Increased Human RORγT Expression in *KRAS* and *STK11* Driven Non-Small Cell Lung Cancer Tumours

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Background

Non-small cell lung cancer (NSCLC) development is driven by genetic mutations (MTs) that dysregulate cellular pathways. The mTOR pathway regulates cell growth, metabolism and survival. It is frequently altered by MTs in *KRAS* (36% NSCLCs) and *STK11* (19%), which can be concomitant (11%). Differences in cancer genetics can be linked to different immune contextures within the tumour microenvironment. This heterogeneity has important implications for the success of immunotherapies.

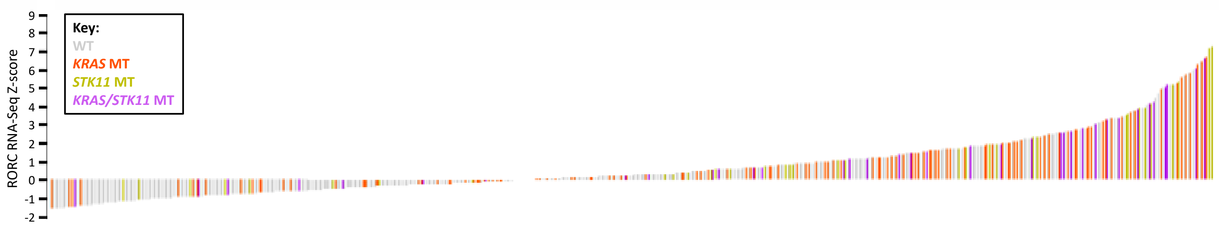
Methods

- The Cancer Genome Atlas (**TCGA**) 'PanCancer' lung adenocarcinoma **MT and RNA expression data** (*n* = 566) were merged using methods by Lal *et al* (2014). **Median expression Z-scores were compared** in MT and wild type (WT) groups by unpaired Wilcoxon test.
- HEK293Ts were **transfected with *RORC2-GFP*** plasmid and either fixed for IHC staining or stained by flow cytometry using a RORγT antibody (6F3.1, Merck).
- **Stimulated PBMCs were flow cytometry stained** for RORγT and IL-17A.
- Cell pellets and tissue sections were RORγT **IHC stained** using a pH 9 antigen retrieval protocol and DAB chromogenic development.
- IHC staining was **quantified by an in-house algorithm** taught to detect positively stained lymphocyte nuclei and segment tissue.
- **Multiplex IF** of CD4 and RORγT was performed using tyramide signal amplification using the Opal fluorophore kit (Akoya).

1. Identifying *RORC* Gene Upregulation

RNA expression of immune genes related to immune subset, function and recruitment in TCGA NSCLC cases with common driver mutations. Tumours with *KRAS* and *STK11* MTs have low immune gene expression compared to other MT groups; contrasted by upregulations in *RORC* which codes for the Th17 transcription factor RORγT (**Figures 1a.**).

Figure 1a. Case-by-case analysis of *RORC* expression scores in WT and MT patient groups



KRAS and *STK11* MTs had significantly higher *RORC* expression than WT tumours. *RORC* upregulation was synergistically enhanced in the cases with concomitant *KRAS/STK11* MTs (**Figure 1b.**).

The *RORC* gene is located on chromosome 1q21.3, which is frequently amplified in NSCLC. To ensure this observation was immunological and not a result of genetic instability, we removed putative 1q21.3 amplified cases from the analysis and saw the same effect (**Figure 1c.**).

Figure 1b. *RORC* expression statistical comparison

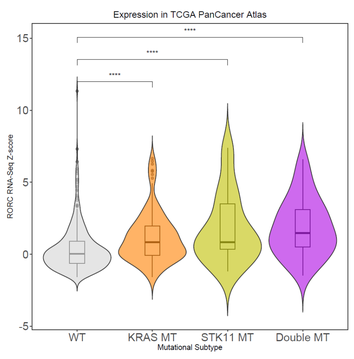
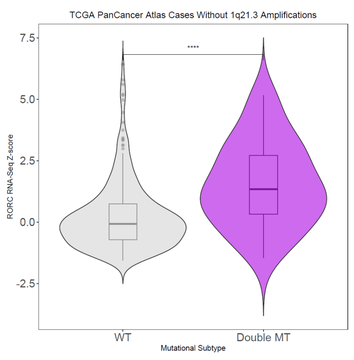


Figure 1c. *RORC* expression in cases without 1q21.3 amplifications



2. RORγT Antibody Validation

We transfected HEK293Ts with *RORC2*-GFP plasmid and stained cells by IHC and flow cytometry (**Figure 2a.**). To test this antibody on lymphocytes, we analysed stimulated PBMCs for RORγT^{+}IL-17A^{+} expressing cells and stained tonsil (**Figure 2b.**).

Figure 2a. *RORC2* GFP Plasmid Transfection System Staining

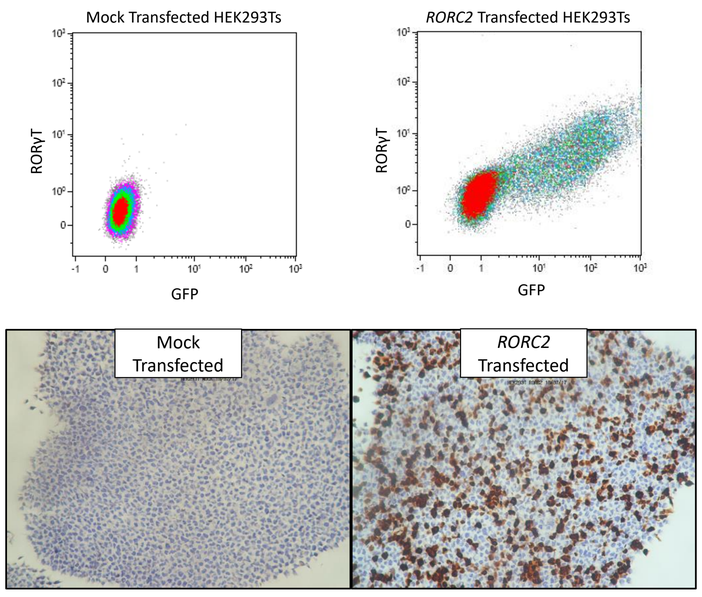
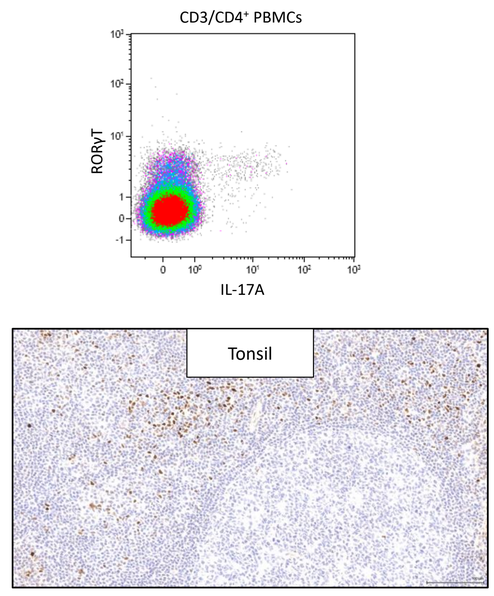


Figure 2b. Staining Human Lymphocytes with RORγT



3. NSCLC RORγT Protein Expression

We sourced patients resections with and without *KRAS* and *STK11* MTs as screened by the TRACERx study (Jamal-Hanjani *et al.*, 2014). Sections were IHC stained for RORγT and expression was quantified using an algorithm developed in-house (**Figure 3a.**). WT tumours had low expression compared to *KRAS* and *STK11* MT positive tumours (**Figure 3b.**).

Figure 3a. Positive RORγT Lymphocyte Nuclei Detection Algorithm

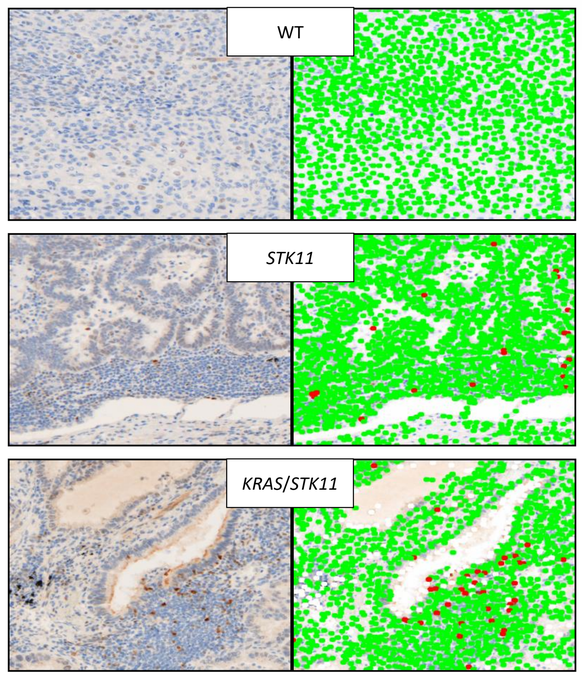
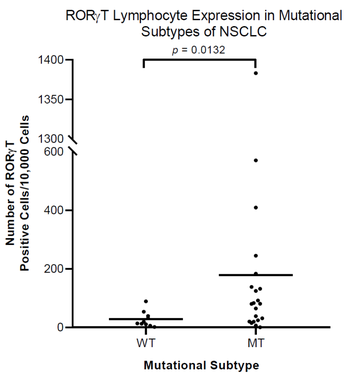


Figure 3b. Increased RORγT Expression in *KRAS* and *STK11* MT NSCLC



This algorithm compartmentalises cellular morphologies including stroma and intra-tumour lymphoid aggregates (**Figure 3c.**). Spatial quantification showed a higher number of RORγT^{+} lymphocytes in stroma and lymphoid aggregates of MT tumours (**Figure 3d.**).

Figure 3c. Tissue Segmentation Algorithm

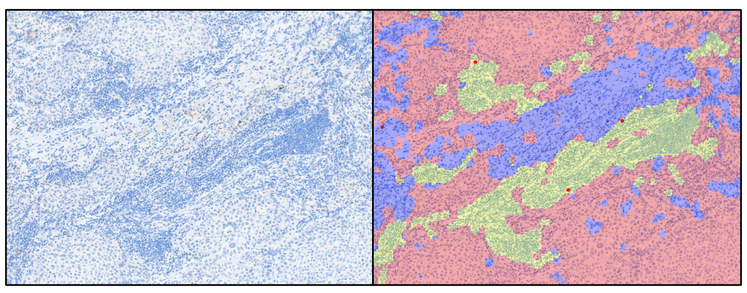
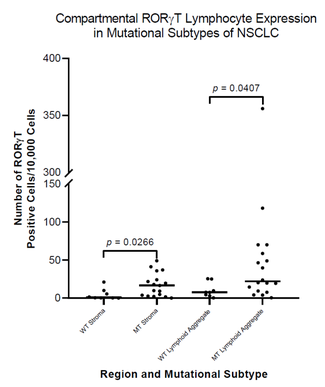
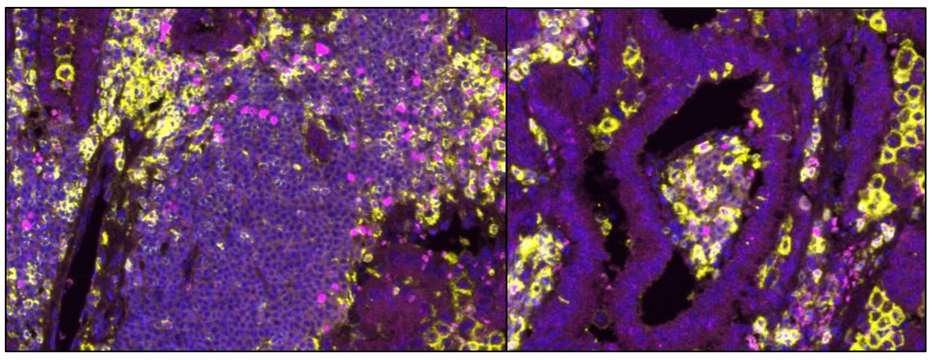


Figure 3d. Increased RORγT Expression in Stroma and Lymphoid Aggregates



Multiplex IF lymphoid aggregate staining showed that CD4^{+}RORγT^{+} Th17 cells, are distally localised within lymphoid aggregates and CD4^{-}RORγT^{+} cells are marginally more proximal. High numbers of RORγT^{+} cells in MT tumours were both CD4^{+} and CD4^{-} (**Figure 3e.**).

Figure 3e. Multiplex Immunofluorescence on Intra-Tumour Lymphoid Aggregates



Summary and Future Direction

- *STK11* and *KRAS* MT NSCLC show upregulations in the RNA and protein of the Th17 transcription factor RORγT.
- RORγT^{+} lymphocytes are CD4$^{+/-}$ and are predominantly in lymphoid aggregates.
- Understanding mechanisms by lymphocyte co-cultures with tumour cell lines.

We thank the following for their support:

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