



# Small Cell Size Circulating Aneuploid Cells as a Biomarker of Prognosis in Resectable Non-Small Cell Lung Cancer

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**Objective:** The size distribution of circulating aneuploid cells (CACs) and its clinical significance were investigated in resectable non-small cell lung cancer (NSCLC).

**Patients and Methods:** A total of 50 patients with resectable NSCLC were enrolled in this study. Blood samples (50 pre-surgery and 35 post-surgery) were collected and used for the detection of CAC chromosome 8 heteroploidy through the subtraction enrichment and immunostaining fluorescence *in situ* hybridization (SE-iFISH) method.

**Results:** Less than 20% small cell size and more than 80% large cell size CACs were detected. Karyotypes, including triploid, tetraploid, and multiploid, had varying distributions. The triploid subtype accounted for the majority of small cell size CACs, whereas the multiploid subtype accounted for the majority of large cell size CACs. We found that total small cell size and triploid small cell size CACs, but not large cell size CACs, derived from pre-surgery samples, were associated with shorter disease-free survival. Moreover, total small cell size and triploid small cell size CACs were associated with higher TNM stage and recurrence. Nevertheless, the variation between pre- and post-surgery CACs was not related to survival among patients with resectable NSCLC.

**Conclusions:** Pre-surgery small cell size CACs, especially the triploid subtype, could be regarded as a potential prognostic biomarker for patients with resectable NSCLC.

**Keywords:** non-small cell lung cancer, circulating aneuploid cells, prognosis, resection, biomarker

## INTRODUCTION

Lung cancer is the leading cause of cancer-associated death worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases, with a low 5-year survival rate of less than 20% (2–4). A total of 45% of early-stage NSCLC patients experience postoperative recurrence, including those who have already undergone primary tumor resection (5). Therefore, biomarkers for predicting relapse in patients with resectable tumors are extremely important for dynamic clinical evaluation and treatment choice. Lower airway bacterial microbiomes, tumorspheres, circulating cell-free DNA, urine cell-free DNA, and circulating non-hematopoietic cells such as circulating tumor cells (CTCs), circulating endothelial cells (CECs), or circulating aneuploid cells (CACs) could reportedly be used as biomarkers for NSCLC diagnosis, relapse prediction, and drug resistance evaluation (6–11).

CTCs, CECs, and CACs are well-known for their association with metastasis and progression and provide information for individual therapy and prediction of prognosis in several cancer types such as breast, colorectal, prostate, and lung (7, 12–17). Epithelial markers such as EpCAM and cytokeratin are often used to enrich CTCs (18). However, this approach may miss an aggressive and clinically relevant subpopulation of tumor cells, partially because of the epithelial-mesenchymal transition (EMT) that occurs with a reduction in or loss of epithelial markers in the tumors (19, 20). This kind of EMT<sup>+</sup> CTC is helpful in predicting poor outcome and managing therapy, which is important for patients (19). In addition, CTCs are a heterogeneous population; hence, clarification of its characteristics can shed light on tumor heterogeneity, recurrence mechanism, treatment efficacy, or poor prognosis for cancer patients (21, 22).

In addition to the numbers of CTCs, CECs, and CACs, the specific characteristics of these cells seem to be more significant as biomarkers. Aneuploidy is a common trait in solid tumors (23, 24); moreover, chromosomal redistribution contributes to proliferation during the evolution of tumor cells (25, 26). Examination of aneuploidy in chromosome 8 can identify CTCs in epithelial and glioma tumors with high sensitivity (20, 27, 28). Moreover, chromosomal instability status can reveal the heterogeneous phenotype of CTCs, CECs, and CACs (21). Li et al. suggested that an increased percentage of triploid CTCs was associated with chemotherapy resistance in advanced gastric cancer (27). Moreover, quantified chromosome ploidy may be a predictor of therapeutic efficacy and disease progression (29).

Herein, we analyzed the clinical significance of CACs and their aneuploidy subtypes in samples of peripheral blood from patients with resectable NSCLC. We found that small cell size CACs, especially the triploid small cell size CAC subtype, were correlated with the prognosis of patients with resectable NSCLC.

## MATERIALS AND METHODS

### Patient Enrollment and Specimen Collection

A total of 50 patients who were newly diagnosed with NSCLC between December 2014 and December 2015 and received R0

resection at Peking University Cancer Hospital were enrolled in this study. These patients were histologically confirmed as having stage I to IIIA NSCLC, including 30 cases of adenocarcinoma, 18 of squamous carcinoma, and two of large cell lung cancer (**Table 1**). Peripheral blood samples (7.5mL) were collected from all patients before the surgery and again from 35 patients one week after resection. All blood samples were processed within 24 h of collection. Each patient provided written informed consent, and the Institutional Ethics Committee of Peking University Cancer Hospital approved this study. The study was conducted according to the principles of the Declaration of Helsinki.

### CAC Detection by SE-iFISH

To identify CACs, we performed subtraction enrichment and immunostaining fluorescence *in situ* hybridization (SE-iFISH) on the samples as previously described (30, 31). CAC enrichment was performed using the subtraction enrichment method. A 7.5mL blood sample from each patient was centrifuged at 600 × g for 5 min to separate the plasma. The sedimented cells were placed on top of 3 mL of anon-hematopoietic cell separation matrix (Cytelligen, San Diego, CA, USA) and then centrifuged at 400 × g for 5 min to deplete the red blood cells. To separate the leukocytes, immune-magnetic particles conjugated with anti-CD45 monoclonal antibodies were added and incubated with

**TABLE 1** | Characteristics of patients (n = 50).

	Variable	No. of cases	Percentage (%)
<b>Age</b>	≤60	16	32
	>60	34	68
<b>Gender</b>	Male	28	56
	Female	22	44
<b>Smoking</b>	No	25	50
	Yes	25	50
<b>Histology</b>	Adenocarcinoma	30	60
	Squamous carcinoma	18	36
	Large cell lung cancer	2	4
<b>Pre-treatment clinical TNM stage</b>	I	22	44
	II	3	6
	IIIA	25	50
<b>Pathological TNM stage</b>	I	28	56
	II	12	24
	IIIA	10	20
<b>T stage</b>	T1	18	36
	T2	27	54
	T3	5	10
<b>Lymph node station</b>	N0	31	62
	N1	10	20
	N2	9	18
<b>Lymph-vascular invasion</b>	No	46	92
	Yes	4	8
<b>Recurrence</b>	No	34	68
	Yes	16	32
<b>DFS range (months)</b>	0–12	5	10
	12–24	6	12
	24–36	7	14
	36–48	26	52
	48–60	6	12

the supernatant obtained above at 25°C for 15 min. Next, the entire solution was added on the top of separation matrix again, followed by centrifuging at  $400 \times g$  for 5 min. Next, the supernatants were collected from above the magnetic beads and magnetic separation was performed; then, the bead-free solution was centrifuged again at  $500 \times g$  for 2 min. The cell pellet was mixed with 100  $\mu$ L of cell fixative, then applied to the CAC slides. These slides underwent air-drying and were then suitable for iFISH.

Next, we performed iFISH on the resulting samples according to the kit's instructions (Cytelligen). Prepared samples on the coated slides were hybridized for 4 h with the Vysis Centromere Probe (CEP8) Spectrum Orange (Abbott Laboratories, Abbott Park, IL, USA), followed by incubation with Alexa Fluor 594-conjugated monoclonal anti-CD45 antibodies (Cytelligen) at room temperature for 30 min. Finally, we used 4-6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA, USA) to stain the nuclei. At least two pathologists performed CAC counting for DAPI<sup>+</sup> and CD45<sup>+</sup> cells, identified chromosome 8 aneuploidy under fluorescence, and calculated cell size. CACs of  $\leq 5 \mu$ m (approximately the size of a WBC or less) were considered small cell size CACs, whereas those  $>5 \mu$ m were considered large cell size CACs.

## Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics software version 23.0. Correlations of CACs with clinical or pathological characteristics were calculated and analyzed using the chi-square test or Fisher's exact test, and logistic proportional hazards regression analysis was further used to analyze the multivariate hazard ratios. Disease-free survival (DFS) was defined as the duration from surgery to cancer relapse. Kaplan-Meier survival plots for 3-year DFS were generated based on whether patients were positive or negative for CACs pre- and post-surgery, and the log-rank test was used to compare survival curves.  $P < 0.05$  was considered statistically significant. All  $P$  values were two-sided.

## RESULTS

### Patient Characteristics

This study included 50 cases of NSCLC, of which 28 patients were male and 22 were female. The patients had a median age of 62 years and an average age of 61.5 years (range 39–81). Patient characteristics are presented in **Table 1**. For the pre-treatment clinical stage, 22 (44%), 3 (6%), and 25 (50%) patients were at stage I, II, and IIIA, respectively. In contrast, the numbers of patients at pathological TNM stages I, II, and IIIA were 28 (56%), 12 (24%), and 10 (20%), respectively. Pathological examination confirmed that 18 (36%), 27 (54%), and 5 (10%) patients were diagnosed with T1, T2, and T3 stages, respectively. In addition, 31 patients were diagnosed without lymph node metastasis (N0 stage), and 10 and 9 patients were diagnosed with N1 and N2

stages with lymph node metastasis, respectively. Among the 50 cases, four (8%) had lymph-vascular invasion. As for the follow-up data (DFS data are also shown in **Table 1**), tumor recurrence and progression occurred in 16 (32%) patients, whereas the status of the 34 (68%) other patients remained unchanged until the time of reporting.

### CAC Detection

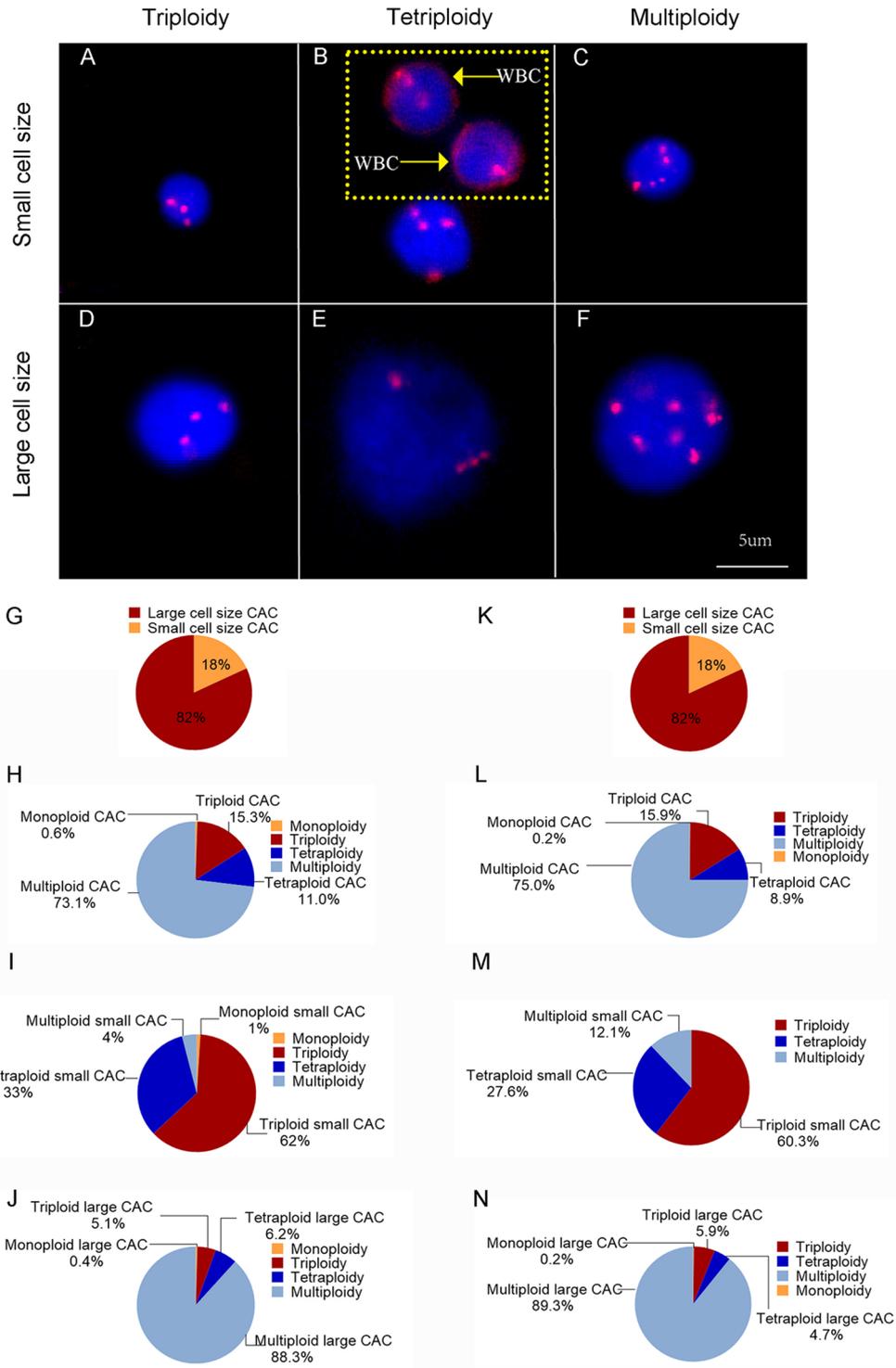
CACs were identified as having an abnormal chromosome 8 karyotype (**Figure 1**). With the general size of WBCs as the threshold, CACs were identified as either small ( $\leq 5 \mu$ m) (**Figures 1A–C**) or large ( $>5 \mu$ m) (**Figures 1D–F**). As in previous studies (32, 33), CACs were further divided into triploid, tetraploid, and multiploid subtypes.

First, we analyzed the number and characteristics of CACs derived before surgery from 50 patients with resectable NSCLC. We found 554 CACs in our patient cohort, including 454 (82%) large cell size CACs and 100 (18%) small cell size CACs (**Figure 1G**). The heteroploid features were as shown in **Figures 1H–J**. The total and large cell size CACs contained more multiploid (73% and 88%) than triploid (15% and 5%) or tetraploid cells (11% and 6%). However, small cell size CACs had the largest proportion of triploid cells (62%), compared to tetraploid (33%) and multiploid cells (4%) (**Figures 1H–J**). In addition, total, small cell size, and large cell size CACs were detected in 88% (44/50), 66% (33/50), and 86% (43/50) of patients, respectively (**Table 2**). There were 62%, 60%, and 82% positive rates for triploidy, tetraploidy, and multiploidy in the total CACs, respectively. For small cell size CACs, 56%, 44%, and 8% of patients had triploidy, tetraploidy, and multiploidy, respectively. For large cell size CACs, 22%, 34%, and 82% of patients had triploidy, tetraploidy, and multiploidy, respectively (**Table 2**).

Next, we detected 628 CACs derived from 35 patients post-surgery. Compared to the proportion of CACs in pre-surgery samples, the same percentages of large cell size CACs (82%, 512) and small cell size CACs (18%, 116) were observed in post-surgery samples (**Figure 1K**). We observed a similar distribution of the different CAC heteroploidies between the pre-treatment and post-surgery samples (**Figures 1L–N**). Multiploid CACs were the major subtype among the total and large cell size CACs, with proportions of 75% and 89%, respectively, whereas the triploid subtype was the main component (60%) among small cell size CACs (**Figures 1L–N**). Furthermore, the positive rates were 94%, 74%, and 94% for total, small cell size, and large cell size CACs, with rates for triploidy, tetraploidy, and multiploidy similar to those detected pre-surgery (**Table 2**).

### CAC Subtypes and DFS

We analyzed the correlations between histoclinical characteristics and pre-surgery CACs among our patients (**Table 3**). Unsurprisingly, triploid CACs were related to later-stage TNM and recurrence ( $p < 0.05$ ). Moreover, 90% of TNM stage III patients had triploid CACs, whereas 55% of TNM stage I/II patients had



**FIGURE 1 |** CAC detection (A–F) Representative images of circulating aneuploid cells (CACs). (A–C) Small cell size CAC. (A) Triploid small cell size CAC. (B) Tetraploid small cell size CAC. The WBC (CD45<sup>+</sup>) is indicated by a yellow arrow. (C) Multiploid small cell size CAC. (D–F) Large cell size CAC. (D) Triploid large cell size CAC. (E) Tetraploid large cell size CAC. (F) Multiploid large cell size CAC. (G–J) Pre-surgery CAC subtype distribution in 50 patients. (G) Proportion of small cell size and large cell size CACs. (H) Proportion of heteroploid subtypes of total CACs. (I) Proportion of heteroploid subtypes of small cell size CACs. (J) Proportion of heteroploid subtypes of large cell size CACs. (K–N) Post-surgery CAC subtype distribution in 35 patients. (K) Proportion of small cell size and large cell size CACs. (L) Proportion of heteroploid subtypes of total CACs. (M) Proportion of heteroploid subtypes of small cell size CACs. (N) Proportion of heteroploid subtypes of large cell size CACs.

**TABLE 2** | CAC detection before and after the surgery.

Variation	Pre-surgery (n = 50)			Post-surgery (n=35)		
	No. of CAC cases (%)	No. of small cell size CAC cases (%)	No. of large cell size CAC cases (%)	No. of CAC cases (%)	No. of small cell size CAC cases (%)	No. of large cell size CAC cases (%)
<b>Monoploidy</b>	1 (2%)	1 (2%)	1 (2%)	1 (3%)	0 (0%)	1 (3%)
<b>Triploidy</b>	31(62%)	28(56%)	11 (22%)	28 (80%)	25 (71%)	10 (29%)
<b>Tetraploidy</b>	30(60%)	22(44%)	17 (34%)	21 (60%)	12 (34%)	14 (40%)
<b>Multiploidy</b>	41(82%)	4 (8%)	41 (82%)	32 (91%)	8 (23%)	32 (91%)
<b>Total</b>	44(88%)	33(66%)	43 (86%)	33 (94%)	26 (74%)	33 (94%)

triploid CACs (**Figure 2A**, left panel). Triploid CACs were present in 88% of patients who had relapsed and 50% of patients who had not relapsed (**Figure 2A**, right panel). More importantly, small cell size CACs were associated with a higher recurrence rate and were present in 88% of patients who had relapsed and 56% of patients who had not relapsed (**Figure 2B**). Furthermore, 90% of TNM stage III patients had triploid small cell size CACs; however, only 38% of TNM stage I/II patients had triploid small cell size CACs (**Figure 2C**, left panel). Triploid small cell size CACs were present in 81% of patients who had relapsed, and in 44% of patients who had not relapsed (**Figure 2C**, right panel). Nevertheless, there was no significant correlation between clinical features and large cell size CACs (**Table 3**). The multivariate hazard ratios for CACs are shown in **Table 4**. Recurrence was an independent predictive factor for positive triploid CACs, small cell size CACs, and triploid small cell size CACs; however, gender, age, and TNM were not independent predictive factors for positive CACs.

The relationship between DFS and total, small cell size, and large cell size CACs was further clarified (**Figures 2D–O**). Among these analyses, we found that pre-surgery triploid CACs, small cell size CACs, and triploid small cell size CACs were associated with shorter DFS (**Figures 2E, H, I**). However, the Kaplan-Meier curves showed no significant differences between the other CAC subtypes and outcome in NSCLC (**Figures 2D, F, G, J–O**). We also found no significant relationship between clinical characteristics or DFS and total, small cell size, or large cell size CACs collected one week after surgery (**Table 5** and **Figures 3A–M**).

These data suggest that in resectable NSCLC before surgery, small cell size CACs, especially of the triploid subtype, are correlated to poor prognosis.

## Variations in CAC Values

We further studied CAC variation in samples derived from 35 patients pre- and post-surgery (454 and 628 CACs, respectively). The average numbers of total, small cell size, and large cell size CACs and their aneuploidy subtypes were slightly increased, with an increase in number from 1 to 5 per patient (**Figures 4A–C** and

**Table 6**). Moreover, 54%, 57%, and 54% of patients had more total, small cell size, and large cell size CACs after surgery. For small cell size CACs, the counts of the triploid, tetraploid, and multiploid subtypes increased after surgery in 57%, 23%, and 23% of patients, respectively. For large cell size CACs, the counts of the triploid, tetraploid, and multiploid subtypes increased after surgery in 26%, 29%, and 57% of patients, respectively. Based on the CAC variations pre- and post-surgery, we divided patients into the “Increased” and “Not increased” groups, as shown in **Table 7**. Nevertheless, the variation in number of total, small cell size, and large cell size CACs, as well as all their heteroploid subtypes, was not significantly correlated with clinical features or DFS (**Table 8**) (**Figures 4D–O**) ( $P>0.05$ ). These data suggest that the variation in post-surgery CAC counts was not associated with tumor recurrence among patients with resectable NSCLC.

## DISCUSSION

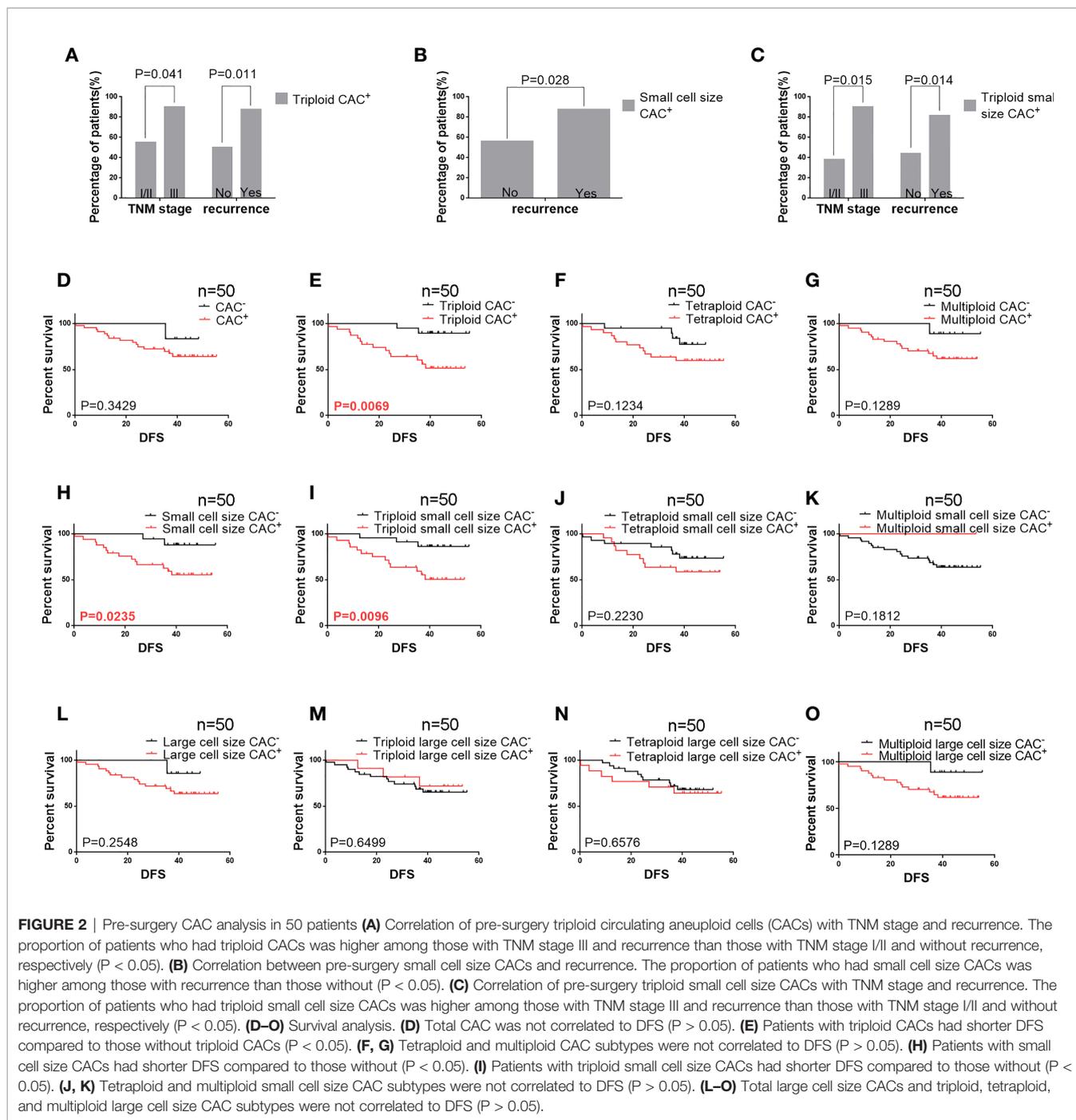
The detection of CTCs to monitor the prognosis of cancer patients has been previously reported (34, 35). Based on the pervasiveness of aneuploidy in solid tumors, we used the SE-iFISH system to detect chromosome 8 ploidy, identifying CACs in 50 patients with stage I to IIIA NSCLC who underwent surgery. In this study, we investigated a variety of correlations between clinical significance and karyotypic subtype or CAC size.

The CellSearch system has been approved for the detection of CTCs as a novel clinical marker and prognostic factor in metastasized breast, colorectal, prostate, and lung cancers (14, 17, 36, 37). However, the incidence of CTCs in NSCLC was reportedly lower than that in other cancers such as prostate, breast, ovarian, and colorectal cancer, as determined using the EpCAM-dependent method (38). Lin’s previous study validated the SE-iFISH method with over 80% recovery efficiency in lung cancer cells (20), and no CAC was found in 30 healthy donors (29). Previous studies using the conventional CellSearch method reported positive rates of only 23%–36% among NSCLC patients (37, 39, 40). By contrast, the SE-iFISH

**TABLE 3** | Correlation between pre-surgery CAC and clinical characteristics (n = 50).

Variables	CAC				Small cell size CAC				Large cell size CAC															
	total		Triploidy		Tetraploidy		Multiploidy		total		Triploidy		Tetraploidy		Multiploidy									
	+	p	+	p	+	p	+	p	+	p	+	p	+	p	+	p								
<b>Gender</b>																								
Male (n=28)	25	0.752	19	0.336	20	0.063	23	0.976	21	0.130	17	0.449	14	0.335	2	0.801	25	0.450	6	0.912	12	0.136	23	0.976
Female (n=22)	19		12		10		18		12		11		8		2		18		5		5		18	
<b>Age</b>																								
≤60 (n=16)	15	0.391	11	0.500	12	0.137	12	0.377	12	0.357	9	0.981	8	0.558	0	0.153	14	0.834	4	0.725	8	0.101	12	0.377
>60 (n=34)	29		20		18		29		21		19		14		4		29		7		9		29	
<b>Smoking history</b>																								
Never (n=25)	21	0.384	13	0.145	12	0.083	20	0.713	14	0.136	12	0.254	10	0.569	2	1.000	20	0.221	6	0.733	6	0.136	20	0.713
Yes (n=25)	23		18		18		21		19		16		12		2		23		5		11		21	
<b>Histology</b>																								
ADC (n=30)	26	0.845	17	0.527	15	0.157	24	0.763	19	0.728	14	0.222	12	0.254	1	0.235	25	0.731	8	0.537	8	0.402	24	0.763
SCC (n=18)	16		13		14		15		13		13		10		3		16		3		8		15	
LCC (n=2)	2		1		1		2		1		1		0		0		2		0		1		2	
<b>TNM classification</b>																								
I/II (n=40)	34	0.192	22	<b>0.041</b>	22	0.149	32	0.462	24	0.073	19	<b>0.015</b>	17	0.669	3	0.794	33	0.154	10	0.306	13	0.654	32	0.462
III (n=10)	10		9		8		9		9		9		5		1		10		1		4		9	
<b>Recurrence</b>																								
No (n=34)	29	0.391	17	<b>0.011</b>	18	0.137	26	0.138	19	<b>0.028</b>	15	<b>0.014</b>	13	0.231	4	0.153	28	0.279	8	0.704	11	0.720	26	0.138
Yes (n=16)	15		14		12		15		14		13		9		0		15		3		6		15	

The bold values were P values less than 0.05, which were considered statistically significant.



method used in this study previously yielded a 92% (24/26) CTC-positive rate among lung cancer patients (20). Similarly, Ye et al. evaluated CTCs in 594 blood samples from 19 various carcinomas, suggesting that the overall positive rate of CTCs in 594 samples was 89.0%, with the CTC-positive rate of lung cancer being 92.9% (79/85) through the use of SE-iFISH (29). Consistent with these data, we found that 88% and 94% of patients were positive for total CACs pre- and post-surgery,

respectively, although total CACs did not significantly correlate with DFS in resectable NSCLC.

In addition to the high CAC-positive rate in NSCLC detected using the SE-iFISH method, we then wondered whether all the CACs were malignant cells. Therefore, we divided CACs into small cell size and large cell size groups. It was shown that small cell size CACs ( $\leq 5 \mu\text{m}$ ) accounted for only a small proportion of total CACs ( $\leq 20\%$ ), whereas large cell size CACs ( $> 5 \mu\text{m}$ ) were

**TABLE 4 |** Logistic regression analysis of CAC and clinicopathologic characteristics among 50 patients with resectable NSCLC.

Variable	Triploid CAC		Small CAC		Triploid small CAC				
	Positive case number	Multivariate analysis		Positive case number	Multivariate analysis		Positive case number	Multivariate analysis	
		HR	P		HR	P		HR	P
<b>Gender</b>									
Male (n=28)	19	–	0.432	21	–	0.166	17	–	0.567
Female (n=22)	12			12			11		
<b>Age (year)</b>									
≤60 (n=15)	11	–	0.611	12	–	0.428	9	–	0.85
>60 (n=35)	20			21			19		
<b>TNM Stage</b>									
I/II (n=40)	22	–	0.193	24	–	0.254	19	–	0.078
III A (n=10)	9			9			9		
<b>Recurrence</b>									
No	17	7	<b>0.019</b>	19	5.526	<b>0.04</b>	15	5.489	<b>0.019</b>
Yes	14			14			13		

The bold values were P values less than 0.05, which were considered statistically significant.

the majority (≥80%) among the 50 cases. However, small cell size CACs, but not large cell size CACs, were related to poor outcomes in resectable NSCLC patients in our study. A previous study of advanced lung cancer showed that small cell size CTCs accounted for 52.8% of the absolute CTC number and were related to progression-free survival (40). Evidently, small cell size CACs were significantly fewer in resectable NSCLC cases than in advanced NSCLC. Another study suggested that tumor cells undergoing EMT were smaller in size than those without EMT characteristics (41) and that EMT is generally regarded as the reason for cancer relapse, metastasis, and poor prognosis (19). Wang et al. further showed that the majority of Vimentin<sup>+</sup> (a marker for mesenchymal cells) CACs were small in size and completely different from Vimentin<sup>-</sup> cells (32). All of these studies suggest that small cell size CACs with more malignant behavior are closely related to tumor progression and poor prognosis.

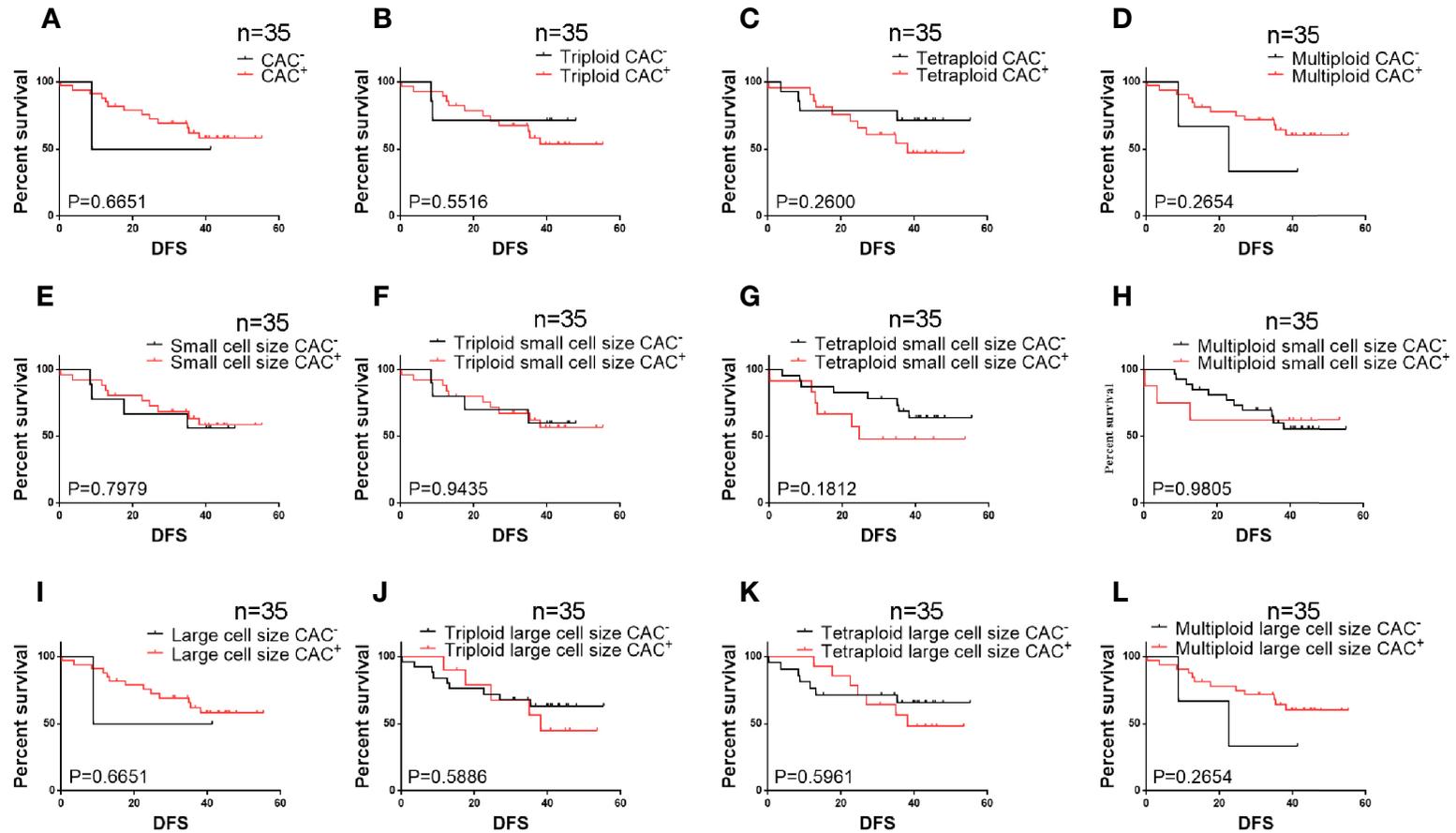
Aneuploidy, which can cause tumor formation, remains the most common feature of chromosomal instability in solid cancer (25). Coward et al. further explained this by suggesting that polyploid tumor cells, with significantly elevated genomic content, facilitate rapid tumor evolution and the acquisition of therapy resistance (26). A previous study clarified that SE-iFISH was feasible for efficient co-detection and *in situ* phenotypic and karyotypic characterization, as well as for quantification of CACs, which made further classification into diverse subtypes possible based on chromosome ploidy and biomarker expression (30). Numerous papers have already described the clinical significance of aneuploid CACs (15, 27, 30, 33, 42, 43). Ye et al. even studied 594 blood samples from 19 different carcinomas using the SE-iFISH method and found that patients at stage III–IV had more tetraploid, polyploid, large cell size, and total CTCs compared with those in patients at stage I–II (29). In addition, the latest research in 18 healthy volunteers and 34 early-stage and 24 advanced lung adenocarcinoma patients suggested that combined detection of specific aneuploid subtypes of circulating tumor endothelial cells and

CTCs may facilitate diagnosis in early-stage patients with a higher sensitivity and specificity (15). Therefore, we focused on changes in the different CAC ploidies before and after surgery. We classified CACs into the karyotypic triploid, tetraploid, and multiploid subtypes. We found that small cell size CACs had a higher percentage of triploidy, whereas large cell size CACs possessed a higher multiploidy ratio. Triploid CACs and triploid small cell size CACs were related to shorter DFS and poor clinical features. We reviewed related articles from PubMed and found that the triploid CTC subtype is considered to be related to therapeutic resistance in several solid tumors. Chen et al. found that in patients with esophageal cancer, those without triploid cells were more sensitive to chemotherapy than those with them (33). Similarly, Li et al. also suggested that an increased percentage of triploid CTCs was associated with chemotherapy resistance in advanced gastric cancer (27). As for pancreatic cancer, Xu et al. found that triploid CTC number could not only predict chemo-sensitivity but was also associated with reduced 1-year survival (43). Our study, focusing on 3-year DFS in resectable NSCLC, further suggested that triploid small cell size CACs, not triploid large cell size CACs, might be more helpful for predicting prognosis. The above data may suggest that triploid cells are more malignant and can hence lead to proliferation, progression, and chemotherapy resistance. Triploid CACs or triploid small cell size CACs may have potential as new prognostic predictors and therapeutic targets in tumors, including NSCLC.

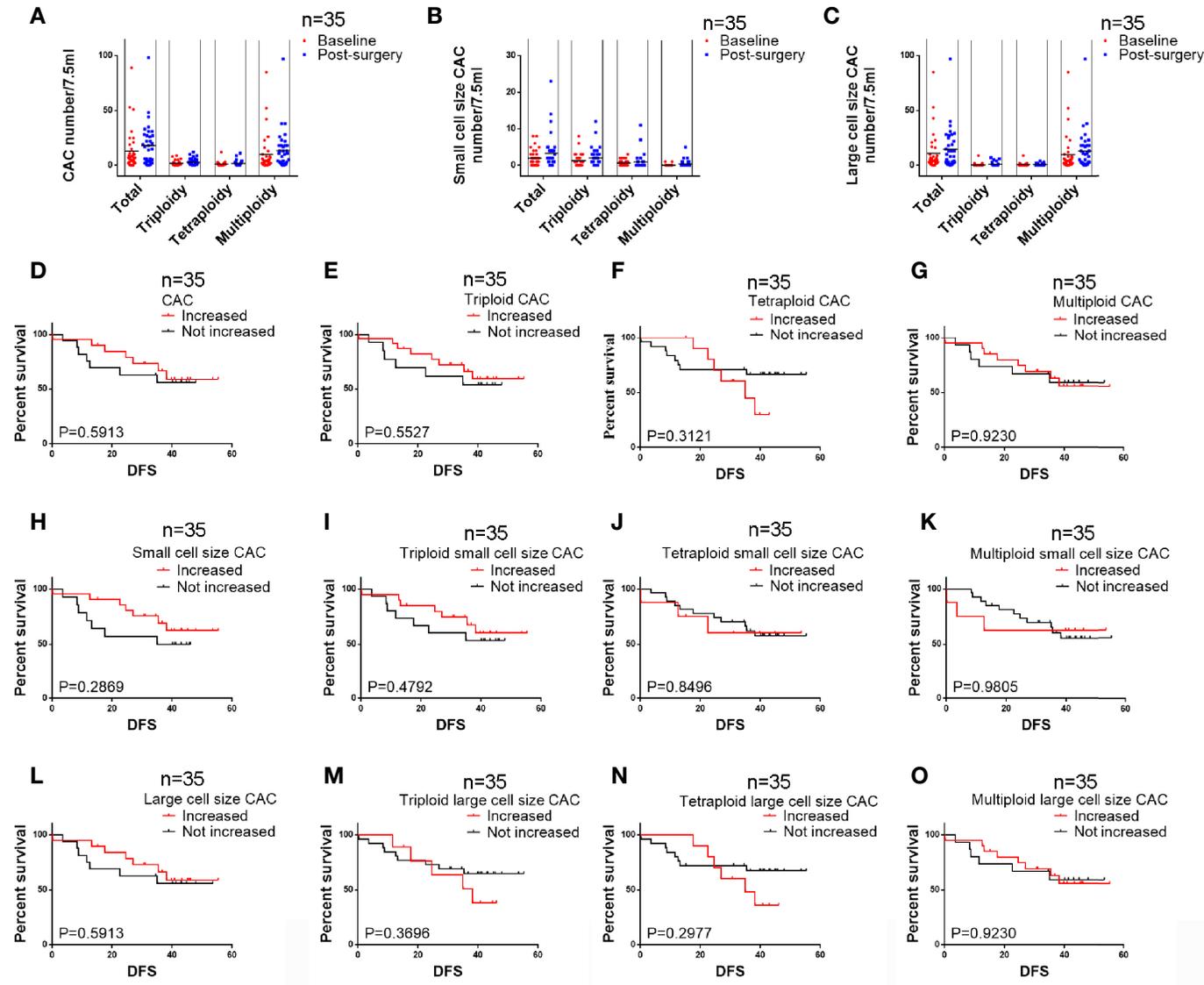
We also investigated how CAC number and CAC ploidy changed before and after surgical treatment. After resection, the CAC number and subtypes increased slightly, but no significant correlation was found between DFS and the variation pre- and post-surgery. Treatment through surgery may facilitate the entry of CACs into the general circulation, and these cells were mostly nonmalignant CACs or endothelial cells, which could be gradually eliminated by circulating immune cells (44–46). Among patients who underwent breast cancer resection, the CAC number was reportedly elevated 3–4 days after surgery but

**TABLE 5 |** Correlation of post-surgery CAC to clinical characteristics (n=35).

Variables	CAC																							
	Small cell size CAC								Large cell size CAC															
	total		Triploidy		Tetraploidy		Multiploidy		total		Triploidy		Tetraploidy		Multiploidy									
+	p	+	p	+	p	+	p	+	p	+	p	+	p	+	p	+	p							
<b>Gender</b>																								
Male (n=23)	23	0.111	20	0.200	16	0.153	22	0.266	18	0.685	17	0.706	9	0.476	7	0.216	23	0.111	7	1.000	10	0.721	22	0.266
Female (n=12)	10		8		5		10		8		8		3		1		10		3		4		10	
<b>Age</b>																								
≤60 (n=10)	10	1.000	8	1.000	5	0.474	10	0.542	9	0.235	8	0.686	4	0.706	3	0.661	10	1.000	1	0.218	2	0.252	10	0.542
>60 (n=25)	24		20		16		22		17		17		8		5		23		9		12		22	
<b>Smoking history</b>																								
Never (n=14)	12	0.153	10	0.401	6	0.159	12	0.551	10	1.000	10	1.000	4	0.721	3	1.000	12	0.153	3	0.704	4	0.311	12	0.551
Yes (n=21)	21		18		15		20		16		15		8		5		21		7		10		20	
<b>Histology</b>																								
ADC (n=18)	16	0.546	13	0.627	9	0.464	16	1.000	13	1.000	12	0.860	6	0.752	6	0.340	16	0.546	4	0.402	5	0.112	16	1.000
SCC (n=15)	15		13		10		14		11		11		6		2		15		6		7		14	
LCC (n=2)	2		2		2		2		2		2		0		0		2		0		2		2	
<b>TNM classification</b>																								
I/II (n=29)	28	0.318	23	1.000	17	1.000	27	0.442	22	0.635	21	1.000	9	0.391	6	0.602	28	0.318	8	1.000	12	1.000	27	0.442
III (n=6)	5		5		4		5		4		4		3		2		5		2		2		5	
<b>Recurrence</b>																								
No (n=21)	20	1.000	16	0.676	11	0.311	20	0.551	16	1.000	15	1.000	6	0.477	5	1.000	20	1.000	5	0.474	7	0.483	20	0.551
Yes (n=14)	13		12		10		12		10		10		6		3		13		5		7		12	



**FIGURE 3** | Survival analysis of post-surgery CACs among 35 patients **(A–D)** Total circulating aneuploid cells (CACs) and triploid, tetraploid, and multiploid CAC subtypes were not correlated to DFS ( $P > 0.05$ ). **(E–H)** Total small cell size CACs and triploid, tetraploid, and multiploid small cell size CAC subtypes were not correlated to DFS ( $P > 0.05$ ). **(I–L)** Total large cell size CACs and triploid, tetraploid, and multiploid large cell size CAC subtypes were not correlated to DFS ( $P > 0.05$ ).



**FIGURE 4** | Analysis of CAC pre- and post-surgery variation among 35 patients. **(A)** Variation in numbers of total CACs and the three subtypes. **(B)** Variation in numbers of total small cell size CACs and the three subtypes. **(C)** Variation in numbers of total large cell size CACs and the three subtypes. **(D–G)** Variations in total CACs and the triploid, tetraploid, and multiploid subtypes were not correlated to DFS ( $P > 0.05$ ). **(H–K)** Variations in total small cell size CACs and the triploid, tetraploid, and multiploid small cell size CAC subtypes were not correlated to DFS ( $P > 0.05$ ). **(L–O)** Variations in total large cell size CACs and the triploid, tetraploid, and multiploid large cell size CAC subtypes were not correlated to DFS ( $P > 0.05$ ).

**TABLE 6 |** Average CAC number in 35 pairs of patients before and after the surgery.

Variable	CAC		Small cell size CAC		Large cell size CAC	
	Pre-surgery	Post-surgery	Pre-surgery	Post-surgery	Pre-surgery	Post-surgery
	No. of CACs	No. of CACs	No. of CACs	No. of CACs	No. of CACs	No. of CACs
Monoploid	0 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Triploid	2 ± 2	3 ± 3	1 ± 2	2 ± 3	0 ± 2	1 ± 2
Tetraploid	1 ± 2	2 ± 2	1 ± 1	1 ± 2	1 ± 2	1 ± 1
Multiploid	10 ± 17	13 ± 18	0 ± 0	0 ± 1	10 ± 17	13 ± 18
Total	13 ± 18	18 ± 19	2 ± 2	3 ± 5	11 ± 18	15 ± 19

**TABLE 7 |** CAC variation in the 35 NSCLC cases before and after surgery.

Variations		No. of increased cases	No. of not increased cases
CAC	Total	19 (54%)	16 (46%)
	Triploidy	22 (63%)	13 (37%)
	Tetraploidy	11 (31%)	24 (69%)
	Multiploidy	20 (57%)	15 (43%)
Small cell size CAC	Total	20 (57%)	15 (43%)
	Triploidy	20 (57%)	15 (43%)
	Tetraploidy	8 (23%)	27 (77%)
Large cell size CAC	Total	8 (23%)	27 (77%)
	Triploidy	19 (54%)	16 (46%)
	Multiploidy	9 (26%)	26 (74%)
Large cell size CAC	Triploidy	10 (29%)	25 (71%)
	Tetraploidy	20 (57%)	15 (43%)
	Multiploidy		

fell back to the pre-surgery condition one week post-surgery (47). However, no publication has yet illustrated the proper time for post-surgery CTC or CAC blood collection in lung cancer. In our study, CACs kept increasing among patients with resectable NSCLC one week after surgery, suggesting that blood collection may need to be performed much later. A previous study illustrated that an operation was not able to reduce CTCs in all cases, and a considerable number of CTCs remained in circulation following resection of the primary tumor. Moreover, some CTCs may already be pre-existing in the patients' circulation, and not derived from the resection of primary malignant tumors, influencing later metastasis and recurrence (34, 48, 49). This may be why post-surgery CACs had a weak influence on clinical outcome in our study.

### CONCLUSIONS

In summary, we used SE-iFISH to detect CACs in patients with resectable NSCLC. We first divided total CACs into small and large sizes and further classified these CACs into three heteroploid subtypes based on chromosome 8 ploidy. We found that small cell size CACs accounted for no more than 20% of total CACs and were significantly related to shorter DFS in patients with resectable NSCLC. Pre-surgery small cell size CACs, especially of the triploid subtype, were significantly correlated with later TNM stage and recurrence, suggesting that this subtype might be a good biomarker for poor prognosis in patients with resectable NSCLC.

**TABLE 8 |** Correlations between clinical characteristics and CAC variations pre- and post-surgery in 35 NSCLC patients.

Variables	CAC				Small cell size CAC				Large cell size CAC															
	total		Triploidy		Tetraploidy		Multiploidy		total		Triploidy		Tetraploidy		Multiploidy									
	IG	p	IG	p	IG	p	IG	p	IG	p	IG	p	IG	p	IG	p								
<b>Gender</b>																								
Male (n=23)	10	0.152	14	1.000	8	1.000	12	0.489	13	1.000	12	0.489	7	0.216	7	0.216	11	0.476	7	0.450	7	1.000	12	0.489
Female (n=12)	9		8		3		8		7		8		1		1		8		2		3		8	
<b>Age</b>																								
≤60 (n=10)	3	0.132	5	0.444	1	0.120	5	0.712	4	0.266	5	0.712	1	0.390	3	0.661	4	0.454	1	0.235	1	0.218	5	0.712
>60 (n=25)	16		17		10		15		16		15		7		5		15		8		9		15	
<b>Smoking history</b>																								
Never (n=14)	9	0.491	10	0.488	3	1.000	8	1.000	9	0.728	10	0.296	2	0.431	3	1.000	8	1.000	2	0.262	2	0.252	8	1.000
Yes (n=21)	10		12		8		12		11		10		6		5		11		7		8		12	
<b>Histology</b>																								
ADC (n=18)	10	1.000	11	1.000	5	0.177	12	0.488	12	0.488	12	0.488	4	1.000	6	0.340	11	0.741	4	0.834	4	0.105	12	0.488
SCC (n=15)	8		10		4		7		7		7		4		2		7		5		4		7	
LCC (n=2)	1		1		2		1		1		1		0		0		1		0		2		1	
<b>TNM classification</b>																								
I/II (n=29)	15	0.666	18	1.000	9	1.000	16	0.680	18	0.367	17	1.000	7	1.000	6	0.602	15	0.666	7	0.635	8	1.000	16	0.680
III (n=6)	4		4		2		4		2		3		1		2		4		2		2		4	
<b>Recurrence</b>																								
No (n=21)	12	0.739	14	0.724	5	0.283	12	1.000	13	0.511	13	0.511	5	1.000	5	1.000	12	0.739	4	0.432	4	0.151	12	1.000
Yes (n=14)	7		8		6		8		7		7		3		3		7		5		6		8	

IG: case number of "increased group" after surgery.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the medical ethics committee of Beijing Cancer Hospital. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

YH, YM, and CL contributed to conception and design of the study. Medical practitioners YY, CL, JieZ, YX, JiaZ, and JF

provided patients and patient data for the study. YH, YM, and CL organized the database, and YH performed the statistical analysis. JS supported the literature and statistical methods. PL supported the detection method. YH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** Author PPL was employed by Cytelligen, San Diego, CA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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