Supplementary Material

Materials and Methods

Human tumor samples

By performing left upper lobectomy, we have collected 3 nodules including one solid nodule and two mixed ground glass nodules (mGGN). Each resected nodule contained samples of freshly resected, frozen-section and formalin-fixed and parrffin-embedded (FFPE). Written informed consent of tumor acquisition for research has been obtained before surgery and approved by internal review board from Guangdong Lung Cancer Institute and Guangdong Provincial People's Hospital (Guangzhou, China, IRB approved protocol number GDREC2016175H).

Pathology evaluation and immunohistochemistry analysis

Comprehensive pathological evaluation was performed in 3 resected nodules. 4-um non-stained formalin-fixed and parrffin-embedded slides were set for immune staining to assess infiltrating immune cell subsets. The antibodies used in this study included: anti-CD3, anti-CD4, anti-CD20, anti-CD38, anti-CD163, anti-Foxp3. Staining results were manually evaluated and confirmed by specialized pathologist. Expression score was assessed based on staining quantity and intensity as follows: -, absence; +, low intensity; ++, intermediate intensity; +++, high intensity. Negative controls were used for all antibodies.

Expression of PD-L1 (22C3 pharmDx; Agilent Technologies Inc., Santa Clara, CA, USA) of tissue section was scored by specialized pathologist. PD-L1 expression was evaluated by defining the proportion of PD-L1 positive cells among tumor cells. Positive expression of PD-L1 was defined as≥1% for tumor cell while negative expression was opposite.

Multiplex Immunohistochemistry Staining and Analysis

5 slides of 5-um non-stained formalin-fixed and parrffin-embedded samples were obtained for multiplex immunohistochemistry staining. To identify the cell subsets expressing PD-L1 in the TME and multiplex immunofluorescence staining, we used PANO 7-plex IHC kit (cat 0004100100, Panovue, Beijing, China) including CD8, CD56, CD68, HLA-DR and PANCK. Different primary antibodies were sequentially applied, followed by horseradish peroxidase-conjugated secondary antibody incubation and tyramide signal amplification. The slides were microwave heat-treated after each TSA operation. Nuclei were stained with 4′-6′-diamidino-2-phenylindole (DAPI, SIGMA-ALDRICH) after all the human antigens had been labelled. To obtain multispectral images, the stained slides were scanned using the Mantra System (PerkinElmer, Waltham, Massachusetts, US), which captures the fluorescent spectra at 20-nm wavelength intervals from 420 to 720 nm with identical exposure time; the scans were combined to build a single stack image.

Images of unstained and single-stained sections were used to extract the spectrum of autofluorescence of tissues and each fluorescein, respectively. The extracted images were further used to establish a spectral library required for multispectral unmixing by inForm image analysis software 2.2.1. (PerkinElmer, Waltham, Massachusetts, US). Using this spectral library, we obtained reconstructed images of sections with the autofluorescence removed.

Fresh Tissues Processing for scRNA-seq

Fresh resected tissues within half hour after surgical resection were kept in MACS Tissue Storage Solution (Miltenyi Biotec) until processing. Samples were first washed with phosphate-buffered

saline (PBS), minced into small pieces (approximately 1mm^3) on ice and enzymatically digested with 250U/mL collagenase I (Worthington), 100U/mL collagenase IV (Worthington) and 30U/mL DNase I (Worthington) for 45 min at 37°C, with agitation. After digestion, samples were sieved through a 70μm cell strainer, and centrifuged at 300g for 5 min. After the supernatant was removed, the pelleted cells were suspended in red blood cell lysis buffer (Miltenyi Biotec) to lyse red blood cells. After washing with PBS containing 0.04% BSA, the cell pellets were re-suspended in PBS containing 0.04% BSA and re-filtered through a 35μm cell strainer. Dissociated single cells were then stained with AO/PI for viability assessment using Countstar Fluorescence Cell Analyzer for quality assessment.

Single-Cell RNA Sequencing

The scRNA-Seq libraries were generated using the 10X Genomics Chromium Controller Instrument and Chromium Single Cell 3' V3 Reagent Kits (10X Genomics, Pleasanton, CA). Briefly, cells were concentrated to 1000 cells/uL and approximately 8,000 cells were loaded into each channel to generate single-cell Gel Bead-In-Emulsions (GEMs), which results into expected mRNA barcoding of 5,000 single-cells for each sample. After the RT step, GEMs were broken and barcoded-cDNA was purified and amplified. The amplified barcoded cDNA was fragmented, A-tailed, ligated with adaptors and index PCR amplified. The final libraries were quantified using the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific) and the size distribution of the libraries were determined using a High Sensitivity DNA chip on a Bioanalyzer 2200 (Agilent). All libraries were sequenced by HiSeq Xten (Illumina, San Diego, CA) on a 150 bp paired-end run.

Single-Cell RNA Data Analysis

Fastq data was applied with default parameter filtering the adaptor sequence and removed the low-quality reads to achieve the clean data. Then the feature-barcode matrices were obtained by aligning reads to the human genome (GRCh38) using CellRanger v3.1.0. Cells contained over 200 expressed genes and mitochondria UMI rate below 20% passed the cell quality filtering. Mitochondria genes were removed in the expression table but used for cell expression regression to avoid the effect of the cell status for clustering analysis and marker analysis of each cluster.

Seurat package (version: 2.3.4, https://satijalab.org/seurat/) was used for cell normalization and regression based on the expression table according to the UMI counts of each sample and percent of mitochondria rate to obtain the scaled data. PCA was constructed based on the scaled data with all high variable genes and top 10 principals were used for tSNE construction. The fastMNN function (k = 5, d = 50, approximate = TRUE, auto.order = TRUE) from R package scran (v1.10.2) was used to apply mutual nearest neighbor method to correct for batch effect among samples. Utilizing graph-based cluster method, we acquired the unsupervised the cell cluster result based the PCA top 10 principal and we calculated the marker genes by FindAllMarkers function with wilcox rank sum test algorithm under following criteria:1. logFC > 0.25; 2. P value<0.05; 3. min.pct>0.1. In order to identify the cell type detailed, the clusters of same cell type were selected for re-tSNE analysis, graph-based clustering and marker analysis.

Single-Cell Trajectories analysis was calculated with Monocle2 (http://cole-trapnell-lab.github.io/monocle-release) using DDR-Tree and default parameter. Before Monocle analysis, we select marker genes of the Seurat clustering result and raw expression counts of the cell passed filtering. Based on the pseudo-time analysis, branch expression analysis modeling (BEAM Analysis)

was applied for branch fate determined gene analysis. The function FindMarkers with wilcox rank sum test algorithm was used under following criteria:1. logFC > 0.25; 2. P value<0.05; 3. min.pct>0.1 was used to identify differentially expressed genes among samples.

Targeted Panel Sequencing and Whole Exome Sequencing

1μg of genomic DNA from 3 resected nodules were used for whole-exome sequencing. Genomic DNA from tumor frozen sections or the whole blood control sample was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen) and DNeasy Blood and tissue kit (Qiagen), respectively, and fragmented by M220 Focused-ultrasonicator (Covaris) into approximately 250bp. Whole genome library was prepared using KAPA Hyper Prep Kit (KAPA Biosystems). For WES, exome capture was performed using the Illumina Rapid Capture Extended Exome Kit (Illumina Inc). For the targeted-panel, capture-based enrichment was performed using a customized panel targeting 425 cancer-related genes with Dynabeads M-270 (Life Technologies) and xGen Lockdown hybridization and Wash Reagent kit (Integrated DNA Technologies) according to the manufacturers' protocols. The captured libraries were then amplified with Illumina p5 (50 AAT GAT ACG GCG ACC ACC GA 30) and p7 primers (50 CAA GCA GAA GAC GGC ATA CGA GAT 30) in KAPA HiFi HotStart ReadyMix (KAPA Biosystems), and subsequently purified using Agencourt AMPure XP beads. Sequencing libraries were quantified by qPCR using KAPA Library Quantification kit (KAPA Biosystems). Library fragment size was determined by Bioanalyzer 2100 (Agilent Technologies). Both WES and target-enriched libraries were sequenced using the Illumina HiSeq 4000 platform (Illumina) according to the manufacturer's instructions.

Variant Filtering and Mutation Calling

Trimmomatic was used to trim adapters and remove low quality reads (quality reading below 20) or N bases from FASTQ files. Burrows-Wheeler Aligner (BWA) was then used to align clean paired-end reads to the reference human genome (hs37d5). PCR deduplication was performed using Picard and indel realignment and base quality score recalibration were performed using Genome Analysis Toolkit (GATK 3.4.0 (McKenna et al.,2010). Matching of tumor and whole blood control samples was assessed using VCF2LR (GeneTalk) for same SNP fingerprint. Cross sample contamination was estimated using ContEst (Broad Institute) by evaluating the likelihood of detecting alternate alleles of SNPs reported in the1000g database. Somatic SNV and insertion/deletions (INDELs) were called using Vardict (V 1.5.4). SNVs and INDELs were further filtered using previously reported criteria (Zhang et al. 2019). Final mutations were annotated using vcf2maf.

HLA Alleles Typing

Typing for Class I HLA alleles was done through alignment with the IGMT/HLA database running Opitype (V1.3.1) with default parameters. Neoantigen calling was performed using the identified HLA types with netMHCpan (V4.0) for binding prediction under the NeoPrePipe pipeline.

Copy-Number Variants Analysis

Copy number analysis was performed using FACETS (Ver 0.5.13). Somatic CNA events were assigned based on sample-ploidy values calculated in the FACETS algorithm (Riaz et al., 2017). Chromosome arm-level CNA gain (>sample average ploidy +1) was defined if segments of amplification and deep amplification events account for more than 60% of total segments for the

corresponding chromosome arm. Similarly, arm-level CNA loss (<sample average ploidy -1) was identified if segments of deletion and deep deletion events account for more than 60% of total segments for the given chromosome.

TCR library preparation and sequencing

Genomic DNA from frozen section was extracted and quantified as described above. Minimum 1ug of DNA was required for subsequent experiments. Multiplex PCR reaction was prepared using QIAGEN Multiplex PCR Plus Kit with customized TCR primer mixture comprising 51 forward primers complementary to the V gene segments and 13 reverse primers complementary to the J gene segment. To correct amplification bias from multiplex PCR primers, 663 barcoded synthetic templates (i.e. a synthetic repertoire of all possible V-J combinations) were used to calibrate PCR efficiency. These templates contain universal P5 and P7 ends for standard primer recognition, barcodes and V gene and J gene segments flanking barcoded internal markers. Amplified synthetic products and tumor samples were then purified using AxyPrep MAG FragmentSelect-I Kit (Axygen). Subsequently, tumor TCR library was prepared with KAPA Hyper Prep Kit (KAPA Biosystems). Briefly, A-trailing and end-repair of fragments was performed before ligation of index adaptors in the TruSeq DNA PCR-free Library Prep Kit (Illumina). Purified ligation product was then amplified with Illumina p5 and p7 primers in the KAPA HiFi HotStart ReadyMix (KAPA Biosystems), followed by a final purification step using the Axygen beads. TCR library was sequenced using the Illumina HiSeq 4000 platform according to the manufacturer's instructions.

TCR analysis and profiling

Trimmomatic was used to remove adaptors and filter low quality reads from FASTQ files. Non-V-J paired reads was further removed using Cutadapt (V 1.18). Next, paired-end read merger (PEAR, V 0.9.10) was employed to merge paired reads and non-barcoded reads were removed for synthetic standards, while barcoded reads were filtered for tumor samples. Clean reads were subsequently assembled using MiXCR (V 2.1.11). Reads were aligned to the reference V or J gene segments according to the international ImMunoGeneTics (IMGT) database. Clonotypes were then built from alignments using the assemble pipeline of the software. For final repertoire profiling, sample V/J counts, CDR3 counts and clonality counts were calculated with normalization using corresponding counts from synthetic standards. Statistical analyses and graphical visualization were performed with R packages tcR and Vdjtools.

Signature Establishment and External Datasets

In this study, we established two signatures related to Trm cluster, MDSC-like macrophage cluster and TAM-like macrophage cluster using principal component analysis (PCA) to identify specific genes for different signatures. Trm signature contained CXCR6, ZNF683 and ITGAE. MDSC-like macrophages signature contained CCL2, PLA2G7, AQP9, VCAN, CD36 and FCN1. TAM-like macrophages signature contained GPNMB, C1QC, CCL13, SLC40A1 and FOLR2.

To validate prognostic value of the signatures we established in this study, we collected five different cohorts which have already published (referenced in the figure) or available from The Cancer Genome Atlas (TCGA) datasets (https://gdc.cancer.gov/).

Statistical analysis

All statistical analysis was performed in SPSS version 25.0 software (SPSS, Inc., Chicago, IL, USA). t-test was used for two group comparison and ANOVA or Kruskal–Wallis test was used for more than two group comparisons. Fisher's exact test was used to compare two categorical variables. Kaplan Meier analysis was used to compare survival between groups and log-rank test p value was calculated. All reported p-value were 2-tailed, and the statistical significance was defined as $p<0.05$.