Organ-specific heterogeneity in tumor-infiltrating immune cells and cancer antigen expression in primary and autologous metastatic lung adenocarcinoma

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ABSTRACT

Background Tumor immune microenvironment (TIME) and cancer antigen expression, key factors for the development of immunotherapies, are usually based on the data from primary tumors due to availability of tissue for analysis; data from metastatic sites and their concordance with primary tumor are lacking. Although of the same origin from primary tumor, organ-specific differences in the TIME in metastases may contribute to discordant responses to immune checkpoint inhibitor agents. In immunologically ‘cold’ tumors, cancer antigen-targeted chimeric antigen receptor (CAR) T-cell therapy can promote tumor-infiltrating lymphocytes; however, data on distribution and intensity of cancer antigen expression in primary tumor and matched metastases are unavailable.

Methods We performed a retrospective review of a prospectively maintained database of patients who had undergone curative resection of pathological stage I–III primary lung adenocarcinoma from January 1995 to December 2012 followed by metastatic recurrence and resection of metastatic tumor (n=87). We investigated the relationship between the primary tumor and metastasis TIME (ie, tumor-infiltrating lymphocytes, tumor-associated macrophages, and programmed death-ligand 1 (PD-L1)) and cancer antigen expression (ie, mesothelin, CA125, and CEACAM6) using multiplex immunofluorescence.

Results Brain metastases (n=36) were observed to have fewer tumor-infiltrating lymphocytes and greater PD-L1-negative tumor-associated macrophages compared with the primary tumor (p<0.0001); this relatively inhibitory TIME was not observed in other metastatic sites. In one in three patients, expression of PD-L1 is discordant between primary and metastases. Effector-to-suppressor (E:S) cell ratio, median effector cells (CD20+ and CD3+) to suppressor cells (CD68/CD163+) ratio, in metastases was not significantly different between patients with varying E:S ratios in primary tumors. Cancer antigen distribution was comparable between primary and metastases; among patients with mesothelin, cancer antigen 125, or carcinoembryonic antigen expression in the primary tumor, the majority (51%–75%) had antigen expression in the metastases; however, antigen-expression intensity was heterogeneous.

Conclusions In patients with lung adenocarcinoma, brain metastases, but not other sites of metastases, exhibited a relatively immune-suppressive TIME; this should be considered in the context of differential response to immunotherapies.
immunotherapy in brain metastases. Among patients with cancer antigen expression in the primary tumor, the majority had antigen expression in metastases; these data can inform the selection of antigen-targeted CARs to treat patients with metastatic lung adenocarcinoma.

BACKGROUND

Despite the success of immune checkpoint inhibitor (ICI) agents in patients with ‘hot’ tumor immune microenvironments (TIMEs), discordant responses to ICI agents at different disease sites have been observed in 22%–44% of patients with metastatic non-small cell lung cancer.1–4 These discordant responses may be due to organ-specific differences in the TIME; factors—including tumor-infiltrating lymphocyte (TIL) density5,6 and programmed death-ligand 1 (PD-L1) expression on tumor cells7–9 and macrophages8—are associated with responses to ICI agents. Investigations into the TIME of brain metastasis have shown relatively fewer TILs compared with paired primary tumors10–11; however, other metastatic disease sites have not been investigated, and the association between the host organ and the metastatic TIME is unknown.

To promote TILs in immunologically ‘cold’ tumors, we and others have investigated adoptive cell therapy, specifically investigating the use of a patient’s own T cells transduced with a chimeric antigen receptor (CAR).12 CARs are genetically engineered synthetic receptors, and when transduced into T cells, CARs can recognize and bind to a cancer cell-surface antigen to induce target cell lysis.13–15 CAR T-cell therapy has achieved durable responses in hematological malignancies.16,17 A hurdle to CAR T-cell therapy for solid tumors is heterogeneous target antigen expression.18–20 The selection of a cell-surface antigen that is overexpressed in patients with solid tumors is currently based on antigen expression patterns in early stage cancer,21 where resected tumors are available to investigate. Even though clinical trials of CAR T-cell therapy are conducted in patients with advanced metastatic disease, data are lacking for antigen expression in metastases due to the limited availability of tissue from metastatic disease sites. For example, in The Cancer Genome Atlas—a source where many investigators derive antigen-expression data to develop a specific cancer antigen-targeted CAR—only 9 of 230 patients in the lung adenocarcinoma (ADC) cohort had stage 4 disease22; lung ADC, subtype of non-small cell lung cancer, comprise the largest of the cohort in lung cancer. Candidate antigen targets in solid tumors include mesothelin (MSLN), cancer antigen 125 (CA125), and carcinoembryonic antigen adhesion molecule 6 (CEACAM6), which have been evaluated as antigen targets in phase I clinical trials23,24 or in preclinical studies of antigen-targeted immunotherapy.25

Here, we address the paucity of data on the TIME and target antigen expression in metastatic lung ADC by evaluating patients with paired primary lung ADC tumors and metastases. While a single-site biopsy results typically determine antigen expression and TIME and the selection of specific immunotherapy as the treatment of choice for patients with solid tumors, our study provides insight into interpretation of differential response at metastatic sites. Furthermore, our data on antigen expression distribution and intensity in lung ADC can inform the selection of single-antigen-targeted and dual-antigen-targeted CARs to treat patients with metastatic lung ADC.

METHODS

Patients

We performed a retrospective review of a prospectively maintained database of patients who had undergone curative resection of pathological stage I–III primary lung ADC from January 1995 to December 2012 and subsequently developed metastases. Inclusion criteria included the development of metastases following a disease-free interval and surgical resection of metastatic disease with adequate specimen for analysis. Exclusion criteria included induction therapy, local recurrence, or second primary lung cancer (online supplemental figure 1).

Histological examination and tissue microarray construction

Tumors were staged according to the eighth edition of the International Association for the Study of Lung Cancer classification.25 Histological evaluation of H&E-stained tumor slides was performed by a pathologist, and the tumor areas were marked. From both primary and metastatic tumors, six tumor cores were identified; three tumor cores each from the predominant histological subtypes on the corresponding formalin-fixed, paraffin-embedded tumor blocks. Cylindrical 0.6 mm tissue cores were arrayed into a recipient block to create a tissue microarray (online supplemental figure 2).

Multiplex immunofluorescence staining and image analysis

Multiplex immunofluorescence staining of 4 μm thick tissue microarray sections were stained with three antibody panels (panel 1: CD20, CD3, CD68/CD163, myeloperoxidase (MPO), and Pan-C; panel 2: CD4, CD8, CD68/CD163, PD-L1, and Pan-C; panel 3: MSLN, CA125, CEACAM6, and Pan-C), using the Opal 7-color multiplex immunofluorescence kit (Akoya Biosciences, Marlborough, Massachusetts, USA) (online supplemental table 1). High-powered images of individual cores were captured using the Vectra 3.0 multispectral imaging system.

Multiplex immunofluorescence quantification

Multiplex immunofluorescence images were analyzed using inForm V.2.4.1 software (online supplemental figures 3,4). Manual quality control was performed to exclude missing, folded, or out-of-focus cores and non-specific staining. Enumeration of CD20, CD3, CD68/CD163, MPO, Pan-C, and PD-L1 cell markers was performed using cell segmentation and phenotyping algorithms. Following cell segmentation and phenotyping of Pan-C-positive cells, a threshold differentiating...
cell-specific, cell-surface MSLN, CA125, and CEACAM6 antigen expression from background immunofluorescence and from normal tissue was determined and validated by concordance with pathologist assessment (online supplemental figure 5). Cell-surface expression of MSLN, CA125, and CEACAM6 antigens was then determined based on threshold membrane-intensity values, using inForm V.2.4.1 software and R V.3.6.0 software. Immune cell populations were calculated as relative percentages of total immune cells (CD3, CD20, CD68/163, and MPO) present in each core. Tumor and macrophage PD-L1 expression was determined based on the percentage of Pan-Ck+ (tumor) and CD68/163+ (macrophage) cells expressing PD-L1. Cancer antigen cell-surface expression was determined based on the percentage of Pan-Ck (tumor) cells expressing MSLN, CA125, or CEACAM6. Relative antigen intensity was calculated as the fold-change from background fluorescence.

**Statistical analysis**

Immune cell populations and antigen expression were compared using two-sided Student’s t-test, and tumor and macrophage PD-L1 and antigen-expression intensity was compared using Spearman’s correlation. Spearman’s rank coefficient (ρ) was measured using the following equation:

\[ \rho = 1 - \frac{6 \sum d^2}{n(n^2-1)} \]

Statistical analysis was conducted using GraphPad Prism V.9.0.0 software (GraphPad Software, San Diego, California, USA).

To quantify CD3+ T cell and CD68/C163 macrophage co-localization using cell counts, we apply the Morisita-Horn similarity index (MH index).26 The value of MH index ranges from 0 (indicating no similarity or overlap between two community structures) to 1 (an equal proportion of each of the two cell types in each cluster), indicating that the two cell types are highly co-localized. The distribution of MH index was summarized using box plots and compared between primary and metastatic cores and between primary and stroma cores using Wilcoxon signed rank test with continuity correction for paired data.

Effect-to-suppressor (E:S) cell ratio was calculated by dividing effector cell count (CD20+ and CD3+ cells) by suppressor (CD68/C163+ cells) cell count. The E:S ratios in the primary tumor were plotted against the E:S ratios in metastases. Based on the primary tumor E:S ratio, samples were categorized as being immunologically ‘hot’ (at or above median E:S ratio) or ‘cold’ (below median E:S ratio). The E:S ratio in the metastatic samples were compared between ‘hot’ and ‘cold’ primary samples using Wilcoxon rank sum test. Metastatic samples were categorized as being ‘hot’ (at or above median E:S ratio) or ‘cold’ (below median E:S ratio). The combination of ‘hot’ and ‘cold’ in the primary and in the metastatic samples results in four distinct categories: ‘cold’ primary and ‘cold’ metastasis, ‘cold’ primary and ‘hot’ metastasis, ‘hot’ primary and ‘cold’ metastasis, and ‘hot’ primary and ‘hot’ metastasis. The average cell counts of each cell type (CD3, CD20, C68/C163, and MPO) in the primary and metastatic samples were visualized as a heatmap, stratified by the four distinct categories based on ‘hot’ and ‘cold’ primary and metastasis combinations. Immune cell density [(CD3+CD20+CD68/C163+ MPO)/(total nucleated cells)] was quantified for each primary and metastatic core.

**Procedure for managing conflicts of interest**

Any conflicts of interest will be presented to the institution’s Conflicts Committee and designated Research Oversight Committee and resolved appropriately.

**RESULTS**

**Patient characteristics**

Among 87 patients with paired primary lung ADC and autologous metastases evaluated for cancer antigen expression, the site of metastasis was in the brain in 34 patients (39%), in the lung in 27 patients (31%), and in other sites (lymph node, pleura, bone, adrenal, kidney, colon, and soft tissue) in 26 patients (30%) (table 1). Following multiplex immunofluorescence staining and quality control, data from 84 patients were available for infiltrating immune cells, and data from 79 patients were available for PD-L1 expression.

**Metastasis site-specific heterogeneity in tumor-infiltrating immune cells**

Tumor-infiltrating immune cells were compared between paired primary and metastatic tumors. Among all patients, the primary tumor had a higher percentage of CD20+ B cells (p<0.01), CD3+ T cells (p<0.01), and CD4+ T cells (p<0.01), and a lower percentage of CD68/C163+ macrophages (p<0.001), compared with paired metastases. There was no difference observed in MPO+ neutrophils between the primary tumor and metastases (figure 1A).

Patients were stratified by site of metastasis to evaluate site-specific heterogeneity in immune cell composition. Compared with the primary tumor, in brain metastases (n=36), we observed a lower percentage of CD20+ B cells (p<0.001), CD3+, CD4+, and CD8+ T cells (p<0.001), and a higher percentage of CD68/C163+ macrophages (p<0.001). In patients with lung (n=24) and other sites of metastases (n=24), however, there were no differences in immune cell populations between the primary tumor and paired metastases (figure 1A).

In advanced NSCLC, PD-L1 expression on macrophages has been associated with both CD8+ T cell infiltration and prognosis following immune checkpoint blockade, suggesting PD-L1 expression on macrophages may be a characteristic of immunologically ‘hot’ tumors.8 Given the higher percentage of CD68/C163 macrophages observed in brain metastases, we further evaluated macrophage by stratifying CD68/C163 macrophages...
We observed no difference in PD-L1+ macrophage percentages between primary and metastatic tumors in patients with brain, lung, or other sites of disease. In patients with brain metastases, we observed higher percentages of PD-L1− macrophages in metastases compared with primary tumor (p<0.001) (online supplemental figure 6). This result is consistent with a relatively immunologically ‘cold’ TIME in brain metastases.

Heterogeneity in PD-L1 expression between primary tumor and paired metastases

The expression of PD-L1 was evaluated both on tumor cells and macrophages as a surrogate marker for immune activation in the tumor. PD-L1 expression on tumor cells was stratified based on clinical practice cut-off of ≥1%, and for macrophages based on the cohort’s median value of ≥27%. When analyzed as a categorical variable, concordance of tumor PD-L1 expression between paired primary tumor and metastases was 61%; 28% were PD-L1 positive (≥1%) in both the primary tumor and metastases, and 33% were PD-L1 negative in both the primary tumor and metastases (figure 1B). When stratifying tumor PD-L1 expression using clinically relevant cut-off values (<1%, 1%–49%, or ≥50%), PD-L1 expression percentage in the primary tumor and metastases remained similar (figure 1C), as did tumor PD-L1 expression in brain metastases (online supplemental figure 7A). However, even among patients with PD-L1 expression ≥50% or 1%–49% in primary tumor, there is discordant PD-L1 expression in metastases (figure 1C; online supplemental figure 7B). Concordance of macrophage PD-L1 expression between paired primary tumor and metastases was 58%; 32% were PD-L1 positive in both the primary tumor and metastasis, and 27% were PD-L1 negative in both the primary tumor and metastasis (figure 1D). Discordance of PD-L1 expression between the primary tumor and metastases was 39% for tumor-cell PD-L1 (figure 1B) and 42% for macrophage PD-L1 (figure 1D).

When analyzed as a continuous variable, Spearman’s correlation demonstrated strong correlation between tumor cell PD-L1 and macrophage PD-L1 expression within each anatomical site in both the primary tumor (p=0.72) and metastases (p=0.67) (figure 1E). In contrast, when tumor and macrophage PD-L1 was analyzed between the primary tumor and paired metastases, weak correlation was observed for both tumor PD-L1 (p=0.33) and macrophage PD-L1 (p=0.29) (figure 1F).

Co-localization of T cells and macrophages in paired primary and metastatic tumors

Given the finding of site-specific heterogeneity in T-cell and macrophage populations in metastatic tumors, we next investigated T-cell and macrophage co-localization in primary tumor and metastases. Co-localization was quantified using the MH index,26 based on T-cell and macrophage cell counts within the six tumorous cores. The MH index ranges from 1 (an equal proportion into PD-L1+ and PD-L1− macrophage subpopulations. We observed no difference in PD-L1+ macrophage percentages between primary and metastatic tumors in patients with brain, lung, or other sites of disease. In patients with brain metastases, we observed higher percentages of PD-L1− macrophages in metastases compared with primary tumor (p<0.001) (online supplemental figure 6). This result is consistent with a relatively immunologically ‘cold’ TIME in brain metastases.

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*Mutation status known in 67 patients.

EGFR, epidermal growth factor receptor; KRAS, Kirsten rat sarcoma viral oncogene homolog.
Figure 1  Metastasis site-specific differences in tumor immune microenvironment. (A) CD20+ (B cells), CD3+ (T cells), CD4+ and CD8+ (T-cell subpopulations), CD68/CD163+ (macrophages), and myeloperoxidase+ (MPO; neutrophils) were compared (as a percentage of total immune cells) between paired primary and metastatic tumors. Among all patients, the primary tumor had higher percentages of B cells, CD3+ and CD4+ T cells, and lower percentages of macrophages. Among patients with brain metastases, percentages of B cells and CD3+, CD4+, and CD8+ T cells were lower, and percentages of macrophages higher compared with primary tumor. Among patients with lung or other sites of metastasis (lymph nodes, pleura, bone, adrenal, kidney, colon, or soft tissue), there was no difference in immune cell populations between the primary tumor and metastases. Median and IQR is displayed. *P<0.01; **p<0.001. (B) Programmed death-ligand 1 (PD-L1) expression on tumor cells (≥1% of tumor cells) differed between the primary tumor and metastatic tumor in 39% of patients; 19% of patients had high (≥1% of tumor cells) PD-L1 expression on the primary tumor but low (<1%) PD-L1 expression on the metastatic tumor, while 20% had low PD-L1 (<1%) PD-L1 expression on the primary tumor but high (≥1%) PD-L1 expression on the metastatic tumor. (C) Tumor-cell PD-L1 expression was stratified into <1%, 1%–49%, and ≥50% of tumor cells. The percentage of primary tumors (n=79) with tumor-cell PD-L1 expression <1% was 53%, 1%–49% was 35%, and ≥50% was 11%. The percentage of metastases (n=79) with PD-L1 expression <1% was 52%, 1%–49% was 41%, and ≥50% was 8%. (D) PD-L1 expression on macrophages (≥27% of macrophages) differed between the primary tumor and the metastatic tumor in 42% of patients; 20% of patients had high (≥27%) macrophage PD-L1 expression on the primary tumor but low (<27%, median value) macrophage PD-L1 expression on the metastatic tumor, while 22% had low macrophage PD-L1 expression on the primary tumor but high macrophage PD-L1 expression on the metastatic tumor. (E) The correlation between tumor cell PD-L1 expression (as a percentage of Pan-CK+ tumor cells) and macrophage PD-L1 expression (as a percentage of CD68/CD163+ macrophages) was assessed within each tumor specimen for both primary and metastatic tumors. The correlation (Spearman’s ρ) between tumor cell PD-L1 expression and macrophage PD-L1 expression was 0.72 within primary tumors, and 0.67 within metastatic tumors. (F) PD-L1 expression on tumor cells and PD-L1 expression on macrophages is shown for 79 paired primary and metastatic tumors, with each horizontal line representing one tumor. The correlation (Spearman’s ρ) between paired primary tumor and metastatic tumors was 0.33 for tumor PD-L1 expression, and 0.29 for macrophage PD-L1 expression. MPO, myeloperoxidase; PD-L1, programmed death-ligand 1.
of two cell types in each cluster) to 0 (no similarity or overlap between two cell types).\textsuperscript{26} MH index indicated a high degree of clustering in both the primary tumor (MH=0.83) and metastases (MH=0.84) (figure 2A). Co-localization between T cells and macrophages, based on MH index, was similar between primary and metastatic tumors (p=0.13, Wilcoxon signed rank test).

**Heterogeneity in effector:suppressor cell ratio between primary tumor and metastases**

Given the observation that T cells and macrophages exhibited co-localization in both primary and metastatic tumors, we next investigated the relationship between immune cell populations in primary and metastatic tumors. Both primary and metastatic tumors were divided into ‘hot’ and ‘cold’ tumors based on median effector cell (CD20+ and CD3+ cells) to suppressor cell (CD68/CD163+ cells) ratio (E:S ratio). High E:S ratio was defined as a ‘hot’ tumor. Patients were then divided into four groups based on E:S ratio in primary and metastatic tumor as displayed in the correlation plot (figure 2B); ‘cold’ primary and ‘cold’ metastasis (blue quadrant); ‘cold’ primary and ‘hot’ metastasis (purple quadrant); ‘hot’ primary and ‘cold’ metastasis (orange quadrant); and ‘hot’ primary and ‘hot’ metastasis (red quadrant).

Among ‘cold’ primary tumors, the median E:S ratio in corresponding metastases was 0.3 (IQR 0.1, 1.2), and among ‘hot’ primary tumors, the median E:S ratio was 0.8 (IQR 0.3, 2.4) (p=0.082, Wilcoxon rank sum exact test) (figure 2C). This indicates that metastasis E:S ratio was not significantly different between patients with ‘hot’ and ‘cold’ primary tumors.

To investigate immune cell populations in ‘hot’ and ‘cold’ tumors in individual patients across different sites of metastatic disease, we stratified patients into four groups based on ‘hot’ and ‘cold’ primary and metastatic tumors and produced a heatmap visualizing immune cell populations within each tumor. Average cell counts for each immune cell type were included (CD3, CD20, CD68/CD163, and MPO) (figure 2D). Among patients with a ‘hot’ primary tumor and a ‘cold’ metastasis, the site of metastasis was in the brain in 16 of 19 patients. This is consistent with an immunologically ‘cold’ tumor micro-environment in brain metastasis relative to the corresponding primary tumor from the same patient. Among ‘hot’ metastatic sites, lymph nodes, lung and other tissues are predominant.

**Cancer antigen expression between paired primary tumors and metastases**

We compared cancer antigen cell surface expression between paired primary and metastatic tumors (figure 3A; online supplemental figure 8). Antigen expression was assessed as a percentage of tumor cells for each patient, and patients were stratified into high (≥10% of tumor cells) or low (<10% of tumor cells) expression levels based on previously reported inclusion criteria for antigen-targeted immunotherapies.\textsuperscript{12} 27 Among patients with high CEACAM6 expression in the primary tumor (80%), 75% also had high CEACAM6 expression in the metastases. Similarly, among patients with high CA125 expression in the primary tumor (67%), 73% had high CA125 expression in the metastases. Among patients with high MSLN expression in the primary tumor (59%), 51% had high MSLN in the metastases (figure 3A).

**Antigen co-expression patterns in paired primary tumors and metastases**

Dual antigen-targeted CARs are in development to translate to solid tumors cell therapy with a rationale that targeting two cell-surface antigens can expand the eligible patient population for treatment as well as overcome the therapy efficacy limitation from antigen escape seen with single antigen-targeted CAR T cells.\textsuperscript{19} 28 29 To evaluate the potential to target multiple tumor antigens in lung ADC, we investigated cell surface antigen co-expression in primary and metastatic tumors. Eighty-eight per cent of patients expressed (in ≥10% of tumor cells) at least one antigen (MSLN, CA125, or CEACAM6) in the primary tumor, and 86% expressed at least one antigen in metastatic tumors (figure 3B). Antigen co-expression did not differ by site of metastasis (online supplemental figure 9A). We then evaluated the potential for dual antigen targeting for metastatic tumors. Expression of MSLN, CA125, or both was observed in 69% of metastases; expression of MSLN, CEACAM6, or both was observed in 73% of metastases; and expression of CA125, CEACAM6, or both was observed in 86% of metastases. MSLN expression was accompanied by expression of either CA125 or CEACAM6 (online supplemental figure 9B).

Antigen co-localization within the primary and metastatic tumor was investigated for each patient. The median percentage of tumor cells that were positive for at least one antigen was 62% in primary tumors, compared with 72% in metastatic tumors (n=87) (figure 3C; online supplemental figure 9C). The median percentage of tumor cells that were positive for CEACAM in lung ADC was 52%, for CA125 alone was 21%, and for MSLN alone was 6% (figure 3D).

**Antigen intensity heterogeneity between the primary tumor and metastases**

The intensity of surface antigen expression between the primary tumor and paired metastases was evaluated, as CAR T-cell efficacy is dependent on antigen density.\textsuperscript{10} 31 Among patients with high (≥10% of tumor cells) antigen expression, median relative CEACAM6 antigen intensity (calculated as fold-change from background fluorescence) was 1.21 (range 0.45–2.86) in primary tumors and 1.49 (0.43–3.60) in metastatic tumors. For CA125, median relative antigen intensity was 0.89 (0.45–3.87) in primary tumors and 0.99 (0.32–2.90) in metastases, and for MSLN, median relative antigen intensity was 0.79 (0.49–7.97) in primary tumors and 0.88 (0.44–22.97) in metastases (figure 3E). For paired primary and metastatic tumors with high (≥10% of tumor cells) antigen expression,
Figure 2  Co-localization of CD3+ T cells and CD68/CD163+ macrophages and patient stratification by effector:suppressor cell ratio in paired primary and metastatic tumors. (A) Co-localization of CD3+ T cells and CD68/CD163+ macrophages was quantified through the Morisita-Horn (MH) index, based on immune cell populations in the tumorous tissue microarray cores. MH index indicated a high degree of co-localization in both primary (MH=0.83) and metastatic tumors (MH=0.84). There was no difference in CD3 and macrophage cell co-localization between primary and metastatic tumors (p=0.13, Wilcoxon signed rank test). (B) Patients were divided into four groups based on median in primary and metastatic tumor. Tumors with greater than median effector cell (CD20+ and CD3+):suppressor cell (CD68/CD163+) ratio (E:S ratio) were considered ‘hot’, and tumors with less than median E:S ratio were considered ‘cold’. (C) Among ‘cold’ primary tumors, the median E:S ratio in corresponding metastases was 0.3 (IQR 0.1, 1.2), and among ‘hot’ primary tumors, the median metastatic E:S ratio was 0.8 (0.3, 2.4) (p=0.082, Wilcoxon rank sum exact test). (D) Immune cell abundance (cells/core) and site of metastasis (brain, lung, lymph node, and other) is displayed for each primary and metastatic tumor, following stratification of patients into groups based on E:S ratio in primary and metastatic tumor. The data are represented with metastatic tumors increasing E:S ratio (cold to hot). ICD, immunogenic cell death; MPO, myeloperoxidase.
Figure 3  Comparison of cancer antigen surface expression between the primary tumor and metastases. (A) Carcinoembryonic antigen adhesion molecule 6 (CEACAM6), cancer antigen 125 (CA125), and mesothelin (MSLN) surface expression is compared between paired primary and metastatic tumors (cut-off value ≥10% of tumor cells with antigen expression). CEACAM6 expression differed between the primary tumor and the metastatic site in 33% of patients; 20% of patients had high CEACAM6 expression (≥10%) in the primary tumor and low CEACAM6 expression in the metastatic tumor, while 13% of patients had low CEACAM6 expression in the primary tumor and high CEACAM6 expression in the metastatic tumor. Similarly, CA125 expression differed between the primary tumor and the metastatic site in 38% of patients, and CA125 expression differed between the primary tumor and the metastatic site in 42% of patients. (B) Cell surface antigen co-expression was compared between the primary tumor and metastases. (C) Cell surface antigen co-localization was assessed for primary and metastatic tumors (n=87, each bar represents one patient). For each tumor, the percentage of tumor cells positive for a single antigen, two antigens (double), and three antigens (triple) is shown. The median expression of at least one antigen was 62% for primary tumors and 72% for metastatic tumors. (D) In metastatic tumors, the percentage of tumor cells positive for a single antigen (n=87) for CEACAM6, CA125, and MSLN is shown (cell surface expression). Median antigen expression (as a percentage of tumor cells) was 52% for CEACAM6, 21% for CA125, and 6% for MSLN. (E) Waterfall plots demonstrating relative cell-surface antigen intensity for CEACAM6, CA125, and MSLN in primary and metastatic tumors with high (≥10% of tumor cells) antigen expression only are shown. Each bar represents one patient. Relative antigen intensity is calculated as the fold-change from background fluorescence.
Spearman’s correlation demonstrated weak correlation between primary tumor and metastasis antigen intensity (CEACAM6, ρ=0.34, n=52; CA125, p=−0.01, n=43; MSLN, ρ=0.05, n=26). There was no correlation between the TIME (as evaluated by E:S cell ratio) and cell-surface antigen expression, either in the primary tumor or metastases (online supplemental figure 10).

**DISCUSSION**

To our knowledge, the current study assessed the largest cohort of paired primary and metastatic lung ADC for TIME and antigen expression. While other studies have focused on brain metastases, our study included additional sites of metastases, and in addition to TIL density and PD-L1 expression, we investigated additional immune markers as well as co-localization of TIME factors by use of multiplex immunofluorescence. In addition, we compared antigen-expression density and intensity across primary and metastatic tumors for multiple cancer cell-surface antigens. Most importantly, to account for tumor heterogeneity, we selected tumor cores from six different sites of multiple tumor blocks, representing diverse histological patterns within the lung ADC—a methodology that we developed that has been highly reproduced.21−32–37

Our report, for the first time, delineates the concordance and discordance between primary and metastatic lung ADC, and further demonstrates differential TIME across metastatic sites.

Consistent with previous reports,9,10 we observed relatively fewer number of T cells and B cells and higher number of macrophages in the TIME of brain metastases. There was no difference observed in immune cell populations between the primary tumor and other sites of metastatic disease, which is a previously unreported finding. This raises the possibility of a relatively immune inhibitory TIME in brain metastases specifically, which may contribute to discordant responses to ICI agents in patients with brain metastases.4,4 It was recently shown that CD20+ B cells in germinal centers in tumors are associated with better prognosis with immunotherapy.38 39

One limitation in our comparison of TIME factors in primary tumors and metastases was that intermediate therapies received between resection of the primary tumor and metastatic tumor resection or biopsy might have influenced the TIME in metastases.32 We previously published that the benefit of adjuvant chemotherapy is enhanced in patients with immune-activated stage II–III lung ADC (identified by PD-L1 expression in tumor cells and macrophages) with enriched myeloid-derived suppressor cells (MDSCs); this led to the hypothesis that chemotherapy-mediated MDSC and macrophage elimination may have provided fertile ground for endogenous immune responses to eliminate micrometastases.32

For immunologically ‘cold’ tumors that are resistant to ICI agents, antigen-targeted CAR T-cell therapy is under investigation.15,26 46 Due to the limited availability of tissue from metastatic disease sites, current data on cancer antigen expression are based on early stage disease,21,22 although clinical trials of CAR T-cell therapy have been conducted in advanced metastatic disease. To our knowledge, the present study represents the largest cohort of paired primary and metastatic lung ADC tumors assessed for cancer antigen expression. Our data indicate that among patients with high (≥10% of tumor cells) cell surface antigen expression in the primary tumor, antigen expression was also high in the metastases in the majority (51%−75%) of patients. CAR T-cell efficacy is associated with antigen density,30,31 and the use of multiplex immunofluorescence allowed us to investigate cell-surface antigen expression intensity. Among tumors with high (≥10% of tumor cells) cell-surface antigen expression, antigen intensity was similar for both the primary tumor and metastases; however, there was heterogeneity in antigen-expression intensity between primary and metastatic sites.

A potential strategy to overcome heterogeneous antigen expression41 is to target a combination of cancer antigens as well as reduce the occurrence of antigen escape.28 42 The use of multiplex immunofluorescence to assess the co-localization of three tumor-associated antigens (MSLN, CA125, or CEACAM6) allowed us to determine the proportion of tumor cells that can be targeted by selection of multiple cancer antigens as targets. To our knowledge, co-localization of multiple cancer antigens has not been previously assessed in patients with lung ADC. Although targeting multiple cancer antigens has been described as a strategy to overcome heterogeneous antigen expression in solid tumors, we observed only a modest increase in the percentage of tumor cells that could be targeted by a combination of antigens, as compared with targeting a single tumor-associated antigen. A median of 72% of tumor cells could be targeted with a combination of MSLN, CA125, and CEACAM6, compared with a median of 52% of lung ADC metastatic tumor cells that could be targeted by CEACAM6 alone.

The limitations of our study include the fewer sites of metastases where tissue is available. The patients included in this study had surgical resection of metastasis, which
may not have been representative of all patients with metastatic lung ADC. However, the use of surgically resected tissue allowed for the inclusion of six tissue microarray cores from each primary and metastatic tumor, facilitating for a more representative tumor heterogeneity than a single biopsy. Other limitations include the number of cancer antigens or TIME factors investigated. The tissue microarray we developed provides an infrastructure to investigate additional antigens or TIME factors as a next step.

In summary, brain metastases in patients with lung ADC exhibited a relatively immune-suppressive TIME compared with the primary tumor, while other metastatic disease sites exhibited a similar immune cell population compared with the primary tumor. This observation should be considered in the setting of discordant responses to ICI agents in brain metastases. E:S ratio, median effector cells (CD20+ and CD3+) to suppressor cells (CD68/CD163+) ratio, in metastases was not significantly different between patients with varying E:S ratios in primary tumors. In patients with cancer antigen expression in the primary tumor, antigens were expressed in metastases in the majority of patients with expression heterogeneity by antigen or site of metastases, providing an infrastructure to select cancer-associated antigens for targeting in metastatic lung ADC.

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