Favourable diffuse prognostic pattern of FOXP3+ and CD69+ T cells in follicular lymphoma demonstrated using automated imaging and analysis

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Abstract
Background: In many cancers, tumour-infiltrating lymphocytes (TILs) indicate levels of tumour immunity and are a strong predictor of survival. In particular, increased levels of regulatory T cells (Tregs) are associated with poorer prognosis in some cancers. However, visual TIL assessment cannot easily determine the phenotype of lymphocytes in situ. An understanding of both the phenotypes and spatial distribution of TILs in vivo within tumor regions would be advantageous. Here we present a multi-marker, computer-aided quantification method for analysing the distributions of CD3/FOXP3 (Treg) and CD3/CD69 (Tact) T cells in follicular lymphomas using a multiplexed imaging (MSI) and automated analysis approach. An hypothesised interaction domain (HID) analysis was used to determine whether the spatial patterns of Tregs and Tact was prognostically significant.

Design: A single section of a tissue microarray containing triple follicular lymphomas cores from 40 subjects (24 male, 16 female, age 35 to 75 years at diagnosis, median age 64 years, 2-171 months follow-up) was stained for CD3, FOXP3, CD69 and hematoxylin. Each core was imaged using MSI and the individual staining of each marker acquired from each area using spatial unmixing. CD3+ and CD69+ TILs were used as automated analysis workflow.

Results: Multiplexed staining, MSI and automated per-cell quantitation analysis was successful. Kaplan-Meier analysis demonstrated favourable outcome with higher numbers of CD3+ and CD69+ cells and CD3/CD69+ cells. This analysis demonstrated the association of favourable outcome with a high entropy/diffuse pattern of CD3+ and FOXP3+ T cells.

Conclusions: In this study we report that higher Treg cell counts in a diffuse pattern was associated with favourable outcome. This supports the importance of Tregs in the tumour microenvironment. It is pertinent to mention that contradictory findings are routinely reported from studies investigating the role of Tregs in solid and haematological malignancies. This is due to the complex interactions between pro-anti-tumour immune factors present in the tumour microenvironment. The resultant effects are due to the summation of the activities of these factors. It is therefore even more relevant that a method such as exhibited here, capable of defining, and measuring the effect on biological behaviour, in this case patient outcome, of cellular pattern is available, as demonstrated for the HID method in the present study.

Methodology

Key Component #1: Multiplexed staining

**Brightfield (H&E) up to 3 Abs**

- Multiplexed staining is a process in which multiple markers are stained on a single section and then studied with a microscope. This allows for the simultaneous visualization of multiple cellular components.
- The use of multiplexed imaging (MSI) and linear mixing makes it possible to be able to acquire four or more primary markers at a time. MSI analyses multiplexed single sections with four or more primary markers at a time.
- **T**vox fluorescence multiplexed based signal, faster scanning, amplification in false color signals, and easy multiplexing.

Key Component #2: Automated per-cell quantitation

- **Pre-cell and per-compartment quantitation of multidrug markers**
  - Automated workflow replicates the tasks a human would do in finding areas of interest on a high-content image, which can be further processed. This can greatly improve throughput saving time in autometry.

Methodology example: PD-L1 in melanoma

- **Fluorescence H/E up to 8 Abs**
  - The use of multiplexed imaging (MSI) and linear mixing makes it possible to be able to acquire four or more primary markers at a time. MSI analyses multiplexed single sections with four or more primary markers at a time.
  - **T**vox fluorescence multiplexed based signal, faster scanning, amplification in false color signals, and easy multiplexing.

Clinical correlation of results in follicular lymphoma

- **Overview of Kaplan-Meier Analysis**
  - Higher numbers of CD3+ single positive cells were significantly associated with a favourable outcome by Kaplan-Meier analysis for cell survival split at the lower quartile (p=0.017), the median (p=0.017) and the upper quartile (p=0.017). Higher numbers of FOXP3+ single positive cells were associated at the upper quartile (p=0.017). Higher numbers of FOXP3+/CD69+ double positive cells were significantly associated with a favourable outcome by Kaplan-Meier analysis for cell survival split at the median (p=0.017) and at the upper quartile (p=0.017).
  - These examples of these Kaplan-Meier plots are shown above.

- **Overview of Higher-dimensional Interaction Domains (HID) analysis used to identify prognostic patterns of multiple markers**
  - The HID analysis can be performed in a single stage and can be used to identify patterns of markers that are associated with a specific outcome.
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Conclusions

- **Multispectral imaging enabled the per-cell quantitation of these immunostains (CD3, CD69 & FOXP3) in intra- and extra-follicular compartments in follicular lymphoma.**
- The enumeration of FOXP3+ and CD69+ T cells (CD3+) in these clinical samples was effective and easy to perform.
- **Increased numbers of FOXP3+/CD69+ lymphocytes all correlated with improved outcomes.**
- **HID analysis showed a correlation between high-entropy T cell distributions and overall survival.**
- **This panel of immune markers can easily be changed to a panel of whichever markers are of interest (CD4, CD8, CD20, CD68, PD-L1, Ki67, etc).**