In situ Biomarker analysis in cancer Immunotherapy: development of quantitative multiplex IHC

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There is now growing evidence that the immune contexture influences cancer progression and clinical outcome of patients. The tumors microenvironment (TME) is the bed of cancer progression and the target of increasing drugs in development. The objective is to develop and partially validate multiplex IHC panels to analyze the human immune TME. The developed panels enabled to analyze the lymphocyte compartment (TIL), the macrophage status (M1 versus M2) and the presence of Tertiary Lymphoid Structures (TLS) in clinical sample. Multiplex method and quantitative analyses allowed to obtain maximum information about TME in precious clinical sample (biopsies).

Material and Method

Single and Multi-stainings were performed using OPAL system from Perkin Elmer on FFPE Human tumor section: Breast carcinoma, Lung carcinoma ...

Assessment of three panels:
- TILs (Tumors infiltrating Lymphocytes): CD4-CD8-CD20
- TLS (Tertiary Lymphoid Structure) CD3-CD20-DClamp
- TAMs (Macrophages differentiation) CD163-CD68

Used Antibodies

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>References</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>NCL-CD4-368</td>
<td>Novocastra</td>
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<tr>
<td>CD8</td>
<td>M 7103</td>
<td>Dako</td>
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<td>CD20</td>
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<td>CD3</td>
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<td>CD20 DClamp</td>
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<td>Dendritics</td>
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<td>CD68</td>
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<tr>
<td>CD163</td>
<td>NCL-CD163</td>
<td>Leica</td>
</tr>
</tbody>
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IHC process OPAL

- Pretreatment
- Chemical Antigen Unmasking at 95° C
- Primary Antibody
- Secondary Antibody
- Secondary Antibody + Plus
- Revelation TSA OR TSA+
- Nuclear Counterstain

Image Analysis assay

- Microscopic observation
- Hamamatsu Nanozoomer
- Triken Calypix (Stained Objects by Learning)

Results

TILs CD4-CD8-CD20

Lung Carcinoma

Breast Carcinoma

Lung Carcinoma

Breast Carcinoma

Lung Carcinoma

Quantitative analysis on TILs

Validation process (ongoing on TIL)
- Objectives
  - Multiplex versus simplex labelling
  - Quantification method via Calipix
  - Repeatability: repeat process by one operator
  - Reproducibility: repeat process by two operators

- First observations (raw data): Variability induced by methods:
  - Between simplex and multiplex
  - ROA definition: tumor versus parenchyma/necrosis ...
  - Tumor type: difficult for HCC, easier for NSCLC ...

Conclusion and Next Steps

Development of new in situ biomarkers is essential to understand the influence of TME on tumor progression for immunotherapy. The assessment of multiplex IHC panels allows the immune "phenotyping" of this TME. In addition, we start to develop a validated process to quantify these biomarkers.

Next step:
- Complete validation of quantification: increasing the repeatability and the reproducibility data (including a third operator)
- Describe an harmonized quantitation process for future analysis
- Other markers:
  - Tumor architecture via Pan Cytokeratin, CD31, MHC I...
  - Innate immunity: NK, Neutrophil, regulatory cells Treg, Marker M1 : INOS