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ORIGINAL REPORT

Clinical Significance and Molecular Characteristics of Circulating Tumor Cells and Circulating Tumor Microemboli in Patients With Small-Cell Lung Cancer

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ABSTRACT

Purpose
Circulating tumor cells (CTCs) may have utility as surrogate biomarkers and "virtual" biopsies. We report the clinical significance and molecular characteristics of CTCs and CTC clusters, termed circulating tumor microemboli (CTM), detected in patients with small-cell lung cancer (SCLC) undergoing standard treatment.

Patients and Methods
Serial blood samples from 97 patients receiving chemotherapy were analyzed using EpCam-based immunomagnetic detection and a filtration-based technique. Proliferation status (Ki67) and apoptotic morphology were examined. Associations of CTC and CTM number with clinical factors and prognosis were determined.

Results
CTCs were present in 85% of patients (77 of 97 patients) and were abundant (mean ± standard deviation = 1,589 ± 5,565). CTM and apoptotic CTCs were correlated with total CTC number and were detected in 32% and 57% of patients, respectively. Pretreatment CTCs, change in CTC number after one cycle of chemotherapy, CTM, and apoptotic CTCs were independent prognostic factors. Overall survival was 5.4 months for patients with ≥ 50 CTCs/7.5 mL of blood and 11.5 months ($P < .0001$) for patients with less than 50 CTCs/7.5 mL of blood before chemotherapy (hazard ratio = 2.45; 95% CI, 1.39 to 4.30; $P = .002$). Subpopulations of apoptotic and of proliferating solitary CTCs were detected, whereas neither were observed within cell clusters (CTM), implicating both protection from anoikis and relative resistance to cytotoxic drugs for cells within CTM.

Conclusion
Both baseline CTC number and change in CTC number after one cycle of chemotherapy are independent prognostic factors for SCLC. Molecular comparison of CTCs to cells in CTM may provide novel insights into SCLC biology.

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INTRODUCTION

Small-cell lung cancer (SCLC) is characterized by early development of widespread metastases and initial response to chemotherapy but high relapse rates.[1] Study of SCLC biology is hindered by insufficient tissue for research, because surgical resection and/or serial biopsies are rare. Improved technology for detection, enumeration, and characterization of circulating tumor cells (CTCs) has identified CTCs to be prognostic and pharmacodynamic biomarkers in various solid tumors.[2] CTC number, determined using the US Food and Drug Administration–approved CellSearch system (Veridex, Raritan, NJ), is prognostic in metastatic breast, prostate, colorectal, and non–small-cell lung cancer (NSCLC).[3-6] Our previous pilot study demonstrated detection of CTCs in patients with SCLC in whom CTC number fell during the first cycle of chemotherapy,[7] and in addition to solitary CTCs, we also reported CTC clusters, termed circulating tumor microemboli (CTM).[8] This study was conducted to establish the prevalence and clinical significance of CTCs and CTM in patients with SCLC and explore their molecular characteristics with respect to proliferative status (Ki67) and propensity for apoptosis (morphology, Bcl-2, Mcl-1).

PATIENTS AND METHODS

Study Design

This was a prospective, single-center study conducted at the Christie Hospital, Manchester, United Kingdom. Eligible patients had histologically or cytopathologically confirmed chemotherapy-naive SCLC, staged and managed using standard treatment protocols according to international guidelines.[9] All patients gave written, informed consent to ethically approved protocols. Blood samples were collected for analysis within 7 days before commencing treatment (baseline) and after one chemotherapy cycle. Analyses were conducted to good clinical practice standard. Data were collected for clinical/biochemical factors (Table 1).

CTC Analysis

CTC analysis was performed using CellSearch, as previously described.[10,11] CTCs were defined as cells coexpressing EpCam and cytokeratins (8, 18, and 19) without expression of the WBC surface marker CD45 and had a 4′,6-diamidino-2-phenylindole (DAPI) –stained nucleus. Apoptotic cells were identified via characteristic fragmented, condensed DAPI-stained nuclear morphology.[12] Cross-validation of apoptosis on the basis of nuclear morphology versus caspase activation was performed using an antibody to caspase-cleaved cytokeratin (M30, Peviva) after cisplatin treatment of Hela cells (Data Supplement).

Table 1. Patient Characteristics

Characteristic	Evaluable Patients (n = 97) No.	%
Age at baseline, years		
Median	68	
Range	28-84	
Sex		
Female	54	56
Male	43	44
Stage at diagnosis		
Limited	31	32
Extensive	66	68
Baseline WHO PS		
0	12	12
1	46	47
2	31	32
3 and 4	8	8
Treatment received		
Single-agent regimen (carboplatin)	14	15
Platinum doublet regimen	81	83
Carboplatin + etoposide	69	71
Cisplatin + etoposide	12	12
VAC	1	1
No treatment	1	1
Baseline laboratory values		
Na		
Median	137	
Range	113-145	
Na < 132	14	14
LDH		
Median	588	
Range	259-14,131	
LDH > 450	60	62

Abbreviations: LDH, lactate dehydrogenase; PS, performance status; VAC, vincristine, actinomycin, and cyclophosphamide.

To prevent false assignment of a mitotic CTC as a microembolus,[13] CTM were defined as groups of CTCs containing three or more distinct nuclei. Blood-spiking experiments with Hela cells expressing histone H2B-GFP were also performed to confirm that CTM were not artifacts caused by sample manipulation (Data Supplement). Bcl-2 expression was performed using fluorescein isothiocyanate–conjugated mouse antihuman Bcl-2 antibody (BD PharMingen, San Diego, CA) and analyzed in the fourth channel of the CellSearch system. Mcl-1 expression was similarly explored using customized Alexa Flour 488-conjugated mouse antihuman Mcl-1 antibody (BD PharMingen).

For immunohistochemistry, samples were processed using the Isolation by Size of Epithelial Tumor cells (ISET; RareCell Diagnostics, Paris, France) platform according to manufacturer's instructions[14] and our previous study[8] (Data Supplement).

Statistical Analysis

Associations of baseline CTC number, apoptotic CTCs, and CTM with individual clinical and biochemical factors were compared using Fisher's exact test. Correlations between baseline CTC number, apoptotic CTC number, and CTM number were compared using Spearman's rho analysis. The sample size was calculated for the primary end point of survival assuming two populations of patients with favorable and unfavorable CTC number (as previously described[3-6]) having 0.65 and 0.35 survival probabilities at 6 months, respectively, such that a log-rank test with a one-sided significance level of .05 has 80% power to detect a difference between the survival curves, equal to a hazard ratio (HR) of 2.4 after a minimum of 42 events with at least 88 evaluable patients equating to 51 favorable and 37 unfavorable. To determine the most appropriate CTC cutoff, a series of baseline CTC values between 1 and 5,000 were tested for their estimate of survival using the Kaplan-Meier method. After Bonferroni correction for multiple testing, 50 CTCs showed most significant discrimination in survival estimation (Data Supplement; Table 1).[15] Receiver operating characteristic curves were analyzed, confirming 50 CTCs as the optimal cutoff (Data Supplement; Table 2). CTM and apoptotic CTCs were exploratory end points and were analyzed as presence or absence of the event.

CTC number, presence of apoptotic CTCs, presence of CTM at baseline, CTC number after one cycle of chemotherapy (second CTC number), and standard clinical/biochemical factors (Table 1) were subjected to univariate Cox proportional hazards regression analysis for progression-free survival (PFS) and overall survival (OS). Univariately significant parameters were included in a multivariate Cox regression analysis (forward stepwise selection [Wald] method; P value of .05 was selected for entry into the model and P value of .1 was selected for removal). A paired t test was used to analyze CTC counts before and after chemotherapy.

In evaluating absolute change in CTC number, an important consideration is the confounding effect of baseline CTC number on the absolute change. There is strong positive correlation between high baseline CTC number and size of decline (to have a larger decline, a larger baseline CTC number is necessary). Also, using percent change "flattens" the data such that, for example, a 20% change with a baseline CTC number and second time point CTC number of less than 50 becomes equal to a 20% change with a baseline CTC number and then second time point CTC number of more than 50. Therefore, patients were categorized into three groups according to baseline CTC number and residual CTC number after one chemotherapy cycle (group 1, <50 to <50; group 2, ≥ 50 to <50; and group 3, ≥ 50 to ≥ 50) to use as covariates in the multivariate survival model that also included baseline CTC number. PFS and OS were measured from date of baseline blood sample to date of confirmed clinical progression, death, or censoring at last follow-up. Statistical analysis was performed using SPSS for Windows (release 13.0.2004, SPSS, Chicago, IL), where P values of $\leq .05$ were considered significant. Results are reported according to REMARK (Reporting Recommendations for Tumour Marker Prognostic Studies) guidelines.[16]

Table 2. Prevalence of CTCs, CTM, Apoptotic CTCs, and Association With Clinical Characteristics (N = 97)

Variable	Patients With CTC ≥ Threshold		Patients With CTM		Patients With Apoptotic CTCs	
	< 50	≥ 50	No CTM	CTM	No Apoptotic CTCs	Apoptotic CTCs
Stage						
Limited (n = 31)	29	2	30	1	27	4
Extensive (n = 66)	27	39	42	24	26	40
Fisher's exact *P*	< .001		< .001		< .001	
PS						
0 or 1 (n = 58)	40	18	46	12	36	22
2 or 3 or 4 (n = 39)	16	23	26	13	17	22
Fisher's exact *P*	.0114		.2362		.0966	
LDH						
< 450 (n = 42)	38	4	41	1	33	9
> 450 (n = 54)	18	37	31	24	20	35
Fisher's exact *P*	< .001		< .001		< .001	
Na						
> 132 (n = 83)	47	36	60	23	43	40
< 132 (n = 14)	9	5	12	2	10	4
Fisher's exact *P*	.7716		.5089		.2475	
Sites of metastases						
Liver						
Yes (n = 43)	10	33	21	22	11	32
No (n = 54)	46	8	51	3	42	12
Fisher's exact *P*	< .001		< .001		< .001	
Bone						
Yes (n = 12)	4	8	7	5	4	8
No (n = 85)	52	33	65	20	49	36
Fisher's exact *P*	.1158		.2873		.1325	
Adrenal						
Yes (n = 11)	7	4	10	1	7	4
No (n = 86)	49	37	62	24	46	40
Fisher's exact *P*	.7554		.2792		.7493	
No. of sites of metastases						
0 (n = 33)	30	3	32	1	27	6
1 (n = 32)	12	20	18	14	13	19
2 (n = 21)	9	12	13	8	9	12
3+ (n = 11)	5	6	9	2	4	7
Fisher's exact *P*	< .001		< .001		.0012	
CTC cutoff = 50						
≥ 50 (n = 41)			17	24	5	36
< 50 (n = 56)			55	1	48	8
Fisher's exact *P*			< .001		< .001	
CTM						
Presence of CTM (n = 25)	0	25			3	22
Absence of CTM (n = 72)	56	16			50	22
Fisher's exact *P*	< .001				< .001	
Apoptotic CTCs						
Presence of Apoptotic CTCs (n = 44)	8	36	22	22		
Absence of Apoptotic CTCs (n = 53)	48	5	50	3		
Fisher's exact *P*	< .001		< .001			

Abbreviations: CTCs, circulating tumor cells; CTM, circulating tumor microemboli; LDH, lactate dehydrogenase; PS, performance status.

RESULTS

Patient Demographics

A total of 102 patients were enrolled between June 2007 and March 2010 (Table 1), of whom five were nonevaluable (Data Supplement). At the time of analysis, 70 (72%) of the 97 evaluable patients had experienced disease progression and 63 (65%) of the 97 evaluable patients had died, resulting in a median PFS of 7.4 months (95% CI, 5.9 to 8.9 months) and median OS of 9.0 months (95% CI, 7.3 to 10.7 months). The average length of follow-up time for the 34 patients still alive was 7.4 ± 5.9 months (range, 0.7 to 23.9 months).

Numbers of CTCs, CTM, and Apoptotic CTCs at Baseline

CTCs were present in 85% of patients (77 of 97 patients) at baseline before chemotherapy. Median CTC number was 24 (range, 0 to 44,896; mean ± standard deviation [SD], 1,589 ± 5,565). CTM and CTCs with apoptotic morphology were observed in 25 of 77 (32%) and 44 of 77 patients (57%) with CTCs, respectively. There was significant correlation between CTC number, CTM number, and apoptotic CTC number (Data Supplement). On the basis of a cutoff ≥ 50 CTCs/7.5 mL of blood, 43% patients had an unfavorable CTC number at baseline. An unfavorable CTC number was significantly associated with stage, lactate dehydrogenase, presence of liver metastases, and number of sites of metastasis. These clinical factors were also significantly associated with CTM and apoptotic CTCs (Table 2).

Prognostic Significance of CTCs at Baseline

In univariate analysis for CTC number, patients were categorized into favorable and unfavorable groups (< 50 CTCs *v* ≥ 50 CTCs). For patients with an unfavorable CTC number, there was a significantly shorter median PFS (4.6 months; 95% CI, 3.8 to 5.3 months) and OS (5.4 months; 95% CI, 3.1 to 7.7 months) compared with patients with less than 50 CTCs/7.5 mL of blood (median PFS, 8.8 months; 95% CI, 6.9 to 10.6 months; median OS, 11.5 months; 95% CI, 10.3 to 12.7 months; Figs 1A and 1B).

The sensitivity and specificity of ≥ 50 CTCs in predicting survival at 6 months were 67% and 70% with positive and negative predictive values of 50% and 82%, respectively. Considering likelihood ratios, on the basis of a 35% probability of death within 6 months, there is a 55% chance that a patient with ≥ 50 CTCs will die within 6 months as compared with a 20% chance for a patient with less than 50 CTCs. CTM were present in 25 of 97 patients. Presence of CTM at baseline demonstrated significantly shorter PFS (4.6 months; 95% CI, 2.4 to 6.7 months) and OS (4.3 months; 95% CI, 0.87 to 7.7 months) compared with absence of CTM (median PFS, 8.2 months; 95% CI, 7.3 to 9.0 months; median OS, 10.4 months; 95% CI, 9.0 to 11.7 months; Figs 1C and 1D). Apoptotic CTCs (assigned by nuclear morphology) were detected in 44 of 97 patients, and presence of apoptotic CTCs at baseline was associated with worse PFS (4.2 months; 95% CI, 2.9 to 5.4 months) and OS (5.7 months; 95% CI, 3.3 to 8.0 months) compared with their absence (median PFS, 9.0 months; 95% CI, 7.2 to 10.9 months; median OS, 11.8 months; 95% CI, 10.6 to 12.9 months; Figs 1E and 1F).

CTC Number After One Cycle of Chemotherapy

A second blood sample for CTC analysis was obtained from 53 patients after one chemotherapy cycle at a median of 3 weeks from

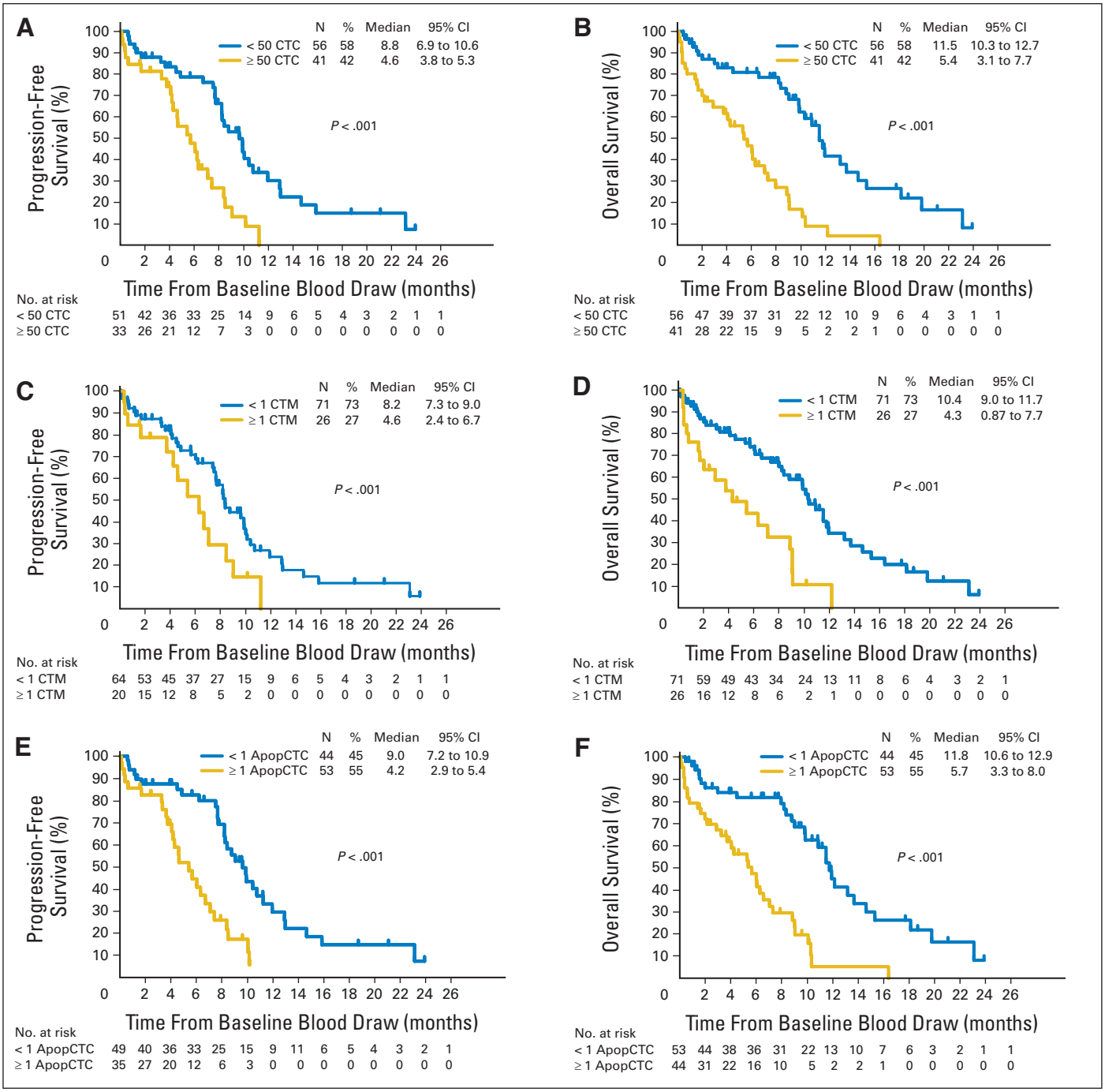

Fig 1. Kaplan-Meier curves for progression-free survival and overall survival of patients (A, B) with fewer than 50 and ≥ 50 circulating tumor cells (CTCs) per 7.5 mL of blood at baseline, (C, D) without the presence of circulating tumor microemboli (CTM) and with the presence of CTM at baseline, and (E, F) without the presence of apoptotic CTCs (ApopCTC) and with the presence of ApopCTCs at baseline (n = 97).

the pretreatment sample. CTC number fell in 43 patients and increased in two patients, and there was no change in nine patients (0 at both time points). Median CTC numbers before and after treatment were significantly different at 24 (range, 0 to 44,896) and 1 (range, 0 to 2,960) respectively ($P = .006$). A favorable CTC number (< 50) after one chemotherapy cycle was associated with significantly longer PFS (9.6 months; 95% CI, 7.8 to 11.5 months) and OS (10.4 months; 95% CI, 8.8 to 11.9 months) compared with an unfavorable CTC number (≥ 50; median PFS, 4.1 months; 95% CI, 0 to 9.2 months; median OS, 4.1 months; 95% CI, 0 to 8.5 months; Figs 2A and 2B). Considering baseline and post-treatment time points together, patients with less than 50 CTCs at both time points (group 1) had significantly better survival (PFS, 8.3 months; 95% CI, 6.4 to 10.2 months; OS, 9.2 months; 95% CI, 7.2 to 11.2 months) compared with group 2 (≥ 50 CTCs at baseline, < 50 CTCs at second time point; PFS, 4.3 months; 95% CI, 2.8 to 5.7 months; OS, 5.0 months; 95% CI, 3.5 to 6.5 months) and group 3 (≥ 50 CTCs at both time points; PFS, 4.1 months; 95% CI, 1.7 to

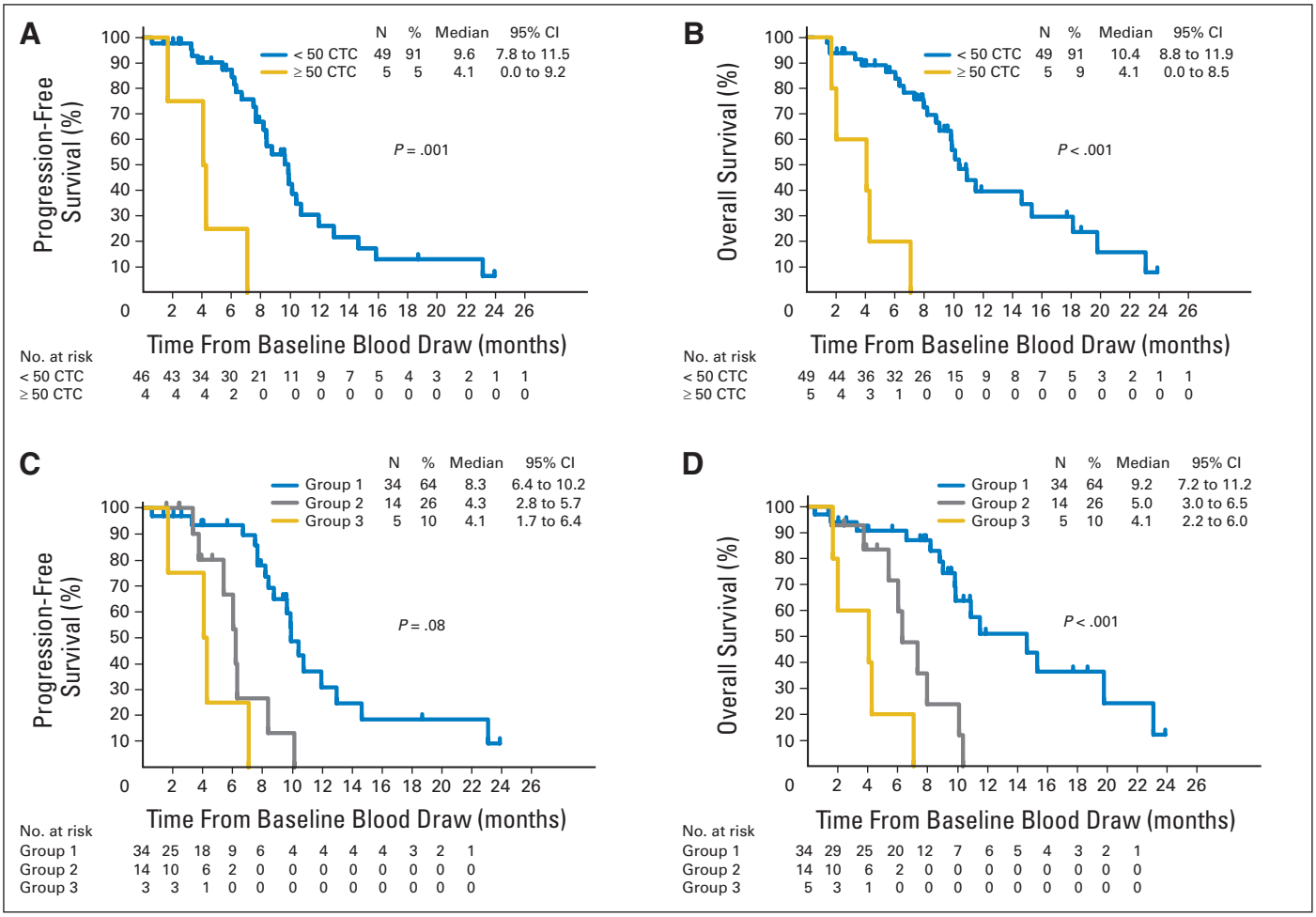

Fig 2. Kaplan-Meier curves for progression-free survival (PFS) and overall survival (OS) of patients (A, B) with fewer than 50 and ≥ 50 circulating tumor cells (CTCs)/7.5 mL of blood at second time point (after one cycle of standard chemotherapy). When both time points (before and after one cycle of chemotherapy) are considered, (C) PFS and (D) OS of patients with fewer than 50 CTCs at both time points (group 1), ≥ 50 CTCs at baseline, fewer than 50 CTCs at second time point (group 2), and ≥ 50 CTCs at both time points (group 3) are shown.

6.4 months; OS, 4.1 months; 95% CI, 2.2 to 6.0 months; Figs 2C and 2D).

Multivariate Cox Proportional Hazards Regression Analysis

The clinical factors significant for survival in univariate analysis were stage, performance status, number of metastatic sites, treatment, and lactate dehydrogenase. In multivariate analysis adjusting for these factors, CTC number at baseline was an independent prognostic factor for PFS (HR = 2.01; 95% CI, 1.17 to 3.46, $P = .011$) and OS (HR = 2.45; 95% CI, 1.39 to 4.30, $P = .002$). Presence of CTM and presence of apoptotic CTCs before chemotherapy were also independent prognostic factors (Tables 3 and 4). When CTC number was removed from the models so that only clinically significant factors remained, there was a statistically significant loss in model performance for both OS ($P = .03$) and PFS ($P = .017$), confirming additional impact of CTC number. In an exploratory analysis of 53 patients grouped according to CTC number change using data from two time points before and after chemotherapy, CTC number change was the most significant variable for survival (HR = 4.1; 95% CI, 1.1 to 15.1, $P = .03$), adjusting for the baseline CTC count and other clinical prognostic factors (performance status, stage, number of metastatic sites).

Molecular Characteristics of CTCs and CTM

Examples of apoptotic CTCs with classical fragmented and condensed DAPI-stained nuclei within intact cells are shown in Appendix Figure A1A (online only). Apoptotic CTCs (detected in 44 patients) ranged from 0.2% to 20% of overall CTC number. CTM were present in 25 patients. In contrast, and even in patients with apoptotic solitary CTCs, none of the cells (> 15,795) comprising CTM (n = 5,265) exhibited apoptotic morphology (Appendix Fig A1B). Six of seven patients with apoptotic CTCs at both time points demonstrated an increased ratio of apoptotic CTC number to overall CTC number after treatment compared with pretreatment.

Overexpression of the antiapoptotic protein Bcl-2 has been reported in 55% to 90% of SCLC biopsies.[17] On an exploratory basis, Bcl-2 was detected in CTCs and CTM isolated by CellSearch in 18 of 30 patients (Appendix Fig A1C), and percentage of Bcl-2–positive CTCs to overall CTC number ranged from 0% to 100% (Median, 1.5%; mean ± SD = 13.2% ± 24.1%). In addition, no Bcl-2–positive CTCs

Table 3. Univariate Cox Regression Analysis for Prediction of PFS and OS: Statistically Significant Factors in Univariate Analysis

Parameter	At-Risk Group		PFS Risk			OS Risk		
	Positive	Negative	*P*	HR	95% CI	*P*	HR	95% CI
Stage	Extensive	Limited	< .001	2.69	1.54 to 4.68	< .001	3.95	2.17 to 7.19
Performance status	Continuous variable		.003	2.07	1.29 to 3.31	< .001	2.50	1.52 to 4.12
Metastases								
Liver	Yes	No	< .001	2.35	1.42 to 3.90	< .001	3.46	2.03 to 5.89
Adrenal	Yes	No	< .004	3.21	1.45 to 7.09	.019	2.68	1.17 to 6.14
Bone	Yes	No	< .002	3.03	1.49 to 6.16	< .001	3.69	1.77 to 7.71
No. of metastatic mites	Continuous variable		.001	1.46	1.18 to 1.81	< .001	1.70	1.36 to 2.14
Treatment received	No/singlet	Doublet	.012	2.36	1.21 to 4.62	.005	2.61	1.34 to 5.11
LDH	Continuous variable		.048	1.00	1.00 to 1.00	.018	1.00	1.00 to 1.00
CTCs at baseline	≥ 50	< 50	< .001	2.70	1.62 to 4.50	< .001	3.55	2.08 to 6.04
CTM at baseline	Yes	No	.008	2.07	1.21 to 3.54	< .001	2.94	1.67 to 5.19
Apoptotic CTCs at baseline	Yes	No	< .001	3.03	1.79 to 5.12	< .001	3.75	2.16 to 6.51
CTCs at second time point	≥ 50	< 50	.001	6.28	2.17 to 18.20	< .001	8.63	2.81 to 26.58

Abbreviations: CTCs, circulating tumor cells; CTM, circulating tumor microemboli; HR, hazard ratio; LDH, lactate dehydrogenase; OS, overall survival; PFS, progression-free survival.

or cells within CTM exhibited apoptotic nuclear morphology. Mcl-1, another Bcl-2 family member, was detected in one third patients screened using the fourth channel of the CellSearch system (Appendix Fig A1C).

To assess the proliferation status of solitary CTCs and CTM, Ki67 analysis was performed by immunohistochemistry on cells isolated using the ISET filter technique (Appendix Fig A1D). Ki67 expression was detected in solitary CTCs from 20 patient samples in variable proportions of cells (Appendix Fig A1E, left panel). In contrast, all CTMs (n = 34) were negative for Ki67, even in patients with Ki67(+) solitary CTCs (Appendix Fig A1E, middle and right panels).

Table 4. Stepwise Multivariate Cox Regression Analysis for Prediction of PFS and OS

Parameter	Categories		PFS Risk			OS Risk		
	Positive	Negative	*P*	HR	95% CI	*P*	HR	95% CI
Prognostic factors considering CTCs at baseline								
CTCs at baseline	≥ 50	< 50	.011	2.01	1.17 to 3.46	.002	2.45	1.39 to 4.30
No. of mets involved	Continuous variable		.008	1.39	1.09 to 1.78	< .001	1.61	1.24 to 2.10
Treatment received	No/singlet	Doublet	.025	2.24	1.10 to 4.55	.006	2.75	1.34 to 5.61
Prognostic factors considering CTM at baseline								
CTM at baseline	≥ 1	< 1	—	—	—	.006	2.25	1.26 to 4.01
No. of mets Involved	Continuous variable		—	—	—	< .001	1.76	1.38 to 2.26
Treatment received	No/singlet	Doublet	—	—	—	.006	2.74	1.34 to 5.62
Prognostic factors considering apoptotic CTCs at baseline								
Apoptotic CTCs at baseline	≥ 1	< 1	.003	2.31	1.33 to 4.03	.001	2.66	1.49 to 4.74
No. of mets involved	Continuous variable		.013	1.37	1.07 to 1.76	< .001	1.61	1.24 to 2.09
Treatment received	No/singlet	Doublet	.027	2.22	1.10 to 4.51	.005	2.76	1.35 to 5.63
Prognostic factors considering CTCs at second time point								
CTCs at second time point	≥ 50	< 50	.008	4.20	1.44 to 12.25	.003	5.49	1.78 to 16.91
Stage	Extensive	Limited	.003	3.63	1.56 to 8.44	.001	4.75	1.90 to 11.86
Prognostic factors considering CTC numbers at 2 time points*								
CTC change	1 < 50–< 50 2 ≥ 50–< 50 3 ≥ 50–≥ 50		.08	2.96	0.9 to 9.9	.03	4.10	1.1 to 15.1
CTCs at baseline	≥ 50	< 50	.97	0.91	0.2 to 5.7	.68	0.67	0.1 to 4.3
Performance status	Continuous variable		.40	0.80	0.5 to 1.4	.67	0.88	0.5 to 1.6
Stage	Extensive	Limited	.17	2.52	0.6 to 9.7	.14	2.92	0.7 to 12.0
No. of mets involved	Continuous variable		.88	1.04	0.6 to 1.8	.58	1.17	0.7 to 2.0

Abbreviations: CTCs, circulating tumor cells; CTM, circulating tumor microemboli; HR, hazard ratio; mets, metastases; OS, overall survival; PFS, progression-free survival.
*Change in CTCs, adjusted for baseline CTCs and other clinical factors.

DISCUSSION

The prognostic value of CTC number using CellSearch was demonstrated in metastatic breast, prostate, and colorectal cancer.[3-5] Consistent with these data and our recent study of CTCs in advanced NSCLC,[18] we demonstrate the prognostic value of CTC number in SCLC. The presence of ≥ 50 SCLC CTCs/7.5 mL of blood detected before chemotherapy was highly significant for inferior PFS and OS and was independent of clinical prognostic factors. Furthermore, the CTC number change defined according to pre- and post-treatment CTC counts is the most significant variable in a multivariate model that adjusts for the baseline CTC and other clinical factors. We also report for the first time on the prevalence of groups of CTCs in SCLC, termed CTM, and demonstrate their association with worse survival. Similarly, the presence of CTCs with apoptotic nuclear morphology appears to confer a poorer prognosis.

The high prevalence of CTCs (77 of 97 patients, 85%) and CTM (25 of 97 patients, 26%) as well as the abundance of CTCs (mean ± SD = 1,589 ± 5,565) in the present study concurs with the highly malignant nature of SCLC. Although CTC number is prognostic for other types of cancer, CTC prevalence is reported to be less (20% to 57% patients) with lower mean abundance (mean ± SD = 60 ± 693).[19] In most cancers studied so far, CTCs are rarely detectable in early stages of disease, including NSCLC.[18] Here, CTCs were detected in 19 of 31 of patients with SCLC (61%) with limited disease stage (median, 1; range, 0 to 91; mean ± SD = 11 ± 22), and two CTM were detected in one of these patients. The disparity in the range of CTC number between SCLC and other cancer types and a prognostic cutoff of 50 CTCs compared with 5 CTCs/7.5 mL of blood for breast, prostate, and NSCLC cancer, and 3 CTCs/7.5 mL of blood for colorectal cancer, highlights the importance of statistically defining a disease-specific cutoff and not automatically extrapolating a cutoff from another type of cancer.

In addition to prognostication, we demonstrated a pharmacodynamic role for CTCs in SCLC. The CTC number after one chemotherapy cycle and persistence of more than 50 CTCs after one chemotherapy cycle were highly prognostic, which could be of clinical utility to guide a change in management or in early clinical trials of novel agents. Likewise, CTC number at baseline could potentially upstage patients with limited disease and occult metastases and, conversely, downstage patients with extensive disease and equivocal lesions.

The evaluation of CTCs will be fully realized if molecular characteristics can be measured and monitored in real time. The ease with which serial blood samples can be obtained provides realistic potential to study the dynamic change(s) in CTCs expressing a drug target before and after therapy and provides proof of mechanism and proof of concept data to inform drug development. In keeping with reports of heterogeneous expression of Bcl-2 in primary tumors in patients with SCLC,[17] SCLC CTCs are also heterogeneous for Bcl-2, although the dynamic range of this assay is narrow. Our observed increase in CTCs expressing Bcl-2 after chemotherapy highlights the feasibility of CTCs for stratification and pharmacodynamic monitoring in trials of Bcl-2 family antagonists.[20] Similarly, Mcl-1 expression may predict for resistance to the BH-3 mimetic ABT-263 that targets Bcl-2 and Bcl-x_L[21] (Appendix Fig A1C).

We previously showed a positive correlation ($P < .05$) between DAPI-defined apoptotic CTC number and circulating levels of the caspase-cleaved cytokeratin 18.[7] Here we found an adverse prognostic significance and correlation of apoptotic CTCs at baseline with extensive disease stage and number of metastatic sites. This is potentially counterintuitive but consistent with the central tenet that increased spontaneous tumor cell death is associated with increased proliferation and high turnover of primary tumor cells. Several oncogenes, including *c-Myc*, which is amplified in 16% of the tumors of patients with SCLC, drive both apoptosis and proliferation.[22,23]

Although CTM have been reported for various cancers by different technologies, including the recently developed Herringbone CTC-chip,[24-27] this is, to our knowledge, the first analysis of CTM detected using CellSearch and ISET in SCLC. Blood spiking experiments excluded the possibility that CTM might be an artifact caused by sample manipulation. Moreover, all clinical samples were processed identically, yet only a subset of clinical samples demonstrated CTM. The molecular characteristics of CTM compared with solitary CTCs are intriguing. Groups or clusters of tumor cells, when injected into the circulation of mice, demonstrate higher metastatic potential than solitary tumor cells.[28,29] Absence of apoptotic cells and of proliferating cells within CTM supports speculation that cells within CTM have a survival advantage, protected from anoikis (apoptosis resulting from cell–cell and cell–matrix contact).[29-31] The lack of proliferation would, theoretically, also make them relatively resistant to chemotherapy compared with proliferating single CTCs. Furthermore, it implies that CTM are not groups of cells actively dividing during transit in the blood; rather, they are cell clusters breaking off from the primary tumor, intravasating via "leaky," chaotic tumor vessels and appearing in the blood as a result of collective migration. We did not identify platelets within CTM, and it remains unclear whether CTM originate in a coagulation-driven manner. Interestingly, an alternative model for metastasis involving tumor cell cooperativity has been postulated in which mesenchymal cells provide invasive capability to allow "passenger" noninvasive epithelial cells access to the blood, where they survive and subsequently form metastases.[32] The heterogeneity of CTM regarding epithelial versus mesenchymal cell phenotypes was demonstrated in our previous study.[8]

In summary, we report CTC number to be an independent prognostic factor for SCLC and demonstrate that failure of CTC number to decrease to less than 50 after one cycle of chemotherapy is associated with worse prognosis. Our finding that CTCs frequently coexist with CTM provides new insights into SCLC biology and new biomarkers for this disease.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Appendix

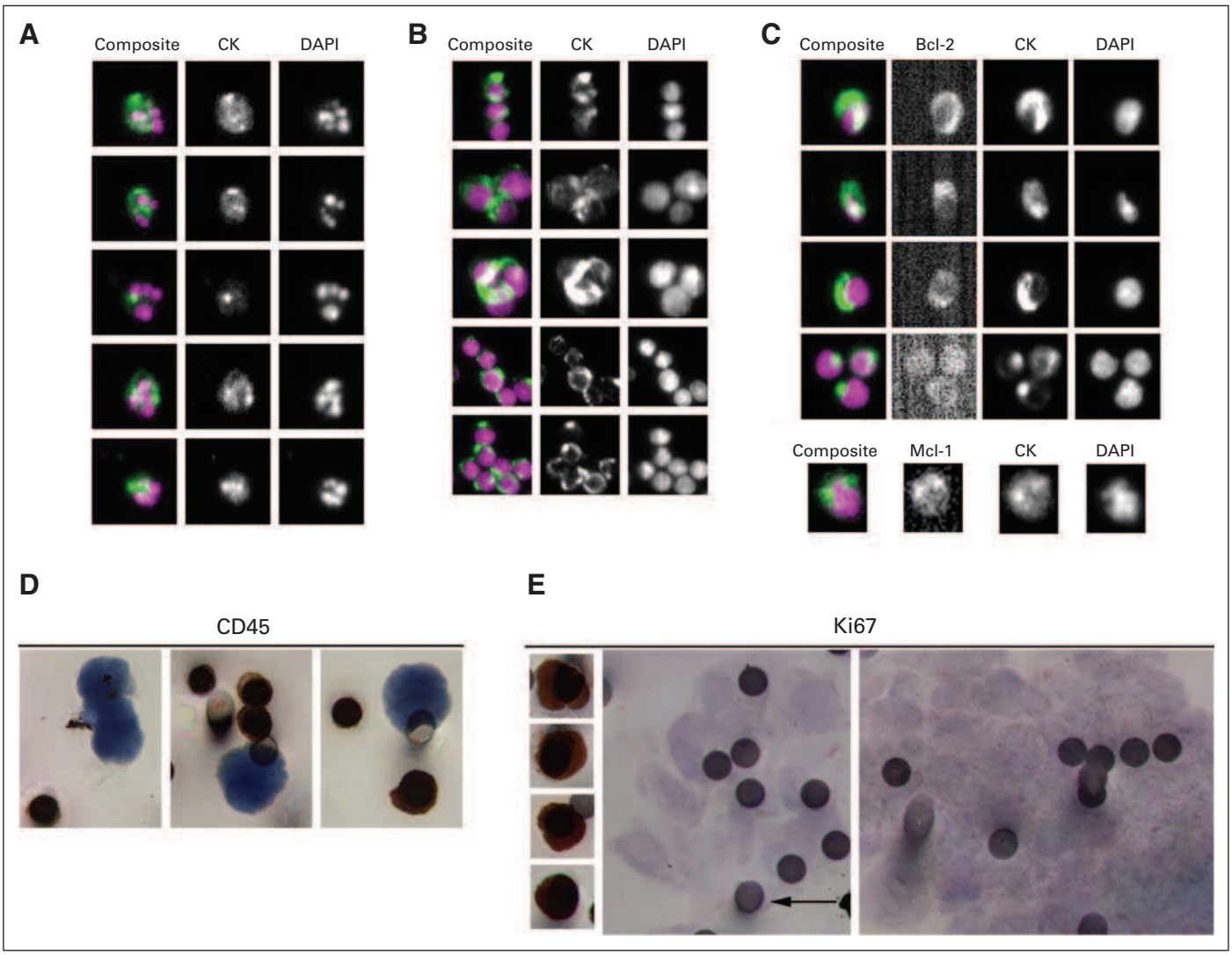

Fig A1. Molecular characteristics of circulating tumor cells (CTCs) and circulating tumor microemboli (CTM). Representative images of apoptotic profile of CTC and CTM are shown in A and B, respectively. Characteristic morphology of fragmented or condensed nuclei can be observed (A) in CTCs but not (B) in cells forming CTM. (C) Representative images of CTCs/CTM with detectable Bcl-2 and Mcl-1 are shown. (D) Examples of ISET-isolated CD45-negative CTCs with irregularly shaped and hyperchromatic nuclei (blue) and CD45-positive stained WBCs (brown) are shown. (E) Single CTC with strongly stained nuclear by Ki67 (brown; left panel) and negative staining of Ki67 for CTM (middle and right panel; ×40) are shown; black arrow indicates 8-μm pore. CK, cytokeratin; DAPI, 4′,6-diamidino-2-phenylindole.