

Differential and longitudinal immune gene patterns associated with reprogrammed microenvironment and viral mimicry in response to neoadjuvant radiotherapy in rectal cancer

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Supplementary Information

Supplementary Methods:

Immunohistochemistry for CD68 and CD163

After heat-mediated antigen retrieval in 10mM citric acid buffer, pH 6.0, for 10 minutes in a 900-watt microwave, slides were washed under running water. To block endogenous peroxidase activity 100µL of dual endogenous enzyme block was pipetted onto each slide for 5 minutes followed by a rinsing in Tris-buffered saline (TBS) for 5 minutes.

100µL of antibody diluent was applied to all specimens to block nonspecific binding. 100µL of CD163 primary antibody (mouse monoclonal clone immunoglobulin (IgG1)/EDHu-1), diluted at 1:200 was added to each slide. All slides were incubated for 60 minutes after which they were washed in TBS with Tween 20 (TBS-T) (2 x 5 minutes) and TBS (5 minutes). 100µL of horseradish peroxidase conjugated polymer was applied as a secondary antibody to the sections and incubated for 10 minutes, and washed as above. 3, 3'

diaminobenzidine tetrahydrochloride (DAB+) chromogen solution (100 μ L, 10 minutes) was applied to allow visualisation of the first primary antibody. Slides were washed in running water for 5 minutes to remove unbound DAB+ chromogen. 100 μ L of double stain block was incubated on the slides for 3 minutes after which it was washed off with TBS-T (2 x 5 minutes) and TBS (5 minutes).

100 μ L of the antibody CD68 (mouse monoclonal clone IgG1/KP1) diluted at 1:3000 was added to each slide and incubated for 60 minutes at room temperature. Washing steps were performed as mentioned above. Following the washing of the slides, 100 μ L of a secondary antibody rabbit/mouse link solution was pipetted on for 10 minutes, followed by washing as above. A final 100 μ L of alkaline phosphatase polymer was applied for 10 minutes and washed as above. The second dye-stain to aid in visualisation of the immunohistochemical reaction, permanent red working solution, was applied to all specimens (100 μ L, 15 minutes). Following this final staining, the slides were washed in running water for 5 minutes, and counterstained with Mayer's Haematoxylin for 1 minute. The slides were dehydrated on a hotplate at 70 $^{\circ}$ C for 20 minutes, before being immersed in xylene in preparation of being mounted with cover-slips using DePex mountant (VWR BDH Prolabo).

Supplementary Table 1: Genes upregulated in intermediate responding tumours versus tumours showing a good response (false discovery rate <0.04).

| Gene Name |
|-----------|
| ITGAX |
| ICAM1 |
| CDH5 |
| TNFSF13B |
| TLR1 |
| CCL28 |
| ENTPD1 |
| PLAU |

Supplementary Table 2: The Molecular Signature Database (MsigDB's) Investigate Gene Sets of hallmark gene sets - Overlap Results - Enrichment analysis of Chauvin's publication (1): See attached excel spreadsheet

Supplementary Table 3: ARCHS4 table of cell populations. This table shows a statistical comparison of the cell populations in the ARCHS4 tissue database that are enriched in the baseline biopsies of poorly responding tumours versus tumours showing a good response to radiotherapy: See attached excel spreadsheet.

Supplementary Table 4: Gene lists from the ARCHS4 database that were used to

| Cell Population (ARCHS4) | Genes |
|-----------------------------|---|
| MACROPHAGE | CD53;IL15RA;MSR1;CD274;CD40;CD163;ITGAM;IL1R1;IL1R2;ITGB3;IL24;LILRB2;LILRB3;LILRA5;ICAM1;TLR1;CLEC4A;HCK;FCGR3A;PLAU;SPP1;ITGAX;CCL2;CCL18 |
| ALVEOLAR-LIKE MACROPHAGE | CD53;MSR1;CD274;CD163;ITGAM;ITGA4;IL1R2;LILRB2;LILRB3;ICAM1;TLR1;CLEC4A;HCK;FCGR3A;PLAU;ITGAX;CCL2;CCL18 |
| NEUTROPHIL | CD53;CD274;CSF3R;ITGAM;IL1R1;IL1R2;IL24;LILRB2;LILRB3;LILRA5;ICAM1;TLR1;CLEC4A;HCK;FCGR3A;PLAU;ITGAX;IL6R |
| GRANULOCYTE | CD53;CD274;ENTPD1;CSF3R;ITGAM;IL1R2;LILRB2;LILRB3;LILRA5;TLR1;CLEC4A;HCK;FCGR3A;ITGAX;IL6R |
| DENDRITIC CELL | CD53;ENTPD1;ITGAM;ITGA4;IL1R2;LILRB2;LILRB3;TNFSF13B;TLR1;CLEC4A;HCK;ITGAX;CCL18;IL6R |
| VASCULAR SMOOTH MUSCLE | PDGFRB;IL11;CDH5;CD274;C1S;IL1R1;PLAU;ITGB3;VEGFC;CCL2;ICAM1;JAM3 |
| PLASMACYTOID DENDRITIC CELL | CD53;TLR1;IL15RA;HCK;CD274;CD40;LILRB2;LILRB3;LILRA5;TNFSF13B;ICAM1 |
| KUPFFER-LIKE CELL | PDGFRB;IL15RA;C1S;IL1R1;ITGB3;ITGA1;VEGFC;CCL2;ICAM1;JAM3 |
| FIBROBLAST | PDGFRB;IL11;C1S;IL1R1;PLAU;ITGB3;ITGA1;VEGFC;CCL2;JAM3 |
| STROMAL CELL | PDGFRB;IL11;C1S;IL1R1;PLAU;ITGB3;VEGFC;CCL2;JAM3 |

define the ten cell populations shown in Figure 5A.

Supplementary Table 5: Human Gene Atlas table of cell populations. This table shows a statistical comparison of the cell populations in the Human Gene Atlas tissue database that are enriched in the baseline biopsies of poorly responding tumours versus tumours showing a good response to radiotherapy.

See attached excel spreadsheet

Supplementary Table 6: Gene lists from the Human Gene Atlas tissue database that were used to define the three cell populations shown in Figure 5B.

| Cell Population (Human Gene Atlas) | Genes |
|------------------------------------|--|
| CD14+_Monocytes | CLEC4A;HCK;CD163;ITGAX;LILRB2;LILRB3;LILRA5 |
| CD33+_Myeloid | CLEC4A;HCK;CD163;ITGAM;LILRB2;TYK2;LILRB3;IL6R |
| SmoothMuscle | IL11;IL24;VEGFC;CCL2;ICAM1 |

Supplementary Figure 1: A. Boxplot of mean expression of the 40 genes expressed in the poor responding samples in high and low expressors of the third validation cohort samples. **B.** Heatmap to show individual gene expression in the third validation cohort (based on 40 upregulated genes in our study cohort).

See separate pdf.

Supplementary Figure 2: Heatmap to show 198 genes with a significant difference in gene expression between matched pre- and post-radiotherapy tumours showing a good response to radiotherapy.

Supplementary Figure 3: Boxplots to show immune enrichment scores (as per Rooney et al. (2)) in pre-radiotherapy biopsies and post-radiotherapy resection specimens for **A:** Co-inhibition of T-cell scores, **B:** type I IFN scores and **C:** macrophage scores

References

1. Chauvin A, Wang CS, Geha S, Garde-Granger P, Mathieu AA, Lacasse V, et al. The response to neoadjuvant chemoradiotherapy with 5-fluorouracil in locally advanced rectal cancer patients: a predictive proteomic signature. *Clin Proteomics*. 2018;15:16.
2. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell*. 2015;160(1-2):48-61.