Co-expression and Frequency of Immune Checkpoint Proteins in the Tumor Microenvironment Analyzed via Multiplex Immunohistochemistry

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INTRODUCTION

Antibody blockade of the PD-1/PD-L1 axis is approved for clinical use in multiple tumor types in an effort to block tumor immune evasion. The high number of emerging cancer immunotherapy agents targeting this pathway indicates that this axis has significant therapeutic potential. To support research in this area, we are developing tools to help investigators analyze the immunosuppressive tumor microenvironment. In this study, we applied a 7-color multiplex immunohistochemistry panel to a lung tumor tissue microarray consisting of FFPE adenocarcinoma and squamous cell carcinoma cores. The panel was composed of immune checkpoint receptors PD-1 and TIM-3; PD-L1, a PD-1 ligand; CD68 and CD8, macrophage and cytotoxic T cell markers, respectively; as well as cytokeratin as the tumor mask and DAPI as the nuclear counterstain. The array included three cores per patient, and the concordance between these triplicate cores was analyzed. Additionally, the frequency and co-localization of these markers and checkpoint proteins in the tumor and tumor microenvironment were quantified. TIM-3 expression was found not only on CD8+ T cells, but also on CD68+ macrophages, where it is thought to have a distinct function involved in phagocytosis of tumor cells. Furthermore, the significant role that macrophages play in tumor progression via promoting angiogenesis and inhibiting anti tumor immunity makes these data increasingly relevant. These data demonstrate how antibody-based multiplex analysis in tissue can provide insight into the various cell types involved in tumor-mediated immune evasion. Multiplex IHC with highly specific antibodies will serve to improve our understanding of how immunotherapies can be used to affect the innate and adaptive branches of the immune system in the course of cancer treatment.

METHODS

A seven-plex panel consisting of the antibodies listed below was optimized and applied to a formalin fixed paraffin embedded (FFPE) lung tumor array purchased from US Biomax, Inc (Rockville, MD). The array included triplicate lung adenocarcinoma and squamous cell carcinoma samples. This array was deparaffinized and rehydrated. After antigen retrieval, tissue was stained with a primary antibody, followed by SignalStain Boost #8114 (rabbit) or #8125 (mouse) from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies were applied serially, with heat mediated stripping in between each. Each core was then imaged with a Mantra™ quantitative pathology workstation and analyzed via the InForm™ image analysis software (both made by PerkinElmer, Waltham, MA). One case was excluded from the analysis due to a lack of CK+ tumor cells. Images were spectrally unmixed and quantitative data were obtained. The following antibodies from Cell Signaling Technology, Inc. were used with the listed conditions:

Target	Dilution	Order	Fluorophore
PD-1 (D4W2J) XP® Rabbit mAb #86163	1:400	1st	FITC
PD-L1 (E1L3N®) XP® Rabbit mAb #13684	1:1400	2nd	Cy5
CD68 (D4B9C) XP® Rabbit mAb #76437	1:100,000	3rd	AF594
TIM-3 (D5D5R™) XP® Rabbit mAb #45298	1:400	4th	AF555
CD8 (C8/144B) Mouse mAb #70306	1:200	5th	Cy5.5
Pan-Keratin (C11) Mouse mAb #4545	1:50	6th	AF350

CONCLUSIONS

- Concordance between tumor cores from the same patients with respect to numbers of CD8+ T cells, CD68+ macrophages, and CK+ tumor cells was low, with a Coefficient of Variance of 85.17%, 64.25%, and 42.1%, respectively.
- TIM-3 was expressed more frequently on CD68+ macrophages than on CD8+ T cells.
- CD8+ T cells positive for both PD-1 and TIM-3 were also observed, indicating the presence of exhausted T cells in some tumors.
- 71% of CD68+ macrophages expressed TIM-3, whereas only 29% of CD8+ T cells expressed TIM-3.
- PD-L1 expression was found to localize primarily on CK+ tumor cells, while a subset was also present on T cells and macrophages.
- Further investigation is required to understand the role of PD-1, TIM-3 and PD-L1 on multiple immune cell types and how this may impact the efficacy of immunotherapeutic agents targeting these receptors.

REFERENCES

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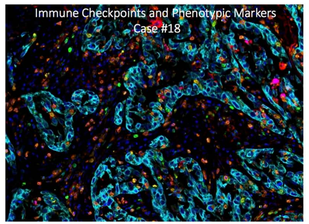

Figure 1A. Phenotypic markers as well as immune checkpoint receptors and ligands in the tumor microenvironment of a lung adenocarcinoma core. CK – cyan, DAPI – blue, PD-1 – green, TIM-3 – yellow, CD68 – magenta, PD-L1 – red, CD8 – orange.

7-Color mIHC

Figure 1B

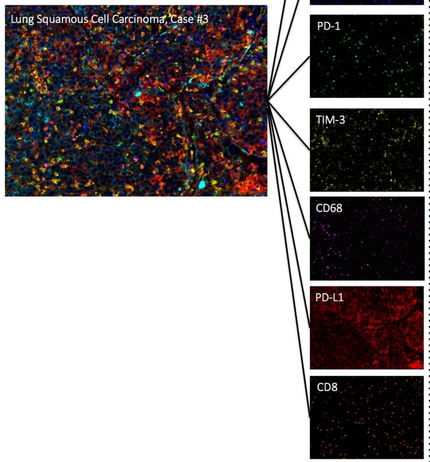

Figure 1B. Lung squamous cell carcinoma stained with the mIHC panel described, demonstrating that seven fluorescent signals can be clearly unmixed via spectral imaging.

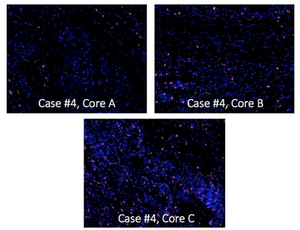

Figure 2A. CD8 expression among three different cores taken from the same lung adenocarcinoma. DAPI – blue, CD8 – orange.

Low Concordance Among Tumor Cores

Figure 2B

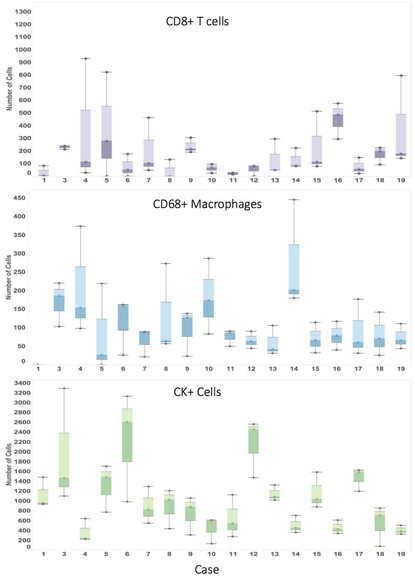

Figure 2B. Concordance among the three cores taken from the tumor with respect to numbers of CD8+ T cells (top), CD68+ macrophages (middle), or CK+ tumor cells (bottom).

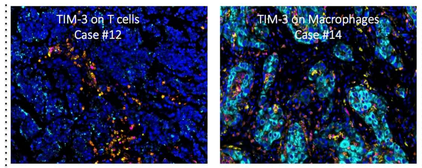

Figure 3A. TIM-3 is expressed by CD8+ T cells (A) and CD68+ macrophages (B). CK – cyan, DAPI – blue, TIM-3 – yellow, CD68 – magenta, CD8 – orange.

Expression and Co-Localization of Immune Checkpoint Receptors

Figure 3B

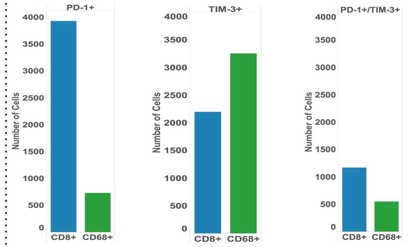

Figure 3C

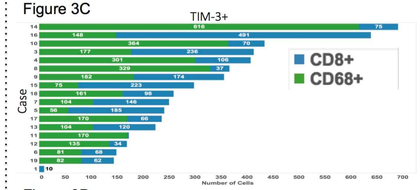

Figure 3D

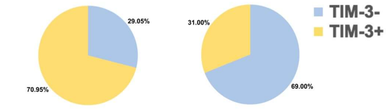

Figure 3B. PD-1 is primarily expressed on CD8+ T cells, while TIM-3 is more frequently expressed on CD68+ macrophages.
Figure 3C. A case by case analysis of TIM-3 expression on CK- cells, either CD8+ T cells or CD68+ macrophages.
Figure 3D. CD68+ macrophages in the TME (L) express TIM-3 more frequently than CD8+ T cells (R).

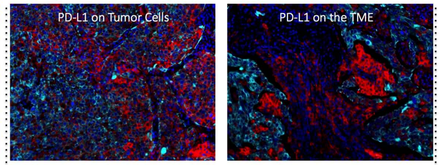

Figure 4A. PD-L1 localization varies among cores. Images depicting PD-L1 primarily on tumor cells (L) or within the TME (R).
CK – cyan, DAPI – blue, PD-L1 – red.

Heterogeneous PD-L1 Localization

Figure 4B

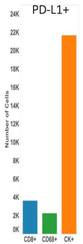

Figure 4C

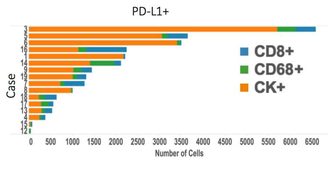

Figure 4D

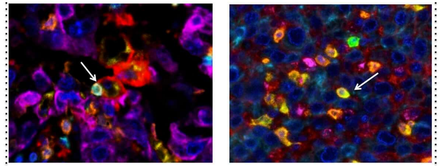

Figure 4B. PD-L1 is expressed primarily on CK+ tumor cells, compared to CD8+ T cells or CD68+ macrophages.

Figure 4C. PD-L1 expression broken down by case. Heterogeneity of the cores is apparent as PD-L1 localization varies from primarily on CK+ tumor cells in many cases to significant expression on cells of the TME in other cases.

Figure 4D. CD8+PD-1+ T cells proximal to PD-L1 expressed on macrophages (L) or tumor cells (R). On left: CK – magenta, DAPI – blue, PD-1 – cyan, CD68 – yellow, PD-L1 – red, CD8 – orange. On right: CK – cyan, DAPI- blue, PD-1 – green, CD68 – magenta, PD-L1 – red, and CD8 – orange.

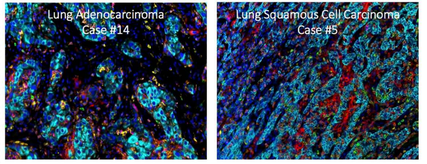

Figure 5A. Lung adenocarcinoma (L) and squamous cell carcinoma (R) cores stained with the described multiplex panel. CK – cyan, DAPI – blue, PD-1 – green, TIM_3 – yellow, CD68 – magenta, PD-L1 – red, and CD8 – orange.

Similar Immune Cell Checkpoint Receptor Phenotypes Among Different Tumor Types

Figure 5B

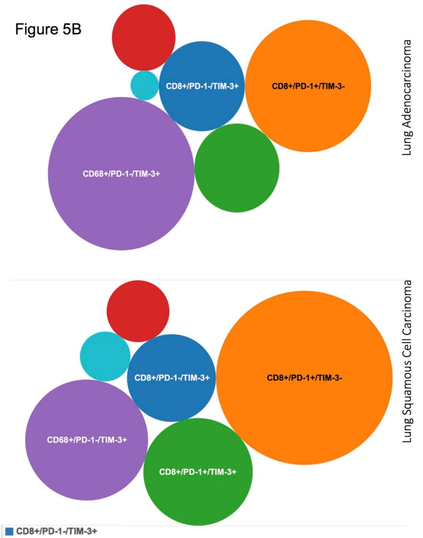

Figure 5B. Distribution of immune cell phenotypes present in lung adenocarcinoma vs. squamous cell carcinoma cores.