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Immune evasion before tumour invasion in early lung squamous carcinogenesis

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Supplementary Information:

Study population and sample collection

Based on the fact that high-grade lesions were rare and based on Dobbin *et al*.'s report[1], we included at least twelve biopsies from each histological stage. The histopathological classification was performed based on the 2004 histological WHO/IASLC classification[2] by one pathologist (AH) on three independent blinded occasions. Any discordant diagnoses between successive evaluations were re-evaluated by the local team of pathologists using a multi-head microscope to obtain a consensus. The CIS included in this study were pure CIS and no CIS having adjacent invasive squamous cell carcinomas was included.

In order to avoid batch and tissue-preparation effects. During bronchoscopy, two biopsies were taken with clean forceps in the same area: one for routine histopathology and the second, immediately dropped in Tripure Isolation Reagent on ice, homogenized and frozen at –80°C (Roche Diagnostics, Indianapolis, IN, USA), for molecular studies.

Detailed protocol of acquisition of gene expression profiles

RNA extraction was performed using previously reported protocols[3]. Isolated RNAs were assessed for quantity and purity on the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and for quality on the Agilent 2100 bioanalyser with RNA 6000 NanoAssay (Agilent Technologies, Palo Alto, CA, USA). Normal bronchial biopsies from 16 never-smokers were pooled (same amount of RNA for each) for use as reference RNA. mRNAs were retrotranscribed using a T7 primer coupled with oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The cDNA was then *in vitro* transcribed in labelled cRNA by the T7 RNA polymerase with fluorescent nucleotides, Cy5 for samples or Cy3 for reference RNA dyes, by using the Low input RNA Fluorescent Linear Amplification Plus kit (Agilent Technologies). Spike-in RNAs (Agilent Technologies) were included as positive

controls to monitor the whole microarray workflow (sample amplification, labelling and microarray processing). For each sample, an amount of 100 ng of total RNA was amplified in parallel with 100 ng of pooled reference RNAs for each experiment with the same Master-Mix. The different histological subtypes were randomized between the amplification runs. Labelled cRNAs were checked for quantity and dye incorporation by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and the Gaussian distribution of sample sizes was assessed by migration profiles on the Agilent 2100 bioanalyser with RNA 6000 NanoAssay (Agilent Technologies). Thereafter, the labelled cRNAs were hybridized on an Agilent oligonucleotide microarray (Two Colours Whole Human Genome 4x44K arrays, Agilent Technologies) for 17 hours at 60°C in a rotating oven according to the provider's protocol (Agilent Technologies). The samples were randomized between the different hybridization runs according to their histological stage and their amplification runs. Moreover, a self-self was included in each run. After disassembling the hybridization chambers, the slides were washed and the signal was read by confocal laser scanning (Agilent Technologies). Grid positioning, spots localization, outliers flagging, fluorescence intensities quantification, background level assessment, correction of the values according to the background followed by linear and Lowess data normalization were all performed according to the default protocol for two colours Agilent customized arrays (GE2_v5_95_Feb07 protocol, Feature Extraction software version 9.5.3.1, Agilent Technologies). Quality control (QC) metrics were calculated (GE2_QCMT_Feb07 QC metrics set, Feature Extraction software version 9.5.3.1, Agilent Technologies) and the arrays which reached the 12/12 points among the 12 qualitative experimental aspects assessed (reproducibility of spike-in RNAs, maximum acceptable background, quantification of the outliers, reproducibility of the replicated spots on the arrays, etc) were included in the analysis.

Batch effect control and quality assessment

We carefully controlled batch effect at each step of tissue preparation, data generation, and data processing. The process for handling and freezing the biopsies was carefully standardized. The principal investigator was present in the bronchoscopy room and handled the samples immediately. The biopsies were immediately removed from the forceps by using a needle that was directly dropped into Tripure in an RNAse-free eppendorf on ice, then immediately homogenized in Tripure and frozen in liquid nitrogen in the bronchoscopy room. All samples were thus frozen less than 5 minutes after sampling (**Table S2**).

Regarding data generation, the samples were randomized between the different hybridization runs according to their histological stage and their amplification runs (**Table S2**). Spike-in RNAs (Agilent Technologies) were included as positive controls to monitor the whole workflow of sample amplification, labeling and data pre-processing. Moreover, a self-self hybridization design was included in each run. Background correction and data normalization were performed following Agilent's protocol for two-color microarrays.

Finally, we performed several steps of quality assessment of the microarray expression. First, we confirmed that the relative abundance of each probe, *i.e.*, the ratio between the red and green color intensity (Cy5/Cy3) for all probes followed Gaussian-like distribution (**Fig. S8A**). We observed equal gene expression distribution for each hybridization and amplification run (**Fig. S8B**, left). Randomized principal component analysis also showed that there was no batch effect introduced by hybridization and amplification runs, and there were no outliers among the samples (**Fig. S8B**, right). Finally, we classified the samples based on the expression of sex-chromosome genes and the patient's sex was correctly assigned to all samples (**Fig. S8C**).

Weighted gene correlation network analysis

We sought to detect co-expression of genes, which may indicate a potential biological relationship. A module of co-expressed genes could thus reveal active biological processes. Weighted correlation networks have been widely used for identification of co-expression modules across diverse biological systems[4–9],

including cancer[10,11]. By incorporating the topology of the co-expression network, network-based approaches can detect cohesive gene modules with sensitivity to subtle changes in connectivity patterns [12].

To determine the number of gene expression trajectories, we first selected for genes that were associated with the nine developmental stages (linear mixed-effects model), accounting for the confounding effects of smoking history, previous cancer status, age, sex, and between-patient variation. The expression of all of the associated 4734 genes is now illustrated in Figure S9A-B. We then ran WGCNA using these genes. To identify trajectories of gene expression during development, we applied weighted gene correlation network analysis with the tool WGCNA[13] on the genes significantly associated with developmental stages. WGCNA network construction and module detection was performed using the function “blockwiseModules” configured for signed network type, soft-thresholded power of 12, and a dendrogram cut height of 0.3 for merging modules.

To functionally describe the gene modules, we used the cancer hallmark[14] definitions from the mSigDB database (v6.2)[15] and applied the over-representation hypergeometric test using the R package clusterProfiler.

Of note, three hallmark definitions associated with the gene module Ascending were not shown in Fig. 1B (the highest adj. p-values, p>0.003): PI3K_AKT_MTOR_SIGNALING (proliferation), UV_RESPONSE_UP (DNA damage, confirmed by the UV_RESPONSE_DN in the module Descending), and HYPOXIA (pathway, proliferation). Supported by all significant cancer hallmarks, proliferation best describes the gene module Ascending. In the Molecular Signatures Database, there are two gene signatures related to DNA damage among the hallmark gene sets: DNA Repair and UV response. Therefore, the gene definition of UV response likely subsumes the genes that are involved in response to DNA stress stimulus such as UV, and the gene definition of UV response was considered a surrogate of DNA damage response. In addition, we also used single-sample gene set enrichment analysis (ssGSEA)[16] on the full gene expression assay to determine whether the cancer hallmark genesets were

enriched among the up-regulated or down-regulated genes of each sample, regardless of gene module (**Fig. S4**).

Here, we describe the details and rationale behind parameter selection. The similarity measure that was used to build the adjacency matrix was Pearson correlation. The adjacency matrix was then raised to a power to construct the weighted network of genes (**Fig. S9C**), ensuring highly robust network results[17]. The value to which the adjacency matrix was raised is referred to as the power for soft thresholding. The value of the power for soft thresholding was selected to satisfy the scale-free topology criterion[17]. The lowest power term where the network approximately fits a scale-free topology was 12 (R^2<=0.85, close to red line, **Fig. S9C**). Next, topological dissimilarity measures were calculated and subjected to average-linkage hierarchical clustering for module detection. A dendrogram cut height of 0.3 was chosen to merge similar modules. The parameter for ratio threshold is considered after module detection as the p-value ratio threshold for reassigning genes between modules.

WGCNA identified seven gene modules. Only 18 out of the 4734 genes were not assigned to a gene module and did not follow any of the seven illustrated expression patterns (**Fig. S9B**). This number of 18 genes was considered too low to underpin novel expression trajectories that were not already similar to the seven identified patterns. Based on the expression of genes associated with the 9 developmental stages (**Fig. S2**) and based on the seven expression trajectories (**Fig. 1B**), gene expression discerned four rather than nine stages of carcinogenesis, grouped as normal, low grade, high grade, and SCC. While there was a continuum of expression changes from stage 0 to stage 8 (**Fig. S3B**, randomized principal component analysis on the full expression profile and on cancer hallmarks), there were four distinct steps of gene expression evolution from normal, to low grade, to high grade, and SCC.

Gene module stability and preservation

We performed additional analysis to show the stability and robustness or preservation of the detected modules (**Fig. S9D-E**). First, we split randomly the

full dataset into reference and test set and iteratively evaluated module preservation across the respective networks[18]. In Figure S9D, we showed that all of the modules were robust between the reference and the test dataset having a preservation statistic significantly higher than expected by chance (**Fig. S9D**). Specifically, the Zsummary statistic provides evidence that the observed value of the preservation statistic is significantly higher than expected by chance (strong evidence if Zsummary>10; weak to moderate if 2<Zsummary<10; no evidence Zsummary<2). The grey module is unclassified and expectedly showed no preservation (Zsummary<2). All of the modules were preserved between the reference and the test dataset (Zsummary>10), except the SCC increase module (Zsummary=9.1) which is also the smallest module with gene expression in small number of samples after resampling (increase specific to SCC). The median rank preservation statistic showed that independently of the module size there is stronger preservation for all modules compared to the grey unclassified set of genes (bottom). Moreover, to demonstrate module stability, we applied the same parameters for weighted gene correlation network analysis on a resampled subset of the full dataset (randomly selected 2/3rd of the full dataset). The resampling was performed without replacement and ensured proportional representation of each developmental stage. In Figure S9E, color rows indicated the module assignments obtained on the full dataset (first row) and on the resampled subsets of samples (n=50). All of the seven gene modules identified in the full dataset appear in almost every resampling, indicating module stability[19]. Lastly, we showed that the findings that rely on the gene module detection were also confirmed independently of the gene modules (**Fig. S3A**).

Altogether, we showed evidence that the detected trajectories rather robust and preserved across data resampling and across multiple random data splits.

HD immune signature

To explore a large number of different immune cell subtypes and, even more, to examine their activation status, we compiled a large number of carefully annotated microarray gene expression profiles from 1769 publicly available microarrays (**Fig. S1**) normalized with the frozen Robust Multi-array Averaging

(fRMA) method. Building on our previous methods for defining the immune copendium[20,21,22], we identified genes with specific expression for immune cell types, considering their naive, resting, and activated status. Thereby, we provided the largest to our knowledge compilation of manually-annotated microarray dataset of purified immune cell types, as well as normal tissue and a diverse set of cancer cell lines. Pan-celltype signatures were defined as genes expressed at similar levels in multiple cell types. For examples, the Myeloid-derived category comprised all subtypes of dendritic cells, eosinophils, monocytes, macrophages, neutrophils, and mast cells, while Macrophages-DC was a gene signature comprising common genes expressed in all studied subtypes of both macrophages and dendritic cells. The full expression dataset is available upon request. We first used WGCNA to identify gene modules with similar expression patterns. Within each module, we selected for genes with significantly high expression in one immune cell type compared to the all the others, conditioning at least a fold change of 1.5 increase over the next highest expressed cell type. We additionally included the compendium of immune genes used in this study (**Table S4**).

Comparison of deconvolution methods

We evaluated five additional methods for deconvolving the immune signal from gene expression data. We demonstrated concordance across different methods and independent immune signatures for the majority of the immune cell types. We reported the results derived from the method CIBERSORT[22] with the LM22 gene signature and the method single-sample GeneSet Enrichment analysis (ssGSEA) on our independent in-house developed immune signature (HD signature, **Table S4**). In addition, we applied the deconvolution methods mcpCounter[23], TIMER[24], EPIC[25], and xCell[26], and more, executed the method CIBERSORT on our HD signature.

First, we showed that the immune-cell estimates from CIBERSORT applied on the LM22 gene signature correlated positively with the estimates from the same method applied with an independent immune gene signature (HD), and we confirmed the same immune trajectories across developmental stages (**Fig.**

S4A-B). Even more, positive correlations were confirmed for five additional independent deconvolution tools for the common immune cell types (**Fig. S4C**). Uniform positive correlation was observed with all tested methods with significance ($p<0.0003$) for Neutrophils (Spearman correlation coefficients 0.42-0.78, n=5 tools), DC resting (0.46-0.74, n=6 tools), T cells CD8 (0.56-0.72, n=6), Plasma cells (0.53-0.84, n=3), Total macrophages (0.32-0.69, n=6), B cells (0.39-0.58, n=6), T cells (0.45-0.71, n=6), Mast cells resting (0.42-0.61, n=3), and T cells activated (0.59-0.63, n=2). At least one tool showed positive correlation for Tfh (0.48, CIBERSROT with HD), Monocytes (0.41, xCell), and NK cells resting (0.35-0.57, n=3). The agreement among the methods was weaker on Eosinophils (0.31, xCell), Monocytes (0.41, xCell), T cells CD4 naive (0.31, Cibersort HD). There was no concordance among the tools only for the cell type DC activated. Alogether, comparable immune estimates were estimated across six deconvolution approaches: Cibersort on HD, ssGSEA on HD, mcpCounter, EPIC, TIMER, and xCell.

Tumor heterogeneity

Pre-invasive lesions change over time through an evolutionary process at cell level, driven by inherent genetic and epigenetic alterations. As a result, we observed that the normal-cell component was reducing continually with each developmental stage (**Fig. S10A**, ssGSEA using pan-normal tissue and normal lung tissue from HD gene signature, rho=-0.61, p=8e-14). Despite that the proliferation gene marker *MKI67* increased proportionally with the developmental stages (rho=0.56, p=2e-11, **Fig. S10C**), the change in the abundance of epithelial cells showed a weak positive correlation with the histological stages (rho=0.24, p=0.008, **Fig. S10B**). In each developmental stage, there was a wide variation of the epithelial cell estimate.

Based on cytokeratin (CK^+) cell quantification using multispectral IHC, we did not observe significant changes in the density of epithelial cells among the four molecular steps (**Fig. 4A**). However, the absolute number of detected epithelial cells had an increasing trend with the developmental stage. The results were consistent with both the functional and the phenotype panel, for all epithelial cells

(rho=0.3, p=0.002) and for $CK^{+}Ki67^{-}PDL1^{-}$ (rho=0.37, p=9e-05). The observation that there was no difference in the epithelial cell densities was likely due to the fact that we did not microdissect the epithelial area to exclude the stroma for RNA extraction, which, at the same time, gave us the unique opportunity to study the immune response.

The total yield of RNA that was extracted reflects the size of the biopsy and its cellularity and was, as expected, weakly correlated with stage (R=0.28, the RNA yield in SCC is significantly higher than in low-grade, high-grade, and normal). This is due to the bigger size of the SCC biopsies. There was no correlation between the total yield of extracted RNA and the epithelial cell density estimate (Spearman correlation). But mainly, as the same quantity of RNA was used for amplification for each biopsy, the quantity of RNA used for gene expression analyses was not related total yield of RNA that was extracted or to the size biopsy or its cellularity.

We do not expect the findings related to the immune response to be biased by the tumor heterogeneity, whether they were derived from gene expression or multispectral imaging quantification (**Figure 2-4**). To verify that tumor heterogeneity does not affect the gene module detection (**Figure 1**), we first calculated the gene expression weighted by the log_2-transformed CK^{+} epithelial cell density and confirmed the same expression patterns for each gene module (Spearman correlation between average module expression, rho>0.98, p<2e-16, for all modules).

Supplemental Information Table Legends

Table S1. **Clinicopathological characteristics of the cohort summarized per developmental stage.**

Table S2. A list of all studied samples of the corresponding patients of the cohort. Samples whose blocks could not be analyzed with multispectral imaging were marked with X, concerning both the phenotype and the functional panel.

Table S3. **Functional analysis of differential gene expression.** Immune-related Gene Ontology biological processes significantly enriched among differentially regulated genes in low-grade, high-grade, and SCC compared to normal.

Table S4. **Compendium of immune genes referred to as the HD gene signature.**

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