accounts for nearly 85% of all lung cancers. To date, reliable predictive and prognostic factors for NSCLC are still needed. Thus, finding new predictive and prognostic factors is of great importance to help diagnosing and treating specific patients with NSCLC. In the present study, we explored the significance of circulating tumor cells (CTCs) in NSCLC, which help discovering additional factors for treating NSCLC. Methods: Firstly, we applied CanPatrol™ CTC enrichment technique to isolate CTCs. In addition, through combining with tri-color RNA in situ hybridization assay we analyzed the relationship between NSCLC and CTCs or BCL2L1. Finally, we explored the potential relationship between NSCLC curative effect and CTCs or BCL2L1. Results: CTCs were highly expressed in the occurrence and development of NSCLC. Meanwhile, the positive ratio of mesenchymal CTCs in stage III was highest during NSCLC. In addition, CTCs with epithelial characteristics were strongly correlated with differentiation and biomarkers levels of NSCLC. Moreover, we confirmed that BCL2L1 was highly expressed in CTCs. Finally, our results showed that decreased CTCs and BCL2L1 levels indicated a favorable curative effect. Conclusion: Taken together, CTCs and BCL2L1 were close correlated with NSCLC characteristics. Of note, decreased CTCs suggested a good curative effect for NSCLC patients. Thus, analyzing CTCs in NSCLC patients provides a trustworthy reference to identification, therapy, and prognosis of NSCLC.

Keywords: Circulating tumor cells, BCL2L1, cancer cell characteristics non-small-cell lung carcinoma

Introduction

Non-small-cell lung carcinoma (NSCLC), including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, makes up nearly 85% of all lung cancers [1, 2]. Active smoking and passive smoking are blamed for the occurrence and progress of NSCLC [3]. Although new therapies are emerging for specific patient populations, reliable predictive and prognostic factors for NSCLC are still in need [4]. Thus, finding new predictive and prognostic factors is of great importance to help diagnosing and treating specific patients with NSCLC [5, 6].

Circulating tumor cells (CTCs) are generated from primary and metastatic cancer, which actively indwell in circulation system [7, 8]. Actually, CTCs have three subtypes: epithelial, mesenchymal, and hybrid CTCs. What's interesting is that circulation existed CTCs undergo epithelial-to-mesenchymal transition during the development of cancer [9]. Researchers explained this phenomenon was a vital progress to meet the need of cancer invasion and migration through settling and creating secondary lesions [10, 11]. Recently, many studies have reported that CTCs were intimately correlated with progress and characteristics of various cancers including NSCLC [12, 13]. However, more experiments and evidence are still lacking for CTCs application on NSCLC prediction and prognosis.

Therefore, in the present study, we sought to uncover the relationship between CTCs and NSCLC. Firstly, we isolated and analyzed CTCs from NSCLC patients through advanced CanPatrol™ CTC enrichment technique and in situ hybridization assay. Then we explored the relationship between subtypes of CTCs and clinical stages, clinical characteristics, and BCL2L1 expression of NSCLC. Finally, we studied variation of NSCLC CTCs and BCL2L1 expression before and after therapy.

Table 1. Capture probe sequences for MMP9 gene

Gene	Sequence (5'-3')
CD45	TCGCAATTCTTATGCGACTC
	TGTCATGGAGACAGTCATGT
	GTATTTCCAGCTTCAACTTC
	CCATCAATATAGCTGGCATT
	TTGTGCAGCAATGTATTTCC
	TACTTGAACCATCAGGCATC
CK19	AAGTCATCTGCAGCCAGACG
	CTGTTCCGTCTCAAACTTGG
	TTCTTCTTCAGGTAGGCCAG
	CTCAGCGTACTGATTTCCTC
	CTGTAGGAAGTCATGGCGAG
	AAGTCATCTGCAGCCAGACG
Twist	ACAATGACATCTAGGTCTCC
	CTGGTAGAGGAAGTCGATGT
	CAACTGTTCAGACTTCTATC
	CCTCTTGAGAATGCATGCAT
	TTTCAGTGGCTGATTGGCAC
	TTACCATGGGTCCTCAATAA
BCL2L1	ACAATGCGACCCCAGTTTAC
	CCCGCCGAAGGAGAAAAGG
	ACTCCCTTGTCTACGCTTTC
	TGCGATCCGACTCACCAATA
	CTAGGTGGTCATTCAGGTAA
	GAGTTCCACAAAAGTATCCC
	TCAGGAACCAGCGGTTGAAG
	GTCATTTCCGACTGAAGAGT

Patients and methods

Reagents

Reagents including ethylene diamine tetra acetic acid (EDTA), formaldehyde, fetal bovine serum, Tris-HCl, sodium dodecyl sulfate, phosphate buffered saline (PBS), and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). Protease was purchased from MedChemExpress, Inc.

Patients and blood samples

From May 2014 to November 2015, a total of 116 patients (73 males and 43 females) were recruited by First Affiliated Hospital of Guangxi Medical University. Inclusion criteria were carried out as follows: diagnosed with NSCLC, suitable performance status (0-2), and older than 18 years. In addition, 25 healthy volunteers were considered as controls. All blood samples were collected in three periods: 1-3 days before surgery or chemotherapy, 1-3 days after surgery or chemotherapy, and 1 month after discharged from hospital to check. Among which, 5 ml peripheral blood samples from patients were collected with anticoagulation tube for CTCs separation or biochemical detection. Protocol of the trial was approved by the ethical committee of First Affiliated Hospital of Guangxi Medical University.

Size-dependent CTCs isolation

After emendating the deviation of CanPatrol™ CTC enrichment technique, calibrated membrane (8-μm pores, Millipore, Billerica, USA) was applied to filtrate blood samples collected within 4 h. Among which, the appointed filtration system (including a filtration tube containing the membrane (SurExam, Guangzhou, China), a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum manifold (Omega, Norcross, USA), and vacuum pump (Auto Science, Tianjin, China) was applied to satisfied the need of filtration. With FACS Lysing Solution, erythrocytes were removed. Then cells were re-suspended in PBS containing 4% formaldehyde, followed by transferred to the filtration tube. At the same time, the pump valve and the manifold vacuum plate valve was maintained to fulfill filtration.

Tri-color RNA in situ hybridization (ISH) assay

Target sequences were detected by RNA-ISH method [14]. Among which, sequences of CD45 (leukocyte biomarker), CK19 (epithelial biomarker), and twist (the mesenchymal biomarker), were used to help distinguishing epithelial, mesenchymal, and hybrid CTCs [15]. Isolated cells that mentioned above were treated with protease, followed by hybridization of indicated capture probes specific for CK19, twist, or Bcl-2 (**Table 1**). After incubation (42°C for 2 h), unbound probes were washed away with buffer. Then cells were treated to meet signal amplification under incubation of preamplifier solution (30% horse serum, 1.5% sodium dodecyl sulfate, 3 mM Tris-HCl (pH 8.0), and 0.5 fmol of preamplifier (the sequences are shown in **Table 2**). On the other hand, the indicated membranes were washed and incubated with 100 μl of amplifier solution (30% horse serum, 1.5%

Table 2. Sequences for the bDNA signal amplification probes

	Function (copies)	Sequence (5'-3')	Complement
bDNA probes for CD45	Capture probe tail (1)	CTTTATACCTTTCTTTCA	Preamplifer leader (1)
	Preamplifer repeat (5)	GCGCGCTGTAGGG	Amplifer leader (1)
	Amplifer repeat (5)	AGGCGAGGGGAGA	Label probe (1)
bDNA probes for CK19	Capture probe tail (1)	CTACAAACAAACAATATT	Preamplifer leader (1)
	Preamplifer repeat (5)	CGCAGCCTCAGCC	Amplifer leader (1)
	Amplifer repeat (5)	CCCAGACCCTACC	Label probe (1)
bDNA probes for twist	Capture probe tail (1)	CTTCTCAATAACTAACAT	Preamplifer leader (1)
	Preamplifer repeat (5)	CTTCTCAATAACTAACAT	Amplifer leader (1)
	Amplifer repeat (5)	GTCACCGCTCCAC	Label probe (1)
bDNA probes for BCL2L1	Capture probe tail (1)	CTACATGCCTACTATAGT	Preamplifer leader (1)
	Preamplifer repeat (5)	CTGAGTCAGTAGTC	Amplifer leader (1)
	Amplifer repeat (5)	CGCTGGAGATGC	Label probe (1)

The sequences labeled "leader" appear once in the indicated construct, while sequences labeled "repeat" appear the indicated number of times. The tail on the capture probe is a single sequence.

Table 3. Expression of CTCs in NSCLC clinical stages

Clinical stage	PR[a] of CTCs	PR[a] of mesenchymal CTCs	PR[a] of BCL2L1	Median of CTCs	Mean of CTCs	CTCs scope
I (n=42)	88.1%	31.0%	87.8%	4	7	0-54
II (n=27)	92.6%	40.7%	92.6%	7	9	0-43
III (n=29)	96.6%	51.7%	85.7%	6	11	0-64
IV (n=11)	100%	36.4%	81.8%	9	11	1-42
Unknown (n=7)	100%	42.9%	100%	4	5	1-13
Sum (n=116)	93.1%	39.7%	88.4%	5	9	0-64

PR[a], positive ratio.

sodium dodecyl sulfate, 3 mM Tris-HCl (pH 8.0), and 1 fmol of amplifier (the sequences are shown in **Table 3**). Fluorescent dyes Alexa Fluor 594 (for CK19), Alexa Fluor 488 (for twist), or Alexa Fluor 647 (for Bcal-2) were conjugated with fluorescently labeled probes and then applied to treat cells at 42°C for 2 min. Finally, cells were stained with DAPI and analyzed with the fluorescence microscope (Olympus BX53, Tokyo, Japan).

Statistical analysis

Statistical analyses were executed with one-way analysis of variance, followed by Student two-tailed t test. Direction and strength of the relationship between two variables were analyzed through Spearman's rank correlation test. *P* values less than 0.05 were examined statistically significant.

Results

CTCs detection in NSCLC

Firstly, CanPatrol™ CTC enrichment technique was utilized to isolate and analyze CTCs from NSCLC patients. Through incubating with indicated biomarker-probes (CK-19 specified for epithelial cells and twist specified for mesenchymal tissues), epithelial, hybrid, and mesenchymal CTCs were isolated and showed in **Figure 1** (leucocytes were taken as negative control). In addition, relationship between clinical stages and CTCs was analyzed (**Table 3**). Mean positive ratio of CTCs was 93.1% in 116 NSCLC patients. Values of CTCs were ranging from 0 to 64. Median and Mean of CTCs were 5 and 9 respectively. On the other hand, mesenchymal CTCs positive ratios in stage I (n=42), stage II (n=27), stage III (n=29), and stage IV (n=11) groups were 88.1%, 92.6%, 96.6%, and 100% respectively, indicating that the ratio of mesenchymal CTCs increased with the development of NSCLC. Interestingly, we found that total CTCs, hybrid CTCs, and mesenchymal CTCs had a significantly positive correlation with standard tumor classification of NSCLC. ($P<0.05$) (**Table 4**). These results led us to determine whether CTCs subtype was distinctive for NSCLC. We found that only mesenchy-

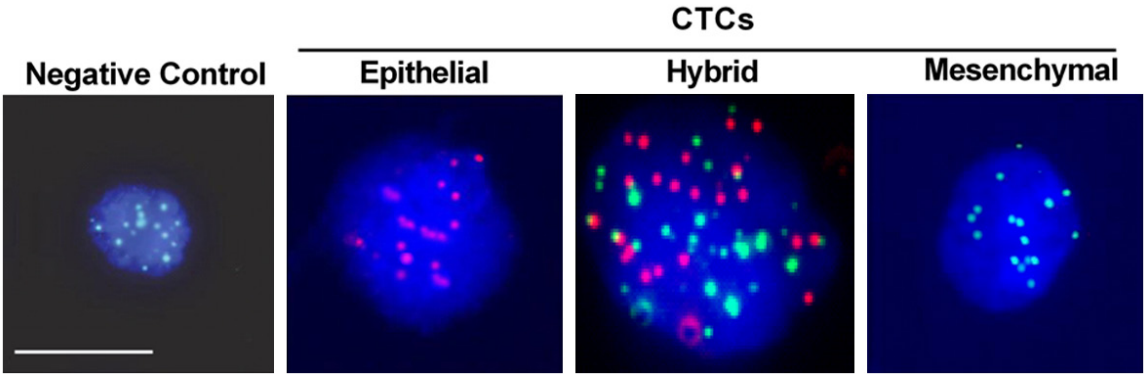

Figure 1. CTCs from NSCLC patients. Leukocytes were stained for CD45 (blue fluorescence). CTCs were stained for CK19 (red fluorescence) and twist (green fluorescence) to help distinguishing CTCs subtypes. The cells were analyzed using a 100× oil objective. CTCs: circulating tumor cells.

Table 4. Correlation between CTCs and NSCLC clinical stage

Spearman's rho		Total CTCs	Epithelial CTCs	Hybrid CTCs	Mesenchymal CTCs	PR of mesenchymal CTCs
T stage	R^a	0.197	-0.031	0.198	0.215	0.137
	P^b	0.042*	0.751	0.041*	0.026*	0.175
N stage	R^a	0.085	0.062	0.041	0.097	0.047
	P^b	0.382	0.526	0.678	0.318	0.646
M stage	R^a	0.007	0.077	-0.005	-0.021	-0.044
	P^b	0.944	0.429	0.957	0.826	0.662
Number		107	107	107	107	107

R^a, Spearman's correlation coefficient; P^b, *P* value; *$P<0.05$.

Table 5. Correlation between CTCs and NSCLC

Spearman's rho		Total CTCs	Epithelial CTCs	Hybrid CTCs	Mesenchymal CTCs	Mesenchymal CTCs ratio
NSCLC	R^a	-0.108	-0.118	0.030	-0.191	-0.170
	P^b	0.256	0.215	0.754	0.044*	0.084
Number		112	112	112	112	112

R^a, Spearman's correlation coefficient; P^b, *P* value; *$P<0.05$.

mal CTCs were strongly related to NSCLC (r=-0.191, *P*<0.05) (**Table 5**). Taken together, CTCs were highly expressed in the occurrence and progress of NSCLC. Also, the positive ratio of mesenchymal CTCs in stage III was highest during NSCLC. However, only CTCs with mesenchymal characteristics were correlated with attack and standard tumor classification of NSCLC.

Correlation between CTCs and NSCLC characteristics

As we found CTCs were intimately related to NSCLC. We next tried to discover the probable link between clinical parameters and CTCs of NSCLC. Firstly, we discovered that only epithelial CTCs were correlated with the differentiation of NSCLC (r=0.215, *P*<0.05) (**Table 6**). Likewise, epithelial CTCs were negatively related to NSE level (NSCLC biomarker) and serum ferritin level (r=-0.196, r=-0.254, *P*<0.05, respectively). As to hybrid CTCs, CYFRA21-1 (NSCLC biomarker) was positively correlated with them (r=0.237, *P*< 0.05). However, mesenchymal CTCs showed no correlation with NSCLC characteristics mentioned above. Taken together, it's seems that only CTCs with epithelial characteristics were closely correlated with differentiation and biomarkers of NSCLC.

Relationship between BCL2L1 and NSCLC

Expression of BCL2L1 in different types of CTCs has not yet been studied. Thus, tri-color RNA in situ hybridization (blue fluorescence) was applied to help indicating BCL2L1 expression in CTCs (**Figure 2**). Firstly, results indicated that BCL2L1 positive rate was 88.4% in total 116 patient samples (**Table 3**). On the other hand, positive ratios of BCL2L1 were especially high in CTCs (**Figure 3A**). Interestingly, percent-

Table 6. Correlation between CTCs and CYFRA21-1, NSE, or serum ferritin

Spearman's rho		Total CTCs	Epithelial CTCs	Hybrid CTCs	Mesenchymal CTCs	PR of mesenchymal CTCs
NSCLC differentiation	R^a	-0.007	0.215	-0.008	-0.117	-0.129
	P^b	0.946	0.049*	0.939	0.285	0.260
CYFRA21-1 level	R^a	0.158	-0.105	0.237	0.049	-0.026
	P^b	0.114	0.295	0.017*	0.628	0.800
NSE level	R^a	0.078	-0.196	0.134	0.061	0.588
	P^b	0.438	0.049*	0.179	0.543	-0.099
Serum ferritin level	R^a	-0.069	-0.254	0.050	-0.124	-0.099
	P^b	0.512	0.015*	0.633	0.239	0.366

CYFRA21-1, cytokeratin 19 fragment; NSE, neuron-specific enolase; R^a, Spearman's correlation coefficient; P^b, *P* value; *$P<0.05$.

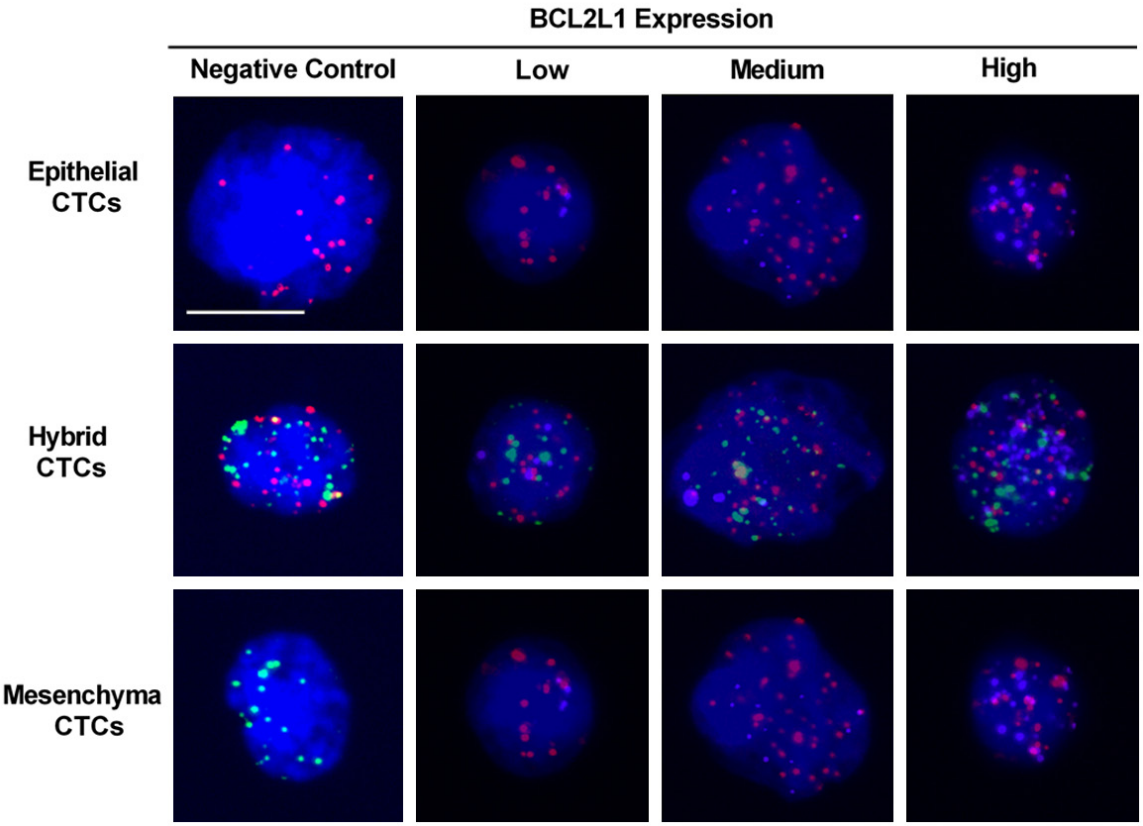

Figure 2. Morphology of CTCs stained for BCL2L1. CTCs were analyzed and classified according to their BCL2L1 expression. CTCs: circulating tumor cells.

age of low BCL2L1 expressed epithelial CTCs (27.1%) was higher than that of hybrid CTCs (14.3%) and mesenchymal CTCs (17.5%) (**Figure 3B**). However, percentage of high BCL2L1 expressed epithelial CTCs (34.6%) was lower than that of hybrid CTCs (55.2%) and mesenchymal CTCs (50%). At the same time, percentages of medial BCL2L1 expression were similar among three CTCs (nearly 35%). Then we attempted to explore the relationship between BCL2L1 level and CTCs. We found that total and hybrid CTCs levels were significantly correlated with BCL2L1 level (r=0.465, r=0.459, *P*<0.01, respectively) (**Figure 3C**). Also, quantity changes of CTCs were closely related to BCL2L1 level (r=0.428, r=0.400, *P*<0.01, respectively)

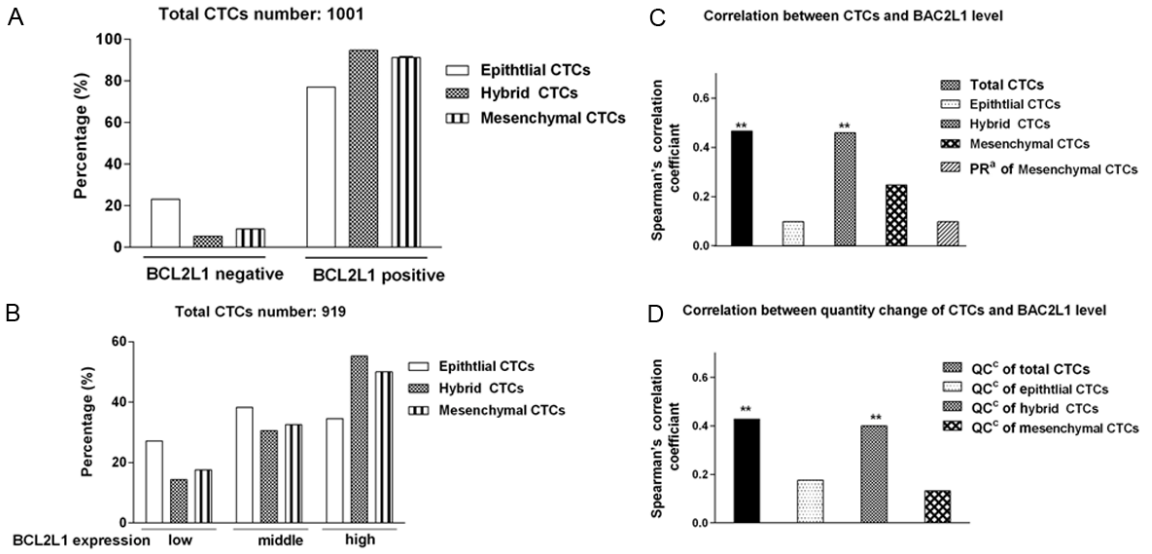

Figure 3. BCL2L1 expression in different types of CTCs. Positive ratios of BCL2L1 in three types of CTCs were tested (A). BCL2L1 level in three types of CTCs were tested (B). (C, D) Correlation between BCL2L1 CTCs. CTCs: circulating tumor cells; PR[a], positive ratio; **P<0.01.

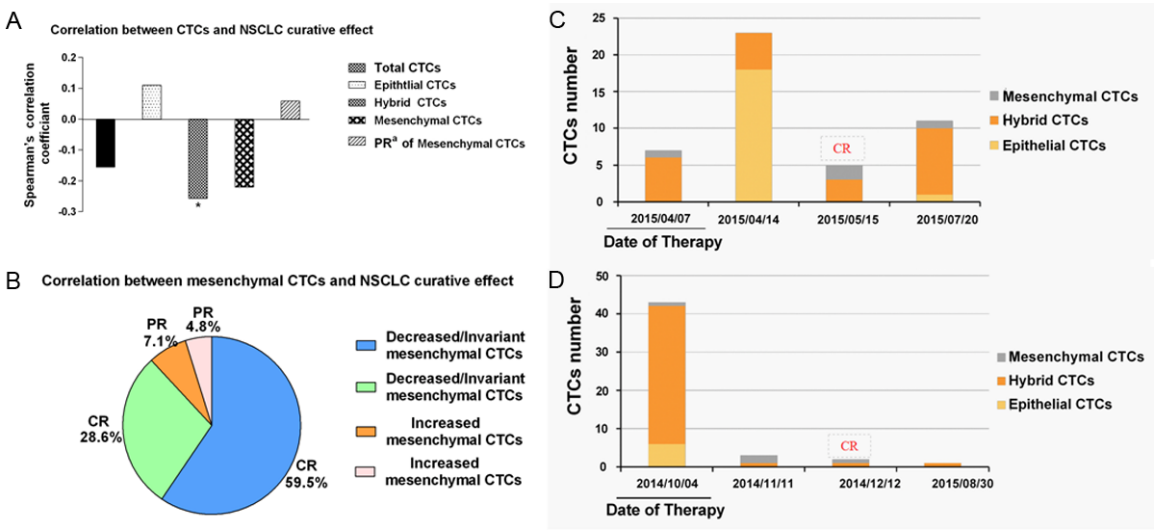

Figure 4. Number variation of CTCs after NPC therapy. CTCs were collected and analyzed from the same patients before and after therapy. A, B. Correlation between curative effect and CTCs. C, D. CTCs variations after treatment. CTCs: circulating tumor cells CR, complete response; PD, progressive disease. PR[a], positive ratio; *P<0.05.

(**Figure 3D**). Of note, only high expression level of BCL2L1 was strongly related to normal CA125 level (r=-0.215, *P*<0.05). However, BCL2L1 did not link to other NSCLC characteristics (data not shown). Taken together, BCL2L1 was highly expressed in CTCs.

Reduced expression of CTCs benefited NSCLC curative effect

Because we found CTCs were closely related to NSCLC, we then tried to determine whether quantity changes of CTCs could indicate NSCLC curative effect. Firstly, we found that only hybrid CTCs level was negatively correlated with NSCLC curative effect (r=-0.257, *P*<0.05) (**Figure 4A**). In addition, decreased and invariant total CTCs level and mesenchymal level were consistent with good curative effect in 42 patients (**Figure 4B**). Next, to study numerical variation of CTCs after NPC therapy, we collected and analyzed samples from the same patients before and after therapy. The results

Table 7. Correlation between NSCLC curative effect and BCL2L1 level

		-BCL2L1 expressed	BCL2L1 high expressed
NSCLC curative effect	R[a]	-0.235	-0.076
	P[b]	0.056	0.543
Cell number		68	68

R[a], Spearman's correlation coefficient; P[b], *P* value; **P*<0.05.

Table 8. Correlation between BCL2L1 level and NSCLC curative effect

Percentage	Complete response	Progressive disease
Decreased/Invariant BCL2L1 level	71.4%	9.5%
Increased BCL2L1 level	16.7%	2.4%
Total number	42	

showed that total numbers of CTCs from two II stage patients (patient nos. K05650 and K01831) were dramatically decreased a month after therapy (**Figure 4C** and **4D**). They acquired obviously complete response in a short time after therapy (1 month and 2 months respectively). Finally, we tried to discover the link between BCL2L1 and curative effect. Although BCL2L1 expression only slightly and negatively correlated with curative effect (not significant), decreased and invariant BCL2L1 level was also consistent with good curative effect (**Tables 7** and **8**). In summary, decreased CTCs and BCL2L1 levels indicated a favorable curative effect.

Discussion

As CTCs were firstly found in peripheral blood of cancer patients, techniques that applied to isolate and analyze CTCs arose at the historic moment [16, 17]. However, some drawbacks (including poor purity, low specificity, low recovery rate, etc) restricted the widespread use of these techniques [18-22]. Recently, CanPatrol™ CTC enrichment technique emerged as an ideal method to isolate and characterize CTCs because of its superiorities: stable monoblast isolation, high collective efficiency, etc. [23]. Herein, we used CanPatrol™ CTC enrichment technique to isolate CTCs from NSCLC patients. Combined with tri-color RNA in situ hybridization assay, we clearly found that morphology of CTCs is nearly twofold bigger than leucocytes (**Figure 1**). Based on the isolated CTCs, we started to study the distribution and clinical significance of CTCs in NSCLC.

Although several combined therapies are applied to treat NSCLC, recurrence rate is relatively high [24]. Thus, finding a reliable and fast criterion for NSCLC diagnosis and therapeutic evaluation is pivotal to treat NSCLC in time. Interestingly, CTCs detection may provide a possibility for addressing this problem [25, 26]. In 2007, American Society of Clinical Oncology has suggested CTCs as a hallmark of cancer. It has been reported that CTCs have close relationship with some cancers and could even be detected in patients without cancer lesions in early stage [27, 28]. In the present study, we explored the clinical significance of CTCs in NSCLC. Firstly, we found that the positive ratio of CTCs is extremely high in all stages of NSCLC (average is 93.1%). Among which, positive ratio of mesenchymal CTCs in stage III were higher than those of other NSCLC stages (**Table 3**). Perhaps, this asymmetric distribution features in NSCLC inclined to progress and metastasize [29]. In addition, total CTCs, hybrid CTCs, and mesenchymal CTCs were only significantly correlated with NSCLC T stage (**Table 4**). What's more, only mesenchymal CTCs were closely related to the occurrence of NSCLC (**Table 5**). On the other hand, only epithelial CTCs dramatically related to NSCLC differentiation (**Table 6**). As to other NSCLC markers such as CYFRA21-1, NSE, and serum ferritin, CTCs with epithelial characteristics seems to be the leading subtypes correlated with them (**Table 6**). Taken together, these results indicated CTCs with mesenchymal characteristics were vital to NSCLC attack and development. Meanwhile, CTCs with epithelial characteristics seems to strongly connect to the differentiation and markers of NSCLC.

Chemotherapy resistance is a universal phenomenon in cancer patients [30, 31]. It is believed that anti-apoptotic gene including BCL2 over expression promotes cancer cells survive under therapies [32-34]. According to BCL2-cytochrome c-caspase axis (cellular intrinsic apoptosis signaling), BCL2 inhibitors emerged and reached obvious clinical and translational advances [35-37]. Thus, in order to forecast the prospect of NSCLC gene intervention in the future, it is of great importance to determine whether BCl2 has a specific relation-

ship with NSCLC CTCs. Firstly, we found that BCL2L1 strongly expressed in CTCs and patient samples (**Figures 2** and **3**; **Table 3**), which was in accordance with strong viability of cancer cells [38]. Meanwhile, only hybrid CTCs levels were significantly correlated with BCL2L1 level. Furthermore, only high expression level of BCL2L1 was strongly related to normal CA125 level. Taken together, BCL2L1 was highly expressed in NSCLC CTCs. On the other hand, BCL2L1 level had close relationship with one NSCLC marker, CA125.

Finally, we tried to confirm that whether decreased CTCs could be an indicator for effective therapy efficiency, which has been reported in other kinds of cancers. We found that decreased CTCs and BCL2L1 were significantly related to good curative effect (**Figure 4**; **Tables 7** and **8**). As showed in **Figure 4**, although CTCs shortly increased after therapy, patients got complete response and decreased CTCs during recovery, indicating that decreased CTCs and BCL2L1 levels indicated a favorable curative effect.

Taken together, CTCs and BCL2L1 were closely correlated with NSCLC characteristics. Of note, decreased CTCs suggested a good curative effect for NSCLC patients. Thus, analyzing CTCs in NSCLC patients provides a dependable reference for identification, therapy, and prognosis of NSCLC.

Disclosure of conflict of interest

None.

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