Cancer Stem Cells, Epithelial to Mesenchymal Markers, and Circulating Tumor Cells in Small Cell Lung Cancer

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Abstract

The prognostic value of markers of cancer stem cells and epithelial to mesenchymal transition in small cell lung cancer is not known. We retrospectively studied these markers in the biopsy tissue of patients with small cell lung cancer and correlated them with overall survival and the strongest known prognostic marker circulating tumor cells.

Background: Small cell lung cancer (SCLC) has a poor prognosis, and even with localized (limited) disease, the 5-year survival has only been around 20%. Elevated levels of circulating tumor cells (CTCs) have been associated with a worse prognosis, and markers of cancer stem cells (CSCs) and epithelial to mesenchymal transition have been associated with increased chemoresistance and metastatic spread in SCLC. **Patients and Methods:** The biopsy specimens of 38 SCLC patients were used for marker evaluation by immunohistochemistry. The markers for CSCs were CD44 and SOX2. The markers for epithelial to mesenchymal transition were E-cadherin, epithelial cell adhesion molecule, cytokeratins 8, 18, and 19, vimentin, and c-MET. Staining was scored as low (weak) or high (strong) intensity for SOX2, epithelial cell adhesion molecule, cytokeratins 8, 18, and 19, and c-MET and using the immunoreactive score for CD44, E-cadherin, and vimentin, expressed as low or high expression. **Results:** High expression of c-MET (c-MET^{H}) and low expression of E-cadherin (E-cad^{L}) showed a trend toward a better prognosis ($P = .07$ and $P = .09$, respectively). The combination of c-MET^{H} and E-cad^{L} resulted in significantly better survival ($P = .007$). The tested markers were not associated with CTCs, although a trend was seen for c-MET^{H}E-cad^{L} ($P = .09$) with low CTCs. The CSC markers SOX2 and CD44 were not associated with overall survival in this patient cohort. **Conclusion:** SCLC with a mesenchymal-like phenotype (c-MET^{H}E-cad^{L}) is associated with longer survival and showed a trend toward lower CTCs.

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Introduction

Small cell lung cancer (SCLC) accounts for 8% to 12% of all lung cancer cases worldwide.[1] With treatment, an initial tumor response to chemotherapy will be observed in most patients. However, resistance to treatment inevitably emerges, usually within several months to 1 year, after therapy. Standard SCLC treatment includes platinum-based chemotherapy; however, only for localized disease (LD), concurrent thoracic radiotherapy is applied. Prophylactic cranial irradiation is indicated for all patients with a response to primary treatment.[2,3] The median survival for treated SCLC LD is 18 months but with extensive disease (ED) is only 9 months. Untreated, the median survival after diagnosis is 2 to 4 months.[4,5] To improve the clinical outcome of patients, greater insight into the mechanisms underlying disease progression and research into novel molecular targets are warranted.

A role for cancer stem cells (CSCs), epithelial to mesenchymal transition (EMT), and circulating tumor cells (CTCs) in the progression of solid tumors, including lung cancer, has been suggested.[6-8] The CSC theory predicts the presence of a unique

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Table 1 Patient and Clinicopathologic Characteristics (n = 38)

Characteristic	n (%)
Age	
Median	65
IQR	60-71
Male gender	20 (53)
ECOG PS	
0	18 (47)
1	14 (37)
2	3 (8)
3	3 (8)
Disease extent	
LD	12 (32)
ED	26 (68)
Baseline CTC count	
Median	19
IQR	2-295
Overall survival (mo)	
Median	10
IQR	5-17

Abbreviations: CTC = circulating tumor cell; ECOG = Eastern Cooperative Oncology Group; ED = extensive disease; IQR = interquartile range (25%-75%); LD = limited disease; PS = performance status.

Table 2 Marker Intensity or Expression and Overall Survival: Cox Regression Analysis

Variable	Patients (n)	HR	95% CI	*P* Value
CD44 expression				.42
Low	35	1.81	0.43-7.60	
High	3	1[a]		
SOX2 intensity				.11
Low	21	1.79	0.88-3.66	
High	17	1		
E-cadherin expression				.09
Low	24	0.54	0.26-1.10	
High	14	1		
EpCAM intensity				.59
Low	18	0.83	0.42-1.64	
High	20	1		
CK intensity				.80
Low	27	1.10	0.53-2.31	
High	11	1		
Vimentin expression				.67
Low	37	1.54	0.21-11.39	
High	1	1		
c-MET intensity				.07
Low	19	1	0.26-1.05	
High	19	0.52		
c-MET and E-cad				.007[b]
Other	27	1		
c-MET^{H}E-cad^{L}	11	0.30	0.13-0.72	
CTCs				.005[b]
Low	9	1		
High	29	3.43	1.46-8.03	
LD versus ED				.02[b]
LD	12	1		
ED	26	2.67	1.19-5.99	

Abbreviations: CI = confidence interval; CK = cytokeratin (8, 18, and 19); c-MET^{H}E-cad^{L} = high intensity c-MET staining combined with low intensity E-cadherin; CTCs = circulating tumor cells; E-cad = E-cadherin; ED = extensive disease; EpCAM = epithelial cell adhesion molecule; HR = hazard ratio; LD = limited disease.
[a]Reference category.
[b]Statistically significant.

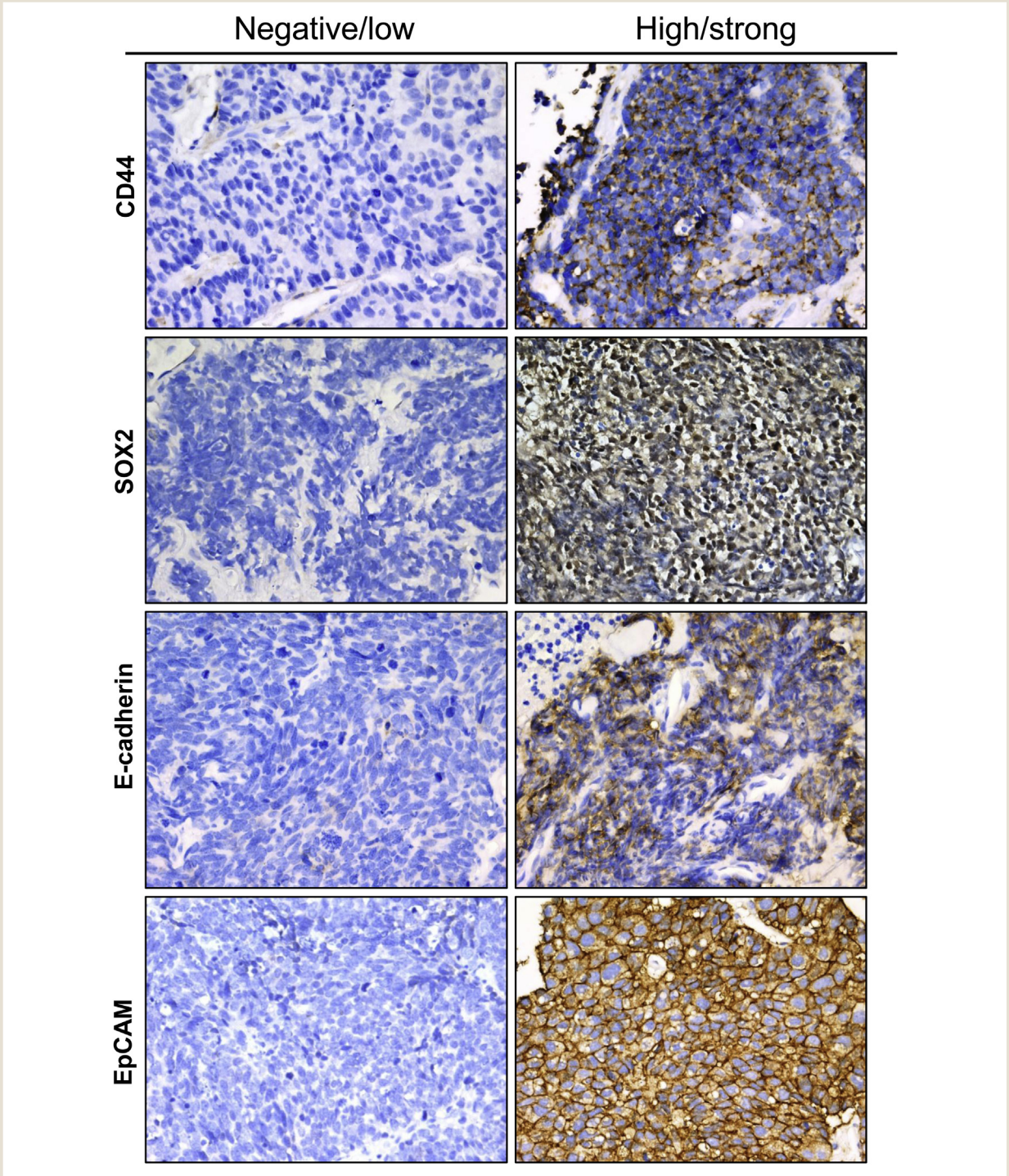
tumor cell population with the ability of self-renewal and differentiation driving tumor growth.[9] CSCs have been linked to resistance to therapy and metastatic spread.[10] Several markers used for identification of CSCs have been identified in lung cancer, including SCLC. These include expression of cell surface markers (eg, CD44), transcription factors (eg, SOX2), and functional properties (eg, a high level of Hoechst exclusion in side population cells).[11] High expression of CD44, a multifunctional class 1 transmembrane glycoprotein, in lung cancer cells was associated with the CSC properties of these cells.[12] The transcription factor SOX2 has been linked to the pluripotency of embryonic stem cells[13] and to mechanisms involving invasion and metastasis in solid cancers.[14] *SOX2* gene amplification and overexpression have been observed more frequently in SCLC.[15,16]

EMT has been increasingly recognized as important in tumor formation and progression and in the invasive and metastatic properties of tumor cells.[17] The hallmarks of EMT include the loss of epithelial markers, such as E-cadherin, epithelial cell adhesion molecule (EpCAM), and cytokeratins, and the gain of mesenchymal markers, such as vimentin, and the overexpression of c-MET.[7,18,19] The expression of EMT markers has been associated with overall survival in lung cancer.[20] EMT can be induced by several growth factors, including hepatocyte growth factor, the natural ligand of c-MET.[21] Exogenous treatment with hepatocyte growth factor induced EMT by activating c-MET–dependent pathways in SCLC cell culture models.[22] Furthermore, amplification and oncogenic mutations in c-MET, leading to overexpression and activation of the receptor, have been linked to EMT activation.[19] Amplification of c-MET has been demonstrated in acquired resistance to gefitinib in lung cancer cell lines.[23]

In SCLC, large numbers of CTCs have been found in the blood of both patients with LD and patients with ED.[24,25] Recent studies of breast and lung cancer have suggested that CTCs in clusters could be responsible for the development of distant metastases.[26-28] Several methods are available to enumerate CTCs, including antibody-based capture assays, the physical characteristics, or nucleic acid-based assays.[6] In a previous study,[24] we used the CellSearch system to identify EpCAM^{+}, cytokeratin-positive, 4′,6-diamidino-2-phenylindole-positive, and CD45^{-} CTCs. We showed that CTCs have both a prognostic and a predictive value in patients with SCLC.[24]

In the present study, we used biopsy material from 38 patients of the patient cohort from our previous study[24] for

Figure 1 Photographs of Representative Immunohistochemical Staining of Negative/Low Versus High/Strong Expression and Intensity Patterns of CD44, SOX2, E-cadherin, Epithelial Cell Adhesion Molecule, Cytokeratin 8, 18, and 19, and Vimentin (High Intensity Indicated by Arrow) and c-MET



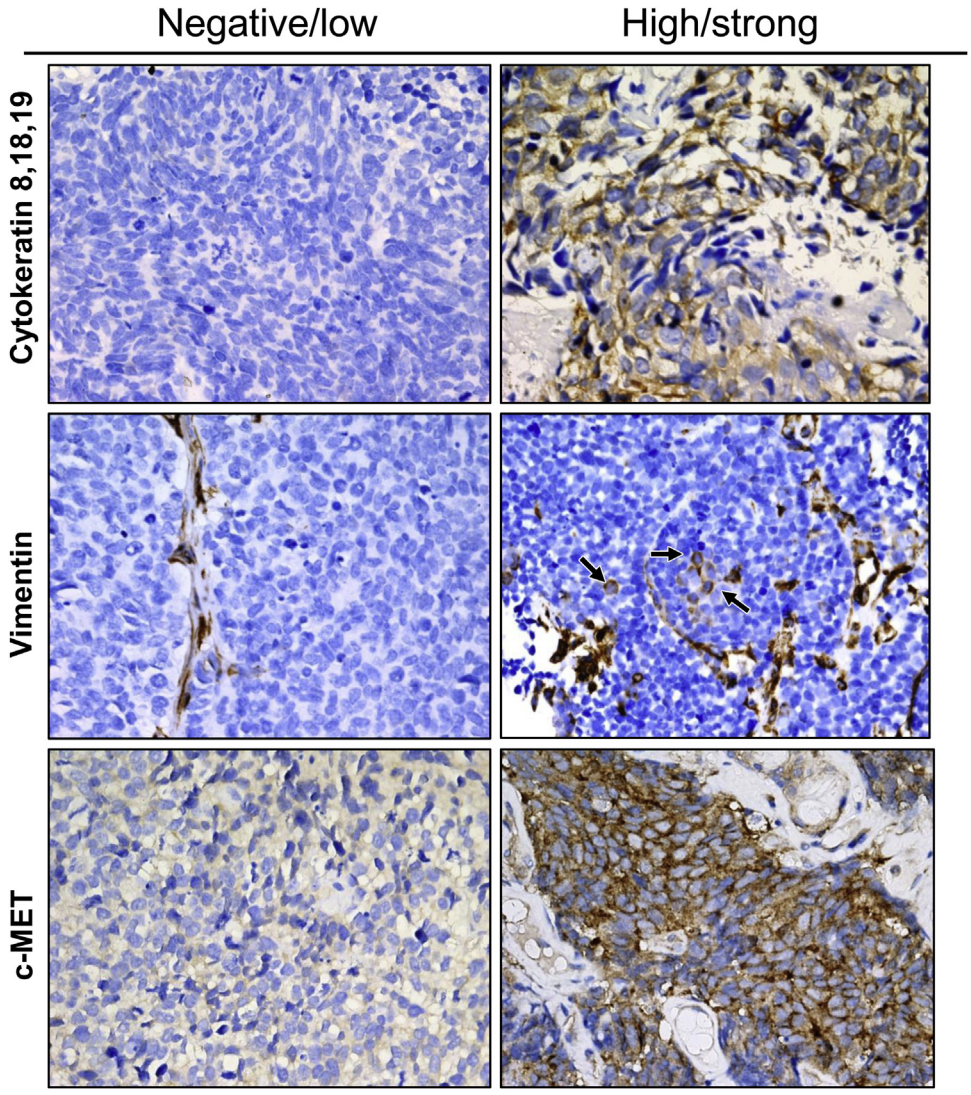
immunohistochemical analyses. We hypothesized that both CSC and EMT marker expression in the primary tumor biopsy tissue of these patients would be associated with both the baseline CTC count and the prognosis.

Patients and Methods

Patient Material

Biopsy specimens were selected from the 59 patients who had participated in a previous SCLC-CTC study.[24] Sufficient tumor

Figure 1 continued



material was available for 38 patients and was used in the present retrospective study.

Immunohistochemistry

Formalin-fixed SCLC tissue biopsies (n = 36) or cytology cell blocks (n = 2) were used for immunohistochemical staining. After deparaffinization of the tissue sections, antigen retrieval was performed using heated citrate buffer (pH 6) for 15 minutes, followed by blocking endogenous peroxidase activity. The sections were incubated with primary antibodies for 1 hour at room temperature. The primary antibodies used were rat monoclonal CD44 1:100 (clone IM7; Biolegend; ITK Diagnostics, Uithoorn, The Netherlands), mouse monoclonal SOX2 1:600 (clone L1D6A2; Cell Signaling; Bioke, Leiden, The Netherlands), mouse monoclonal E-cadherin 1:100 (clone 36/E-cadherin; BD Biosciences, Breda, The Netherlands), mouse monoclonal cytokeratins 8, 18, and 19 (CK) 1:100 (clone 2A4; Abcam, Cambridge, UK), mouse monoclonal EpCAM 1:500 (clone VU1D9; Cell Signaling; Bioke), mouse monoclonal vimentin 1:100 (clone sc-6260; Santa Cruz Biotechnology; Bioconnect, Huissen, The Netherlands), and rabbit monoclonal c-MET 1:200 (clone EP1454Y; Abcam). Subsequently, the tissue sections were incubated with horseradish peroxidase-conjugated secondary and tertiary antibodies (1:100 dilution; all from DAKO, Glostrup, Denmark). Staining was visualized using 3,3′diaminobenzidine and hematoxylin for counterstaining. Positive and negative controls (including immunoglobulin class-matched control sera) were included for each staining. Images were obtained using a light microscope attached to a digital camera (Leica DM 3000; Leica, Rijswijk, The Netherlands).

Analysis of Immunohistochemistry

After immunohistochemical staining, the slides were scanned digitally using the NanoZoomer (Hamamatsu, Shizuoka, Japan)

and scored using the accompanying NDP software. The stained slides were scored by 2 independent observers without knowledge of the clinical outcomes of the patients (M.P., C.M.). As an extra internal control, random samples of different stained slides were checked by a lung pathologist (W.T.) unaware of the study details. All samples were scored on the basis of the following 3 criteria: (1) staining localization (nuclear, cytoplasm, membrane, mixed); (2) percentage of positive cells divided into 6 categories (0, no staining; 1, 1%-5%; 2, 5%-25%, 3, 25%-50%; 4, 50%-75%; and 5, 75%-100%); and (3) intensity (scored as 0, negative; 1, low/weakly positive; 2, normal positive; 3, high/strongly positive). SOX2, EpCAM, CK, and c-MET showed homogeneous staining. Therefore, scoring of these markers was based on the staining intensity. Patients with an intensity score of 0 and 1 were regarded as having negative/low (weak) positive staining and those with an intensity score of 2 and 3 were considered to have high/strong positive staining. The protein expression of CD44, E-cadherin, and vimentin was more heterogeneous. Therefore, we used the immunoreactive score (IRS). The IRS was defined by multiplying the percentage of positive cells (category) with the intensity score (category). This created a scale with a range of a minimal score of 0 and greatest score of 15, which was further divided into 2 subgroups. An IRS in the range of 0 to 5 was regarded as negative/low positive expression and an IRS of 6 of 15 was considered high/strong positive expression.

Statistical Analysis

Overall survival (OS) was measured in months from the day on which the biopsy was taken until the patient died or was lost to follow-up. For the CSCs and EMT markers, the individual staining scores and a combination of epithelial and mesenchymal markers (E-cadherin and c-MET) were compared regarding OS. To estimate the differences in OS, Cox regression analyses were performed, yielding hazard ratios (HRs) and 95% confidence intervals (CIs). To describe the median OS in months, with 95% CIs, Kaplan-Meier survival tables were constructed and log-rank tests performed. Differences in the association between the EMT/CSC markers and baseline CTC count were determined using the Fisher exact test for association. All tests were 2-sided, and $P < .05$ was considered significant. Analyses were performed using the statistical software SPSS, version 22.0 (SPSS Statistics 22; IBM Corp, Armonk, NY).

Results

Patient and Clinicopathologic Characteristics

The clinical characteristics of the 38 included patients are listed in Table 1. The median age of the patients was 65 years (interquartile range [25%-75%] [IQR], 60-71), 20 patients were male, and 12 patients had LD and 26 ED at diagnosis. The performance score (Eastern Cooperative Oncology Group performance status) was 0 for 18, 1 for 14, 2 for 3, and 3 for 3 patients at the start of treatment. The median baseline CTC count was 19 (IQR, 2-295), and the median OS was 10 months (IQR, 5-17; Table 1).

Protein Expression of Markers

The number of patients with low or high levels of the individual markers and the combined expression of the EMT markers with OS in association with the HRs, 95% CIs and *P* values are listed in Table 2. For the CSC marker CD44, only 3 (8%) of the samples showed high protein expression, 35 (92%) showing low protein expression. For SOX2, 17 of the samples (45%) had high SOX2 intensity and 21 (55%), a low staining intensity. For the EMT markers, high E-cadherin expression was detected in 14 patients (37%) and low expression in 24 (63%). For EpCAM, 20 patients (53%) had a high intensity and 18 (47%) had a low staining intensity. For CK, a high staining intensity was observed for 11 patients (29%), but most (n = 27; 71%) patients had low intensity staining. Vimentin expression was high in only 1 patient (3%), with the remaining samples (n = 37; 97%) having low vimentin expression. For c-MET, equal numbers of patients (ie, n = 19; 50%) had high and low intensity staining. Figure 1 shows representative images of the low and high staining levels for all 7 markers tested.

Table 3 Marker Expression in Relation to Overall Survival

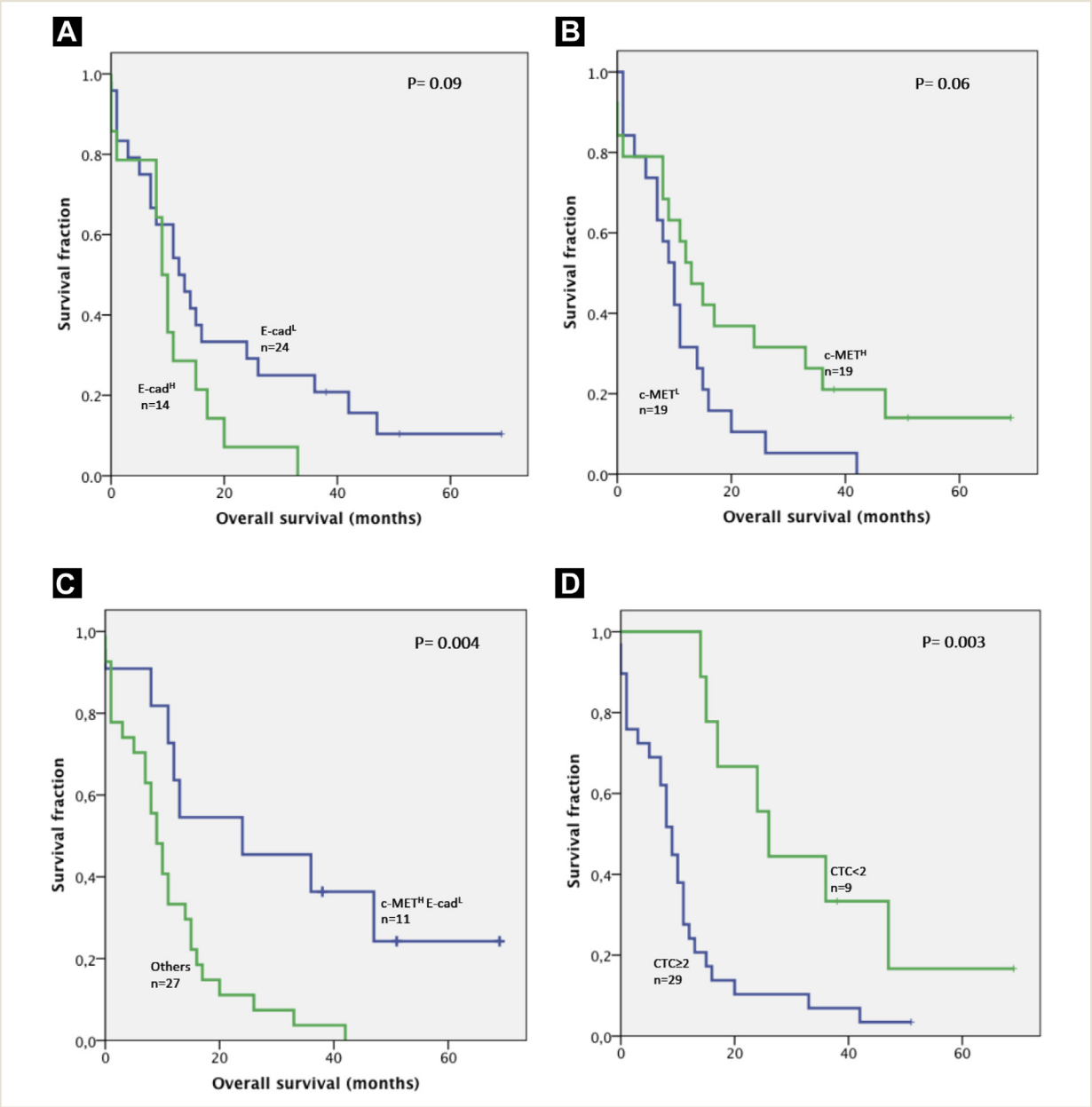
Variable	Overall Survival (mo)		*P* Value[a]
	Low Marker Expression	High Marker Expression	
CD44 expression	11 (9-13)	20 (0-52)	.42
SOX2 intensity	10 (8-12)	15 (11-19)	.10
E-cadherin expression	12 (6-18)	9 (7-11)	.09
EpCAM intensity	10 (4-16)	11 (9-13)	.62
CK intensity	11 (9-13)	13 (1-25)	.84
Vimentin expression	11 (9-13)	24 (NA)[b]	.68
c-MET intensity	10 (7-13)	13 (7-19)	.06
Other vs. c-MET^{H}E-cad^{L}	9 (6-12)	24 (0-50)	.004[c]
CTC < 2 vs. ≥ 2	26 (20-32)	9 (7-11)	.003[c]
LD vs. ED	14 (11-17)	8 (6-10)	.01[c]

Data presented as median (95% CI) from Kaplan-Meier survival analyses.
Abbreviations: CI = confidence interval; CK = cytokeratin (8, 18, and 19); c-MET^{H}E-cad^{L} = high intensity c-MET staining combined with low intensity E-cadherin staining; CTC = circulating tumor cell; ED = extensive disease; EpCAM = epithelial cell adhesion molecule; LD = limited disease.
[a] *P* values from the log-rank test.
[b] One observation only.
[c] Statistically significant.

Prognostic Value of the Tested Markers

The CSC markers SOX2 and CD44 showed no significant differences in OS in this patient cohort (Table 3). Patients with low E-cadherin expression (E-cad^{L}) had a median OS of 12 months (95% CI, 6-18 months) compared with only 9 months (95% CI, 7-11 months) for the patient with high E-cadherin (E-cad^{H}) expression ($P = .09$; Table 3, Figure 2A). Furthermore, high intensity c-MET staining (c-MET^{H}) showed a trend ($P = .06$) toward a better prognosis for these patients (Table 3, Figure 2B). Patients with c-MET^{H} had a median OS of 13 months (95% CI, 7-19 months) compared with 10 months (95% CI, 7-13 months) for the patients with c-MET^{L}. Combining c-MET^{H} and E-cad^{L}, a significant association with a better prognosis was observed (HR, 0.30; 95% CI, 0.13-0.72; $P = .007$; Table 2, Figure 2C). The c-MET^{H}E-cad^{L} patients had a median OS of 24 months (95% CI, 0-50 months) compared with 9 months (95% CI, 6-12 months) for the other patients. In agreement with our earlier study of the larger patient cohort,[24]

Figure 2 Kaplan-Meier Survival Curves Comparing Survival Outcome of (A) Low and High Levels of E-cadherin Expression and (B) Low and High Levels of c-MET Intensity. (C) Survival Outcome of Combination of High c-MET Intensity and Low E-cadherin Expression (c-MET^{H}E-cad^{L}), and (D) Survival Outcome of Low (Circulating Tumor Cells [CTCs] < 2) and High (CTCs ≥ 2) Baseline CTC Count



the patients with high CTC numbers had a significantly worse prognosis (HR, 3.43; 95% CI, 1.46-8.03; $P = .005$; Table 2, Figure 2D), with a median OS of 9 months (95% CI, 7-11 months). In contrast, the patients with low CTC numbers (CTC < 2) had a median OS of 26 months (95% CI, 20-32 months; Table 3). The CTC level was the strongest predictor in this cohort, even surpassing disease stage (ED vs. LD; HR, 2.67; 95% CI, 1.19-5.99; $P = .02$; Table 2), with a median OS of 8 months (95% CI, 6-10 months) for ED and 14 months (95% CI, 11-17 months) for LD (Table 3). Adding immunohistochemistry markers to the CTC number did not change the HRs significantly (data not shown).

Association of EMT Markers With Baseline CTC Count

No significant associations were found between the expression of the tested CSCs and EMT markers and the baseline CTC counts in this population, although a trend was seen for c-MET^{H}E-cad^{L} ($P = .09$) with a low CTC count (Table 4).

Table 4 Association Between EMT/CSC Markers and Baseline CTC Count (n = 38 Patients)

Variable	Patients (n)		*P* Value[a]
	CTCs < 2	CTCs ≥ 2	
CD44 expression			1.0
Low	8	27	
High	1	2	
SOX2 intensity			.70
Low	4	17	
High	5	12	
E-cadherin expression			.44
Low	7	17	
High	2	12	
EpCAM intensity			1.0
Low	4	14	
High	5	15	
CK intensity			1.0
Low	6	21	
High	3	8	
Vimentin expression			.24
Low	8	29	
High	1	0	
c-MET intensity			.12
Low	2	17	
High	7	12	
c-MET and E-cad			.09
Other	4	23	
c-MET^{H}E-cad^{L}	5	6	
LD vs. ED			.42
LD	4	8	
ED	5	21	

Abbreviations: CK = cytokeratin (8, 18, and 19); c-MET^{H}E-cad^{L} = high intensity c-MET staining combined with low intensity E-cadherin staining; CSC = cancer stem cell; CTC = circulating tumor cell; E-cad = E-cadherin; ED = extensive disease; EMT = epithelial to mesenchymal transition; LD = limited disease.
[a] *P* values from 2-sided Fisher's exact test.

Discussion

In the present study, we explored the possible relationship between CSC and EMT markers in relation to CTCs and OS of SCLC patients. We hypothesized that the presence of CSC markers and mesenchymal markers in the tumor would be associated with higher CTC counts and lower OS. Both CSCs and EMT have been associated with greater tumor aggressiveness and metastatic spread. However, the presence of the CSC markers SOX2 and CD44 in SCLC tumors and the level of CTCs was not related nor associated with OS. Of the EMT markers tested, EpCAM, CK, and vimentin expression also did not show a correlation with the CTCs and also were not associated with survival. Somewhat surprisingly, we found that tumors displaying combined c-MET^{H}E-cad^{L}, markers assumed to be indicative of the presence of mesenchymal-like cells, correlated significantly with better prognosis, and a trend toward lower baseline CTCs was found.

In the present study, we did not see any significant differences in the clinical outcome in these patients according to SOX2 and CD44 expression. Our findings increase the contradictory reports on the clinical significance of SOX2 and CD44 expression in SCLC. SOX2 overexpression has been correlated with worse clinical outcomes.[15,29] Also, SOX2-specific antibodies have been detected in SCLC patient sera; however, their presence did not correlate with the prognosis.[30] The loss of CD44 has been correlated with a poor prognosis in SCLC previously.[31] The expression of these proteins might be temporally lost without the loss of CSC properties. Moreover, other CSC markers have been suggested for SCLC, including CD133.[32] Therefore, whether SOX2- and/or CD44-expressing SCLC cells are specifically staining the CSC fraction remains an issue of debate.

Accumulating evidence is suggesting that EMT plays a crucial role in invasion and distant metastasis and chemoresistance.[20,33] Overall, a differentiated epithelial phenotype of tumors is considered to be a favorable property, because differentiated epithelial cells form strong adherent and interconnected cell layers that limit the metastatic spread of tumor cells. In SCLC, for example, elevated E-cadherin expression was associated with a better prognosis.[34] Similarly, c-MET overexpression has been correlated with a poor prognosis in SCLC patients.[35] In contrast to these findings, in our present study, overexpression of c-MET (c-MET^{H}) and low expression of E-cadherin (E-cad^{L}) each separately showed a trend toward better outcomes in the SCLC patient cohort. Moreover, these 2 markers combined (c-MET^{H}E-cad^{L}) were associated with significantly better OS. In line with this finding, the other epithelial and mesenchymal markers that we examined, EpCAM, CKs, and vimentin, did not show correlations with prognosis. Although epithelial markers can be detected in SCLC, SCLC has a neuroendocrine origin. In a recent report, both neuroendocrine and epithelial markers were detected in SCLC, and a neuroendocrine phenotype correlated with liver metastases and poor survival.[36] Thus, in addition to EMT-like events in this tumor type, neuroendocrine properties correlate with the prognosis and might be relevant in explaining the role of the changes in E-cadherin and c-MET in SCLC.

The CellSearch platform was used to count the CTCs, which makes use of EpCAM to capture CTCs and thereby capturing mostly epithelial CTCs. We did not find a correlation with EpCAM levels in the tumor samples and CTCs detected using this method, perhaps illustrating the plasticity of tumor cells that leads to losses and gains of epithelial and mesenchymal features.

Hou et al[27] demonstrated heterogeneous expression of epithelial and mesenchymal markers in CTCs derived from both NSCLC and SCLC patients using ISET (Metagenex, Paris, France). Dual staining of these CTCs for vimentin and E-cadherin showed that all vimentin-positive CTCs were negative for E-cadherin and vice versa.[27] Thus, epithelial-like and mesenchymal-like CTCs appear to exist. It would be interesting to count the CTCs with varying differentiation in SCLC patients and explore the expression of c-MET and E-cadherin.

Conclusion

The results of our study suggest that in SCLC, a mesenchymal-like signature of high c-MET expression coupled with low

E-cadherin expression in the primary tumor is associated with lower (epithelial) CTC counts using CellSearch and a better prognosis. This is different from NSCLC and possibly explained by the neuroendocrine origin of SCLC. However, our results were based on a relative small cohort of patients and need to be validated in a larger study cohort.

Clinical Practice Points

- New targets and insights for treatment of SCLC are needed.
- A mesenchymal-like tumor signature was associated with OS (HR, 0.30; 95% CI, 0.13-0.72).
- This signature showed a trend toward lower CTCs.
- The number of CTCs was the strongest parameter associated with OS in the present patient cohort.
- The expression of stem cell markers CD44 and SOX2 in the tumor biopsy material was not associated with OS.

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