Multiplex immunofluorescence for investigating the prognostic value of immune cells

Chidozie Anyaegbu, PhD

Catholic Health Australia Symposium
Mater Hospital, Brisbane
1st June 2018
Why colorectal cancer?

- Originate from epithelial cells lining digestive tract
- Accounts for 10% of cancer-related deaths - 2nd most deadly in Australia
- Most commonly diagnosed gastrointestinal cancer and the leading cause of cancer-related death for both men and women, with over 14,000 new cases and 4,000 deaths annually
- Incidence expected to increase

Data source: Australian Institute of Health and Welfare
Why colorectal cancer?

- Classified into 4 stages based on TNM (Tumour Node Metastasis) system

- Treatment for advanced disease: a combination of surgical resection, chemotherapy and/or radiotherapy to reduce the risk of relapse

- At least 20% of Stage III and IV patients relapse and die within 5 years
Clinical estimation of prognosis

- No reliable indicators of prognosis

- A selection of clinical and pathological factors are currently used in combination with variable accuracy
  - E.g. Age, TNM Stage of disease, Microsatellite instability, Histological Type and Tumour Antigen Levels

- Superior or complementary markers of prognosis are needed

- Improve identification of patients at high-risk of relapse for proactive personalised treatment
  - Could reduce unnecessary treatments which offer little to no benefit, improving quality of life and providing an economic benefit for the Health Department
The rationale for immunological prognostic markers

- Immune system plays an important role in cancer control
- Infiltration of immune cells into colorectal tumours generally correlate with good prognosis
- The density, phenotype and location of T cells within colorectal tumours have even been shown to be a better indicator of prognosis than pathological staging (Pages et al, J Clin Oncol 2009)
- International collaborative efforts are underway to standardise T cell assessment for inclusion in routine pathology review (Galon et al, J Pathol 2014)
- Various methods can be used for immune cell characterisation
  - Gene expression analysis
  - Flow cytometry
  - Immunohistochemistry
- Immunohistochemistry has an important advantage
  - Sectioned tissue specimens preserves context, allowing spatial understanding of cellular interactions within tumours, which is incredibly complex
The tumour microenvironment: a complex landscape

Lymphatic endothelial cells
- Tumor cells can invade existing lymphatics or stimulate lymphatic vessel sprouting with the production of factors, such as VEGFC or VEGFD.
- Lymphatic vessels are important in the dissemination of malignant cells, but they may also promote tumor growth by mediating the TME and altering the host immune response to the tumor.

T lymphocytes
- Abundant in the majority of human cancers, but not all cells in the tumor.
- Found within and surrounding the tumor.
- Phenotypes of pro- and anti-tumor T cells can vary with disease type and stage. CD8+ cytotoxic T cells, CD4+ Th1 helper T cells, and Th17 T cells are usually associated with a good prognosis.
- PD-1+ T regulatory cells (CD4+ Tregs) within T cells and TME T cells are usually associated with a poor prognosis.

B lymphocytes
- Sometimes found at the invasive margin of some tumors, but more often in secondary lymphoid tissues adjacent to the TME.
- B cell infiltration is associated with a better prognosis in some human cancers. However, deposition of B cells within and immune responses in tumors, promoting in some mouse cancer models.
- Immunosuppressive IL-10 producing subtypes of B cells, B1 cells, and B1a cells also have tumor-promoting activity in mouse models.

Myeloid cells
- Consist of several subsets: probably the most abundant cell lineage in the TME.

- Tumor-associated macrophages (TAMs)
  - Typically tumor-promoting.
  - M1-like, IL-12/Th1 phenotype and macrophage receptor-positive.
  - TAMs also promote angiogenic factors and accumulate in hypoxic or necrotic areas of the TME.

- Myeloid-derived suppressor cells (MDSCs)
  - Immunobiologically producing large amounts of IL-10.
  - Induce cytokines and polypeptides, and TAMs to a tumor-promoting phenotype.

- Tumor-associated neutrophils (TANs)
  - Can have both pro- and anti-tumor activity.

- Terminally differentiated myeloid dendritic cells
  - Might be defective in the TME and cannot adequately stimulate an immune response to tumor-associated antigens.

NK and NKT cells
- Invasive cytotoxic lymphocytes, NK cells, and NKT cells are usually found outside the tumor area.
- For some cancers, they can predict a good prognosis.

Cancer-associated fibroblasts
- Found in many human and experimental cancers, especially at the invasive margin.
- Produce tumor-promoting growth factors, chemokines, cytokines, ECM components, and ECM remodeling enzymes.
- Can also have important immunosuppressive activity.

Vascular endothelial cells
- Angiogenic factors produced by malignant cells, myeloid cells, or TAMs in the TME stimulate sprouting of endothelial cells.
- The new blood vessels have chaotic branching and المهني, an abnormal structure.
- The vessels are also leaky, raising interstitial pressure, with uneven blood flow, oxygenation, nutrient, and drug delivery in the TME.

Mesenchymal stem cells
- Mesenchymal stem cells can be recruited from the bone marrow and give rise to CAFs, pericytes, adipocytes, and smooth muscle cells in the TME.

Adipocytes
- In some cancers, adipocytes actively recruit and promote recruitment of malignant cells through the secretion of adipokines.
- They also promote malignant cell growth by providing fatty acids as fuel for cancer cells.

Pericytes
- Perivascular pericytes, CAFs, provide structural support for blood vessels in the TME.
- The pericyte coverage, increasing the thickness of the TME, may also reduce oxygen diffusion and increase the thickness of the TME.

Balkwill et al, J Cell Sci 2012
Multiplex immunofluorescence (IF)

- Allows assessment of multiple markers on the same tissue section
  - Ideal for profiling immune cells in tumour specimens
IF Workflow

Staining

Image Acquisition

Image Analysis

Primary Antibody

Secondary Ab - HRP

TSA Fluor

MWT Strip

Primary Antibody

Secondary Ab - Fluor

Mounting Medium

Repeat
IF Workflow

Staining → Image Acquisition → Image Analysis

Fluorescence slide scanner

Panoramic MIDI II (3D Histech)
IF Workflow

Staining

Image Acquisition

Image Analysis

Qualitative Analysis - CaseViewer

Quantitative Analysis - StrataQuest
# Multiplex IF immune cell staining panels

<table>
<thead>
<tr>
<th>PANELS &amp; TARGET CELLS</th>
<th>Panel 1: Tregs</th>
<th>Panel 2: CD8+ T cells</th>
<th>Panel 3: NK cells</th>
<th>Panel 4: cDC1s</th>
<th>Panel 5: cDC2s</th>
<th>Panel 6: MoDCs</th>
<th>Panel 7: PD-L1+ DCs &amp; CD8s</th>
</tr>
</thead>
<tbody>
<tr>
<td>MARKERS</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>Nuclei</td>
</tr>
<tr>
<td></td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td></td>
<td>Foxp3</td>
<td>PD-L1</td>
<td>CD56</td>
<td>CD86</td>
<td>CD86</td>
<td>CD86</td>
<td>CD11c</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>PD-1</td>
<td>PD-1</td>
<td>CD11c</td>
<td>CD11c</td>
<td>CD11b</td>
<td>PD-L1</td>
</tr>
<tr>
<td></td>
<td>Ki67</td>
<td>CD8</td>
<td>CD4</td>
<td>CD141</td>
<td>CD1c</td>
<td>CD14</td>
<td>CD8</td>
</tr>
</tbody>
</table>
Panel 1 - Tregs

Tumour CD4 Foxp3 Ki-67 Nuclei
Traditional chromogenic method vs multiplex IF

**Chromogen**

- Foxp3 Hx

**IF**

- Foxp3 Tumour CD4 Ki-67 Nuclei

- Density
- Identification of co-expressing markers
- Functional phenotype
- Spatial relationships
- Intra- and extra-tumoural localisation
Image analysis - StrataQuest

Tissue Detection

Tumour vs Stroma

Nuclear Segmentation

Multiplex marker analysis + scattergrams

Proximity analysis
Summary

Developed a multiplex IF method

- Cutting-edge, multi-parametric profiling of immune cells in tumour specimen
- Discovery of novel immunological biomarkers of prognosis and response to treatment
- Allow better identification of patients with poor prognosis for tailored treatment

Current Work

- Using these panels to profile tumour-infiltrating immune cells in various cohorts of colorectal cancer patients
Acknowledgements

St John of God Subiaco Hospital Colorectal Research Group
- Prof Cameron Platell
- Dr Melanie McCoy
- Ms Tracey Lee-Pullen
- Dr Tim Miller

Telethon Kids Institute
- Dr Tamara Abel
- Dr Raelene Endersby
- Hilary Hii

Community Representative
- Mr Terry Iannello

University of Western Australia
- Dr Paul Rigby
- Alysia Hubbard

Funding
- Tonkinson Foundation for Colorectal Cancer Research
- Cancer Council Western Australia
- St John of God Foundation

Patients
The orchestration of anti-tumour immune responses is incredibly complex

Adapted from Lake & Robinson, Nature 2005