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# Comprehensive multiparametric analysis of Non-Small Cell Lung Cancer describes novel genotype-immunophenotype relationships and provides putative biomarker signatures of response to checkpoint blockade


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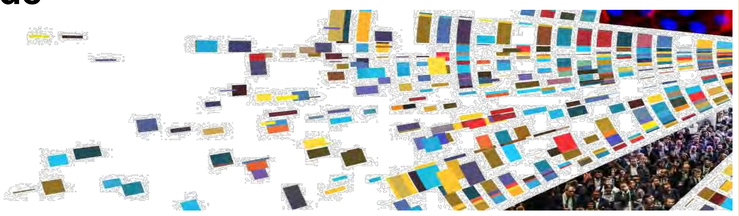


## BACKGROUND

Lung cancer leads cancer mortality worldwide, being Non-Small Cell Lung Cancer (NSCLC) the most prevalent subtype (85% of the cases).

In recent years, immunotherapy with checkpoint inhibitors has showed promising results in this type of tumor, yet this benefit is restricted to a subset of patients. However, robust predictive biomarkers to identify them are lacking.

Several studies suggest that certain immune phenotypes may be associated to specific response outcomes. However, a deeper research is needed in order to define predictive biomarkers of response to immunotherapy beyond PD-L1.

Identifying the key variables that predict the response to immunotherapy in NSCLC remains a major research challenge. In this work, we seek a multiparametric biomarker signature to fulfill this urgent need.

## HYPOTHESIS

Our hypothesis is that the specific molecular aberrations of NSCLC tumor cells have a profound impact on the immune phenotype observed in the tumor microenvironment and, consequently, on the response to immunotherapy. Defining this relationship is critical in order to predict response to immunotherapy.

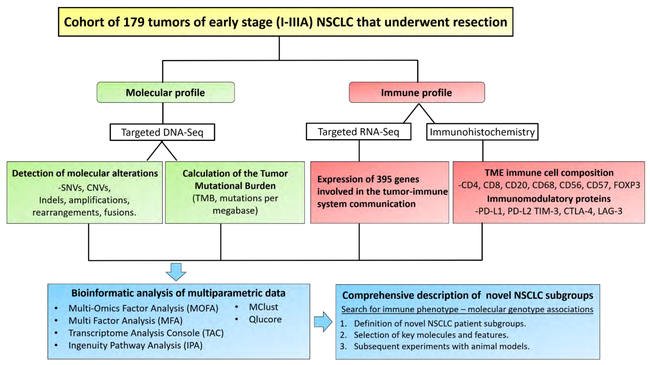
## OBJECTIVES

**1-** Characterization of the molecular alterations and the immune phenotype of early stage (I-IIIA) NSCLC.

**2-** Description of the association between the immune phenotype and the molecular genotype. Definition of novel NSCLC subgroups based on these associations.

**3-** Validation of predictive biomarkers of response to immune checkpoint inhibitors in a cohort of advanced stage NSCLC patients treated with immunotherapy (future work).

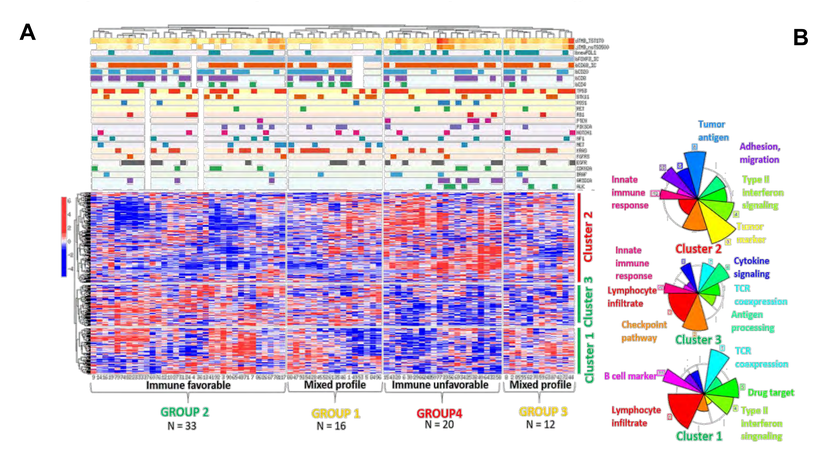
## METHODOLOGY

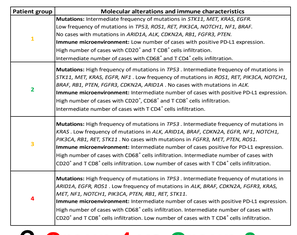



## RESULTS

We performed a multiparametric description of 179 early stage NSCLC tumor samples. It is important to remark that none of these patients were treated with immunotherapy. The Multi-Omics Factor Analysis (MOFA) software determined that the gene expression data was the most significant factor to explain the variance observed in our samples. Therefore, we performed a classification of our cohort based on the expression of genes involved in the tumor-immune system communication, separated by histology. Finally, we described the molecular and immune characteristics of the defined subgroups and identified predictive biomarkers of response to immunotherapy with checkpoint inhibitors.

### Multiparametric comprehensive description of lung adenocarcinoma defines 4 novel tumoral subgroups with specific molecular and immune characteristics

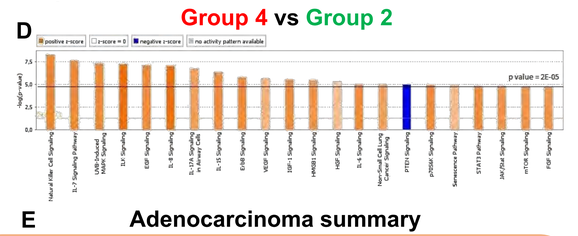


| Patient group | Molecular alterations and immune characteristics |
|---|---|
| 1 | **Mutations:** Intermediate frequency of mutations in *STK11, MET, KRAS, EGFR*. Low frequency of mutations in *TP53, ROS1, RET, PIK3CA, NOTCH1, NF1, BRAF*. No cases with mutations in *ARID1A, ALK, CDKN2A, RB1, FGFR3, PTEN*. **Immune microenvironment:** Low number of cases with positive PD-L1 expression. High number of cases with $CD20^+$ and $T\ CD8^+$ cells infiltration. Intermediate number of cases with $CD68^+$ and $T\ CD4^+$ cells infiltration. |
| 2 | **Mutations:** High frequency of mutations in *TP53* . Intermediate frequency of mutations in *STK11, MET, KRAS, EGFR, NF1* . Low frequency of mutations in *ROS1, RET, PIK3CA, NOTCH1, BRAF, RB1, PTEN, FGFR3, CDKN2A, ARID1A* . No cases with mutations in *ALK*. **Immune microenvironment:** Intermediate number of cases with positive PD-L1 expression. High number of cases with $CD20^+$, $CD68^+$ and $T\ CD8^+$ cells infiltration. Intermediate number of cases with $T\ CD4^+$ cells infiltration. |
| 3 | **Mutations:** High frequency of mutations in *TP53* . Intermediate frequency of mutations in *KRAS* . Low frequency of mutations in *ALK, ARID1A, BRAF, CDKN2A, EGFR, NF1, NOTCH1, PIK3CA, RB1, RET, STK11* . No cases with mutations in *FGFR3, MET, PTEN, ROS1*. **Immune microenvironment:** Intermediate number of cases positive for PD-L1 expression. High number of cases with $CD68^+$ cells infiltration. Intermediate number of cases with $CD20^+$ and $T\ CD8^+$ cells infiltration. Low number of cases with $T\ CD4^+$ cells infiltration. |
| 4 | **Mutations:** High frequency of mutations in *TP53* . Intermediate frequency of mutations in *ARID1A, EGFR, ROS1* . Low frequency of mutations in *ALK, BRAF, CDKN2A, FGFR3, KRAS, MET, NF1, NOTCH1, PIK3CA, PTEN, RB1, RET, STK11*. **Immune microenvironment:** Intermediate number of cases with positive PD-L1 expression. High number of cases with $CD68^+$ cells infiltration. Intermediate number of cases with $CD20^+$ and $T\ CD8^+$ cells infiltration. Low number of cases with $T\ CD4^+$ cells infiltration. |

**C Group 4 vs Group 2**

| Upregulated genes | Downregulated genes |
|---|---|
| *AKT1* | *CD19* |
| *BUB1* | *CD1C* |
| *CCNB2* | *CD27* |
| *CD276* | *CD3D* |
| *CDK1* | *CD48* |
| *CDKN2A* | *CD69* |
| *CDKN3* | *CD79A* |
| *EGFR* | *CD79B* |
| *FOXM1* | *CXCL13* |
| *IGF1R* | *CXCR5* |
| *MAPK1* | *GZMK* |
| *MIF* | *HLA-DQA2* |
| *MKI67* | *HLA-G* |
| *MTOR* | *IL10* |
| *MYC* | *IL2* |
| *TOP2A* | *IL7R* |
| *TRIM29* | *JCHAIN* |
| *TUBB* | *MS4A1* |
| *VEGFA* | *TNFRSF17* |

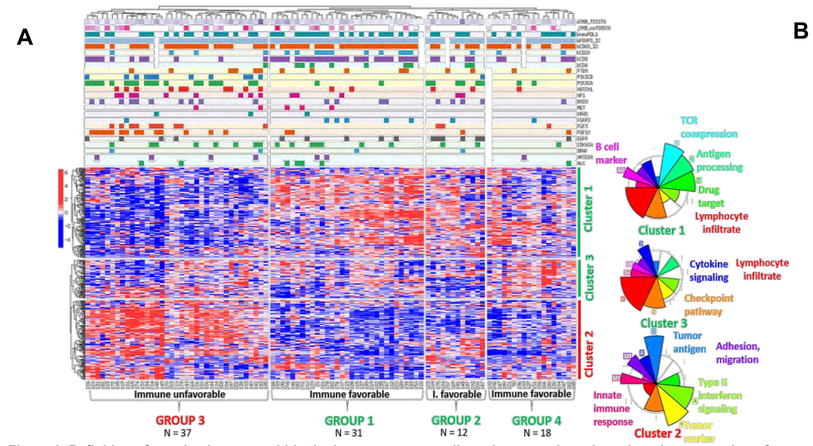


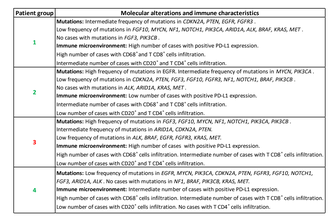

**E Adenocarcinoma summary**

Considering adenocarcinomas, we defined four novel subgroups of tumors with molecular and immune (gene expression + immunohistochemistry) specific patterns. Group 2 showed an immune favorable landscape, group 4 had an immune unfavorable environment and groups 1 and 3 showed a mixed immune profile. These subgroups showed differentially expressed genes related to proliferation, tumor antigen/markers, antigen presentation, immune chemoattraction, B cells, lymphocyte infiltrate and immune signal transduction, among others. Functional pathways associated with these genes involved processes such as cytokine signaling (IL6, IL7, IL8, IL15 IL17A), well-described tumor-related molecules (EGF, FGF, HGF, IGF-1, MAPK, STAT3, JAK, MTOR), tumor suppresors (PTEN), angiogenesis (VEGFA) and innate immune response (NK cells), among others.

**Figure 1. Definition of novel subgroups within the lung adenocarcinoma patients based on the expression of genes involved in the tumor – immune system communication.**
Adenocarcinoma N = 81. **(A)** Heatmap of expression of genes involved in the tumor – immune system communication (left) and Over Representation Analysis (ORA, right) are shown. Groups of patients and clusters of genes were decided by Mclust and defined by hierarchical clustering. Heatmaps scales are expressed as percentils. Green color is associated with an immune favorable landscape. Red color is associated with an immune unfavorable landscape. Yellow color is associated with a mixed immune landscape. **(B)** Molecular patterns and immune infiltration landscape of the described groups. **(C)** Example of the differentially expressed genes between group 4 (immune unfavorable) and group 2 (immune favorable) of adenocarcinomas determined with DESeq2 (FDR ≤ 0.01, Fold Change ≥ |2|). Total number of differentially expressed genes was 137. **(D)** Associated functional pathways determined with Ingenuity Pathway Analysis (z score ≥ |1|, pvalue ≤ 2 E -05). **(E)** Summary of the results of adenocarcinomas.

### Multiparametric comprehensive description of squamous cell carcinoma defines 4 novel tumoral subgroups with specific molecular and immune characteristics

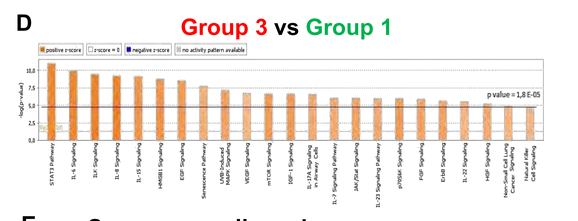


| Patient group | Molecular alterations and immune characteristics |
|---|---|
| 1 | **Mutations:** Intermediate frequency of mutations in *CDKN2A, PTEN, EGFR, FGFR3* . Low frequency of mutations in *FGF10, MYCN, NF1, NOTCH1, PIK3CA, ARID1A, ALK, BRAF, KRAS, MET* . No cases with mutations in *FGF3, PIK3CB* . **Immune microenvironment:** High number of cases with positive PD-L1 expression. High number of cases with $CD68^+$and $T\ CD8^+$ cells infiltration. Intermediate number of cases with $CD20^+$ and $T\ CD4^+$ cells infiltration. |
| 2 | **Mutations:** High frequency of mutations in *EGFR*. Intermediate frequency of mutations in *MYCN, PIK3CA* . Low frequency of mutations in *CDKN2A, PTEN, FGF3, FGF10, FGFR3, NF1, NOTCH1, BRAF, PIK3CB* . No cases with mutations in *ALK, ARID1A, KRAS, MET*. **Immune microenvironment:** Low number of cases with positive PD-L1 expression. Intermediate number of cases with $CD68^+$ and $T\ CD8^+$ cells infiltration. Low number of cases with $CD20^+$ and $T\ CD4^+$ cells infiltration. |
| 3 | **Mutations:** High frequency of mutations in *FGF3, FGF10, MYCN, NF1, NOTCH1, PIK3CA, PIK3CB* . Intermediate frequency of mutations in *ARID1A, CDKN2A, PTEN*. Low frequency of mutations in *ALK, BRAF, EGFR, FGFR3, KRAS, MET*. **Immune microenvironment:** High number of cases with positive PD-L1 expression. High number of cases with $CD68^+$ cells infiltration. Intermediate number of cases with $T\ CD8^+$ cells infiltration. High number of cases with $CD20^+$ and $T\ CD4^+$ cells infiltration. |
| 4 | **Mutations:** Low frequency of mutations in *EGFR, MYCN, PIK3CA, CDKN2A, PTEN, FGFR3, FGF10, NOTCH1, FGF3, ARID1A, ALK* . No cases with mutations in *NF1, BRAF, PIK3CB, KRAS, MET*. **Immune microenvironment:** Intermediate number of cases with positive PD-L1 expression. High number of cases with $CD68^+$ cells infiltration. Intermediate number of cases with $T\ CD8^+$ cells infiltration. Low number of cases with $CD20^+$ cells infiltration. No cases with $T\ CD4^+$ cells infiltration. |

**C Group 3 vs Group 1**

| Upregulated genes | Downregulated genes |
|---|---|
| *AKT1* | *CD19* |
| *BUB1* | *CD1C* |
| *CCNB2* | *CD27* |
| *CD274* | *CD3D* |
| *CD44* | *CD3E* |
| *CDK1* | *CD3G* |
| *FOXM1* | *CD40LG* |
| *IGF1R* | *CD69* |
| *MAD2L1* | *CD79A* |
| *MAPK14* | *CD79B* |
| *MELK* | *CD8A* |
| *MKI67* | *CXCL13* |
| *MTOR* | *HLA-DMB* |
| *MYC* | *IL2* |
| *PIK3CA* | *IL21* |
| *TGFB1* | *IFNG* |
| *TOP2A* | *MS4A1* |
| *TRIM29* | *PDCD1* |
| *VEGFA* | *ZAP70* |




**E Squamous cell carcinoma summary**

Focusing on squamous cell carcinomas, we defined four novel subgroups of tumors. Groups 1, 2 and 4 had an immune favorable profile and group 3 had an immune unfavorable landscape. These subgroups showed differential expression of genes related to proliferation, tumor antigen/markers, antigen presentation, B cells, lymphocyte infiltrate, immune chemoattraction, immunomodulation and immune signal transduction, among others. These genes were involved in pathways related to cytokine signaling (IL6, IL7, IL8, IL15, IL17A, IL22), tumor progression related molecules (STAT3, EGF, MAPK, MTOR, IGF-1, JAK, FGF, HGF), tumor cell migration (HMGB1) and Non-Small Cell Lung Cancer signaling (AKT1, EGFR, MAPK1, PIK3CA, RB1), among others.

**Figure 2. Definition of novel subgroups within the lung squamous cell carcinoma patients based on the expression of genes involved in the tumor – immune system communication.**
Squamous cell carcinoma N = 98. **(A)** Heatmap of expression of genes involved in the tumor – immune system communication (left) and Over Representation Analysis (ORA, right) are shown. Groups of patients and clusters of genes were decided by Mclust and defined by hierarchical clustering. Heatmaps scales are expressed as percentils. Green color is associated with an immune favorable landscape. Red color is associated with an immune unfavorable landscape. **(B)** Molecular patterns and immune infiltration landscape of the described groups. **(C)** Example of differentially expressed genes between group 3 (immune unfavorable) and group 1 (immune favorable) of squamous cell carcinomas determined with DESeq2 (FDR ≤ 0.01, Fold Change ≥ |2|). Total number of differentially expressed genes was 159. **(D)** Associated functional pathways determined with Ingenuity Pathway Analysis (z score ≥ |1|, pvalue ≤ 1,8 E -05). **(E)** Summary of the results of squamous cell carcinomas.

## CONCLUSIONS

**1** – In this work we performed a comprehensive characterization of 179 early stage NSCLC tumors gathering molecular and immune (transcriptomics and immunohistochemistry) data that defined novel NSCLC patient subgroups.

**2** – For both histologies, we defined four novel tumor subgroups with specific molecular and immune features that may influence the response to immunotherapy with checkpoint inhibitors.

**3** – When comparing group 4 and group 2 of adenocarcinomas, we identified differentially expressed genes involved in proliferation, tumor antigens/markers, antigen presentation, immune chemoattraction, B cell-related, lymphocyte infiltrate and immune signal transduction. These genes participated in pathways related to tumor progression, angiogenesis, cytokine signaling and innate immune response.

**4** – When comparing group 3 and group 1 of squamous cell carcinomas, we identified differentially expressed genes participating in proliferation, tumor antigens/markers, antigen presentation, B cell-related, lymphocyte infiltrate, immune chemoattraction, immunomodulation and immune signal transduction. These genes were associated to pathways involved in cytokine signaling, tumor development, tumor cell migration and NSCLC signaling.

**5** – In next steps, we will establish a prediction of the response to immunotherapy with checkpoint inhibitors for each novel NSCLC subgroup we defined. Ultimately, we will validate our results in a cohort of advanced stage NSCLC patients that underwent immunotherapy treatment. These approaches are not included in the shown results.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