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Immune cell profiles of tumor and regional lymph nodes in surgically treated non-small cell lung cancer

presented by:

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Year:

2023

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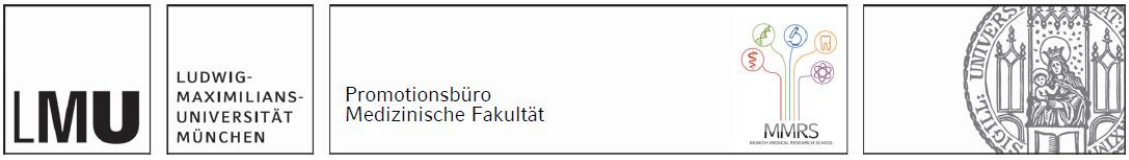
Date of oral defense:

07.03.2023

Affidavit



Affidavit

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I hereby declare, that the submitted thesis entitled:

Immune cell profiles of tumor and regional lymph nodes in surgically treated non-small cell lung cancer

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

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Signature doctoral candidate

Confirmation of congruency

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Confirmation of congruency between printed and electronic version of the doctoral thesis

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I hereby declare, that the submitted thesis entitled:

Immune cell profiles of tumor and regional lymph nodes in surgically treated non-small cell lung cancer

is congruent with the printed version both in content and format.

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Table of content

List of abbreviations

BTLA: B- and T-lymphocyte attenuator

CD: Cluster of differentiation

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

EOMES: Eomesodermin

FFPE: Formalin-fixed paraffin-embedded

FOXP3: Forkhead box P3

GC: Germinal center

H&E: Hematoxylin and eosin

HR: Hazard ratio

IASLC: International Association for the Study of Lung Cancer

LAG-3: Lymphocyte-activation gene 3

LN: Lymph node

MPO: Myeloperoxidase

mRNA: Messenger ribonucleic acid

Ntb: Non-tumor bearing

NSCLC: Non-small cell lung cancer

OS: Overall survival

PD-L1: Programmed death-ligand 1

PFS: Progression-free survival

qPCR: Quantitative polymerase chain reaction

REL: Relapse

REM: Remission

RNA: Ribonucleic acid

Tb: Tumor-bearing

TBP: TATA-binding protein

TIDE: Tumor Immune Dysfunction and Exclusion

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TiL: Tumor-infiltrating lymphocyte

TIM-3: T-cell immunoglobulin and mucin-domain containing-3

List of publications

1. **Sellmer L.**, Kovács J., Neumann J., Walter J., Kauffmann-Guerrero D., Syunyeava Z., Fertmann J., Schneider C., Zimmermann J., Behr J., Tufman A.
   Lymphocytes and sinus histiocytosis in tumor and matched lymph nodes as predictors of survival in NSCLC.
   Future Oncology 2022; doi:10.2217/fon-2021-0402

2. **Sellmer L.**, Kovács J., Walter J., Kumbrink J., Neumann J., Kauffmann-Guerrero D., Kiefl R., Schneider C., Jung A., Behr J., Tufman A.
   Markers of immune cell exhaustion as predictor of survival in surgically-treated early-stage NSCLC
   Frontiers in Immunology 2022; doi.org/10.3389/fimmu.2022.858212

1. Contribution to the publications

1.1 Contribution to paper I

Lymphocytes and sinus histiocytosis in tumor and matched lymph nodes as predictors of survival in NSCLC

During the planning phase of the presented work, I contributed to the study design and methodology. I identified all patients included in the study and extracted their clinical information from hospital records.

I was then responsible for retrieving all tissue blocks from pathology archives and cutting them. Morphological criteria for analysis of H&E stained tissue sections were established by me and Jens Neumann as the expert pathologist. All morphologic analyses of stained tissue sections were performed by me.

Rosemarie Kiefl and I performed mRNA extraction for qPCR. I was then responsible for primer design and establishing preamplification and all qPCR run protocols. All qPCR data was then analyzed and quality control was performed by me.

All statistical analyses were performed by me.

I wrote the manuscript, incorporated feedback from coauthors and was responsible for submission and publication.

1.2 Contribution to paper II

Markers of immune cell exhaustion as predictor of survival in surgically-treated early-stage NSCLC

During the planning phase of the presented work, I contributed to the study design and methodology. I identified all patients included in the study and extracted their clinical information from hospital records.

I was then responsible for retrieving all tissue blocks from pathology archives and cutting them. Rosemarie Kiefl and I performed mRNA extraction. I was responsible for Nano-

string panel selection and design. I pre-processed and normalized Nanostring gene expression data and performed all bioinformatic analyses.

I then selected immune cell exhaustion markers for more in-depth analysis and performed those analyses.

All statistical analyses were performed by me.

I wrote the manuscript, incorporated feedback from coauthors and was responsible for submission and publication.

2. Introductory summary

2.1 Non-small cell lung cancer (NSCLC)

2.1.1 Clinical and pathological aspects of NSCLC

Lung cancer remains one of the most commonly diagnosed types of cancer worldwide, with an estimated global incidence of 2.2 million cases and 1.8 million deaths in 2020 (1). Non-small cell lung cancer (NSCLC) is the most common malignant neoplasm of the lung and makes up approximately 85% of lung cancer cases. Five-year survival after NSCLC diagnosis is ca. 22% according to Survival, Epidemiology, and End Results Program (SEER) data. The poor survival odds of NSCLC patients are largely due to late diagnosis. Over half of all patients are diagnosed after the cancer has already metastasized. Even though the advent of novel therapeutic approaches such as immunotherapy has improved survival, they are not yet approved for all treatment settings and fail to produce a durable response in a subset of patients (2–4).

The four stages of NSCLC were redefined by the IASLC Staging Project in 2017. Each stage is defined by a combination of tumor size (the T classifier), nodal status (the N classifier) and location and number of metastases (the M classifier) (5–8). An overview of TNM status and the respective stages is shown in Figure 1.

T/M	Subcategory	N0	N1	N2	N3
T1	T1a	IA1	IIB	IIIA	IIIB
	T1b	IA2	IIB	IIIA	IIIB
	T1c	IA3	IIB	IIIA	IIIB
T2	T2a	IB	IIB	IIIA	IIIB
	T2b	IIA	IIB	IIIA	IIIB
T3	T3	IIB	IIIA	IIIB	IIIC
T4	T4	IIIA	IIIA	IIIB	IIIC
M1	M1a	IVA	IVA	IVA	IVA
	M1b	IVA	IVA	IVA	IVA
	M1c	IVB	IVB	IVB	IVB

Figure 1: An overview of TNM status and respective NSCLC stages according to the 8th edition of the IASLC Staging Project. Adapted from Detterbeck et al. (2017) (8).

2.1.2 Treatment of NSCLC

Treatment of NSCLC is extremely multifaceted. Depending on tumor location, previous treatment, stage, histology, molecular alterations (including Programmed death-ligand 1 (PD-L1) expression and driver mutations), medical history as well as the patient's performance status and personal preferences, one treatment modality or a combination of surgery, chemotherapy, radiation, immunotherapy or a plethora of targeted agents may be selected by the treating physician.

In stage IV disease, only palliative treatment approaches are available. The appropriate treatment is meant to slow further tumor growth, prevent secondary effects of metastases (such as pain and fractures as a consequence of bone metastases) and increase patients' quality of life. The most commonly used treatments are radiation, chemotherapy and targeted agents depending on molecular testing. In recent years, immunotherapy has drastically increased overall survival and progression-free survival while reducing adverse effects (9–12).

Early stage NSCLC has not yet metastasized and includes stage I, stage II and stage III. Curative surgery is the treatment of choice for these early stage tumors. Depending on lymph node involvement and achieved resection margins, adjuvant treatments such as chemotherapy and radiation may be recommended.

2.1.3 Surgical treatment of NSCLC

Surgery has traditionally played a big role in NSCLC treatment. It is a preferred curative treatment for early stage disease and a central component of multimodal treatment approaches with curative potential. Curative resection methods include segmental resection, lobectomy, cuff resection, bilobectomy and pneumectomy. Radical resection involves removal of the tumor with a varying amount of surrounding healthy tissue depending on the clinical state and functional operability of the patient plus resection of regional lymph nodes.

There has been considerable debate about whether complete lymph node dissection or lymph node sampling is preferable. While complete dissection offers better nodal staging and removal of previously undetected micrometastases, this technique carries a higher risk of mortality and fails to consistently show improved patient survival (13–16). Since complete dissection does not seem to offer a clear advantage, it can be assumed that other factors than just presence of micrometastases play a role in postoperative survival.

2.1.4 Lymph flow in the lung and resection of regional nodes

The purpose of the lymphatic system is to absorb excess interstitial fluid and expose immune cells such as lymphocytes to foreign antigens. In the lung, fluid from capillaries is reabsorbed into lymphatic vessels and transported to lymph nodes, where the activation and proliferation of lymphocytes takes place. In this bronchopulmonary network, lymph flows unidirectionally from peripheral areas to hilar lymph nodes and on to the mediastinum. Here, the lymph passes through mediastinal lymph nodes and eventually drains into the right or left bronchomediastinal duct. This drainage system allows the immune system to recognize foreign antigens and mount a targeted lymphocytic response. Lymphatic vessels are also the primary path through which NSCLC spreads in the lung and are therefore removed during curative surgery. Much attention has focused on lymph node metastases of NSCLC, but very little research has aimed to shed light on immune processes in tumor-free, but tumor-draining lymph nodes (17–19). An overview of the lymph nodes in the lung is given in Figure 2.

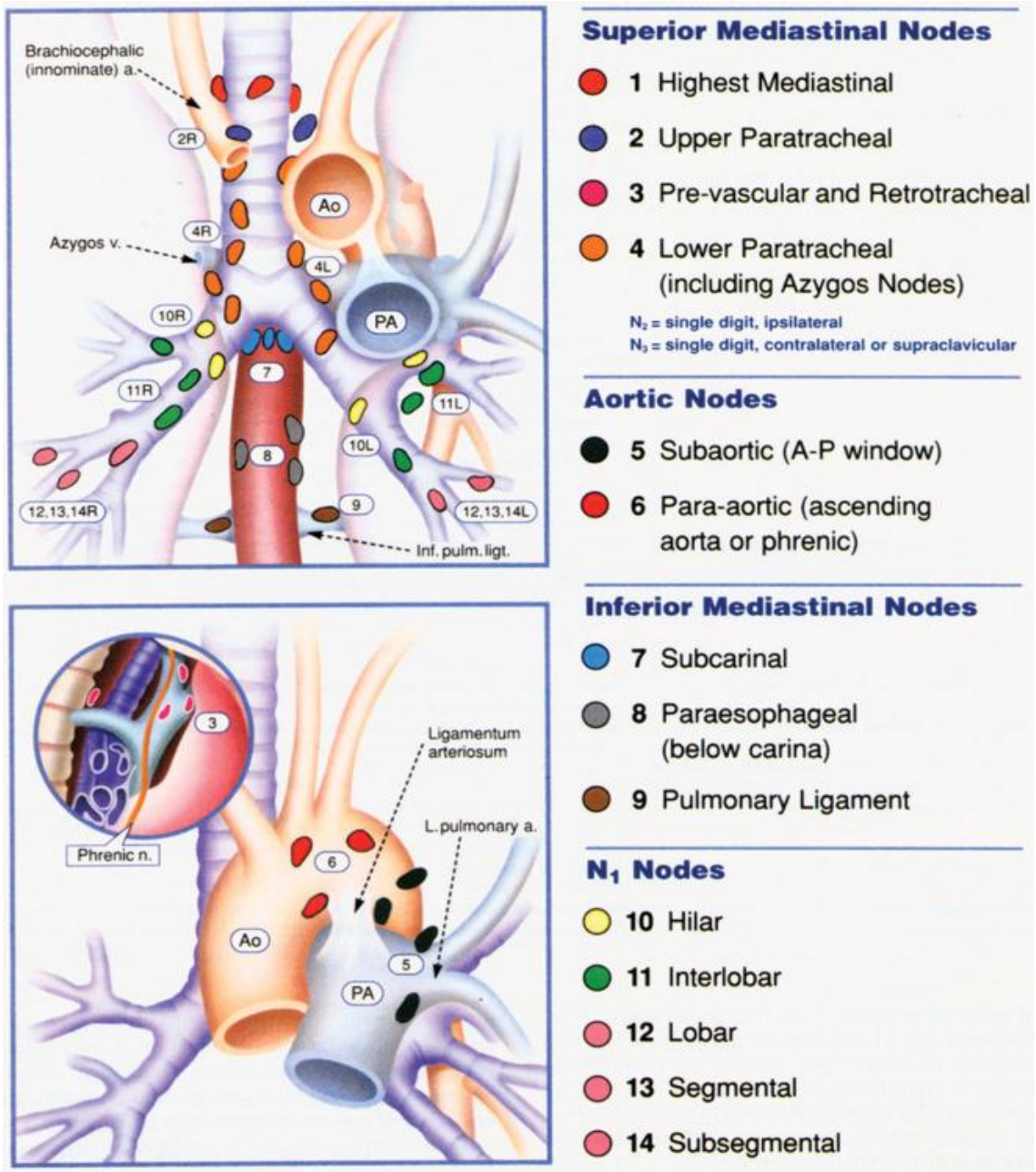

Figure 2: Overview of lymph node stations in the lung. Lymph node stations 1 to 9 are located in the mediastinum. If a primary tumor spreads to one of these nodes one the same side as the tumor itself, it is considered to be N2 status. Lymph node stations 10 to 14 are located in hilar and lobar spaces. If a primary tumor spreads to one of these nodes one the same side as the tumor itself, it is considered N1 status. If a tumor spreads to any node on the other side of the thorax, to supraclavicular nodes or scalene nodes, it is considered N3 status. Adapted from Detterbeck et al. (2017) (8).

2.2 The immune system in cancer

2.2.1 The role of the immune system in cancer

Cancer causes local as well as systemic inflammation (20). These inflammatory processes are context dependent and can either further promote or impede tumor growth. This ability to avoid destruction by the immune system and even use inflammatory processes to aid in tumor growth was added to the hallmarks of cancer in 2011 (Figure 3) (21,22).

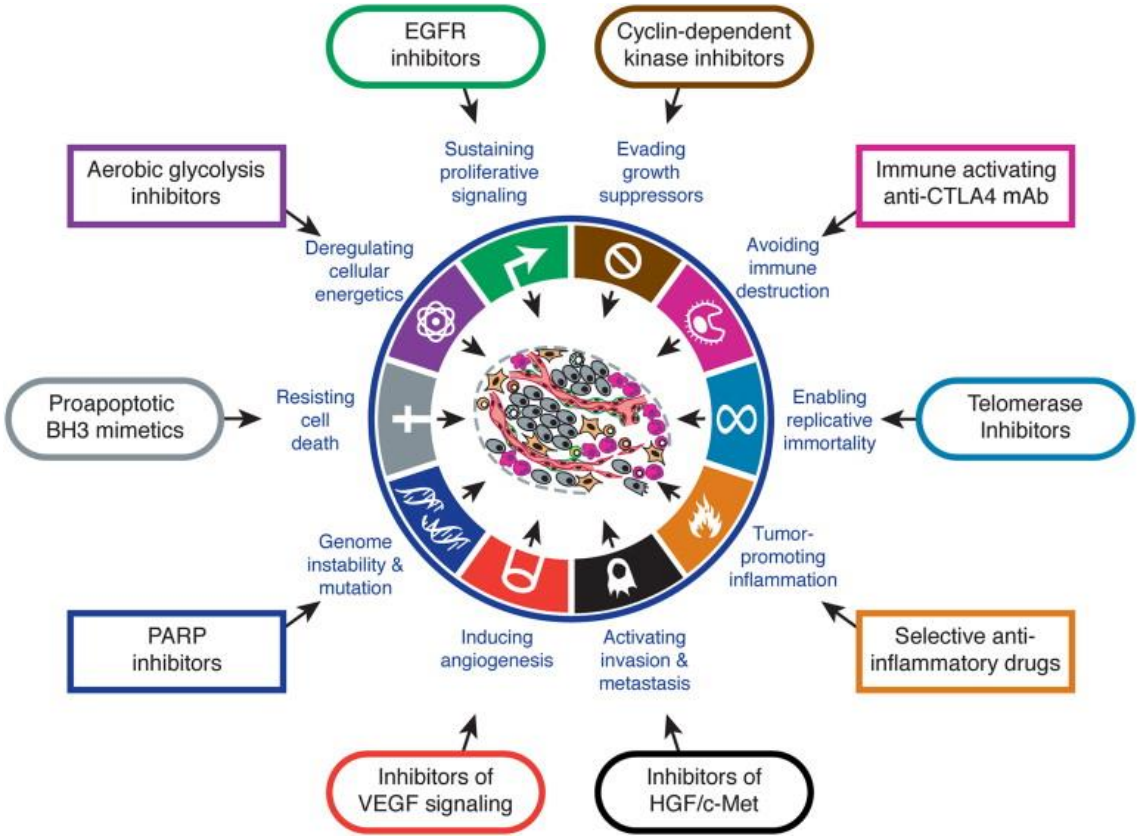

Figure 3: The hallmarks of cancer and potential avenues for therapeutic targeting. Adapted from Hanahan and Weinberg (2011) (21).

There has been a focus on the local immune response in the tumor microenvironment, however, this immunity is initiated and sustained across various tissues. Myeloid cells originating from the bone marrow enter the tumor where they phagocytize and present tumor antigens. These cells then migrate to lymphoid tissues and initiate B- and T-cell priming. Lymphoid cells then find their way back to the tumor via chemokine gradients.

Recently, the idea of targeting immune dysfunction in order to treat tumors has gained traction. This culminated in the introduction of immunotherapies including T-cell therapy, antibody-based therapies or vaccine-based approaches in many entities of cancer such as NSCLC, melanoma, glioblastoma, prostate cancer and others. While these novel therapies have led to pronounced improvements in tolerability and survival, they are not yet approved for all treatment settings and fail to produce a durable response in a subset of patients.

2.2.2 The role of the immune system in NSCLC

The lungs represent one of the sites most targeted by pathogens attempting to infect a host. Due to the importance of the lungs in gaseous exchange, the lymphatic system forms an immunologically dense network. In the case of NSCLC, the lymphatic system is capable of close immune surveillance and subsequent tumor control, at least initially. While therapies such as vaccines and T-cell based approaches have failed to improve patient survival in NSCLC, the introduction of checkpoint inhibitors in NSCLC has led to improvements in tolerability, progression-free survival and overall survival (9,23,24). Immune checkpoint inhibitors work by enabling the recognition and subsequent destruction of cancer cells by tumor-infiltrating cytotoxic CD8+ T-cells. While many patients initially respond very well to immune checkpoint inhibition, tumor progression during treatment is common.

Intratumoral cytotoxic CD8+ cells exist in various functional states with associated levels of ability to perform effector functions (25). It was shown in several cancer entities, that T-cell dysfunction and exhaustion can be the underlying reason of immune checkpoint resistance (26–29).

2.2.3 Immune cell exhaustion in NSCLC

T-cell exhaustion is a functional state of post-thymic T-cells. It results from chronic antigen exposure and manifests in a reduced capacity to release cytokines and an increased expression of inhibitory receptors. While T-cell exhaustion is a highly complex process and considerable debate over sub-phenotypes and remaining functionality exists, it was shown that markers of exhausted CD8+ cells are increasingly expressed with tumor progression and that blocking exhaustion markers can re-establish CD8+ cell proliferation and functionality (25,30–32).

In NSCLC, multiple exhaustion markers, such as PD-L1, TIM-3, BTLA and others, have been identified. They differ based on abundance, timing of their expression during the exhaustion process and changes in expression levels during tumor progression (30).

It was recently shown that the levels of immune cell exhaustion marker TIM-3 in tumor-draining lymph nodes, but not the tumor, is associated with poor prognosis in breast cancer patients (33). While immune cell exhaustion in the tumor clearly plays an important role in tumor control and patient survival, the role of immune cell exhaustion in regional lymph nodes and its interplay with immune cells in the tumor is not completely understood.

2.3 Objectives

Previous work has shown the importance of the local tumor microenvironment as well as the importance of a functional systemic immune response for response to therapy and patient survival. This systemic response is initiated and sustained in regional lymph nodes, and it was shown that local lymph nodes are crucial for tumor control. However, changes in the immune system composition at a cellular and molecular level in matched tumor and lymph nodes have not yet been investigated.

The objectives of this thesis are:

1. To develop and clinically characterize a cohort of surgically-treated NSCLC patients in stage I, II and III containing both long- and short-term survivors
2. To identify factors which are associated with survival in surgically-treated NSCLC patients on a cellular level. Specifically, this work aims to examine morphological features of tumor and matched tumor-bearing and non-tumor-bearing lymph nodes and their relation to survival.
3. To identify factors which are associated with survival in surgically-treated NSCLC patients on a molecular level. Specifically, this work focuses on gene expression levels of immune cell markers in tumor and matched tumor-bearing and non-tumor-bearing lymph nodes. Bioinformatic algorithms are used to determine levels of immune dysfunction (with a focus on exhaustion) and cellular composition.

2.4 Summary

In recent years, improved understanding of the interaction between cancer and the immune system has led to the introduction of various immunotherapy approaches, all harnessing the power of the immune system to impede tumor development, growth and spread. The immune system is a vastly complex network and response to a foreign antigen must be launched in a coordinated fashion both locally as well as systemically. In the case of NSCLC, this requires a response in the lung tissue surrounding the tumor as well as the regional lymph nodes. Most previous studies that attempted to characterize this response were limited by investigating only tumor or lymph nodes, and those who investigated both did not include tumor-free lymph nodes.

To tackle these caveats, I have composed a diverse cohort of surgically-treated NSCLC patients containing both long- and short-term survivors. I investigated morphological features in tumor and matched tumor-bearing and non-tumor-bearing lymph nodes and analyzed their association with survival. I then used these results to inform transcriptomic analyses of these tissues to determine how morphological changes were reflected on a molecular level.

In this thesis, I showed that tumor-infiltrating lymphocytes and macrophages are key components of the immune response in the primary tumor and non-tumor bearing lymph nodes. The importance of lymphocytes in immune mediated tumor control is further corroborated by an association between CD4 expression in non-tumor bearing lymph nodes and survival.

Based on these results, immune transcriptomics showed a negative impact of immune dysfunction measured by Tumor Immune Dysfunction and Exclusion (TIDE) score on patient survival. When comparing patients with high levels to patients with low levels of immune dysfunction, CD8 expression was significantly higher in patients with lower levels of immune dysfunction. A more in-depth analysis explored the relation of the expression of multiple immune cell exhaustion markers and survival. Among these, TIM-3 and PD-L1 were identified as the only markers to be associated with survival in more than one tissue.

Overall, this work presented an integrative approach to assessing immune composition and dysfunction. Levels of immune cell exhaustion markers may indicate a dysfunctional immune status and can predict survival after curative surgery in NSCLC. This work provides the basis for further investigation of the clinical relevance of immune cell exhaustion in early-stage NSCLC.

3. Paper I

Lymphocytes and sinus histiocytosis in tumor and matched lymph nodes as predictors of survival in NSCLC

Published in:

Sellmer L., Kovács J., Neumann J., Walter J., Kauffmann-Guerrero D., Syunyeava Z., Fertmann J., Schneider C., Zimmermann J., Behr J., Tufman A.

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Research Article

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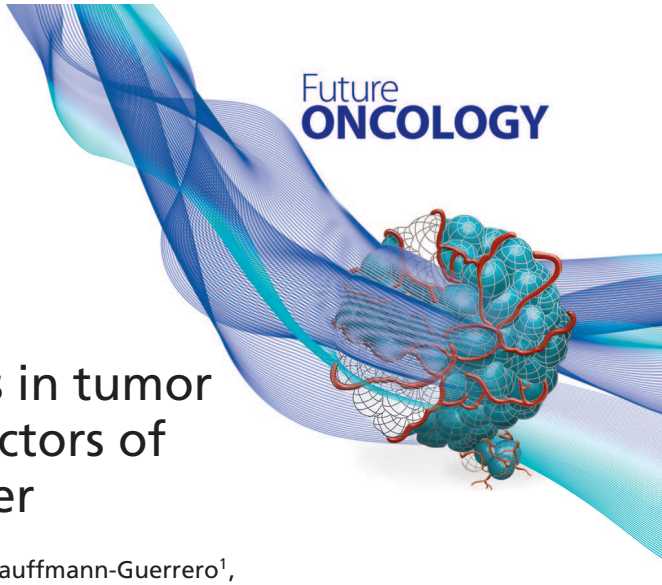

Lymphocytes and sinus histiocytosis in tumor and matched lymph nodes as predictors of survival in non-small-cell lung cancer

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Aim: To analyze immune cell populations in non-small-cell lung cancer (NSCLC) tumors and matched tumor-bearing and non-tumor-bearing lymph nodes (ntbLNs) to predict prognosis. **Patients & methods:** 71 patients with long-term disease-free survival and 80 patients with relapse within 3 years were included in this study. We used Cox regression to identify factors associated with overall survival (OS) and progression-free survival (PFS). **Results:** Sinus histiocytosis and tumor-infiltrating lymphocyte density in the tumor were positively associated with PFS and OS. *CD4* expression in N1 (hazard ratio = 0.72; p = 0.02) and N2 (hazard ratio = 0.91; p = 0.04) ntbLNs were positively correlated with OS and PFS, respectively. **Discussion:** Immunological markers in ntbLNs could be used to predict survival in NSCLC.

Lay abstract: Aim: We analyzed populations of immune cells in non-small-cell lung cancer (NSCLC). In addition, we also investigated lymph nodes from the same patient that contained or did not contain cancer cells. **Patients & methods:** We included 71 patients whose cancer did not return within 3 years and 80 patients whose cancer did return within 3 years after they underwent surgery to remove their tumors. We used various statistical methods to identify factors that can predict survival. **Results:** Sinus histiocytosis (a widening of ducts in the lymph nodes due to an increased number of certain cells) and the density of tumor-infiltrating lymphocytes (immune cells that enter the tumor to destroy it) can predict how long patients can survive after surgery or if their tumor will come back quickly. **Discussion:** Looking at immune cells can help physicians decide which patients need increased follow-up care due to an increased risk for their tumors to return.

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Keywords: *CD4* • immune system • lymph nodes • NSCLC • sinus histiocytosis • tissue morphology

Curative surgical resection is the treatment of choice for early-stage non-small-cell lung cancer (NSCLC). Post-operative survival is highly variable and dependent on many factors, such as stage, perioperative management, postoperative complications, BMI and, perhaps most important, immune system activation [1,2]. The immune system has diverse roles in cancer occurrence and progression, being involved in formation, local control and distant dissemination of tumors [3–5]. This diverse role has drawn attention to the complex interactions between the various immune components, the tumor itself and external stimuli, setting the stage for decades of immunotherapy research.

Recently, there have been important findings regarding the impact of various immune cell populations in the primary NSCLC tumor and their influence on disease course and survival [6–8]. Macrophages are subdivided into M1 and M2 according to their function. M1 macrophages seem to be tumor-inhibiting and associated with improved survival, whereas M2 macrophages are tumorigenic and associated with poor survival. Aside from tumor

Table 1. Histological features investigated in tumor, tumor-bearing lymph nodes and non-tumor-bearing lymph nodes.

Feature	Investigated as	Investigated in
TiL density	% of tumor stroma	Tumor
TiL pattern	Desert, excluded, inflamed	Tumor
Lymph node size	Largest diameter across	TbLNs, N2 and N1 ntbLNs
Extracapsular tumor growth	Yes or no	TbLNs
GC homogeneity	Homogenous or inhomogenous	TbLNs, N2 and N1 ntbLNs
GC activation	Small, enlarged, strongly enlarged	TbLNs, N2 and N1 ntbLNs
T-cell zone	Normal or enlarged	TbLNs, N2 and N1 ntbLNs
Sinus histocytosis	None, mild, strong	TbLNs, N2 and N1 ntbLNs

GC: Germinal center; ntbLN: Non-tumor-bearing lymph node; tbLN: Tumor-bearing lymph node; TiL: Tumor-infiltrating lymphocyte.

cells, M2 macrophages are an important source of PD-L1 and in turn inhibit the movement and antitumoral effects of tumor-infiltrating lymphocytes. A lack of intratumoral effector CD8 lymphocytes is associated with poor survival and immune suppression and evasion by the tumor [9]. Furthermore, tumor-infiltrating mature dendritic cells seem to be beneficial for long-term survival; however, maturation of immature dendritic cells and subsequent antigen presentation in tumor-draining lymph nodes (LNs) is inhibited by tumor-derived signaling [10].

Locoregional N1 and N2 LNs are sites of antigen presentation and T- and B-cell activation. They are removed during surgery and provide a snapshot of the immune cell composition at the time of LN removal. Little is known about the significance of the different immune cell populations in non-tumor-bearing (but tumor-draining) LNs (ntbLNs) in NSCLC patients. Although there is some evidence for the effects of different immune cell populations in ntbLNs in breast cancer and melanoma [11–13], little is known about the role of immune cell populations in ntbLNs in NSCLC and their relation to the immune cell profiles in the respective tumor.

In this study, we investigated immune cell populations in the primary tumor and matched tumor-bearing LNs (tbLNs), N2 ntbLNs and N1 ntbLNs in surgically treated patients with NSCLC.

Patients & methods

Study design & patient characteristics

This is a retrospective study of patients with lung cancer treated between 1999 and 2019 at the Ludwig-Maximilians University in Munich, Germany. Electronic patient records were screened for patients meeting the following inclusion criteria [14]: histologically confirmed diagnosis of NSCLC (adenocarcinoma, squamous cell carcinoma or large cell carcinoma), anatomical resection with curative intent, availability of formalin-fixed paraffin-embedded (FFPE) tissue material and record of either 3-year remission after surgery or documented earlier relapse. Clinical data including gender, age at diagnosis, overall survival and progression-free survival (PFS), the presence of chronic lung diseases as well as tumor (stage and histology) and treatment characteristics (type of surgery and [neo]adjuvant therapy) were extracted from clinical records. Patients were grouped into one of the following two groups according to the duration for which they remained progression free: relapse group (REL; distant metastasis or local relapse within 3 years after surgery) or remission group (REM; no distant or local tumor within 3 years after surgery). FFPE-embedded tissue blocks of primary tumor, tbLNs (N2 or N1) and N2 ntbLNs and N1 ntbLNs were obtained from pathology archives and cut in 3-µm slices for histological staining and 30 µm for mRNA extraction. Samples were stored at room temperature in sealed boxes, the standard storage method in this archive for samples used in clinical diagnostics.

Ethical approval for this study was obtained from the institutional ethics board (reference number: [12-16]) and informed consent was obtained from all patients. This study was conducted in accordance with the Declaration of Helsinki.

Histological immune assessments

Hematoxylin and eosin (H&E) stainings of all samples were performed according to standard protocols. Morphological features and their subcategories were reviewed by a senior pathologist. LN size and tumor-infiltrating lymphocyte (TiL) were evaluated quantitatively on H&E stained sections; all other features were evaluated semi-quantitatively. All features investigated in this study, and their subcategories are shown in Table 1.

TiL scores were obtained according to Salgado's criteria [15]. TiL distribution was subdivided in one of three patterns: desert (≤10% lymphocyte density in tumor stroma), excluded (15–40% density of lymphocyte in tumor stroma with accumulation around tumor) and inflamed (≥50% density of lymphocytes in tumor stroma).

The following features were evaluated in all LNs: sarcoid-like lesions (presence or absence), homogeneity of germinal centers (GCs; homogeneous, with an even distribution of GC size across the LN, or inhomogeneous, with an mix of enlarged and nonenlarged GCs), GC activation (none, small GCs with mantle zone at least 50% of GC diameter or large GCs with mantle zone less than 50% of GC diameter), sinus histiocytosis (none, mild with <4 cells across or strong with >4 cells across) and LN size. In addition, extracapsular growth (presence or absence) was evaluated in tbLNs.

For regression analyses, continuous variables (TiL score and LN size) were categorized for statistical analyses.

Quantitative PCR

A subgroup of the study population (20 REM and 20 REL) was randomly selected (by using computer-generated list of random numbers) for detailed analysis of immune marker expression. All available primary tumor and matched tbLNs, N2 tumor-draining ntbLNs and N1 tumor-draining ntbLNs were obtained. Total mRNA was extracted from FFPE slices using the FFPE RNEasy Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. mRNA was quantified photometrically and converted into cDNA using SuperScript IV VILO (ThermoFisher Scientific; MA, USA). Primers for *CD4*, *CD8*, *CD19*, *CD86*, *CD163*, *CD274*, *MPO* and *TBP* were designed using Roche's proprietary Assay Design Center (https://lifescience.roche.com/en_de/brands/universal-probe-library.html#assay-design-center). To enhance mRNA quality, a preamplication step was introduced using the TaqMan PreAmp Master Mix (ThermoFisher Scientific). Quantitative PCR (qPCR) was performed using the Kapa Probe Fast MasterMix (Merck; Darmstadt, Germany).

All assays were run in triplicate and, after quality control steps, compared in relation to *TBP* as reference gene. Gene expression levels of individual genes as well as ratios of two genes were investigated in all four tissues, compared between patient groups and correlated with PFS and OS.

Statistical analysis

Quality control and comparative analyses between qPCR values in patients with and without 3-year metastases-free survival was performed using Student's t-tests. For group differences, Student's t-tests were used for analyses of continuous variables (TiL density and LN size), and chi-squared tests were used for categorical variables (TiL pattern, extracapsular tumor growth, GC homogeneity, GC size, T-cell zone, sinus histiocytosis).

We used Cox regression models to measure which factors had a significant influence on overall survival (OS) and PFS. Results are reported as hazard ratios (HR). The following variables were included in the regression: sex, age at diagnosis, tumor histology, tumor stage at surgery, chronic lung disease before surgery, acute infection after surgery, presence of metastasis or local recurrence (not used for PFS), smoking status, pack-years, TiL scores in the tumor and sarcoid-like lesions, GC activation and sinus histiocytosis in ntb N2 or N1 LNs. To achieve best model fit, we used forward regression selection with cutoff values for inclusion and exclusion in the models of 0.1 and 0.2, respectively. Linearly dependent variables were not included in regression analyses.

We compared OS and PFS based on gene expression levels (obtained via qPCR) using Kaplan–Meier curves, with p-values obtained from log-rank tests. A threshold of $\alpha \leq 0.05$ for significance was applied in all analyses. qPCR analyses were performed in Python; all other analyses were performed in SPSS.

Results

Demographic description

756 patients with a lung cancer diagnosis who underwent a surgical procedure between 1999 and 2019 were identified in internal hospital databases. Of these, 190 fulfilled all inclusion criteria (histologically confirmed diagnosis of NSCLC (adenocarcinoma, squamous cell carcinoma or large cell carcinoma), anatomical resection with curative intent, availability of FFPE-embedded tissue material and record of either 3-year remission after surgery or documented earlier relapse). Most patients who did not meet inclusion criteria were excluded because they did not undergo anatomical resection with curative intent (e.g., mediastinoscopy or metastasectomy). 39 patients could not be reached for obtaining consent or refused to participate. A total of 151 patients were included in this study. Inclusion criteria and the number of patients included and excluded, respectively, are shown in Figure 1.

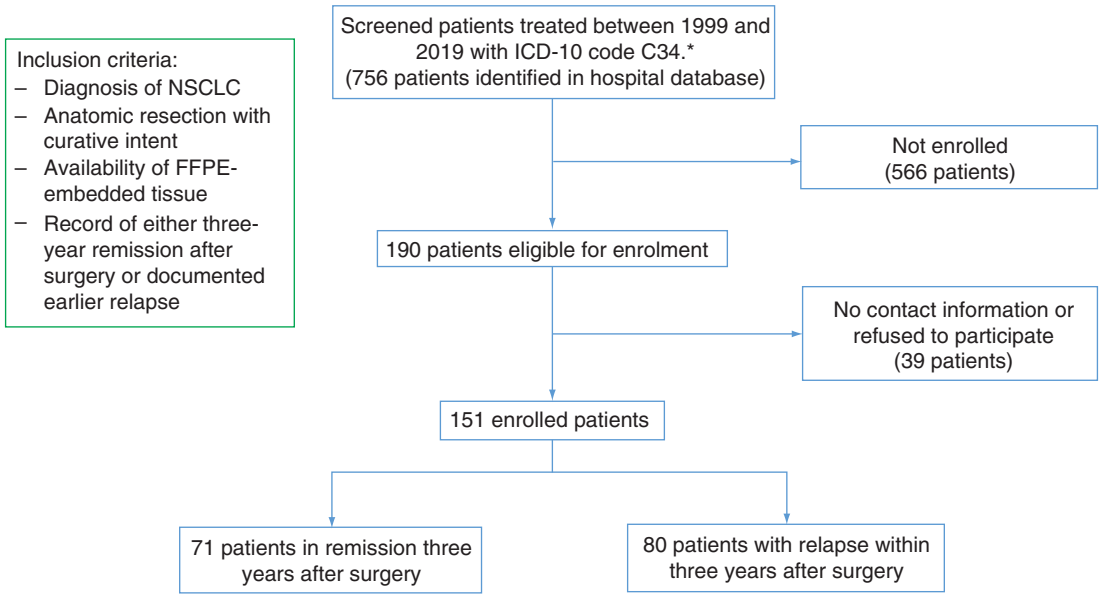

Figure 1. Inclusion criteria and number of patients included or excluded at every stage of the study design.
FFPE: Formalin-fixed paraffin-embedded; NSCLC: Non-small-cell lung cancer.

The average age at diagnosis was 64.7 ± 9.4 years (standard deviation), and 92 (60.9%) of the patients were male. Of these 151 patients, 71 patients were in remission 3 years after curative surgery was performed (REM group), and 80 patients developed distant or local recurrence of their NSCLC (REL group). Baseline characteristics were well balanced in both groups except for gender (p = 0.045), pack years (p = 0.018), stage at diagnosis (p = 0.014) and the presence of chronic lung disease (p = 0.014). All clinical characteristics for both groups are displayed in Supplementary Table 1.

Tissue morphology

For the analysis of tissue morphology, H&E stainings of all available tissues (primary tumor and matched tbLNs, N2 tumor-draining ntbLNs and N1 tumor-draining ntbLNs) of the 151 patients were analyzed. We were able to obtain 142 tumor samples (64 REM and 78 REL patients), 140 tumor-draining N1 ntbLNs (65 REM and 75 REL patients) and 120 tumor-draining N2 ntbLNs (63 REM and 57 REL patients). Only a minority of patients had tumor-bearing LNs; therefore, only 13 tumor-bearing LNs of REM patients and 31 tumor-bearing LNs of REL patients were available and included in the analysis. All morphological features investigated and their frequencies can be found in Supplementary Table 2.

To improve readability, all morphological and clinical variables selected for the final model using multivariate forward Cox regression for OS as well as PFS in all patients are shown in Table 2. The list of variables selected for the final model in REM and REL subgroups can be found in Supplementary Table 3.

First, we investigated whether morphological differences between REM and REL patients existed. None of the features investigated here were significantly different between the two groups. Because we did not identify any features that differed significantly, we aimed to identify factors influencing OS and PFS in both groups combined. In particular, sinus histiocytosis in both non-tumor-bearing N2 and N1 LNs was the only histological feature selected as predictive for OS in all patients. Subsequent subgroup analysis of REM and REL patients separately showed this to be mainly driven by a strong association between sinus histiocytosis in N1 LNs and OS in REL patients. Higher degrees of sinus histiocytosis were associated with improved OS in all patients as well as in the REL subgroup.

Regarding PFS, TiL density in the tumor and sinus histiocytosis in N1 LNs were selected for the final model. Subgroup analysis showed an association between TiL density and PFS in REM patients; however, no association between sinus histiocytosis and PFS was found in the REL subgroup. Higher degrees of sinus histiocytosis as well as TiL density were associated with improved survival.

Table 2. Morphological features selected for the final model of overall and progression-free survival using multivariate forward Cox regression.

Patient group	Outcome variable	Influencing variables	Coef	SE	Exp (coef)	Sig (p-value)
All patients	OS	Presence of distant metastasis	2.06	0.26	7.84	0.0001
		Age at diagnosis	0.04	0.01	1.04	0.001
		Weak degree of sinushistiocytosis vs none in mediastinal LNs	0.40	0.38	1.50	0.29
		Strong degree of sinushistiocytosis vs none in mediastinal LNs	-0.22	0.41	0.80	0.59
		Unknown degree sinushistiocytosis vs none in mediastinal LNs	0.32	0.55	0.40	0.56
		Weak degree of sinushistiocytosis vs none in hilar LNs	-0.32	0.36	0.73	0.38
		Strong degree of sinushistiocytosis vs none in hilar LNs	-0.90	0.40	0.41	0.02
		Unknown degree of sinushistiocytosis vs none in hilar LNs	0.05	0.40	1.05	0.90
All patients	PFS	Stage II at surgery vs stage I	0.32	0.27	1.38	0.23
		Stage III at surgery vs stage I	1.44	0.28	4.21	0.0001
		Chronic lung disease before surgery	0.65	0.21	1.92	0.002
		Between 10% and 50% TiLs vs <10% TiL	-0.79	0.28	0.47	0.004
		>50% TiLs vs <10% TiL	-0.59	0.27	0.55	0.03
		Unknown vs <10% TiL	-1.35	0.74	0.26	0.07
		Weak degree of sinushistiocytosis vs none in hilar LNs	-0.77	0.29	0.46	0.008
		Strong degree of sinushistiocytosis vs none in hilar LNs	-0.80	0.42	0.45	0.06
		Unknown degree of sinushistiocytosis vs none in hilar LNs	-0.13	0.26	1.14	0.61

Results from multivariate Cox regression models of OS and PFS with forward selection in all patients.
HR: Hazard ratio: LN: Lymph node; OS: Overall survival; PFS: Progression-free survival; PY: Pack years; REL: Relapse group; REM: Remission group; SE: Standard error; TiL: Tumor-infiltrating lymphocyte.

In summary, sinus histiocytosis in both non-tumor-bearing N2 and N1 LNs was predictive for OS, whereas TiL density in the tumor and sinus histiocytosis in non-tumor-bearing N1 LNs was predictive for PFS.

Immune marker expression analysis

Twenty REL and 20 REM patients were selected at random for detailed analysis of immune marker expression. Within this subgroup the following tissue samples were available: 40 tumor samples, 14 tbLNs, 40 N1 ntbLNs and 30 N2 ntbLNs. We performed qPCR for *CD4*, *CD8*, *CD19*, *CD86*, *CD163*, *CD274*, *MPO* and *TBP*.

We aimed to identify genes that were differently expressed between REM and REL patients and to investigate whether expression ratios of two genes differed between the groups. No genes were differently expressed between REM and REL patients. Comparison of ratios between different genes showed significant differences between REM and REL patients in *CD19*/*CD274* in N2 ntbLNs ($p = 0.03$) and in *CD4*/*MPO* in N1 ntbLNs ($p = 0.04$). No other genes or ratio of any two genes were significantly different in tumor tissue or tbLNs between REM and REL patients.

In addition to genes being differently expressed between REM and REL groups, we investigated which markers had an influence on OS and PFS in all patients. *CD4* expression in N1 (HR = 0.72; $p = 0.02$) and N2 (HR = 0.91; $p = 0.04$) ntbLNs was correlated with OS and PFS, respectively. No other gene expression levels were significantly associated with OS or PFS in any tissue.

Survival curves for *CD4* High/Low in N1 and N2 LNs can be seen in Figure 2 and Figure 3, respectively.

Discussion

This work represents the first analysis of matched tumor, tumor-bearing LNs and tumor-draining (but non-tumor-bearing) N1 and N2 LNs in NSCLC. Lymph nodes are often the first site of metastatic spread; however, the exact mechanism of tumor infiltration remains unclear. There is considerable premetastatic cellular reorganization as well as molecular change in premetastatic LNs [16,17]. By including affected as well as unaffected tissue, we aimed to investigate both pre- and postmetastatic environments.

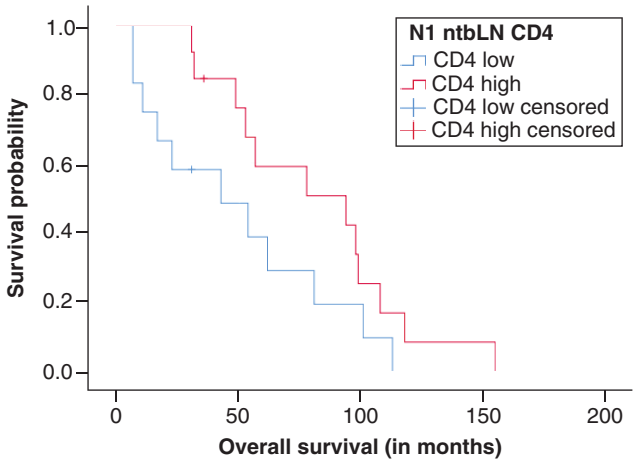

Figure 2. Kaplan–Meier survival plot for *CD4* high/low in N1 non-tumor-bearing lymph nodes.
N: Node; ntbLN: Non-tumor-bearing lymph node.

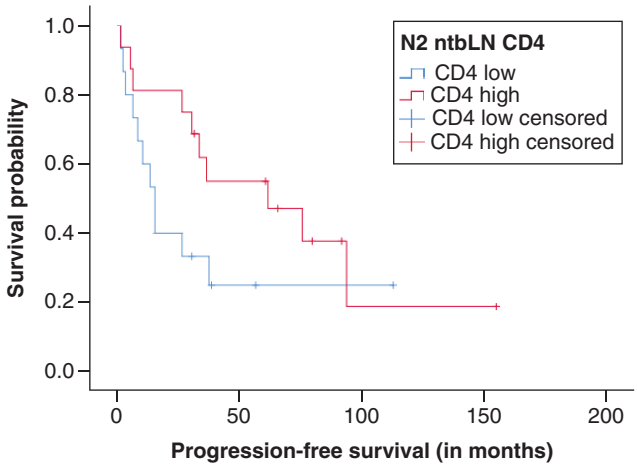

Figure 3. Kaplan–Meier survival plot for *CD4* high/low in N2 non-tumor-bearing lymph nodes.
N: Node; ntbLN: Non-tumor-bearing lymph node.

We found in the morphological analysis that no features differed between REM and REL patients; however, increased degrees of sinus histiocytosis in N1 and N2 LNs as well as increased levels of TiLs in the tumor were beneficial for survival.

Sinus histiocytosis describes the increased proliferation of histiocytes in lymph sinuses as a response to tumor antigens or other infectious agents. Previous research has shown sinus histiocytosis in locoregional lymph nodes to be correlated with improved survival in NSCLC and other entities [18,19]. Prior investigations have found the presence of sinus histiocytosis to be especially beneficial in more advanced disease stages; this corresponds to our study, with the association to OS being driven mainly by the REL patient group [20]. Interestingly, while we found sinus histiocytosis to be predictive for OS in N1 LNs as well as N2 LNs, it was predictive for PFS only in N1 LNs. Tumor draining N1 LNs are the primary site of LN metastasis and immune recognition of NSCLC tumors. Proliferation of sinus histiocytes in N2 LNs may indicate an immune response in more distant sites. It is conceivable that this recognition is not enough to prevent the occurrence of isolated metastases, but may rather lead to a generally reduced metastatic burden during the course of the disease. While no research has investigated a link between sinus histiocytosis in N2 LNs and reduced overall metastatic burden, we are planning to do so in follow-up studies.

In the current study, we found TiL density to be positively associated with longer PFS overall and, in particular, with PFS in the REM group. TiLs and their subsets have recently been used to predict survival as well as response to chemotherapy and immunotherapy [21–23]. Our finding corroborates and extends previous studies because TiL density seems to be of special importance as a predictor for PFS in long-term survivors [24]. Given that the median time to local or systemic relapse is only 14 months and 13 months, respectively, long-term metastasis-free survivors remain a comparatively understudied patient group [25]. In our study we did not see an effect of increased TiL density on overall survival. Although this seems counterintuitive at first, it could be explained by a change in the ability of

the tumor to avoid immune detection. Tumors have the ability to avoid immune detection in various ways, such as loss of antigens or upregulation of PD-L1 and subsequently reduced T-cell proliferation and differentiation [26]. If tumors acquire one of these evasive abilities, they may be able to progress locally and/or systemically despite initially high TiL levels. The ability to identify factors that allow stratification of long-term survivors is a strength of the current study.

CD4 was associated with OS and PFS in the qPCR analysis. CD4 helper T cells play diverse roles in activation, proliferation and differentiation of CD8 T cells, thereby promoting or hindering their ability to recognize and destroy cancerous cells [27]. We did not perform more in-depth analyses to determine which subsets were dominant in our cohort. However, because increased *CD4* expression was associated with better survival in this study, it may follow that they mainly performed an activating role. Other studies have found higher levels of activating CD4 cells to be associated with increased tumor control and survival [27].

The current study presents an investigation of a variety of immune markers in a diverse patient cohort with short- and long-term survivors. It also highlights the importance of not only the different immune cell types but also their locations (N1 LNs vs N2 LNs) when investigating predictors for survival in NSCLC. A major strength of this work is the analysis of matched tumor-bearing and non-tumor-bearing tissues. This unique constellation allowed us to investigate relationships of immune cell populations in different tissues, thus capturing a more accurate snapshot of the patient's immune status at the time of surgery. A potential weakness of this work is sampling bias due to tumor and LN heterogeneity. Even though multiple slices of each tissue were obtained for analysis, we cannot rule out that sampling other areas of the resected tissue would have yielded different results.

Conclusion

This is the first analysis of tumor-bearing and non-tumor-bearing N1 and N2 LNs to investigate patients' immune status at surgery for NSCLC. We found tumor-infiltrating lymphocytes, *CD4* expression levels and the presence of increased sinus histiocytosis to be predictive for PFS and OS, respectively. We are planning to further investigate the mechanisms behind these findings and their interactions.

Summary points

- The non-small-cell lung cancer (NSCLC) tumor, tumor bearing (tb) and non-tb (ntb) lymph nodes (LNs) are removed during curative NSCLC resection.
- Combining immune cell profiles in the NSCLC tumor and tbLNs and ntbLNs provide a snapshot of the immune landscape at the time of surgery and may serve as a predictor of a tumor-specific immune response.
- One hundred fifty-one NSCLC patients were included in this study: 71 patients with long-term disease-free survival and 80 patients with relapse within 3 years.
- We used forward Cox regression to identify factors associated with overall (OS) and progression-free survival (PFS).
- Sinus histiocytosis in both non-tumor-bearing N2 and N1 LNs was the only histological feature selected as being predictive for OS.
- Tumor-infiltrating lymphocyte density as well as sinus histiocytosis in N1 ntbLNs was predictive for PFS.
- *CD4* expression in N1 (hazard ratio = 0.72; p = 0.02) and N2 (hazard ratio = 0.91; p = 0.04) ntbLNs was correlated with OS and PFS, respectively.
- Immunological markers could contribute to identifying the patients at highest risk for relapse; however, further studies are required to understand the role of different immune cell populations in ntbLNs in NSCLC.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fon-2021-0402

Author contributions

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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4. Paper II

Markers of immune cell exhaustion as predictor of survival in surgically-treated early-stage NSCLC

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Markers of Immune Cell Exhaustion as Predictor of Survival in Surgically-Treated Early-Stage NSCLC

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Background: Tumor tissue as well as regional lymph nodes are removed during curative surgery for early-stage non-small cell lung cancer (NSCLC). These tissues provide a unique snapshot of the immune cell composition at the time of surgery. We investigated the immune landscape in matched tumor tissue, tumor bearing (tb) and non-tumor bearing (ntb) N1 as well as N2 lymph nodes (LNs) in patients with NSCLC and its relation to survival.

Methods: Internal hospital databases were screened for surgically treated NSCLC patients for whom tumor tissue, tbLNs as well as N1 and N2 ntbLNs were available. Clinical as well as demographic data were extracted from hospital records. Expression profiling of 770 immune-related genes was performed using the PanCancer IO 360 panel by NanoString Technologies.

Results: We identified 190 surgically treated patients of whom 16 fulfilled inclusion criteria and had sufficient archived tissue. The Tumor Immune Dysfunction and Exclusion (TIDE) score in N1 tumor-free lymph nodes was associated with OS. TIM-3 expression was inversely correlated with TIDE scores in affected LNs, N1 and N2 ntbLNs. Levels of CD8 expression were significantly higher in TIDE High compared to TIDE Low patients. TIM-3 and PD-L1 were selected for the final model for OS in multivariate regression in more than one tissue.

Conclusion: Levels of immune cell exhaustion markers may indicate a dysfunctional immune status and are associated with survival after curative surgery in NSCLC.

Keywords: HAVCR2, immune transcriptomics, NSCLC, TIM-3, immune cell exhaustion

INTRODUCTION

Lung cancer is the leading cause of cancer related deaths worldwide (1). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, making up approximately 85% of cases. In recent years, NSCLC treatment evolved drastically with the introduction of novel radiation techniques, targeted therapies and immunotherapies. However, anatomic resection in early-stage NSCLC remains the preferred curative treatment. While most patients with very early disease are cured by surgical resection, patients with regional lymph node involvement are at higher risk of local or systemic recurrence (2).

Many factors have been shown to impact postoperative survival in NSCLC, such as tumor stage, (neo)adjuvant treatment, extent of surgical resection, perioperative management, postoperative complications or body mass index (3–6). In addition, the importance of the antitumoral immune response has long been recognized in this setting. Recently, the field of immunoncology has gained traction, leading to the introduction of immunotherapies in many entities of cancer such as NSCLC, melanoma, glioblastoma, prostate cancer and others. The introduction of checkpoint inhibitors in NSCLC has led to pronounced improvements in tolerability, progression-free survival and overall survival. The response to these and other immunotherapies (including T-cell therapy, antibody-based therapies or vaccine-based approaches) relies on the immune system's ability to identify foreign antigens and launch a targeted response. This defense is largely mediated by different T-cell subsets, such as cytotoxic CD8+ T-cells (7, 8).

Activation and invasion of tumor-infiltrating lymphocytes (TiLs) has been shown to be a major determinant of response to checkpoint inhibitor treatment and disease-free as well as overall survival (9). However, if activated T-cells fail to entirely eliminate the tumor, they eventually enter a state of exhaustion. In this state, a persistent exposure to antigen will lead to a dysfunctional state in which cytotoxic T-cells can no longer effectively perform effector functions. T-cell exhaustion is common in cancer as well as some other entities, and leads to disease progression despite competent immune function.

Various scoring metrics to quantify the extent of immune dysfunction in tumors have been developed in order to predict survival or response to checkpoint inhibitor treatment. Differences in their predictive value and included genes reflect the diversity of activation/exhaustion pathways as well as tissue-specific processes. Recently, algorithms such as the Tumor Immune Dysfunction and Exclusion (TIDE) were developed that can be applied to a variety of tissues (10). While it has become evident the extent of tumor immune evasion and dysfunction is crucial for predicting survival, it is as of yet unclear whether this also extends to the state of the immune system in tumor bearing and tumor-free lymph nodes.

Various drugs to prevent or revert T-cell exhaustion are currently under development, for example in the phase III COSTAR trial (using Cobolimab, a TIM3 inhibitor) in advanced NSCLC (ClinicalTrials.gov Identifier: NCT04655976) or in the phase III RELATIVITY-047 trial (using Relatlimab, a LAG3 inhibitor) in metastatic melanoma (11).

In this study we examined differences in the immune landscape in matched tumor tissue, affected and unaffected lymph nodes in patients with NSCLC with a focus on markers of immune cell exhaustion and their relation to patient survival.

METHODS

Patient Cohort and Tissue Collection

Patients were identified retrospectively from hospital records. We screened records for the following inclusion criteria: histologically-confirmed diagnosis of NSCLC (adenocarcinoma, squamous cell carcinoma or large cell carcinoma), anatomical resection with curative intent, availability of FFPE-embedded tissue material and at least three years of follow-up. We also documented gender, age at diagnosis, survival, and tumor (stage and histology) and treatment details (type of surgery and (neo) adjuvant therapy). All patients were treated for NSCLC at the LMU Klinikum in Munich, Germany between 1999 and 2019.

We obtained approval from the institutional ethics board (reference number: 12-16) and obtained informed consent from all participants. This study was conducted in accordance with the Declaration of Helsinki.

Tissues and Transcriptomics of Immune-Related Genes

We obtained FFPE-embedded tissues of primary tumor, tumor-bearing lymph nodes (tbLN), and N1 and N2 non-tumor-bearing lymph nodes (ntbLNs) from pathology archives. FFPE blocks containing tumor (primary tumor and lymph nodes, respectively) were identified from pathology reports and reviewed by a senior pathologist based on hematoxylin and eosin stainings. Total RNA was extracted using RNEasy FFPE kits (Qiagen, Hilden, Germany) according to manufacturer's instructions.

We performed gene expression analysis of 770 genes using the nCounter PanCancer IO 360 panel run on a NanoString FLEX platform (including Prep Station and Digital Analyzer) (NanoString Technologies, Seattle, USA). This panel is specifically designed to target genes involved in tumor microenvironment and immune evasion. The NanoString nCounter platform conducts gene expression analysis without amplification steps, therefore being well suited for analysis of potentially degraded RNA extracted from FFPE tissues. Analysis of results including normalization was performed using NanoString's proprietary nCounter Analysis software.

Immune cell composition in all samples based on mRNA expression data was estimated using CIBERSORTx analysis in Relative mode with LM22 signature matrix (https://cibersortx.stanford.edu/) (12). This algorithm determines the abundances of 22 immune cell populations based on 547 markers.

TIDE Score

The Tumor Immune Dysfunction and Exclusion (TIDE) score algorithm was originally developed to assess the immune status in tumor tissues to identify patients who may benefit from immunotherapy. We applied it to immune transcriptomics data from tumor, tbLNs, N1 ntbLNs and N2 ntLNs in order to

determine whether the state of the immune system could predict postoperative survival in early-stage NSCLC even in the absence of immunotherapy. TIDE scores were calculated using a web-based tool with NSCLC as cancer type and no previous immunotherapy (http://tide.dfci.harvard.edu/). Negative TIDE score values represent the presence of immune evasion markers, whereas positive TIDE scores represent a lack of immune evasion. We categorized patients into TIDE High for patients with a positive TIDE score and TIDE Low for patients with a negative score. Since this analysis included different tissues from the same patient, we investigated whether TIDE scores were consistently positive or negative across tissues.

Immune Cell Composition and Exhaustion Markers

In addition to the TIDE score, which incorporates several markers independent of cell of origin, we also investigated cell compositions in our four tissues *via* CIBERSORTx. We compared immune cell composition between patients with TIDE High and TIDE Low patients.

Since a large part of the immunosuppressive environment is mediated through exhaustion of immune cells, we investigated levels of immune exhaustion markers and their association with PFS and OS. In order to obtain the most promising candidates we compiled a list of markers of immune cell exhaustion based on a literature search, investigating TIM-3, CD244, CTLA4, PD-1, PD-L1, BTLA, LAG3, EOMES, TIGIT and FOXP3.

Statistical Analysis

We reported numerical variables as mean with standard deviation and categorical variables as absolute and relative frequencies. We used univariate Cox regression to model the association of TIDE score and OS and PFS. Additionally, we compared OS and PFS between patients with High and Low TIDE score using Kaplan-Meier curves with p-values from log-rank tests. Immune cell composition in TIDE High and TIDE Low patients was non-normally distributed and therefore compared using Mann-Whitney U tests. To identify immune exhaustion markers associated with OS and PFS we used multivariate Cox regression models with forward selection with cut-off values of 0.1 and 0.2 for inclusion and exclusion, respectively. The following variables were used in the forward regression models: Expression levels of TIM-3, CD244, CTLA4, PD-1, PD-L1, BTLA, LAG3, EOMES, TIGIT, FOXP3, age at diagnosis, sex, stage at diagnosis, presence of chronic lung disease and type of (neo)adjuvant therapy. Results are reported as Hazard Ratios (HR).

We compared differences in the 22 cell populations obtained from CIBERSORTx between tumor, affected lymph nodes, N1 and N2 ntbLNs using within-subject ANOVAs.

Correlation between TIDE scores in the different tissues and immune cell exhaustion markers were analyzed using Pearson's correlation coefficient. Correlation coefficients were classified as moderate for an r between 0.5 and 0.7 and strong for an r above 0.7.

A threshold of $\alpha \leq 0.05$ for significance was applied for all analyses. All analyses were performed in SPSS version 26.

RESULTS

Patient Demographics

756 patients with a lung cancer diagnosis who underwent a surgical procedure between 1999 and 2019 were identified in internal hospital databases. Of these, 190 fulfilled all inclusion criteria (histologically-confirmed diagnosis of NSCLC (adenocarcinoma, squamous cell carcinoma or large cell carcinoma), anatomical resection with curative intent and availability of FFPE-embedded tissue material. Most patients who did not meet inclusion criteria were excluded because they did not undergo anatomical resection with curative intent (such as mediastinoscopy or metastasectomy). Of the remaining 190 patients, sufficient tissue for tumor, tbLNs as well as ntb N1 and N2 LNs was available for 16 patients. The vast majority of the 190 patients were excluded because they did not have any tumor-bearing lymph nodes.

The average age at diagnosis was 65.8 ± 9.4 years and 6 (37.5%) of the patients were male. Median follow-up time was 48.5 ± 38.8 months. Clinical characteristics for all 16 patients are displayed in **Table 1**.

TIDE Scores in Hilar Lymph Nodes Are Associated With Survival

Interestingly, while tumor TIDE scores were not significantly associated with OS, the TIDE scores measured in N1 ntbLNs were associated with OS (p=0.03, HR=0.59). Additionally, there was a trend towards significance in N2 ntbLNs (p=0.08, HR=0.57).

No TIDE score in any single tissue was associated with PFS. All results are displayed in **Table 2** and a Kaplan-Meier survival curve for OS (p=0.05) of TIDE high/low in N1ntbLNs is shown in **Figure 1**.

Of the 16 patients in this study, only four patients (25%) showed consistently positive or negative scores across all four tissues. Five patients (31%) showed discrepancies in one tissue and the remaining seven (44%) patients showed two positive and two negative scores.

CD8 Cells Differ Between Patients With Positive and Negative TIDE Scores

When comparing immune cell compositions of all four tissues between patients regardless of TIDE score, we found that M1 macrophages (p=0.02) and neutrophils (p=0.03) had a significantly higher relative abundance in tumor compared to all lymph nodes. In addition, naïve B-cells (p=0.04) and memory B-cells (p=0.004) showed relative lower abundance in tumor tissue compared to lymph nodes. Relative abundances of all cell types are shown in **Supplementary Table 2**.

We then compared patients with positive to patients with negative TIDE scores. CD8 cells were the only cell type with different abundances in patients with positive TIDE scores compared to patients with negative TIDE scores in more than one tissue, with a significantly higher level of CD8 cells in TIDE High patients (**Figure 2**).

TABLE 1 | Overview of clinical features of the 16 patients included in this study.

Patient number	Sex	Age at diagnosis [in years]	Smoking status	Pack years	Stage	(Neo)adjuvant therapy	Other chronic lung disease	Histology	PFS [in months]	OS [in months]
1	Male	68	Former	Unknown	IIIB	RCT	No	Adeno	155	156
2	Female	77	Former	Unknown	IIB	None	No	Adeno	38	45
3	Male	81	Former	Unknown	IIIA	RT	No	Adeno	39	100
4	Male	56	Former	Unknown	IIB	CT	No	SCC	55	98
5	Male	79	Active	Unknown	IIB	CT	No	Adeno	38	50
6	Female	58	Former	30	IIIA	CT	Yes	Adeno	59	73
7	Female	66	Active	50	IIB	None	No	Adeno	41	50
8	Female	61	Former	Unknown	IIIB	RCT	Yes	Adeno	4	7
9	Female	69	Former	40	IIB	None	Yes	SCC	27	102
10	Male	56	Active	100	IIIA	RCT	No	SCC	20	47
11	Female	65	Former	80	IIB	None	Yes	Adeno	9	30
12	Female	50	Active	35	IIIA	None	No	Adeno	24	50
13	Male	68	Former	30	IIB	CT	Yes	Adeno	6	19
14	Female	65	Former	50	IIB	RCT	No	SCC	7	21
15	Female	78	Former	Unknown	IIIA	CT	No	Adeno	17	38
16	Female	56	Never	0	IIIA	CT	No	Adeno	0	40

CT, Chemotherapy; OS, Overall survival; PFS, Progression-free survival; RCT, Radiochemotherapy; SCC, Squamous cell carcinoma.

TIM-3 Is Correlated With TIDE Scores and Is Associated With Survival

Since a large part of the immunosuppressive environment is mediated through exhaustion of immune cells, we investigated levels of exhaustion markers and their association with TIDE scores, PFS and OS.

We found TIM-3 to be inversely correlated with TIDE scores in affected LNs (r=-0.70, p=0.002), N1 ntbLNs (r=-0.61, p=0.01) and N2 ntbLNs (r=-0.74, p=0.001) (see **Figures 3A–D**). In addition, we found TIGIT expression in the tumor (r=-0.52, p=0.04) and FOXP3 in N1 ntbLNs (r=-0.56, p=0.03) to be correlated with TIDE scores (**Supplementary Table 3**).

Expression levels of TIM-3 and PD-L1 in N1 and TIM-3 in N2 ntbLNs were associated with OS in univariate Cox regression (**Figures 4A–D**; **Supplementary Table 1**). We then also performed multivariate regression (**Table 3**). PD-L1 as well as TIM-3 were the only exhaustion markers to be selected for the final model for OS in more than one tissue (even though PD-L1 only reached a p-value of 0.06). The above data support our hypothesis that markers of immune cell exhaustion in ntbLNs may aid in the search for suitable biomarkers for immune exhaustion in NSCLC.

We also performed univariate Cox regression to determine factors which influence PFS. The results of the univariate regression analyses are displayed in **Supplementary Table 1**. Unfortunately, the multivariate regression models failed to converge, so no results could be obtained.

DISCUSSION

To our knowledge, this study presents the first analysis of immunosuppression and immune cell exhaustion in matched tumor, tumor-bearing LNs and ntb N1 and ntb N2 LNs in surgically treated NSCLC. We evaluated the predictive power of the TIDE score on survival in different tissues and showed differences in the relative abundance of CD8 cells in TIDE High and TIDE Low patients. In addition, we showed that exhaustion markers PD-L1 and TIM-3 are associated with survival even in early-stage NSCLC not treated with immunotherapy.

The TIDE algorithm was developed to assess the state of the immune system in tumors and to predict which tumors may respond to immunotherapy. It takes into account T-cell dysfunction as well as their exclusion from the tumor. Our results indicate that patients whose N1 ntbLNs and N2 ntbLNs show high TIDE levels have better overall survival than patients with low TIDE levels. Higher TIDE scores indicate a lack of immune evasion caused by high levels of tumor-infiltrating

TABLE 2 | Results of univariate Cox regression of the TIDE score in tumor, affected LNs, N1 ntbLNs and N2 ntbLNs.

Tissue	Type of survival	coef	SE	HR	p-value
Tumor	OS	0.31	0.32	1.36	0.33
Affected LNs		-0.24	0.18	0.79	0.20
ntb N1 LN		-0.53	0.24	0.59	0.026
ntb N2 LN		-0.56	0.32	0.57	0.081
Tumor	PFS	0.32	0.28	1.38	0.25
Affected LNs		-0.34	0.2	0.72	0.096
ntb N1 LN		-0.42	0.24	0.66	0.076
ntb N2 LN		-0.52	0.31	0.59	0.093

HR, Hazard Ratio; LN, Lymph node; ntb, non-tumor bearing; OS, Overall survival; PFS, Progression-free survival; SE, Standard error; TIDE, Tumor Immune Evasion and Dysfunction.

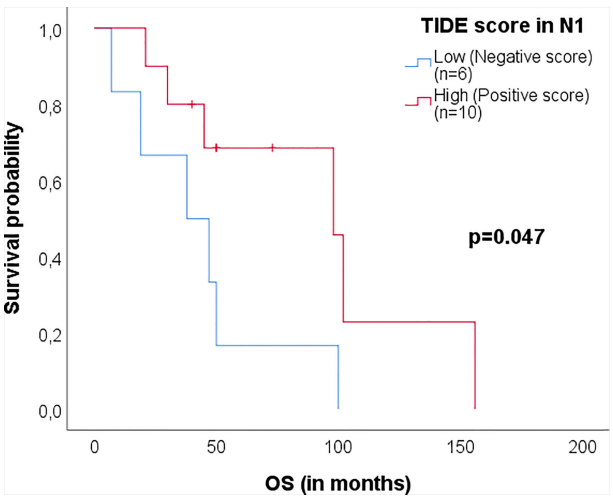

FIGURE 1 | Kaplan-Meier survival plot of patients with TIDE High/Low for OS in N1 ntbLNs. TIDE Low tissues showed negative TIDE values, indicating the presence of immune evasion markers. TIDE High tissues showed positive TIDE values, indicating a lack of immune evasion. P-values were obtained from log-rank tests.

lymphocytes, activating immune cell signatures and permissive surrounding cellular milieu (mediated by cells such as macrophages and fibroblasts). This lack of immune suppression in regional lymph nodes points towards the fact that the immune system was able to retain at least some local control of the tumor. In this study, TIDE scores in N1 and N2 ntbLNs were a better indicator of survival than TIDE scores in the tumor. Since the patients in this study had early-stage NSCLC, it may be the case that their tumors had not yet developed the immunosuppressive environment that is typical of advanced tumors (13, 14). High TIDE scores (and therefore the presence of high numbers of functional cytotoxic lymphocytes) in N1 and N2 ntbLNs may indicate the recognition of the tumor by cytotoxic T-cells as their cognate antigen.

We found markers of immune exhaustion to be negatively correlated with TIDE scores. Since negative TIDE scores indicate the presence of dysfunctional lymphocytes, this dysfunction may be cause by an increased expression of exhaustion markers. However, markers of immune exhaustion such as TIM-3 can be expressed on multiple cell types such as dendritic cells, natural killer (NK) cells or CD8+ T-cells. This diverse pattern can complicate interpretation of results of immune exhaustion marker expression. It was shown that TIM-3 is an important gatekeepers of inflammasome regulation and TIM-3 deletion in dendritic cells led to an increase of anti-tumor activity. However, deletion of TIM-3 on CD4 or CD8 T-cells did not produce the same effect (15). To contrast this, an inducible liver cancer model showed that and TIM-3 expression on cytotoxic CD8 T-cells appeared early and led to loss of antitumor effector function (16). Taken together, the effects of immune exhaustion marker expression is dependent on many aspects such as tissue and cell type as well as timing during tumorigenesis. In the gene expression analysis presented here, we were not able to determine the cells of origin of exhaustion marker expression.

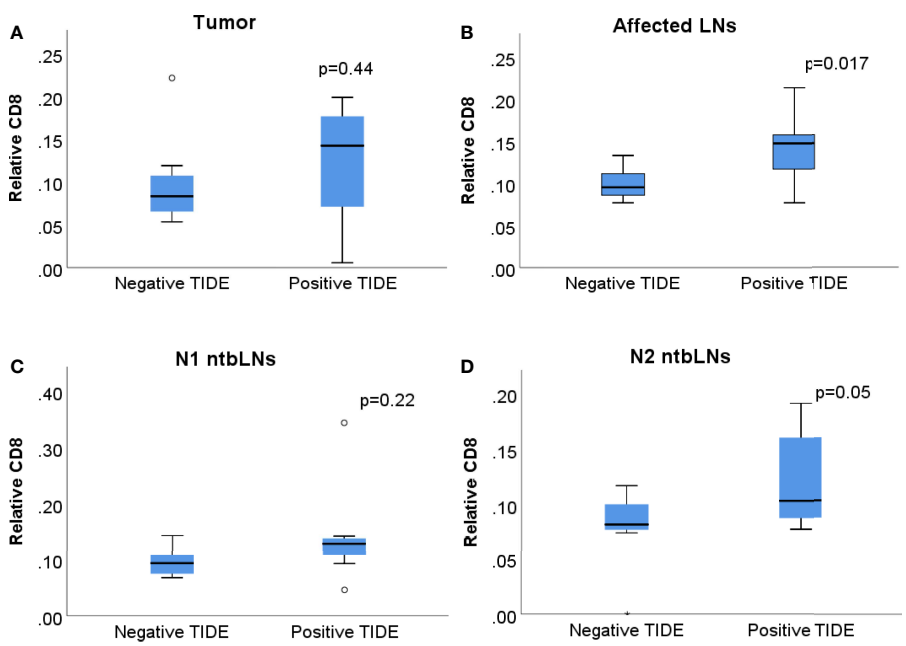

FIGURE 2 | Boxplots of relative abundance of CD8+ cells in patients with negative and positive TIDE scores in **(A)** tumor tissue, **(B)** tbLNs, **(C)** N1 ntbLNs and **(D)** N2 ntbLNs. Boxes show interquartile range, whiskers show lowest and highest quartile.

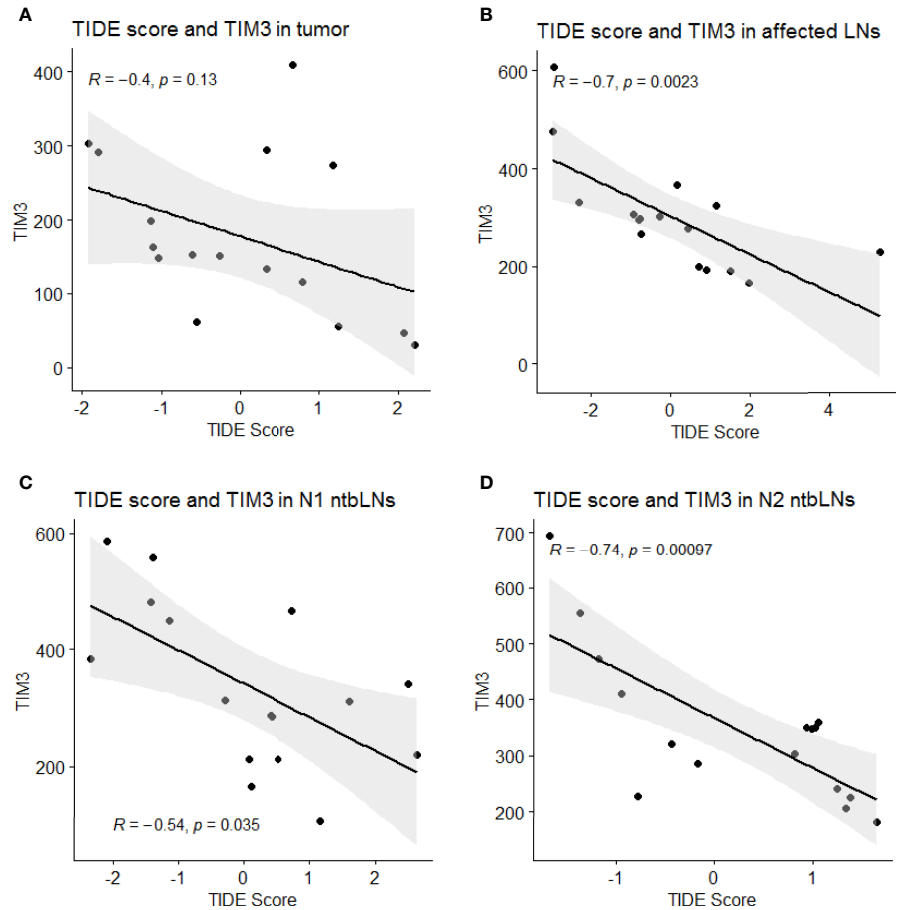

FIGURE 3 | Scatterplots of TIDE scores and TIM-3 expression values in **(A)** tumor, **(B)** affected LNs, **(C)** N1 ntbLNs and **(D)** N2 ntbLNs with regression line and 95% confidence intervals.

However, we were able to show that an increased level of immune exhaustion marker expression overall led to shorter survival and are planning to further investigate the cells of origin.

TIM-3 is a marker of activated and subsequently of terminally exhausted CD8 cells, dendritic cells, NK cells and others and prevents the formation of long-lived memory cells. While the exact mechanism of TIM-3 mediated signalling is currently unknown, binding to one of its multiple interaction partners leads to increased suppressor function and reduces macrophage activation (17). Furthermore, TIM-3 inhibition leads to worsening of autoimmune inflammatory diseases such as inflammatory bowel disease and diabetes (18, 19). In this study, we found increased expression levels of TIM-3 in mediastinal and hilar lymph nodes to be associated with shorter overall survival. This parallels the findings of many other studies

TABLE 3 | Immune cell exhaustion markers selected for the final model of overall and progression-free survival using multivariate forward Cox regression.

Tissue	Type of survival	Variable	coef	SE	HR	p-value
Tumor	OS	PD-L1	0.003	0.002	1.003	0.062
AffLN	OS	CTLA4	0.003	0.001	1.003	0.059
N1 ntbLN	OS	TIM-3	0.005	0.003	1.005	0.064
		PD-L1	0.015	0.007	1.015	0.031
N2 ntbLN	OS	TIM-3	0.006	0.003	1.006	0.035
Tumor	PFS	Failed to converge				
AffLN		Failed to converge				
N1 ntbLN		Failed to converge				
N2 ntbLN		Failed to converge				

HR, Hazard Ratio; LN, Lymph node; ntb, non-tumor bearing; OS, Overall survival; PFS, Progression-free survival; SE, Standard error; TIDE, Tumor Immune Evasion and Dysfunction.

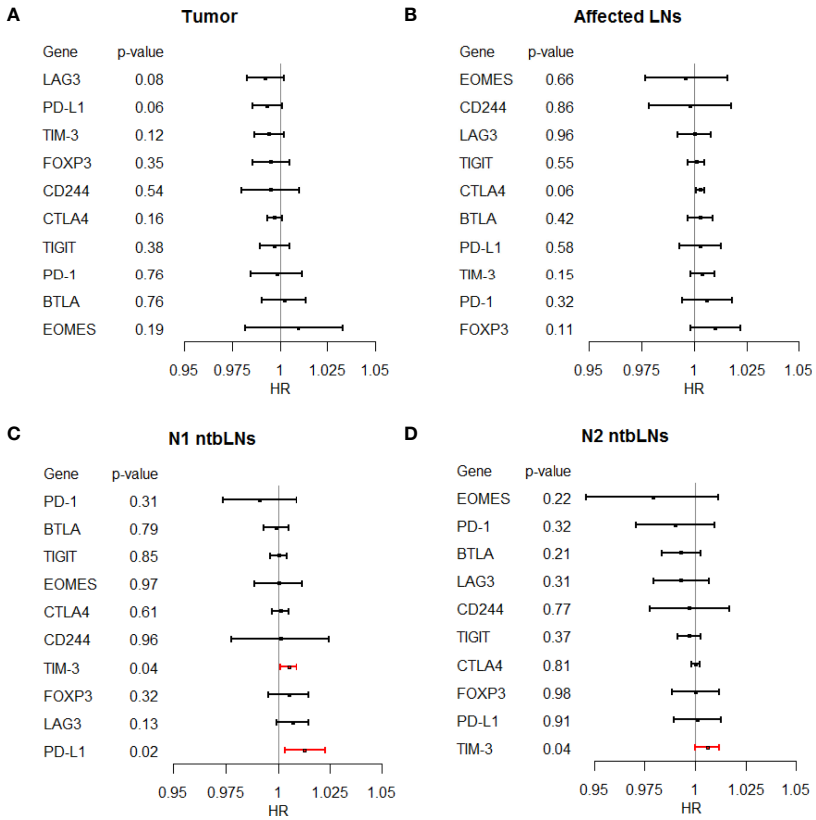

FIGURE 4 | Forest plot of Hazard ratios from univariate Cox regression of T-cell exhaustion markers and overall survival in **(A)** tumor tissue, **(B)** affected LNs, **(C)** N1 ntbLNs and **(D)** N2 ntbLNs.

which have investigated TIM-3 levels in tumors (20–22). It was demonstrated that the tumor-draining lymph nodes of hepatocellular carcinoma acquire an immunosuppressive milieu (23). Taken together, these findings highlight the importance of assessing the state of the immune system in tumor-free local lymph nodes.

This study highlights the role of the immune cell dysfunction in surgically treated early-stage NSCLC patients. By analyzing tumor as well as tissue from tumor-bearing lymph nodes as well as tumor-free N1 and N2 lymph nodes, we were able to identify unique immunosuppressive signatures associated with survival. A limitation of this study is tumor microenvironment and lymph node heterogeneity. There is no consensus about which location in the tumor is best assessed for immune transcriptomics. In addition, there is a potential for sampling bias because of the histological sections that were used in the analysis. Furthermore, since we did not perform single-cell transcriptomics, it was not possible to assign altered expression levels to a cell type of origin.

CONCLUSION

To our knowledge, this is the first study to perform immune transcriptomics in tumor and tumor bearing and non-tumor bearing regional LNs in NSCLC. We showed that immune exhaustion markers are associated with survival in this early-stage surgically treated cohort. Future clinical trials of exhaustion inhibitors should include ntbLNs to help identify patients most likely to benefit from adjuvant approaches and more in-depth postsurgical follow-up.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repositories and accession numbers/links can be found below: GEO, NCBI: GSE197929; Figshare: https://doi.org/10.6084/m9.figshare.18707639.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Medical Faculty (LMU). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LS: Conceptualization, Methodology, Data curation, Formal analysis, Roles/Writing - original draft, Writing - review & editing JKo: Conceptualization, Methodology, Roles/Writing - original draft, Writing - review & editing JW: Formal analysis, Roles/Writing - original draft, Writing - review & editing JKu: Data curation, Writing - review & editing JN: Methodology, Writing - review & editing DK-G: Conceptualization, Writing - review & editing RK: Data curation CS: Conceptualization, Methodology, Writing - review & editing AJ: Conceptualization, Writing - review & editing JB: Conceptualization, Writing - review & editing AT: Conceptualization, Methodology, Funding acquisition, Roles/Writing - original draft, Writing - review & editing. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.858212/full#supplementary-material

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