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SHORT COMMUNICATION

EpCAM-positive disseminated cancer cells in bone marrow impact on survival of early-stage NSCLC patients

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Abstract

Introduction Detection of disseminated cancer cells (DCC) in bone marrow (BM) of patients with early-stage NSCLC has been associated with poor outcome. However, the phenotype, and hence relevant therapy targets, of DCCs in BM are unknown. We therefore compared a classical pan-Cytokeratin (CK) antibody for DCC detection with an anti-EpCAM antibody that may also detect more stem-like cells and tested whether assay positivity impacts on the survival of NSCLC patients.

Materials and methods We prospectively collected BM aspirates from 104 non-metastasized NSCLC patients that underwent potentially curative tumor resection from 2011 to 2016 at the Department of Thoracic Surgery of the University Hospital and Hospital Barmherzige Brüder in Regensburg. DCCs were detected by staining with the pan anti-CK antibody A45-B/B3 and the anti-EpCAM antibody HEA-125. We analyzed the association between detection of DCCs and clinicopathological characteristic and patient outcome.

Results CK-positive and EpCAM-positive DCCs were detected in 45.2% and 52.9% of patients, respectively. Correlation between the two markers was low and neither of them was associated with sex, age, histology, T or N classification, resection status, grading or smoking habit. No significant association with tumor specific survival (TSS) and progression-free survival (PFS) was observed in patients with CK-positive DCCs. In contrast, detection of EpCAM-positive DCCs significantly correlated with reduced PFS (P=0.017) and TSS (P=0.017) and remained an independent prognostic variable for PFS and TSS upon multivariate testing (hazard ratio: 7.506 and 3.551, respectively). Detection of EpCAM-positive DCCs was the only prognostic marker for PFS.

Conclusions EpCAM-positive, but not CK-positive DCCs in BM predict reduced PFS and TSS. This finding suggests that EpCAM-positive DCCs in the BM comprise metastatic founder cells necessitating their in-depth molecular analysis for detection of novel therapy targets.

KEYWORDS disseminated cancer cell; metastasis; early dissemination; bone marrow; lung cancer; biomarker
1. **Introduction**

Prognosis of NSCLC patients is still dismal [1]. Even after diagnosis at early stages of the disease followed by curatively intended tumor resection and adjuvant chemo and/or radiotherapy, up to 50% of the patients relapse within 5 years [2], [3]. Available data indicate that dissemination of lung cancer cells starts years before lung primary tumors reach the size of a T1 stage cancer [4], suggesting that targeting of early disseminated cancer cells (DCCs) is mandatory for the prevention of metachronous metastasis. However, detection of DCCs and particularly their molecular characterization is hampered by the circumstantial knowledge about markers identifying disease-driving cells.

Two major detection markers for DCCs in bone marrow have been widely explored using antibodies directed against epithelial cytokeratins and against EpCAM, an epithelial associated surface marker [5]. Bone marrow as surrogate detection site of hematogenous systemic cancer spread is not only attractive because of the absence of autochthonous epithelial cells, but also because recent experimental data indicate that metastatic precursor cells receive important instructive signals enabling metastasis formation elsewhere [6].

We therefore decided to directly compare Cytokeratin and EpCAM as markers for bone marrow DCCs. Several studies have demonstrated an association of reduced overall (OS) or progression-free survival (PFS) in NSCLC patients with detection of CK-positive DCCs in the bone marrow at the time of primary surgery [7]–[9], however, others did not [10]–[12]. In contrast, no correlation with EpCAM-positive DCCs and patient outcome has been reported so far [10], [11], [13].

Assuming that Cytokeratin expression generally marks more differentiated epithelial cells whereas EpCAM is also a marker for pluripotent stem cells [14], we directly compared their detection in bone marrow of 104 non-metastasized and histologically verified NSCLC patients. The simultaneous analysis of the two most widely used markers for DCC detection in NSCLC should allow assessment of their prognostic significance for non-metastasized NSCLC patients.
2. Materials and Methods

2.1. Patient and bone marrow samples

From 2011 to 2016, patients undergoing potentially curative surgery for presumed or histologically verified Non-Small Cell Lung Cancer (NSCLC) at the Department for Thoracic Surgery of the University Hospital Regensburg or the Hospital Barmherzige Brüder Regensburg were prospectively enrolled in this study. Bone marrow aspirates were collected as described previously [15]. All aspects of this study were approved by the local ethics committee at the University of Regensburg (protocol number 07-079) and the patients provided written, informed consent.

2.2. Sample processing

Bone marrow aspirates were processed as described in [15]. Briefly, the aspirates were washed twice in Hank’s solution and split into two equal halves. For CK-staining, density gradient centrifugation was performed using Percoll 60% (ρ = 1.077 g/ml) to enrich mononucleated cells (MNCs); for enrichment of EpCAM-positive cells the other BM halve was placed on a Percoll 65% gradient (ρ = 1.083 g/ml) before depletion antibodies were added (see section 2.4.).

2.3. Cytokeratin-staining

CK-staining was performed according to the consensus protocol of DCC detection [16]. Briefly, MNCs isolated from the bone marrow aspirates were put on positively charged adhesion slides (Menzel, Braunschweig, Germany) and stained against CK using the monoclonal pan anti-CK antibody A45-B/B3 (Micromet, AS Diagnostik, Germany) at a final concentration of 2 μg/ml as described in [17] or using avidin-biotin complex (ABC) method coupled with alkaline phosphatase Vectastain ABC kit (Vector Laboratories, USA). Isotype controls were performed on a slide from the same sample using the MOPC-21 antibody (Sigma Aldrich, USA).

2.4. Immunomagnetic depletion and EpCAM-staining

To remove weakly EpCAM-positive erythroblastic cells [18] and other BM populations, EpCAM-high DCCs were enriched using negative immunomagnetic depletion as described in [15]. Briefly, autochthonous BM cells were captured after anti-APC staining on a LS MACS column after incubation with APC-conjugated CD11b, CD33 and CD45 antibodies (Miltenyi Biotec, Germany) and anti-CD235 (glycophorin A) microbeads. The flow-through cell fraction was counted using a hemocytometer. DCC-positivity was assigned only for clearly bright and intensively stained cells, as we had noted previously that weakly positive cells could confound the analysis [15], [19].
2.5. Screening of bone marrow for disseminated cancer cells

Stained adhesion slides or immunofluorescently labelled cell suspensions were manually screened for CK or EpCAM-positive single cells using a microscope (Olympus) or inverted fluorescent microscope (Olympus or Zeiss), respectively. The total number of positively stained cells was documented as the number of DCCs per million screened MNCs (disseminated cancer cell density, DCCD).

2.6. Study inclusion criteria

NSCLC patients that presented with overt metastases at the time of surgery (n=13) were excluded from this study. Furthermore, patients with positively stained cells in the isotype control were excluded from the analysis (n=24). Lastly, only patients with results for both CK and EpCAM-staining were included, which resulted in a total of n=104 patients.

2.7. Statistical analysis

Statistical testing for associations of categorical variables was performed using the Chi-square ($\chi^2$) test. Correlation analysis was performed using the nonparametric Spearman correlation method. Survival curves were created with the Kaplan-Meier method and compared using the univariate log-rank test. When analyzing more than two groups, the results were corrected for multiple testing and considered statistically significant using the family-wise significance level of 0.05. Multivariate testing was performed using the Cox regression model. All statistical analysis was performed by using the software GraphPad Prism 6 (GraphPad Software, San Diego, California USA) or IBM SPSS Statistics (Version 25, IBM Corp., Chicago, IL). A $P$-value of <0.05 was considered statistically significant.
3. Results

Overview of the patient cohort

Overall, bone marrow aspirates from 104 non-metastasized (n= 50, 22, 32 for UICC stage I, II, III, respectively) and histologically confirmed NSCLC patients met the study inclusion criteria. An overview of the patient cohort and the clinicopathological characteristics can be found in Supplementary Table 1.

3.1. Patient outcome and survival

The median follow-up time was 40.3 months (range 1.1 - 80.4 months). During the follow-up period 29 of the 104 (27.9%) patients died, while the death of 18 (17.3%) patients was directly caused by NSCLC. Overall, 17 (16.3%) patients developed a local relapse or metastasis during the follow-up period.

3.2. Associations of clinicopathological characteristics with patient outcome and survival

Statistical associations between clinicopathological characteristics and patient outcome are shown in Suppl. Figures 1+2. Univariate analysis revealed no significant association between sex, age, histology, tumor size, grading or smoking habits and PFS (Suppl. Figure 1a-d, g-h) or TSS (Suppl. Figure 2a-d, g-h). Detection of lymph node metastasis (pN1-3) resulted in decreased TSS ($P=0.0002$) when compared to patients without lymph node involvement (pN0) as shown in Suppl. Figure 2e. For PFS, a separation between pN0 and pN1-3 patients could be seen, but was not significant ($P=0.2911$; Suppl. Figure 1e). Complete tumor resection (microscopically tumor-free resection margin; R0) resulted in significantly improved PFS ($P=0.0252$) and TSS ($P=0.0009$) when compared to patients with microscopic or macroscopic residual tumor (R1 and R2; Suppl. Figure 1f, 2f).

3.3. Detection of DCCs in bone marrow by Cytokeratin and EpCAM staining

All 104 patients were analyzed for DCCs detected by the antibodies against Cytokeratin and EpCAM. At least one CK-positive DCC could be found in the bone marrow of 47 (45.2%) patients. The median number of screened cells was $2 \times 10^6$ MNCs (mean: $2.28 \times 10^6$; range: $2 \times 10^6 - 6 \times 10^6$ MNCs). EpCAM-staining with the PE-conjugated monoclonal antibody clone HEA-125 (Miltenyi Biotec, Germany) after immunomagnetic depletion of hematopoietic cells revealed at least one EpCAM-positive DCC in the bone marrow aspirates of 55 (52.9%) patients. The median number of screened cells was $2 \times 10^6$ depleted MNCs (mean: $1.61 \times 10^6$; range: $0.10 \times 10^6 - 4 \times 10^6$ depleted MNCs). Neither for the detection of CK-positive nor for EpCAM-positive cells in bone marrow, a significant association with analyzed clinicopathological variables could be noted ($P>0.05$; Supplementary Table 2).
3.4. Cytokeratin and EpCAM positivity define subgroups of patients with different outcome

We first compared patients positive by either or both markers with patients that were negative for both assays. Patients with no DCCs displayed excellent survival, whereas DCC-positivity imposed a significant risk for PFS and TSS (PFS, \( P = 0.026 \); TSS, \( P = 0.065 \); Figure 1a). When we analyzed the impact of CK-positivity and EpCAM-positivity separately (Figure 1b-c), only EpCAM-positive patients displayed worse outcome when compared to negative patients (PFS and TSS in univariate log-rank testing \( P = 0.0170 \) and \( P = 0.0170 \), respectively), whereas detection of CK-positive cells in BM was not associated with PFS or TSS (\( P = 0.5365 \) and \( P = 0.2948 \), respectively; Figure 1b).

We did not find a significant correlation between detection (\( P = 0.267 \), \( \chi^2 \) test) or numbers of EpCAM and CK-positive DCCs (Spearman \( r = 0.0424 \); \( P = 0.669 \); Suppl. Figure 3), suggesting that the two antigens may define different subsets of DCCs. Therefore, we interrogated whether single-positivity (i.e. either EpCAM-positive or CK-positive assay) or double-positivity (detection of cells in both assays) had a stronger impact on outcome and compared DCC-negative, CK and EpCAM double-positive patients and CK and EpCAM single-positive patients. Using a univariate log-rank test corrected for multiple comparisons only the EpCAM single-positive group demonstrated a significantly reduced PFS (\( P = 0.0132 \)) and TSS (\( P = 0.0042 \)) using the family-wise significance level of 0.05 (Figure 1d).

3.5. Detection of EpCAM-positive cells is an independent prognosticactor of outcome

Finally, we adjusted the analysis for confounding (clinicopathological) variables and performed a multivariate Cox regression analysis. Sex, age, histology, tumor size, presence of lymph node metastasis, resection status, grading, smoking habits, and the results of the EpCAM-staining were included in this analysis. For TSS both the N classification [hazard ratio (95% confidence interval): 3.499 (1.671 - 7.327)] and detection of EpCAM-positive DCCs [hazard ratio (95% confidence interval): 7.506 (1.476 – 38.182)] remained independent and significant prognostic variables, while the presence of EpCAM-positive DCCs was the only independent and significant prognostic variable for PFS in these patients (HR (95% CI): 3.551 (1.126–11.195); Table 1).
4. Discussion

Here we show that the detection of DCCs in bone marrow of non-metastasized NSCLC patients correlates with reduced patient outcome and survival. Surprisingly, the prognostic impact apparently depends on the DCC detection marker used. While the presence of CK-positive DCCs was not associated with patient outcome, detection of EpCAM-positive DCCs resulted in reduced PFS and TSS. This effect remained significant upon multivariate testing and EpCAM-positive DCCs remained the only independent prognostic marker for PFS.

Our detection rates are consistent with published data [8], [10]–[12], however clinical impact varied in the different studies [7], [8], [10]–[12]. Comparability between studies is limited due to the heterogeneity of patient inclusion criteria, sample origin, processing methods and antibody clones used for detection antibodies of DCCs.

We deem particularly interesting that DCCs expressing EpCAM represent a clinically relevant subpopulation of bone marrow DCCs, potentially representing cancer cells able to initiate secondary growths. Consistently, EpCAM-positive, but not EpCAM-negative CTCs in metastatic lung cancer correlated with decreased survival [20]. Of note, EpCAM has been reported to be highly expressed in embryonic development as well as on cancer stem cells (CSC) [5]. It was recently reported that EpCAM, a multifunctional surface protein with nuclear signaling function after cleavage [21], is able to induce pluripotency when combined with the two Yamanaka factors Oct4 or Klf4 [22]. Therefore, further research should concentrate on a detailed molecular genetic characterization of BM-derived EpCAM-positive DCCs as prime therapy targets for the prevention of metachronous metastasis.

5. Conclusions

DCC detection in BM of stage I-III NSCLC patients predicts outcome, with EpCAM-positive DCCs being candidate driver cells for establishing systemic cancer. Future studies should include the molecular genetic characterization of DCCs to provide novel rationales for the development of (neo)adjuvant therapies.

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References


Figure 1
Kaplan-Meier plots of the progression-free survival (PFS, left) and tumor-specific survival (TSS, right). (a) shows PFS and TSS for DCC detection by EpCAM and/or CK staining, while (b) and (c) show survival analysis for CK-positive and EpCAM-positive groups versus negative patients, respectively. (d) displays the survival of the different subgroups of EpCAM-positive, CK-positive and EpCAM and CK-positive groups versus negative patients. P-values (univariate log-rank tests) and number of patients in each group (n) are provided. For d) only the statistically significant P-value for the EpCAM-positive group is shown.

Suppl. Figure 1
Kaplan-Meier plots of the progression-specific survival (PFS) based on the clinicopathological variables (a) sex, (b) age, (c) histology, (d) T classification, (e) N classification, (f) resection status, (g) grading and (h) smoking habits. Patients with missing data for resection status (n=2) and grading (n=3) were excluded. Patients with adenosquamous (n=3) or large cell carcinoma (n=1) were not included in the analysis.

Suppl. Figure 2
Kaplan-Meier plots of the tumor-specific survival (TSS) based on the clinicopathological variables (a) sex, (b) age, (c) histology, (d) T classification, (e) N classification, (f) resection status, (g) grading and (h) smoking habits. Patients with missing data for resection status (n=2) and grading (n=3) were excluded. Patients with adenosquamous (n=3) or large cell carcinoma (n=1) were not included in the analysis.

Suppl. Figure 3
Dot plot depicts the correlation of log transformed disseminated cancer cell density (DCCD) of EpCAM (clone: HEA-125) and CK (clone: A45-B/B3) staining. Linear regression was performed and is depicted by the black line. Correlation was analyzed using the nonparametric Spearman correlation. Degree of correlation (r) and significance level (P) is provided.