ImmunoScore/Immune profiling in Melanoma

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Tumor microenvironment

- DC
- Mast cell
- MDSC
- Tumor core
- Immature DC
- Invasive margin
- NK cell
- B cell
- CTL
- FDC
- T<sub>FH</sub> cell
- TLS
- Stroma
In fact the Immune contexture influence multiple aspects of tumor development and present multiple opportunities to gauge this response in order to make the best treatment decisions:

» Prognostic Value: The presence of the right immune effector cells is correlated with better prognosis and survival (staging criteria?)

» Immunotherapy: Manipulate the patient’s own immune system to respond to the tumor (Ipi, etc…)

» Chemotherapy: Response to CDX may also be immune-related
The Immunoscore in colorectal cancer

- Great tool for **Staging**

Courtesy of Jerome Galon
# ImmunoScore and Immunoprofiling

<table>
<thead>
<tr>
<th></th>
<th>Immunoscore</th>
<th>Immunoprofiling</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of immune markers</strong></td>
<td><strong>2-4</strong></td>
<td><strong>1 – Several</strong></td>
</tr>
<tr>
<td><strong>Immunoscore markers</strong></td>
<td>CD3/CD8</td>
<td></td>
</tr>
<tr>
<td><strong>Possible application</strong></td>
<td>• Staging in <strong>Melanoma</strong>, Breast cancer, Ovarian cancer, NSCLC, Prostate cancer, Pancreatic cancer, Head &amp; Neck cancer (to be defined).</td>
<td>• Prognostic assay, Predictive assay, Personalized immune-treatment</td>
</tr>
</tbody>
</table>

- Melanoma ImmunoScore in Lymph Nodes

- MISIPI study: Melanoma ImmunoScore evaluation in patients treated with Ipilimumab

- Melanoma ImmunoScore: Discovery of predictive biomarker for IPILIMUMAB response
Melanoma ImmunoScore in Lymph Nodes
Why the Lymph Nodes Represent an Interesting Model for the ImmunoScore

1. In many cases, the metastatic lymph nodes from lymphectomy is the only available tissue, Lymph nodes are more accessible vs visceral metastases;

2. More patients with lymph node+ (stage III), high risk of distant metastases;

3. Previous works evidenced the importance of the Lymphnodes and associated microenvironment for melanoma progression and outcome:
   - Mohos et al. Immune cell profile of sentinel lymph nodes in patients with malignant melanoma - FOXP3+ cell density in cases with positive sentinel node status is associated with unfavorable clinical outcome. JTM 2013.

The evaluation of TME in the lymph nodes could be important for better classify patient and for predict clinical outcome;
AIM

To explore evidence of correlation between the immuno infiltrate in Lymphnodes and patients outcome;

• On lymphadenectomies from 34 Stage III melanoma patients, for a total of 277 lymph-nodes;
• Analyzing 5 immuno markers (FoxP3, CD3, CD8, CD20, S100) expression;
• Firstly, evaluating manually a multiplex IHC stained slides with the 5 markers all together;
• After the development of an algorithm, evaluating single stained IHC slide for the 5 marker, in an automatic way.
Concerns on ImmunoScore in Lymph Nodes

1. The lymph nodes are the “house” of immune cells, constitutively rich in CD3 and CD20 lymphocytes;

2. The evaluation of the periphery of the tumor is particularly complex (it might be defined as 0.4 mm deep from the tumor but difficult to apply to lymph nodes).

3. Lymph node metastases may be different in terms of immune infiltration compared to other metastatic lesions.

We have walked over!!
Data Analysis *(manual counting in multiplex slides)*

For each patient, we analyzed the cell counts for all markers (summarized as median expression across the different sampled nodes of the same), and the obtained values were then compared between relapse and no relapse groups.

In the reactive lymph nodes, there are no clear differences in expression levels between relapse and no relapse groups, except for CD8, where there are more patients with high expressing cells in the relapse group.

*Capone et al. SITC 2014.*
In metastatic LNs there were significant differences in the peri/intra tumoral ratio for both CD3 and CD8, with the ratio being higher in no relapse patients compared to relapse patients for both proteins.

Similar differences were seen in FoxP3 and CD20, although power to detect statistically significant was low given our small sample size.

All these data have been useful for to develop the algorithm.
The Algorithm

1. Slides scanned
2. Whole slide images (from serial sections stained with different markers)
3. Automatic region identification from tumor marker slide (MTC)
4. Oncor-mure Analysis Report
5. Report of Cell Counts and Region Labels for Each Patient
6. Automatic cell counting for each selected region
7. Transfer region labels to other marker slides via image registration

Capone et al. SITC 2014.
Definition for the Algorithm
Data Analysis (automatic counting in single staining slides)

- Such analysis was performed on a first cohort of 30 patients.
- For each factor patients were divided in two groups: below median value (low-blu line) and over median value (high-green line);
- Correlate markers expression with survival;
- For patients with more than 1 slide evaluation the mean for each parameter was calculated;
- The value 0.0 was considered as a value and not a missing data.
- Overall survival was measured from dissection date. P value refers to the log-rank test;
intratumoral

**CD3**
P = 0.89

**CD8**
P = 0.80

**CD20**
P = 0.64

**FP3**
P = 0.64

peritumoral

**CD3**
P = 0.54

**CD8**
P = 0.80

**CD20**
P = 0.61

**FP3**
P = 0.76
CD3 intra and peri

P=0.97

CD8 intra and peri

P=0.08

FP3 intra and peri

P=0.93

CD20 intra and peri

P=0.57

both_high
both_low
discordant
Results and Comments

Any evidence of correlations between infiltrate and outcome.

Manual count:

• There are differences in expression levels in the reactive lymph nodes for CD8, high in the relapse group.
• There were significant differences in the peri/intra ratio for both CD3 and CD8, with the ratio being higher in no relapse patients.

Automatic count:

• Initial analysis performed in a small cohort of heterogeneous patients (macrometastases, micrometastases, isolated tumoral cells, etc.), power to detect statistically significant differences low.

We need

• to evaluate a larger and homogenous cohort of patients
• to explore other markers.

But...

The Algorithm is ready!
MISIPI Study: Melanoma ImmunoScore evaluation in patients treated with Ipilimumab
AIM
To evaluate the possible prognostic and predictive value of the immunoscore in patients receiving ipilimumab.

- Immunescore based on the analysis of CD3, CD8, CD68, CD163 and FoxP3 may yield a new approach to classify, prognosticate, and potentially predict response to Ipilimumab for metastatic melanoma patients.
MISIPI Study

- 200 FFPE samples from 150 metastatic melanoma patients treated with Ipilimumab;

- characterized the immune infiltrate and the density of different cellular immune population using the immunohistochemically detected expression: CD3, CD8, CD68, FoxP3 and CD163;

- Correlate marker expression profile with clinical outcome;

  ...Study ongoing...

Meanwhile, evaluation of possible correlation between PD-L1 expression and patient outcome.

AIM:
To evaluate correlation between baseline levels of PD-L1 (through DAKO assay) and outcome prediction in melanoma patients treated with Ipilimumab.

CTLA-4 and PD-L1 play different but complementary roles in the regulation of adaptive immunity!
## Patients characteristics

<table>
<thead>
<tr>
<th></th>
<th>61 years (25-90)</th>
<th>57 (50%) 57 (50%)</th>
<th>114/114 pts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male/female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV Melanoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutated</td>
<td>37 pts (32.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>66 pts (57.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>11 pts (9.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of previous therapy, n(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15 (13.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>83 (72.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16 (14.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous therapy type*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDDP+TMZ</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dacarbazine</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fotemustine</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temozolamide</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAGE A3 (PRAME)</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEK 162</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dabrafenib</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrochemotherapy (Bleomicin + Electroporation)</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other (L19-IL2, Paclitaxel, Allovectin, CBDCA + DTC, etc)</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*More than 114 therapies because some patients performed more than 1 line of treatment*
### Ipilimumab Response

<table>
<thead>
<tr>
<th>Patients evaluable</th>
<th>109 Pts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipilimumab cycles (median, range)</td>
<td>(4, 1 – 4)</td>
</tr>
<tr>
<td>4</td>
<td>75 (65.8%)</td>
</tr>
<tr>
<td>3 (2 stopped for toxicity)</td>
<td>16 (14.0%)</td>
</tr>
<tr>
<td>2</td>
<td>14 (12.3%)</td>
</tr>
<tr>
<td>1 (not evaluable – rapid progressors)</td>
<td>9 (7.9%)</td>
</tr>
<tr>
<td>Immuno-Related Disease Control Rate</td>
<td>25 (23%)</td>
</tr>
<tr>
<td>Immune-related DCR</td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>11 (10.1%)</td>
</tr>
<tr>
<td>Partial response</td>
<td>4 (3.7%)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>10 (9.2%)</td>
</tr>
<tr>
<td>Progression of disease</td>
<td>84 (77%)</td>
</tr>
</tbody>
</table>
PD-L1 status and Survival

PD-L1 status using a 5% tumor cell expression cut-off

- Positive (score > 5): 21 pts
- Negative (score <5): 88 pts

PD-L1 status using a 1% tumor cell expression cut-off

- Positive (score > 1): 35 pts
- Negative (score <1): 74 pts
## PD-L1 status and IPILIMUMAB Response

<table>
<thead>
<tr>
<th>IPI_response</th>
<th>Score &lt;5</th>
<th>Score &gt;5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>9 (10.2)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>PR</td>
<td>2 (2.3)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>SD</td>
<td>10 (11.4)</td>
<td>0</td>
</tr>
<tr>
<td>PD</td>
<td>67 (76.1)</td>
<td>17 (81.0)</td>
</tr>
</tbody>
</table>

\[ P = 0.18 \]

<table>
<thead>
<tr>
<th>IPI_response</th>
<th>Score &lt;1</th>
<th>Score &gt;1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>8 (10.8)</td>
<td>3 (8.6)</td>
</tr>
<tr>
<td>PR</td>
<td>2 (2.7)</td>
<td>2 (5.7)</td>
</tr>
<tr>
<td>SD</td>
<td>9 (12.2)</td>
<td>1 (2.9)</td>
</tr>
<tr>
<td>PD</td>
<td>55 (74.3)</td>
<td>29 (82.9)</td>
</tr>
</tbody>
</table>

\[ P = 0.37 \]
PD-L1 was not a predictive biomarker for overall survival of melanoma patients treated with Ipilimumab; PD-L1 status cannot used to select patients for ipilimumab.

- PD-L1+ expression on tumor cells has been associated with favorable response rates in studies of nivolumab. (Topalian SL, N Engl J Med, 2012)
- PD-L1–negative/indeterminate expression had an improvement of overall survival with nivolumab compared with dacarbazine. (Michael A. Postow, The ASCO Post 2015)

PD-L1 not a good predictive biomarker for overall survival and for patients selection for Nivolumab:

Our findings are enhanced!!
Probably due to heterogeneous expression of PD-L1 between patients and within individual patients (Madore J, et al. Pigment Cell Melanoma Res. 2015);

It is possible that a model with more than one immunologic factor, may help us to correlate PD-L1 and some aspects of melanoma disease.

We have an ongoing project where we correlate PD-L1 status and Mutational status with outcome of melanoma patients...interesting initial results!!!
Melanoma ImmunoScore: Discovery of predictive biomarker for IPILIMUMAB response
Discovery of predictive biomarker for IPILIMUMAB response

AIM:
To evaluate correlation between expression levels of CD3, FoxP3 and outcome prediction in melanoma patients treated with Ipilimumab.

Patients evaluated 31
Immune-related DCR 16
Complete response 11
Partial response 1
Stable disease 4
Progression of disease 15

Analysis was performed on serial IHC stained section with:
H&E (morphology, tumor cells)
CD3 (T cells)
FoxP3 (regulatory T cells, Tregs)
Image Analysis and Data Mining Workflow

• Automated alignment of H&E, CD3 and FoxP3 tissue sections;
• Detection of tumor cells, CD3$^+$ and FoxP3$^+$ cells;
• Produce an image overlapping signals;
• On tumor cells regions (red) make automated measurement of CD3$^+$ (green) and FoxP3$^+$ (blue) evaluating center and border;
• Image analysis results was delivered in a scoresheet for the most predictive factors.

Definiens Image Miner®
Automated Alignment of Serial Sections for Co-Expression Analysis

Software works on an image taken by overlapping the signals obtained from the different slides.

Tumor cell: red  CD3: green  FP3: blue
Results and Comments

First results indicate that:

- Tumor cells-FoxP3 spatial relations are the most predictive factors: IPILIMUMAB Therapy benefit if $\text{FoxP3}_{\text{TB}}^+ > 20\% \text{FoxP3}_{\text{TC}}^+$;
- CD3/FoxP3 ratio on tumor margin seems to be a promising additional factor;
- CD3 alone doesn’t provide predictive information.

These early results need further validation with larger cohorts and other biomarkers:

- CD8 (ready, ongoing a re-analysis of the results including any added data derived from CD8 analysis)
- CD45RO, CD163, CD68 and other markers.
Additional investigations with immuno markers predictive for outcome and for response to the therapies remain of great interest.

To evaluate the functionality of the different immune cells in the tumor microenvironment may be a good future prospective.

TO BE CONTINUED...
Acknowledgement

Oncologists, surgeons and nurses and the **PATIENTS** Who consented to these studies
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