Tumor Heterogeneity Confounds Lymphocyte Metrics in Diagnostic Lung Cancer Biopsies

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• ***Context.***—**The immune microenvironment is involved in fundamental aspects of tumorigenesis, and immune scores are now being developed for clinical diagnostics.**

Objective.—**To evaluate how well small diagnostic biopsies and tissue microarrays (TMAs) reflect immune cell infiltration compared to the whole tumor slide, in tissue from patients with non–small cell lung cancer.**

Design.—**A TMA was constructed comprising tissue from surgical resection specimens of 58 patients with non–small cell lung cancer, with available preoperative biopsy material. Whole sections, biopsies, and TMA were stained for the pan-T lymphocyte marker CD3 to determine densities of tumor-infiltrating lymphocytes. Immune cell infiltration was assessed semiquantitatively as well as objectively with a microscopic grid count. For 19 of the cases, RNA sequencing data were available.**

Results.—**The semiquantitative comparison of immune cell infiltration between the whole section and the biopsy displayed fair agreement (intraclass correlation coefficient [ICC], 0.29; $P = .01$; CI, 0.03–0.51). In contrast, the TMA showed substantial agreement compared with the whole slide (ICC, 0.64; $P < .001$; CI, 0.39–0.79). The grid-based method did not enhance the agreement between the different tissue materials. The comparison of CD3 RNA sequencing data with CD3 cell annotations confirmed the poor representativity of biopsies as well as the stronger correlation for the TMA cores.**

Conclusions.—**Although overall lymphocyte infiltration is relatively well represented on TMAs, the representativity in diagnostic lung cancer biopsies is poor. This finding challenges the concept of using biopsies to establish immune scores as prognostic or predictive biomarkers for diagnostic applications.**

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Lung cancer is the most common cause of cancer-related deaths worldwide. In advanced stages, the overall prognosis is poor, with a median survival rate of 12 to 15 months. The introduction of immune checkpoint inhibitors provides, for the first time, a chance for long-term survival in metastasized patients without targetable genetic aberrations, indicating the fundamental role of the immune system in controlling cancer growth.[1–4] The functional role of the immune system in cancer surveillance and prevention is also evident in the absence of any immune-modulating therapy.[5,6] In many cancer forms, specific immune cell infiltration is independently and strongly connected to survival.[7–10] Particularly in colorectal cancer, the spatial lymphocyte infiltration, as quantified in the central tumor part and in the invasion margin, has a higher prognostic impact than tumor stage.[11] Corresponding initiatives to establish biomarkers based on immune infiltration patterns are ongoing in other cancer types.[7,12]

Still, despite enormous efforts in the way of standardization, immune-based classification has not been integrated into clinical cancer diagnostics, which also holds true for lung cancer. Several independent studies have demonstrated the association between the abundance of immune cells in lung cancer and survival.[13–16] Studies in patients after surgical resection demonstrated, in general, a favorable outcome if tumors were rich in lymphocytic infiltration, $CD8^+$ cells, $CD4^+$ cells, plasma cells, and B cells.[8,9,17–19] On the other hand, M2-like macrophages and T regulatory cells were connected to poor prognosis.[18,20,21] However, the results of these studies are not always consistent, and the choice of immune markers, antibody clone, and cutoff complicates the interpretation and consequently the introduction as a reliable assay in routine diagnostics.[22]

A major weakness of many research studies is the use of tissue microarrays (TMAs). The TMA has become an important scientific tool, making it reasonably easy and cost-effective to evaluate a large number of individual tumor samples on a single glass slide.[23] This is possible because each patient sample is represented by 2 or 3 tissue cores,

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representing only a minimal and highly biased area of the whole tumor. Logically, there is debate over how well the small cores of the TMA correspond to the whole tumor.[24] Many studies have confirmed that central tumor features, such as tumor grade, expression of tumor antigens, and immunohistochemical biomarkers, are largely well represented on TMAs.[25–28] For immune cell infiltration, as a notorious heterogeneous tissue reaction, there are only limited data on its representativity in TMAs for some cancer types, but not for lung cancer.[29–31]

In addition, and more problematically, most lung cancer patients receive a diagnosis at an advanced stage by forceps or core needle biopsies. The tissue area is small, often at best comparable to a TMA core. In contrast to TMA tissue, biopsies usually do not provide tissue that is representative of the whole tumor. Thus, the application of immune cell scores as prognostic or predictive biomarkers in this limited material is probably not informative in clinical practice.

Against this background, we aimed to systematically assess how the evaluation of limited tumor material reflects immune infiltration compared with a whole slide approach. Exemplarily, we used the pan-T lymphocyte marker CD3 on whole slide sections, biopsies, and TMAs, and applied 2 different methods of quantification.

MATERIALS AND METHODS

Patient Population

This study comprised patients from the Uppsala Lung Cancer Cohort, consisting of non–small cell lung cancer (NSCLC) patients surgically treated at Uppsala University Hospital (Uppsala, Sweden) from 1995 to 2010. None of the patients had received neoadjuvant chemotherapy. From this larger cohort, 58 patients with available formalin-fixed, paraffin-embedded (FFPE) material in both preoperative biopsies and surgical tumor blocks were selected. From the FFPE tumor blocks, a TMA was constructed as previously described,[32] consisting of two 1-mm cores from each tumor. An FFPE bloc representative for the whole tumor was selected. The area that best represents the tumor with a high tumor cell content was marked on the corresponding glass slide. From this marked area the 2 cores were taken and transferred to a recipient bloc, the TMA. The study was conducted in adherence with the Declaration of Helsinki and was approved by the regional ethics committee in Uppsala (D-nr 2006/325 and 2012/532).

Immunohistochemistry

New 4-μm sections from the tumor blocks, biopsies, and TMA were stained with the pan-T lymphocyte marker CD3 (FLEX Polyclonal Rabbit Anti-Human CD3; Agilent, Santa Clara, California) according to the manufacturer's instructions. Cases with fewer than 50 evaluable tumor cells were excluded. The stained slides were scanned at ×40 on a Hamamatsu NanoZoomer S60 (Hamamatsu photonics, Hamamatsu, Japan) and assessed using the Hamamatsu NDP.view2 viewer (www.hamamatus.com/jp/en.html).

Two pathologists separately and blindly assessed all 3 fractions, tumor whole slide, biopsy, and TMA, according to the number of $CD3^+$ cells. The percentage of stained cells was established for the tumor compartment, the stroma compartment, and both compartments together. The percentage of stained cells in respective compartments, relative to all nucleated cells in that compartment, was scored semiquantitatively using the cutoffs 0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. Semiquantitative evaluation was performed on the scanned slides. A more simplified scoring system was also tested, where scores were generated by converting the percentage of $CD3^+$ immune cells into 5 categories: 0, <1%; 1, 1% to 4%; 2, 5% to 24%; 3, 25% to 49%; and 4, ≥50%. This score was also applied to the tumor compartment, the stroma compartment, and both compartments together.

To assess the number of immune cells more objectively, the absolute numbers of stained cells were manually calculated using a grid (Supplemental Figure 1; see supplemental digital content, containing 4 figures and 5 tables at https://meridian.allenpress.com/aplm in the January 2024 table of contents). The area with the highest and lowest numbers of $CD3^+$ cells was localized in the total tissue area, not according to the stroma and tumor compartments. In these 2 areas, the number of stained cells was manually counted at ×40 using an Olympus microscope and an Olympus ocular grid (UIS2 WHN10X-H/22, Olympus). The grid area measured 0.0625 mm^2 and all positive cells within the grid area were counted.

RNA Sequencing

For 19 cases, RNA sequencing data were available. Fresh frozen tissue from the surgical specimen was used to extract total RNA using an RNeasy mini kit (Qiagen, Hilden, Germany). The RNA sequencing was performed as previously described,[33] and data can be found on Gene Expression Omnibus under accession number GSE81089.

Statistical Analysis

For statistical analysis, R Studio (2021.09.0 +351) was used. The agreement between the different fractions and compartments as well as interrater agreement was assessed with the intraclass correlation coefficient (ICC),[34,35] using the "irr" package and "icc" function (model = "twoway", type = "agreement", unit = "single" and "average"). The level of agreement was rated accordingly[35]: 0, agreement equivalent to chance; 0.10 to 0.20, slight agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80, substantial agreement; 0.81 to 0.99, near-perfect agreement; and 1.00, perfect agreement.

For ordinal data (5-point scoring system), the agreement was assessed with weighted Cohen κ (squared, function "kappa2"). The CI for each given agreement was calculated using the function "CIr." The κ was graded accordingly[36,37]: 0 to 0.1, negligible agreement; 0.1 to 0.39, weak agreement; 0.4 to 0.69, moderate agreement; 0.7 to 0.89, strong agreement; and 0.9 to 1.0, very strong agreement.

The correlation between the semiquantitative evaluation, and both the grid method and the CD3 RNA sequencing data, was calculated using the Spearman test (non–normally distributed data, function "cor"). The correlation was graded accordingly[36,37]: 0 to 0.1, negligible correlation; 0.1 to 0.39, weak correlation; 0.4 to 0.69, moderate correlation; 0.7 to 0.89, strong correlation; and 0.9 to 1.0, very strong correlation.

RESULTS

The study population consisted of 58 patients, 31 female and 27 male patients. Of these, 36 had adenocarcinoma, 17 had squamous cell carcinoma, 4 had large cell carcinoma, and 1 had adenosquamous carcinoma (Table). Immunohistochemical staining for CD3 was performed on 58 whole tumor sections and on a TMA comprising two 1-mm tissue cores from each corresponding tumor block. Additionally, preoperative biopsies of the same tumors were available and stained for CD3. Representative stainings for 2 cases are shown in Figure 1 (patient ID 755: A through C; patient ID 638: D through F; patient ID can be found in Supplemental Tables).

To test the reproducibility of the visual annotation, a pathologist reannotated all tissue specimens with the semiquantitative and grid method (Supplemental Table 1). The interrater agreement for the semiquantitative annotation was generally good (ICC, 0.43–0.90, moderate to near perfect agreement). For the grid annotation the agreement between both pathologists was substantial to near perfect

Patient Characteristics	
Characteristic	**Value**
Total, No.	58
Age, y	40–82
Sex, No. (%)	
Male	27 (47)
Female	31 (53)
Smoking, No. (%)	
Current	27 (47)
Ex >1 yr	22 (38)
Never	9 (15)
Histology, No. (%)	
AD	36 (62)
SCC	17 (29)
LCC	4 (7)
ADSQ	1 (2)
Stage, No. (%)	
1A	18 (31)
1B	20 (34)
2A	7 (12)
2B	5 (9)
3A	8 (14)
KRAS, No. (%)	
Mut	16 (28)
WT	33 (57)
N/A	9 (15)
EGFR, No. (%)	
Mut	7 (12)
WT	42 (72)
N/A	9 (16)

Abbreviations: AD, adenocarcinoma; ADSQ, adenosquamous cancer; LCC, large cell cancer; Mut, mutated; N/A, no available data; SCC, squamous cell cancer; WT, wild type.

(ICC, 0.74–0.98) for TMA and biopsies. However, for the whole sections the grid method provided only slight to fair agreement (ICC, 0.17–0.31) between both observers (Supplemental Table 1). For all further analyses of the study only the primary cell annotation of the specialist pathologist was used.

The semiquantitative estimation of CD3 immune cell infiltrates demonstrated high variability between different cases when assessed on whole tissue sections, the biopsies, and the TMA (Supplemental Figure 2, A through C). The mean percentages of immune cell infiltration were comparable among the 3 tissue types (Supplemental Figure 3, A; Supplemental Table 2).

However, the comparison between the individual cases on the whole sections and biopsies demonstrated only fair agreement (ICC, 0.29; $P = .01$; CI, 0.03–0.51; Figure 2, A). When the lymphocyte infiltration was evaluated separately in the tumor and the stroma compartment, the agreement for the tumor compartment was moderate (ICC, 0.43; $P < .001$; CI, 0.19–0.62) and that of the stroma area fair (ICC, 0.25; $P = .02$; CI, 0.01–0.47; Supplemental Table 2).

The comparison of the whole section and TMA revealed substantial agreement (ICC, 0.64; $P < .001$; CI, 0.39–0.79) between the proportion of $CD3^+$ cells (Figure 2, B). This was also true when the tissue compartments tumor (substantial agreement; ICC, 0.80; $P < .001$; CI, 0.66–0.88) and stroma (moderate agreement; ICC, 0.6; $P < .001$; CI, 0.32–0.76) were examined separately (Supplemental Table 2).

Because many studies use lymphocyte classification with fewer intervals, we also tested a 5-point scoring system for immune cell quantification. However, this simplified method did not lead to better agreement, with only weak agreement for the comparison of whole section and biopsy ($\kappa = 0.17$, $P = .12$) or whole section and TMA ($\kappa = 0.31$, $P = .007$; Supplemental Table 3).

Visual annotation, even by trained pathologists, includes a high degree of subjectivity and a potential selection bias. To overcome some of these problems, we established a grid-based absolute cell number annotation, counting cells in lymphocyte-dense and lymphocyte-sparse areas, serving as a surrogate for the mean numbers of lymphocytes (Supplemental Table 4; Supplemental Figure 3, B). However, the agreements between different tissue sources using the grid method were clearly lower (biopsy versus whole-section dense area: fair agreement, ICC, 0.27; $P = .02$; CI, 0.01–0.49; TMA versus whole-slide dense area: slight agreement, ICC, 0.16; $P = .12$; CI, –0.08 to 0.40) compared with the semiquantitative annotations (Supplemental Figure 4, A and B; Supplemental Table 5).

To independently evaluate how accurately immune cell infiltration can be quantified in the 3 tissue types, we compared the semiquantitative immune cell scores with the RNA sequencing data available for 19 cases. We found a strong correlation between immune scores and CD3 mRNA gene expression for the whole tissue sections ($r = 0.71$; $P < .001$; CI, 0.37–0.88) and TMA ($r = 0.82$; $P < .001$; CI, 0.57–0.93), but a weak correlation for biopsies ($r = 0.35$; $P = .16$; CI, –0.13 to 0.69; Figure 3). This confirms our observation that TMAs are a relatively reliable tissue form for immune cell quantification, whereas diagnostic biopsies are not.

DISCUSSION

Our study confirmed that the notorious heterogeneity of immune cell infiltration in cancer fundamentally impacts biomarker discovery for clinical use. Diagnostic biopsies only poorly represent the actual immune cell profile in advanced lung cancer tissue, raising the question as to whether such analyses are clinically meaningful. In contrast, the representativity of the TMA was considerably better. This finding further provided evidence that TMAs are valuable research tools, also in the field of immuno-oncology.

There have been substantial attempts to introduce immune cell classifications into the clinical algorithms of cancer diagnostics. The most advanced and validated is the so-called immunoscore on colorectal cancer, based on a quotient of the type, density, and location of immune cells, providing independent prognostic information on patients with colorectal cancer after surgery.[11,38] The prognostic information provided by this immunoscore was recapitulated in a large prospective multicenter trial.[39,40] Corresponding initiatives are ongoing in other cancer types.[11] In breast cancer, immune cell infiltrates are of prognostic value,[41,42] and a working group has already proposed a standardized diagnostic evaluation of tumor-infiltrating lymphocytes.[43]

In addition, in lung cancer there is evidence that the type and location of immune cells provide useful prognostic information.[17,22] In particular, the prognostic impact of lymphocytes differs depending on whether they are located in the stroma or tumor cell compartment.[9,22,44] Another

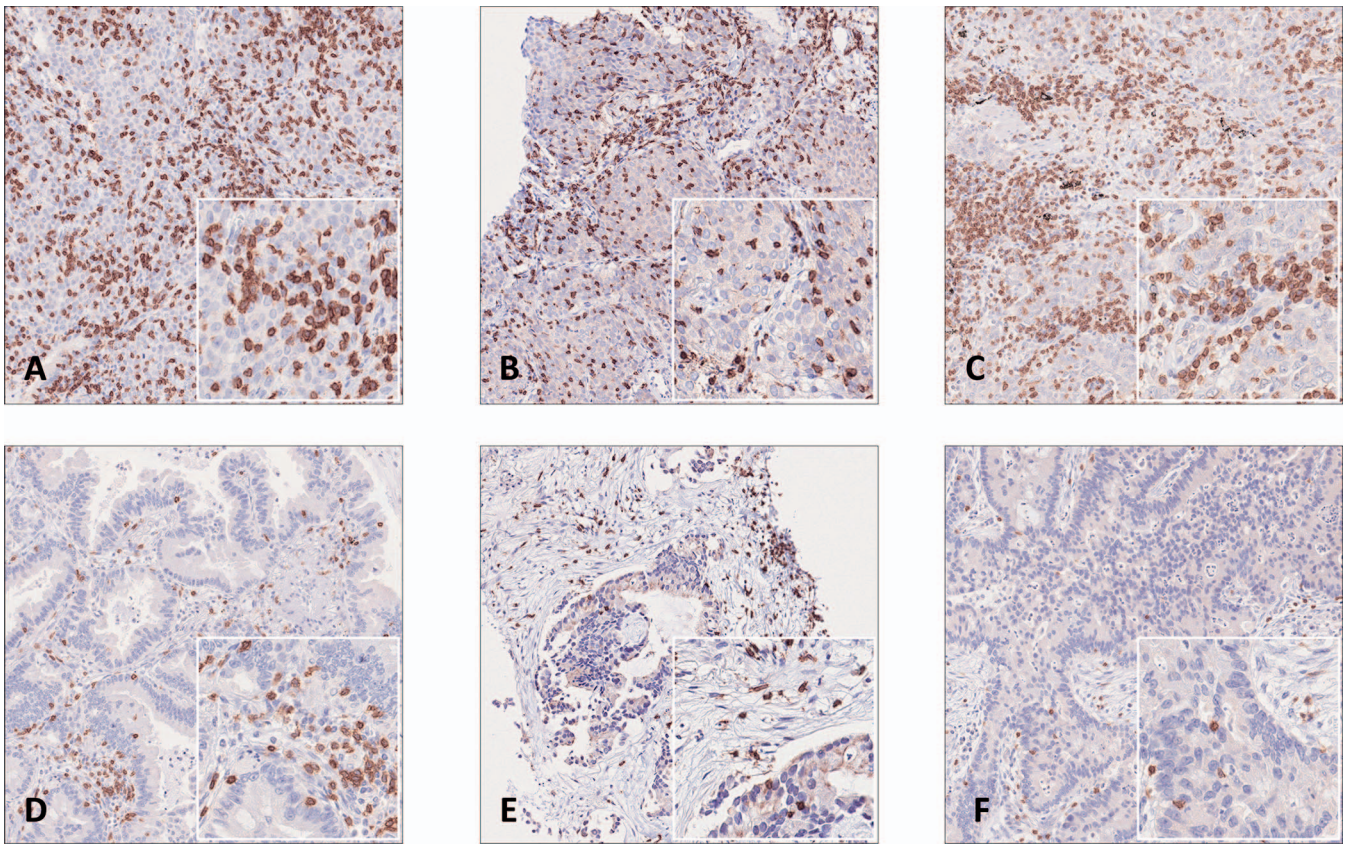

Figure 1. *Representative examples of CD3 staining. A through C represent whole slide (A), biopsy (B), and tissue microarray (C) for patient ID 755. D through F represent whole slide (D), biopsy (E), and tissue microarray (F) for patient ID 638 (original magnifications ×40 [A through F] and ×80 [A through F, insets]). Patient ID can be found in Supplemental Tables.*

study by Gao et al[14] found that a score, based on macrophage infiltration, successfully improved the prognostication for stage I NSCLC. As a main drawback, the findings are based on tissue from patients who have undergone an operation, consequently helping the oncologist to choose adjuvant therapy. However, this patient group represents the minority of lung cancer patients and perhaps does not have the greatest diagnostic need. Most patients with NSCLC receive a diagnosis of advanced disease without the possibility of surgical treatment. Currently, the first-line option for patients without cancer-driver mutations is immunotherapy with or without chemotherapy.[45] The most commonly used biomarker to guide treatment with checkpoint inhibitors is the expression of programmed death ligand-1 (PD-L1) on tumor cells.[46,47] This testing also involves problems of representativity of the diagnostic biopsy compared with the whole tumor. In contrast to our present findings, the tumor cell PD-L1 expression is relatively well presented in biopsies,[48,49] and the evaluation of this small pretreatment material has been approved as a companion diagnostic. The main drawback of PD-L1 immunohistochemistry is that its predictive power is modest and only when checkpoint inhibitors are considered as monotherapy.[1,2,50–52]

Consequently, tremendous efforts have been made to identify better biomarkers for use on patient biopsies with higher accuracy or to determine treatment benefits by combining immune and chemotherapy modalities. One promising tissue feature that has come into focus is local immune cell infiltration. Patients with tumor tissue displaying higher numbers of immune cells, termed "inflamed" phenotypes, experience better responses to immunotherapy than patients with low immune cell infiltrates.[53,54] This concept has been developed mainly in melanoma patients but has also been explored in other cancer types. Advanced multiplex methods that integrate several markers into an immune activation score have been the most successful until now.[55] However, a simple immune cell metric has not yet emerged, and concepts are far from ready for clinical application.

Our study provides a suggestion as to why most of these immune cell–based efforts are determined to fail in lung cancer: the biopsy is not suited to quantify immune cells in the tumor tissue. This assumption is reasonable for the T-lymphocyte population, which we have determined as $CD3^+$ cells. Because many cell types are even more heterogeneously distributed within cancer tissue, we believe that the same problems are likely to be present when other immune cell subsets are evaluated; B-cells, plasma cells, macrophages, and natural killer cells will not be reliably presented in the biopsy. For instance, B cells might cluster in tertiary lymphoid structures, or plasma cells preferentially found in the stroma compartment.[9,56] Against this background, we do not believe that immune scores based on pure cell densities in biopsies will be informative, neither for response prediction nor for prognostication.

However, the association between whole section and TMA was considerably better and indicated that immune cell profiles quantified on TMAs can be a reliable scientific tool. To our knowledge, our study addressed this scientif-

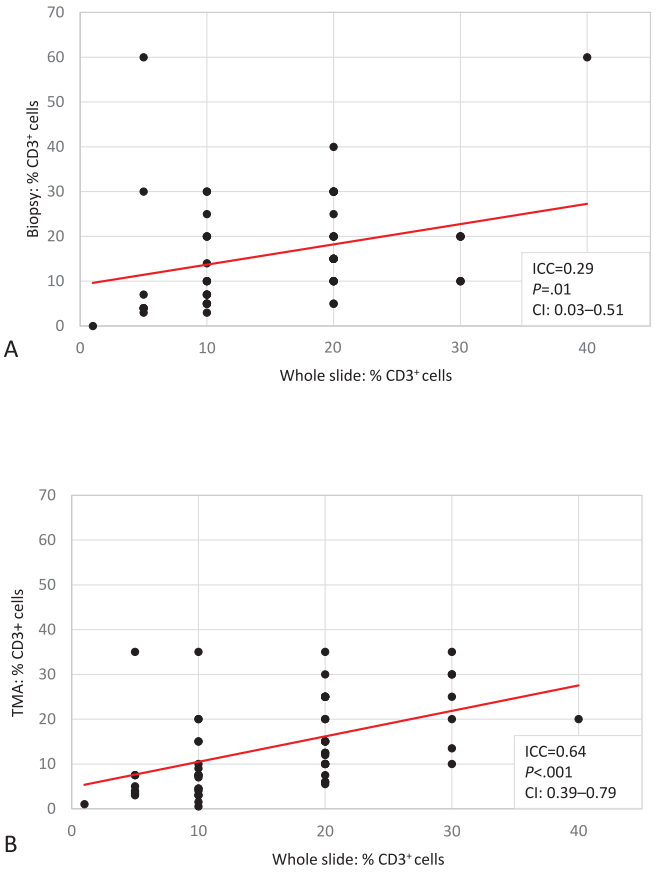

Figure 2. *Intraclass correlation (ICC) between the different fractions for the semiquantitative annotation (percentage of CD3⁺ cells) for whole slide versus biopsy (A) and whole slide versus tissue microarray (TMA) (B).*

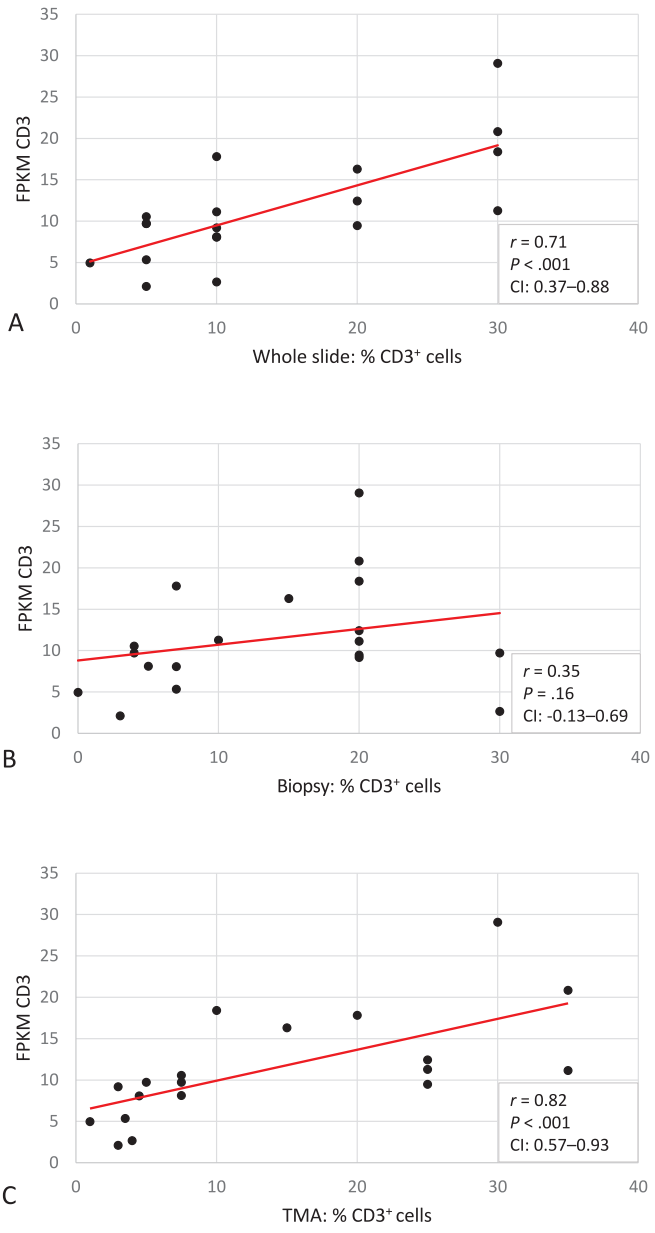

Figure 3. *Correlation between the percentage of CD3⁺ cells (semiquantitative annotation) and CD3 gene expression data (RNA sequencing) for whole slide (A), biopsy (B), and tissue microarray (TMA) (C).*

ically fundamental aspect for the first time. Indeed, most studies that describe the immune landscape of lung cancer are based on TMAs,[8,9,22,57] which comprise representative tumor areas carefully selected by a pathologist. TMAs allow the analysis of dozens of tissue samples at the same time, and they are cost- and time-effective, whereas serious batch effects can be avoided. The major concerns with TMAs are minimal tissue size and selection bias. These concerns were rebutted for many immunohistochemical markers,[58] including estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 scores in breast cancer,[30] and Ki-67 and prostate-specific antigen in prostate cancer.[59] However, most of the tested markers were classical tumor features with relatively homogeneous expression patterns. Studies validating the representativity of cellular components apart from cancer cells are scarce. To the best of our knowledge, only 2 smaller studies suggest that lymphocytes can be reliably accessed on TMAs in ovarian cancer and in leiomyosarcomas.[29,31] The TMA used in this study was constructed to best represent the tumor area of the whole section. This means the selected tissue cores were not selected based on immune cell infiltration or stroma compartment. Thus, our study adds support that TMAs are applicable to studying the cellular microenvironment and that previous TMA studies are generalizable.

Although we believe our findings are highly relevant for biomarker research, some aspects of our study should be considered critically. With 58 tumor samples, the number of cases was still relatively small. The correlations between biopsies and whole sections would be more pronounced if more cases were included. Indeed, most TMA studies usually include more than 100 cases. Furthermore, we evaluated only CD3⁺ T lymphocytes. Other immune cell types could be more or less homogeneously distributed over cancer tissue; thus, the conclusion might not be directly translatable to other immune cells. Finally, the assessment by different pathologists implies a certain amount of variability. However, in our study we found a relatively high interrater agreement between 2 independent pathologists for most of the scorings. Only the grid-based evaluation on the whole tissue section demonstrated poor

agreements between both observers. This uncertainty in scoring probably also impacted the correlation analysis.

In conclusion, our study provides evidence that biopsies of lung cancer patients are not suited for the reliable assessment of tumor-infiltrating lymphocytes. This is of high importance when biopsies are used for the discovery of prognostic and predictive biomarkers, or when companion diagnostics are integrated in the planning of randomized trials.

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