TUMOR INFILTRATING LYMPHOCYTES AS A PREDICTIVE BIOMARKER FOR RESPONSE TO CTLA-4 AND PDL-1 THERAPIES

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Abstract

Purpose: Recently approved immunotherapies capitalize on antitumor mechanisms of the patient's immune system by inhibiting CTLA-4 and PD-1 pathways. Studies have shown better overall survival with increased tumor infiltrating lymphocyte (TIL) across multiple cancers. Recent trials with anti-PDL-1 has shown better response with high PDL-1 expression. However, studies have not evaluated whether TIL level would correlate with anti-PDL-1 or anti-CTLA-4 responses. The aim of this study is to determine if the level of TIL in metastatic melanoma and lung cancer correlates with patient response to modern immunotherapies.

Methods: We identified 10 patients with melanoma or lung cancer treated with an immune checkpoint inhibitor. The biopsy samples were stratified according to level of TIL. The TIL categories ranged from 0 to 3, with 0 indicating no TIL detected and 3 indicating 67-100% TIL infiltration. Survival analysis was achieved with Kaplan-Meier curve, and tumor size change was evaluated with linear mixed model analysis.

Results: Overall survival was significantly longer in patients who had TIL (TIL=1-3 vs. TIL=0, p=0.024). For TIL 0, there was average decrease in size from baseline to first follow up of 0.12cm^2. Tumor size also dramatically decreased based on TIL level (TIL 1-2: 371% greater decrease than TIL 0, $p < 0.01$; TIL 3: 406%; $p<0.01$). The overall survival from time of initial treatment was 0.3 yr for TIL 0 (SD=0.2), 2.45 yr for TIL1-2 (SD=0.71), and 1.56 yr for TIL 3 (SD=1.27). After controlling for baseline tumor size and the type of cancer, the mean difference in the change of tumor size between baseline and the following time point was 0.74cm2 smaller in TIL 1-2 compared to TIL=0 (p=0.002). Furthermore, the estimated mean difference in the change in tumor size between baseline and the following time point was 0.74cm2 lower in patients with TIL=3 compared to TIL=0 (p=0.002)

Conclusion: Our study shows that TIL level may serve as a biomarker of tumor response to immunotherapy, without specific histochemical staining. This study is limited by the low number; a larger review is currently taking place.

Table of Contents

List of Figures and Tables

Introduction

The treatment of cancer has largely relied upon the killing of tumor cells through cytotoxic chemotherapies and radiation. However, these therapies have their limitations: cancer cells can develop resistance to these drugs, and radiation therapy is a localized, not systemic, treatment. Additionally, there is large profile of side effects associated with these therapies. These reasons warrant a closer look at other approaches to cancer therapy, specifically the immune mechanisms modulating cancers.

Cancer Immunology

The cancer immunoediting view is now the prevailing theory regarding the immunobiology of cancer. This process emphasizes both the host-protective and tumor-evolving qualities of the immune system in tumor development. It consists of three phases: elimination, equilibrium, and escape[1]. The elimination phase, or cancer immunosurveillance, is the process in which molecules and cells of the immune system detect the presence of a tumor. These include lymphocytes such as cytotoxic T lymphocytes ($CD8^+$), regulatory T cells ($CD4^+$), B cells, natural killer (NK) cells, and other antigen-presenting cells (APC). They invade and eliminate it before it manifests itself clinically. The equilibrium phase occurs when tumor cells are not completely eliminated, but the immune system controls excessive growth. This results in tumor dormancy. Finally, in the escape phase, transformed cells acquire mechanisms that allow them to grow and evade the immune system. It is this phase that is of particular interest in developing cancer therapies.

Immune checkpoints

While traditional cancer therapies are toxic chemicals that destroy tumor cells, great interest has been growing in stimulating the patient's own immune response to fight cancer. Notably, melanoma and more recently, lung cancer, are considered to be immunogenic cancers, and thus are responsive to immunologic modulation[2]. One obstacle in the immune response is immune checkpoints. These are inhibitory proteins that regulate the immune system, and prevent an active immune response to certain stimuli. Immune checkpoints proteins do this by binding to a specific ligand. This is an important homeostatic mechanism in

preventing tissue injury and autoimmunity, however cancer cells can capitalize on this mechanism to evade immune destruction. Two specific immune checkpoint proteins that are expressed on T-lymphocytes have become of interest in recent cancer immunotherapy research: programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA4). When interacting with their ligands, PD-L1 and B7 respectively, these checkpoint proteins inhibit an active T-cell response[3]. For example, the signaling of PD-1 prevents T-cell activation, and leads to reduced proliferation, cytokine production, and T-cell cytolysis[4]. It has been found that tumor cells express PD-L1 following infiltration of T-lymphocytes[5], leading to the hypothesis that blocking the PD-1/PD-L1 pathway can lead to an active immune response against cancer cells[6]. Rodent studies of PD-L1 blockade and PD-1 knockout mice show increased immune response[7,8]. This led to the development of immune checkpoint inhibitors as a novel cancer therapy, with the first anti-CTLA4 antibody, ipilimumab, being FDA approved in 2011[9]. Pembrolizumab and nivolumab are two other monoclonal antibodies, specifically targeting PD-1. These immune checkpoint inhibitors thwart the interaction between tumor cells and T-lymphocytes to release immune inhibition, and thus promote an antitumor immune response against the cancer cells. Recent clinical trials on immune checkpoint inhibitors (ipilimumab, pembrolizumab, nivolumab) have yielded incredible success in patients with melanoma and lung cancer where durable remission and responses have been achieved, along with a lower profile of side effects[2,8,10,11]. Immune checkpoint inhibitors have become the standard of care for many cancer types, but there is still a high variability in patient response rates with emerging resistance to these therapies[12]. This necessitates the identification of biomarkers that can inform the use of these agents in patients.

Immune checkpoint inhibitors and tumor-infiltrating lymphocytes

Although tumors with high PD-L1 expression have shown an enhanced response to immune checkpoint inhibitors, even tumors without PD-L1 expression can respond to these therapies. This raises question as to whether other pathways are also influenced by these drugs. Recently, research efforts on immune checkpoint inhibitors are focusing on the investigation of predictive biomarkers that would aid in patient selection and response prediction.

Studies have shown that PD-L1 expression was correlated with overall survival, but was not independent, suggesting T-cell content as a confounding variable[8]. Thus, the lymphocytic density in the tumor may be a factor. In fact, the level of PD-L1 has been highly correlated with the level of tumor infiltrating lymphocytes (TIL), both with uni- and multivariable analysis[9]. This supports an adaptive resistance mechanism for immune escape[13]. Given the high correlation between PD-L1 and TIL, this lends to the hypothesis that patients with a high TIL level would have improved response to immune checkpoint therapies. Researchers have identified TIL as an important prognostic factor in melanomas and other tumors[14,15]. Additionally, median survival period was significantly correlated with immune cell infiltrate, specifically $CD8^+$ T cells, in metastatic tumors of melanoma[15]. Furthermore, $CD8^+$ T-lymphocytes correlated with response of melanoma to pembrolizumab in metastatic melanoma patients[3]. Thus, this study aims to further explore the possibility that tumor response to these therapies is correlated to TIL level in tumor samples. This study will be a retrospective review of adult patients who were diagnosed with metastatic melanoma or lung cancer, and were subsequently treated with an immune checkpoint inhibitor. Biopsies of the metastases will be assessed histologically for TIL concentration. Levels of TIL will be correlated with patient response. We hypothesize that the level of TIL in metastases of melanoma or lung cancer in adult patients who received immune checkpoint inhibitor treatment will positively correlate with overall survival.

Materials and Methods

Chart Review

Analysis of specimens and collection of data was approved by the Banner Institutional Review Board. Patients were treated at some point between 2012 and 2015. Subjects were identified through a report generated from the Cerner electronic medical record system. Inclusion criteria included patients between ages 18-99 years who were diagnosed with metastatic melanoma or lung cancer treated with an immune checkpoint inhibitor at our institution between January 1, 2012 and December 31, 2015. Immune checkpoint inhibitors included ipilimumab, pembrolizumab, or nivolumab. Exclusion criteria included patients who were not treated with immune checkpoint inhibitors, or patients who were not evaluated at our institution. These criteria yielded a total of 104 subjects.

Information retrieved from patient records included diagnosis, treatment, response to treatment, timeline of treatment, follow-up reports, pathology reports, and radiology reports.

Determination of TIL expression

One pathologist examined the biopsies of subjects and selected a representative slide of the collected specimens. The cohort included a total of 10 patients. The biopsies were collected from either primary or metastatic lesions, and stained with hematoxylin and eosin. The level of TIL was analyzed microscopically and quantified according to a 4-tiered system, correlating with the percentage of lymphocytic infiltrate by area of the biopsy specimen (Table 1). TIL level of 0 represented 0% TIL, TIL level of 1 represented a TIL area of 1-33%, TIL level of 2 represented a TIL area of 34-67%, and TIL level of 3 represented a TIL area of 68-100%. All histologic analysis and categorization was completed by the same pathologist.

Statistical Analysis

One of the primary endpoints for analysis was the overall survival and its association with TIL level. Overall survival was analyzed using the Kaplan-Meier method. Patients were stratified according to variations of the TIL level and survival curves were constructed. A linear mixed model analysis was used to test the association between TIL level and tumor size over time.

TIL level	Level of lymphocytic infiltrate in biopsy sample
0	0% (no lymphocytes)
1	1-33%
2	34-67%
3	68-100%

Table 1. The level of TIL was quantified according to a 4-tiered system, correlating with the percentage of lymphocytic infiltrate by area of the biopsy specimen

Results

Patient Demographics

The study included a total of ten patients, comprised of four males and six females with mean age of 62.8 years (Range: 48-88) at time of the start of treatment. Of these patients, six were diagnosed with melanoma, and four were diagnosed with lung cancer. Eight patients were deceased at time of analysis, and two were alive. Overall survival (OS) was defined as time of treatment start to time of death or time of last clinical encounter. The average OS was 16.4 months, with a range of 1-39 months. Four patients had a TIL level of 0, two patients had a TIL level of 1, one patient had a TIL level of 2, and three patients had a TIL level of 3. Other patient characteristics are outlined in Table 2.

Overall survival and TIL level

One of the primary analyses of this study was to analyze the level of TIL and its relationship with overall survival. Kaplan-Meier analysis was performed, measuring overall survival from treatment start date to death (or last encounter) and TIL. The first analysis categorized patients with and without any lymphocytic infiltrate in the biopsy sample. Patients with TIL exhibited a significantly longer OS than patients with TIL 0 (TIL 1-3 vs. TIL 0, p=0.024), with no TIL 0 patients alive after 6 months (Figure 1). A further comparison of TIL level 0 with TIL levels 1-2 and 3 revealed a significantly longer OS for patients with TIL 1-2 and TIL 3 compared with TIL 0 (TIL 0 vs. TIL 1-2 vs. TIL 3, p=0.04) (Figure 2). The overall survival from time of initial treatment was 0.3 yr for TIL 0 (SD=0.2), 2.45 yr for TIL1-2 (SD=0.71), and 1.56 yr for TIL 3 (SD=1.27).

Tumor size and TIL level

For TIL 0, there was average decrease in size from baseline to first follow up of 0.12cm^2. A linear mixed model was used to evaluate change in tumor size across TIL levels. Patients with TIL 1-2 level showed a 371% decrease in size compared to TIL 0 ($p < 0.01$), while TIL level 3 showed a 406% decrease in size compared to TIL 0 ($p < 0.01$). After controlling for baseline tumor size and the type of cancer, the mean difference in the change of tumor size between baseline and the following time point was 0.74 cm^2 smaller in TIL 1-2 compared to TIL 0

(p=0.002). Furthermore, the estimated mean difference in the change in tumor size between baseline and the following time point was 0.74 cm^2 lower in patients with TIL 3 compared to TIL 0 (p=0.002).

Patient	Sex	Cancer type	Age at tx start	TIL level	Location of mass	OS (in days)
A	M	Melanoma	50	1	Brain	1015
B	M	Melanoma	54	3	Femur	895
C	M	Melanoma	48	3	Nose	777
D	F	Melanoma	70	0	Liver	179
E	F	Melanoma	54	1	Parietal scalp	594
F	M	Melanoma	88	2	Temporal scalp	1175
G	F	Lung Cancer	63	0	Mainstem bronchus	168
H	F	Lung Cancer	73	0	Lung	50
I	F	Lung Cancer	57	0	Vertebral body	41
J	F	Lung Cancer	71	3	Lung	35

Table 2. Characteristics of patients included in this study

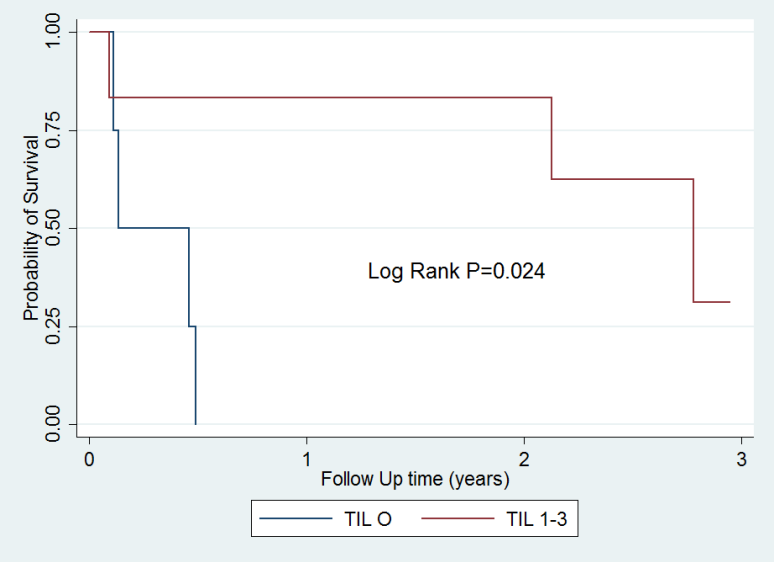

Figure 1. Kaplan-Meier curve for probability for survival by TIL 0 vs 1-3. Time=0 is the treatment start date.

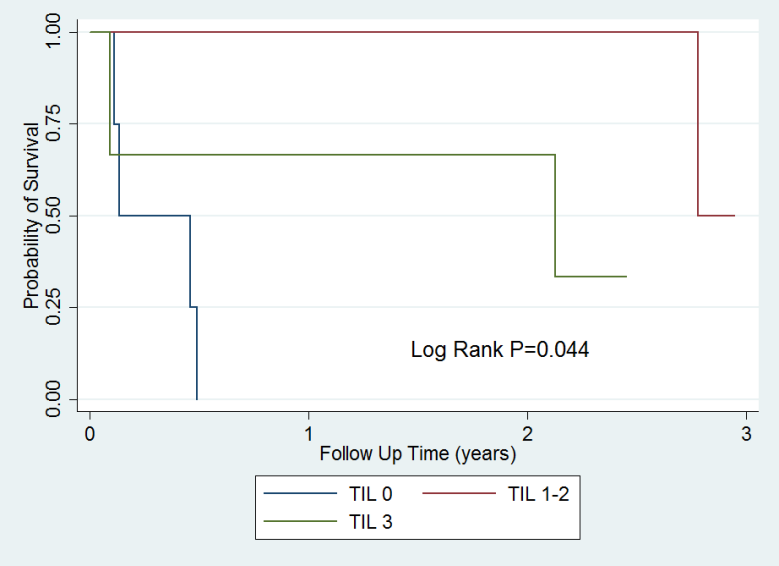

Figure 1. Kaplan-Meier curve for probability for survival by TIL 0 vs 1-2 vs 3. Time=0 is the treatment start date.

Discussion

While immune checkpoint therapies have been shown to be more effective than other therapies, there are still many patients that do not respond to treatment and continue to progress. Studies have established a greater response to immune checkpoint therapy in more immunogenic tumors. Tissue samples with a greater presence of immune checkpoint proteins correlate with longer survival compared to those that lack them. It is crucial to have a reliable biomarker that allows physicians to predict response to immune checkpoint blockades, and thus tailor treatments to the characteristics of the tumor.

Our study supports those of others in identifying TIL density as a predictor for immune checkpoint inhibitor response. We studied 10 patients who were treated with a form of immune checkpoint inhibitor for either metastatic melanoma or lung cancer. We confirmed the association between higher TIL levels and increased overall survival. In fact, when comparing patients with and without any TIL, there was a stark difference in overall survival. Comparing tier-based levels of TIL further supported the association, with patients with higher TIL levels having a longer OS. While this study included a low number of subjects, it can be concluded with statistical significance that a higher level of TIL was associated with a longer overall survival compared to groups with lower TIL levels in patients on immune checkpoint inhibitors. This ultimately lends to the concept of utilizing TIL level as a predictive biomarker to guide patient treatment. With precision oncology on the rise, the incorporation of predictive markers for response to immunotherapy is critical to identify which patients are most likely to benefit. Efforts have focused on profiling of the immune microenvironment and predictive immune-associated biomarkers. Thus far, PD-L1 expression has been the most studied biomarker thus far. It is the only biomarker of its type to have FDA approval for its immunohistochemistry staining as a companion diagnostic[16].

There are other immuno-oncologic biomarkers that have been studied; however, their clinical utility and availability is limited. These include mutation load, neoantigens, and ratio of CD8+ T cells to FoxP3+ regulatory T cells[16]. Other predictors of patient response to immunotherapy include age, smoking status, tumor histology, and treatment history[17]. It is

important to note that there are some obstacles in using immunologic markers. There is great variability in tumor samples within an individual patient, making some biopsy samples less reliable for these types of biomarkers[18]. Additionally, the immune environment of the tumor is dynamic in that it can be upregulated in general inflammatory processes and can change as treatment progresses. More research is needed to develop a companion diagnostic for these translational biomarkers, like that of PD-L1.

Perhaps the most dramatic outcomes of our study lie in the radiologic data, showing a greater decrease in tumor size in patients with higher TIL levels. Patients with any TIL density had a greater than 350% decrease in tumor size compared to those patients with no TIL's visualized on biopsy sample. Not only does this support existing evidence for enhanced patient response in tumors with high TIL levels, but it provides an objective measure by which to assess level of progression. There have not been any published studies utilizing imaging data to assess patient response to immunotherapy. A stronger establishment of the relationship between TIL, PD-L1, and patient response can ideally lend to the future development of a radiological biomarker for PD-L1 and/or TIL. More research is warranted to identify radiologic characteristics of responders vs. non-responders. This will allow for less invasive characterization of the tumor, as well as a more effective and efficient decision of therapy.

Future Directions

While this study showed strong and statistically significant correlations, it was limited by sample size. The inclusion criteria warranted over 100 patients, however many had to be excluded due to development of toxicities and adverse events from the immune therapies, and thus continued with palliative care. While leaving us with a sample size of 10, this speaks volumes to the need for a predictive biomarker to identify patients in whom the risks of the immune therapies are outweighed by the potential benefits. A larger sample size would undeniably provide a greater body of evidence to the findings in this study. A larger sample size for each TIL tier would provide more insight. There were other limitations to the study design, such as the limitations in the histologic staining. Because this was a retrospective review, biopsy slides could not be stained for specific T-cell markers, such as CD8+ and CD4+. Level of TIL was dependent upon the pathologist's experience in identifying lymphocytic infiltrate on the biopsy slides under light microscopy.

Newer technologies, including multispectral imaging and detection systems, can provide greater detail about the specific protein antigens and immune cell types in a single biopsy sample[12]. Future studies should utilize these technologies for better immune cell profiling and identification of predictive biomarkers. Furthermore, using this technology would address the need for criteria to quantify TIL and establish a threshold for predicted patient response. Future research should focus on development of diagnostic companions for research biomarkers as well as utilizing imaging for less invasive tumor characterization.

Conclusions

Despite the limitations, our study contributes to the body of evidence and movement towards establishing predictive translational biomarkers for immune therapies. We supported existing evidence showing increased overall survival in patients receiving immune checkpoint inhibitor therapy with higher TIL levels in the biopsied samples. More importantly, we showed radiologic data to support this, with greater tumor response in patients with higher TIL levels. There is promise in utilizing radiologic parameters for tumor characterization and follow-up.

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