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Research Article

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Abstract

Background. Immune checkpoint inhibitors (ICIs) are a therapeutic strategy for various cancers although only a subset of patients respond to the therapy. Identifying patients more prone to respond to ICIs may increase the therapeutic benefit and allow studying new approaches for resistant patients.

Methods. We analyzed the TCGA cohort of HNSCC patients in relation to their activation of 26 immune gene expression signatures, as well as their cell type composition, in order to define signaling pathways associated with resistance to ICIs. Results were validated on a cohort of 102 HNSCC patients under treatment with PD-L1 inhibitors and by in vitro experiments in HNSCC cell lines.

Results. We observed a significant association between the gene set and TP53 gene status and other predictors of the response to ICI in HNSCC patients. Surprisingly, the presence of a TP53 mutation together with another co-driver mutation was associated with significantly higher levels of the immune gene expression, in comparison to tumors in which the TP53 gene was mutated alone. In addition, the higher level of TP53 mutated-dependent MYC signature was associated with lower levels of the immune gene expression signature. In vitro and a patient cohort validation corroborated these findings.

Conclusions. Immune gene signature sets may classify with more accuracy HNSCC patients responsive to immunotherapy. These biomarkers may be easily implemented in clinical setting.

Background

Every year, almost one million people are affected by Head and Neck Squamous Cell Carcinoma (HNSCC) in the world [1]. HNSCC is a biologically diverse and genomically heterogeneous disease that emerges from the squamous mucosal lining of the upper aerodigestive tract, including the lip and oral cavity, nasal cavity, paranasal sinuses, nasopharynx, oropharynx, larynx and hypopharynx [2]. Most patients present with a locally advanced disease with a high risk of recurrence, and approximately 10% of them present with a metastatic disease [3]. The 5-year survival for HNSCC patients across all stages is ~ 40–50% [4, 5], while the median overall survival for recurrent/metastatic (R-M) patients is 10–14 months only [6, 7].

Immune checkpoint inhibitors (ICIs) are an active category of immunotherapies that block inhibitory immune check-point pathways in order to reactivate immune responses against cancer. In 2016, the US Food and Drug Administration (FDA) approved two ICIs, the anti-programmed cell death protein (PD-1) monoclonal antibodies, nivolumab (Opdivo, Bristol-Myers Squibb) and pembrolizumab (Keytruda, Merck), for the treatment of R-M HNSCC patients refractory to platinum-based therapy. The same agency then approved pembrolizumab for the first-line treatment of patients with unresectable R-M HNSCC. Understanding what determines the response of HNSCC to ICIs is therefore of utmost clinical importance.

The use of immune checkpoint inhibitors (ICIs) is increasing in several cancer settings, both as monotherapy and as combinations with another ICI, chemotherapy, or targeted agents. The benefit of this new class of drugs seems to be large but limited to a subgroup of patients, thus an efficient patient characterization is needed to guide improvements in treatment. Predictors of response to ICIs are critical
to ensure optimal selection of patients to be offered these drugs, thus achieving higher response, preserving patients from unnecessary toxicities, and saving economic resources. Also, this could allow an early activation of other therapeutic strategies, including clinical trials, for patients whose probability of response to ICIs is predicted to be very low. However, there are currently very few markers available, the most important of which are PDL1, microsatellite instability and tumour mutational burden (TMB) assessment; of these, only the assessment of PDL1 as a combined positive score (CPS) is utilized as a predictive marker in first-line R-M HNSCC. Several clinical, molecular, and microbiological factors are assumed to have a role as influencing response to ICIs. Recently, the ASCO and the College of American Pathologists discussed the challenges and opportunities of using biomarkers to predict the benefit from ICI, underlying the need for a more comprehensive evaluation, including testing of group of biomarkers, standardizing assays and generating more data to address the open questions [8].

The aim of the present report is to assess the role of $TP53$ gene status together with additional genetic and non-genetic aberrations as predictors of the response to ICI in HNSCC patients, adjusting for potential confounders. We applied a bioinformatic approach and validated the results in experimental studies with human cancer cell lines and in analyses of independent cohorts of HNSCC patients.

**Methods**

Data were derived from the “The Cancer Genome Atlas” (TCGA - the HNSCC-TCGA, Nature 2015), and the analyses included 520 HNSCC patients. We gathered the normalized TCGA HNSCC gene expression data from Broad Institute TCGA Genome Data Analysis Center (http://gdac.broadinstitute.org/): Firehose stddata__2016_01_28 and Broad Institute of MIT and Harvard. doi:10.7908/C11G0KM9.

Clinical information for cohorts was collected from cBioPortal (https://www.cbioportal.org/datasets), according to the data published by Liu et al. [9].

To identify HNSCC patients responsive to immunotherapy, for each gene set and immune checkpoint protein we initially developed a logistic regression model based on the average expression of the immune signature genes. Specifically, the mean expression values of the genes belonging to the specific immunological signature were used to build linear regression models and to assess their associations with several clinical variables. Odds ratios with confidence intervals at 95% were evaluated for each gene set by including age, gender, tumor size, limphnode status, stage, HPV status, smoking history, TMB, and the mutational status of $TP53$, $PIK3CA$, $FAT1$ and $CDKN2A$. Significance was defined at the 5% level ($p < 0.05$). Results are presented as Odds Ratio values (OR) with 95% confidence intervals. The significance of gene/signature modulation between different subgroups of samples was assessed by Wilcoxon test or ANOVA test, as appropriate. The analyses were conducted with Matlab R2022a.

Kaplan-Meier curves of HNSCC patients with high or low immune scores were conducted in order to assess the overall survival (OS) and progression free survival (PFS). Differences between curves were evaluated by the Logrank test. Hazard ratios with 95% confidence intervals were assessed by Cox Hazard
regression models. Immune scores were evaluated as the positive and negative z-scores of the average expression of the 125 genes composing the 26 immune gene sets.

To investigate the cellular heterogeneity landscape of the tissue expression profiles, we performed a cell type enrichment analysis using XCell (https://xcell.ucsf.edu/), a gene signature-based method to associate gene expression profiles with 64 immune and stroma cell types [10].

A validation cohort of 102 HNSCC patients treated with PDL1 inhibitors was gathered from GEO database with accession ID GSE159067 [11].

**Cell cultures and transfection**

Cal27, FaDu and Detroit 562 cell lines were obtained from ATCC (Rockville, MD, USA). These cells were cultured in RPMI-1640 (Cal27, FaDu) and DMEM (Detroit 562) medium (Invitrogen-GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum and 20% for Detroit 562, penicillin (100 U/mL), and streptomycin (100 mg/mL; Invitrogen-GIBCO). All cell lines were grown at 37°C in a balanced air humidified incubator with 5% CO2. The transfections were performed with Lipofectamine RNAiMax. All experiments were conducted according to the manufacturer’s recommendations. siRNAs were purchased from Eurofins MWG (Ebersberg, Germany) and sequences are as follows: si-SCR: 5′-AAGUUCAGCGUGUCCGGAG-3′; si-YAP: 5′-GACAUCUUCUGUGUCAGAGA-3′; Si-p53: 5′-GACUCCAGUGUUAAUCUC-3′. The cells were transfected for 48 hours according to the cell line and the experiments (see results).

**RNA processing and qRT-PCR**

Total RNA from cell lines differently treated or not was extracted by using Trizol Reagent following manufacturer’s instructions (Ambion). cDNA was synthesized according to the manufacturer’s instructions (M-MLV RT kit, Invitrogen). Gene expression was measured by real-time PCR using the FastStart SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 5 (Applied Biosystems). Sequences of qPCR primers are ACTIN Fw: 5′-GGCATGGGTCAGAAGGATT-3′, Rv: 5′-CACACGCGACTCATTGCTAGAG-3′; PD-L1 Fw: 5′-CATCTTTATTATGCCTTGGTGTGCA-3′, Rv 5′-GGATTACGTCTCCTCCAAATGTG-3′.

**Results**

The study was conducted to investigate the association between 26 immune gene sets (listed in Table S1) and immune checkpoint proteins expressed in HNSCC and their effect on patient survival. The 26 immune gene-set signatures tested in the present report were reported in a previous publication to be associated with the response to ICI in Triple Negative Breast Cancer [9]. In the current study, we assessed these signatures in HNSCC, as 20 of the 26 gene sets were also found to be modulated in HNSCC [12]. In particular, in the present analysis we assessed the effect of the 26 immunosignature on both the programmed cell death protein ligand −1 (PDL-1) axis and the T-lymphocyte associated protein 4 (CTLA-
4) axis, controlling for potential confounders and effect modifiers [13]. The 520 participants of the TCGA sample were equally distributed for age, gender (males/females) and smoking. 19% (N = 97) of the patients were HPV positive and only 4% (N = 20) had a stage 1 lesion at diagnosis. Patients had cancer lesions characterized by mutated TP53 (70%) and mutation of CDKN2A (22%), FAT1 (22%) and PIK3CA (18%) genes (Table S2). The 26 immune gene expression signatures, the related analysis of the genes that comprise these signatures, and the analysis of the 15 immune cell types, are described in Table S1.

To test the hypothesis that TP53 gene status and other genetic and non-genetic aberrations could be combined to predict the response to ICI in HNSCC patients, we used a step-by-step approach depicted in the Fig. 1 flowchart.

**Step 1** of the flowchart includes the computational analysis of the clinical genomics data from TCGA. Phase (A) describes the analysis of 26 immune gene expression signatures as prognosis predictors within the TCGA cohort of 520 HNSCC patients. A global immuno score (IS) for the 26 gene sets considered was obtained as the z-scores of the average expression of the genes included in the gene sets. Phase (B) describes the analysis of the immunosignature prediction performance by HNSCC TCGA subgroups, such as TP53 wild type (TP53-WT) versus mutated (TP53-mut), alone (i) and with other co-mutated genes (ii). A cell type enrichment among these groups was also performed (iii). Phase (C) describes the analysis of association between IS and a 22 MYC-related genes expression. In our previous work we identified a 22-gene MYC-related signature in HNSCC cancer, which is specifically activated by TP53 mutations with a gain of function activity, and could therefore serve as a proxy for such mutations [14]. In **Step 2**, we conducted an in-vitro validation of Phase B and a general result validation on 102 HNSCC patients from the GEO database.

**STEP 1 – phase A: Regression and survival analysis**

In Fig. 2, panel A, we aimed to identify subgroups of HNSCC patients with a significant difference of IS levels. The first set of results are represented by the forest plot with Odds ratio with 95% CI of demographic and prognostic predictors of the immune signature expression by using regression models in the HNSCC dataset from TCGA. All the analyses were performed at univariate level considering the effect of only one independent variable (demographic or prognostic variables) on the signature (dependent variable). We observed that only the HPV status, with HPV negative versus the positive lesions, lymphnode status (N0 vs N+) and TP53-mut plus additional mutations versus TP53-mut-only lesions, were statistically associated with a higher immune signature expression. Details of regression analyses on clinical factors for each immune gene set are shown in S-Fig. 1 and S-Fig. 2. Notably, building a multivariable regression model, TP53 mutational status resulted in the main clinical factor significantly associated with the immune signature (Table S3). To provide clinical meaning to the described results, in Fig. 2, panel B, we reported overall-survival (OS) and progression-free-survival (PFS) curves assessed in the TCGA cohort of 520 patients. In that cohort, high immune signature expression was significantly associated with both overall and progression-free survival.
Phase B: Immunosignature in TP53 mutated (i) and TP53 co-mutated patients (ii) and cell types enrichment (iii).

Because the mutational \textit{TP53} status was so important, we assessed the expression distribution of the immune signature by three groups of patients with \textit{TP53}-WT status, \textit{TP53}-mut status, and \textit{TP53}-mut in combination with one of the other three most frequent mutations observed in HNSCC cancer patients (FAT1, CDKN2A, and PI3K genes), hereinafter denoted as \textit{TP53}-mut$^+$. \textit{TP53}-WT patients were characterized by a higher IS expression level compared with TP53-mut and \textit{TP53}-mut$^+$ patients (Fig. 2C). Surprisingly, \textit{TP53}-mut$^+$ patients had a significantly higher IS score in comparison to the \textit{TP53}-mut patients (Tukey's post-hoc test, p < 0.05) though significantly lower than WT patients (Tukey's post-hoc test, p < 0.01) (Fig. 2C).

In line with the importance of the TP53 status for the response to ICI, an analysis of cell type composition, performed with Xcell software [10], revealed distinct immune cell composition across the three TP53 groups (Fig. 2D). In support of a fundamental difference between TP53-mut tumors with vs. Without additional mutations, the abundance of 7 immune cell types was statistically different between TP53-mut and TP53mut$^+$ patients (Fig. 2E). To further detail the functional link between \textit{TP53} gene mutations with gain of function activity and immune signature, we assessed the role of the \textit{TP53} mutated-dependent MYC signature identified in our previously work [14]. In Fig. 3, we assessed the expression of PDL1 and CTLA4 in TCGA patients with high or low expression of MYC-related signature (Fig. 3A and B) and the expression of the immune gene sets in both TCGA and GEO cohort (GSE195832) (Fig. 3C and D) patients. Again, lower expression level of the TP53 mutated-dependent MYC signature was significantly associated with higher levels of the IS score, PDL1 and CTLA4. Furthermore, in TCGA cohort we had sufficient clinical information and sample size to adjust those modulation for potential confounding factors. The multivariate models reported at the bottom of panels A, B and C confirmed that those associations, between genes or IS and the MYC-dependent signature, were independent from other clinical factors. To validate these results, we performed qRT-PCR analysis of PD-L1 (Fig. 3E) and CTLA4 (Fig. 3F) in Cal27 cells, a head and neck cancer cell line carrying a \textit{TP53} mutation, treated with JQ-1. The latter is a small-molecule that inhibits the activity of the BET family proteins by masking their bromodomain acetyl-lysine-binding pockets [15]. JQ-1 has been demonstrated to act as an antineoplastic agent by mainly inhibiting c-MYC functions. Both genes showed increased expression after treatment when compared to their controls, strengthening the potential role of MYC in this immunogenic context (Fig. 3E and F).

\textbf{Step2- - Phase I and II: Analysis of NCI-60 cell lines and in vitro validation. Analysis of the immune gene sets and MYC dependent signature in a cohort of HNSCC patients treated with PD-L1-inhibitors.}

We further investigated the role of the immune gene sets and of the TP53 mutated-dependent MYC dependent gene signature in their well characterized cohort of HNSCC patients under treatment with PD-L1 inhibitors obtained from the GEO database (accession ID: GSE159067).
In Fig. 4A, we report results of our analysis on the prognostic value of the tested immune gene sets in both OS (left panel) and PFS (right panel). These results corroborate our findings from the TCGA cohort (Fig. 2B), demonstrating that our expression-based IS score is indeed associated with improved survival across clinical datasets.

The immune gene sets, we used to define our immune score, was also strongly correlated to the classification (“COLD” and “HOT”) introduced by Foy and colleagues (S-Fig. 3). Notably, In Fig. 4B, low levels of the immune gene sets are significantly associated with stable or progressive disease during immunotherapy. Furthermore, low level of the TP53-dependent MYC signature was significantly associated with the immunologically “HOT” type (Fig. 4C). These results are in line with our finding on TCGA data and cell lines about the potential role of MYC in an immunogenic context. In NCI-60 cell lines, indeed, we found that WT-TP53 HNSCC cell lines exhibit higher expression of CTLA4 and PDL1 when compared to cell lines harbouring TP53 mutations (S-Fig. 4A). Higher levels of PDL1 were also found in cell lines carrying TP53 co-mutations, such as Detroit-562 and FaDu, when compared to those carrying only TP53 mutation (S-Fig. 4B). We also observed that depletion of either mutant p53 protein or its co-factor YAP released PDL1 expression in HNSCC cell lines (S-Fig. 4C). Enhanced expression of PDL1 was also obtained in CAL-27 and Detroit-562 after treatment with alpelisib, a selective inhibitor of p110α-subunit of PI3K (S-Fig. 4D). We have previously identified that mutated p53 and YAP favour c-Myc stability and its transcriptional activity in HNSCC cell lines [14]. In that contest the use of alpelisib has been found to partially impair this pro tumorigenic axis [14].

Finally, we used specific marker genes of the cell types identified in the cell enrichment analysis of TCGA data (Fig. 2C) to evaluate their quantitative expression on immunotherapy treated patients. Six out of the seven investigated cell types resulted strongly up-regulated in HNSCC patients characterized by complete or partial response to the treatment (Fig. 4D). The same cell types showed a significant reduced abundance in patients with low Immune Score (S-Fig. 5).

To study the potential cause of the difference in the response to ICI between TP53-mut and TP53-mut+ HNSCC patients, we considered the aneuploidy score of TCGA HNSCC patients. In general, aneuploidy is strongly associated with TP53 mutations, and is negatively correlated to several immune signatures across various cancers [16, 17]. In line with previous evidence, we observed the negative correlation between aneuploidy scores and our IS (Fig. 5A). As expected, aneuploidy levels were significantly higher in the TP53-mut and TP53-mut+ patients (Fig. 5B). Interestingly, however, aneuploidy levels were significantly lower in the TP53-mut+ group relative to the TP53-mut group. Therefore, aneuploidy levels may underlie the difference in ICI response between the two groups. To establish the association between TP53 mutation, co-mutation and aneuploidy levels in immune gene prediction set, we built multivariate regression models, adjusting the TP53 mutational and co-mutational status for the aneuploidy scores. In the multivariate models TP53 mutation, TP53 co-mutation and aneuploidy were found to be independent predictors of the immune signature (Fig. 5C and D).

Discussion
The successful implementation of precision medicine is highly based on clinically-relevant predictive biomarkers, but very few markers are currently known. The administration of ICIs has significantly improved treatment outcomes and survival of HNSCC patients. However, only a limited subset of HNSCC patients benefits from ICI treatment, highlighting the unmet need to better stratify patients for this treatment [18]. In the present work, we used gene expression profiling of a large well-characterized database of HNSCC patients (TCGA), to identify new biomarkers of immune modulation in HNSCC, a disease in which no biomarkers, except for PD-L1 for pembroluzimab, have been identified to date. On note, while in other tumor types variables such as TMB and tumor stage appear as a noteworthy aspect for the response to ICIs, in HNSCC tumor these variables seem to lose relevance when looking at outcomes adjusted specifically for HPV and TP53 status (Fig. 2A). The application of 26 gene sets associated with immune functions to HNSCC mRNA expression data allowed us to identify patients with higher-vs. lower-likelihood to respond to ICI treatment. We also found that variables such HPV positive versus negative, lymph node status and TP53-WT versus TP53-mut patients were associated with a higher IS and a higher PDL-1 and CTLA4 expression. We also found that HNSCC cell lines carrying WT-TP53 exhibit higher expression levels of both PDL1 and CTLA4 when compared to cell lines bearing TP53 mutations (S-Fig. 4A). We have previously shown that gain of function activity of TP53 missense mutations in HNSCC also acts through the aberrant transcriptional activation of a MYC-responsive 22 gene signature that is curtailed by the PI3K inhibitor alpelisib [14]. We found that HNSCC patients with low expression of this MYC signature expressed higher levels of both PDL1 and CTLA4 in comparison to those high levels of the signature. Interestingly, HNSCC patients with low expression of TP53-dependent MYC signature have higher immunoscore. Of note, HNSCC patients carrying co-mutations such TP53/FAT1, TP53/CDKN2A, TP53/PI3K exhibited a higher immunoscore than those with only TP53 mutations. Consistently, PDL1 expression levels were higher in HNSCC cell lines carrying TP53 co-mutations compared to those carrying only TP53 mutation (S-Fig. 4B). Depletion of either mutant p53 protein or its co-factor YAP released PDL1 expression in HNSCC cell lines (S-Fig. 4C). The treatment with alpelisib, a selective inhibitor of p110α-subunit of PI3K in CAL-27 and Detroit-562 head and neck cell lines enhanced the expression of PDL1 (S-Fig. 4D). To further define differences in immune activity between TP53-mut patients and TP53-mut + patients, we considered the aneuploidy scores available from cBioPortal (https://www.cbioportal.org/) for TCGA HNSCC cohort. Indeed, the association between TP53 mutation and aneuploidy has been reported in several human cancers [16, 17]. Here in we originally broaden this association showing that TP53 mutated patients with higher level of aneuploidy (Fig. 5B) exhibit also lower level of immune gene set expression as described in Fig. 2C. High aneuploidy and TP53 mutational status are both significantly associated to lower immunoscore as shown in the multivariate model (Fig. 5C). In aggregate, our findings unveil a scenario in which gene mutations, aberrant DNA content and altered gene expression might allow defining more precisely than each one per se HNSCC patients who will benefit from immunotherapy

Conclusions
There are few important implications emerging from these findings. Firstly, while TP53 gain of function mutant p53 proteins might directly repress the expression of ICs such as PDL1, the presence of a co-mutation mitigates this effect through a yet unidentified compensatory mechanism. Given that TP53-mut + tumors are less aneuploid than TP53-mut-only tumors, and that high degree of aneuploidy is associated with escape from immune-surveillance [16, 17, 19], aneuploidy levels are likely an additional contributing factor to the drug response differences between the groups. Secondly, HNSCC patients carrying co-mutations TP53/PI3K could benefit from alpelisib plus ICI. Thirdly, HNSCC patients relapsing to the PI3K inhibitor alpelisib might be proposed for immunotherapy treatment. It should be acknowledged that PI3K inhibitors have been studied [20] and are currently in clinical trials also in RM-HNSCC (NCT04338399).

With the due limitations, deconvolution analyses from bulk-RNA-Seq data revealed that high/low immunoscore might contribute to deciphering the immune infiltration cellular landscape of HNSCC patients. Indeed, we found that high-immunoscore HNSCC patients exhibited immune infiltration in which aDC, macrophages, CD8 + T-, macrophages M1, naive B cells, pDC, Th1 cells appear to be significantly more represented than in those with low immunoscore. Notably, HNSCC patients with TP53 and additional mutations showed a putative immune cellular landscape more similar to P53-WT HNSCC patients than to those with TP53 mutations. There are no current therapies that directly promote infiltration of immune cells for HNSCC patients, but the potential of the identified immunoscore to provide insights into the cellular composition of the immune infiltrate is certainly relevant to profile immunologically a given patient.

Our identified immunoscore is significantly associated with OS and PFS across independent studies. Therefore, our findings provide evidence of an immunoscore that holds prognostic and predictive features of a biomarker, and could facilitate the selection of HNSCC patients who can benefit the most from ICI treatment.

Abbreviations

Recurrent/metastatic (R-M); Food and Drug Administration (FDA); the anti-programmed cell death protein (PD-1); Immune checkpoint inhibitors (ICIs); Head and Neck Squamous Cell Carcinoma (HNSCC); the anti-programmed cell death protein ligand -1 (PDL-1); tumour mutational burden (TMB); combined positive score (CPS); T-lymphocyte associated protein 4 (CTLA-4); The Cancer Genome Atlas (TCGA); overall survival (OS); progression free survival (PFS); Activated dendritic cell (aDC); Plasmacytoid dendritic cell (pDC).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
All authors give consent for publication of the manuscript.

**Availability of data and materials**

Data derived from the “The Cancer Genome Atlas” (TCGA - the HNSCC-TCGA, Nature 2015) and the analyses included 520 HNSCC patients. We gathered the normalized TCGA HNSCC gene expression of tumour from Broad Institute TCGA Genome Data Analysis Center (http://gdac.broadinstitute.org/): Firehose stddata__2016_01_28 and Broad Institute of MIT and Harvard. doi:10.7908/C11G0KM9_A. A validation cohort of 102 HNSCC patients treated with PDL1 inhibitors was gathered from GEO database with accession ID GSE159067.

**Competing interests**

The authors declare no conflict of interest.

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**Authors’ Contributions**


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Not applicable.

**References**


Figures

Figure 1

**STEP 1**

**Phase a**

IMMUNE GENE SETS
26 signatures

Regression & survival analysis on TCGA HNSCC 520 patients

prognostic Immune Score (IS)

**Phase b**

i) IS relationship with TP53 mutated patients
ii) IS relationship with TP53 mutated/co-mutated patients
iii) cell types enrichment in TP53 mutated/co-mutated patients

**Phase c**

IS association with a MYC dependent gene signature

**STEP 2**

**Phase I**

Analysis of NCI-cell lines and in vitro validations

**Phase II**

VALIDATION ON A PDL1-INHIBITORS TREATED COHORT 102 patients

Figure 1

A) Workflow of the main analyses.
Figure 2

Regression, survival analysis and cell types enrichment on TCGA HNSCC cohort. A) Forest plot representing the association of average expression of 125 genes included in the 26 immune gene sets and the clinical variables in 520 HNSCC patients from TCGA. Results of the linear regressions are shown as Odds Ratio with confidence intervals at 95%. B) Kaplan-Meier curves of HNSCC patients from TCGA cohort with high or low Immune Scores evaluated for overall survival and progression free survival (upper
and bottom panel, respectively). Differences between curves were evaluated by logrank test. Hazard ratios with 95% confidence intervals were assessed by Cox Hazard regression models. Immune Scores were evaluated as the positive and negative z-scores of the average expression of the 125 genes composing the immune gene sets. C) Distributions of the gene signature composed by the average expression of 125 genes of the immune gene sets by TP53 mutation and TP53 mutation carried on other mutations among FAT1, CDKN2A and PIK3CA in HNSCC patients (106 WT, 171 TP53 and 189 TP53+mutX). P-values were evaluated by Kruskal-Wallis test. D) Cell types enrichment analysis by comparing 64 cell type signatures in subgroups of HNSCC patients with TP53 mutation, TP53 mutation with other mutations and wild type patients. Heatmap representing the normalized average scores obtained from Xcell software, reflecting the cell type abundance of the most significant modulated cell types among the three subgroups. The statistical significance (p<0.05) was assessed by Kruskal-Wallis test. E) Cell types enrichment of TP53 mutated patients and TP53 mutated patients who harboured other mutations. Scores were obtained from Xcell software. P-values were evaluated by Wilcoxon ranksum test.
Figure 3

**Association of a MYC dependent signature with the immune gene sets. A-C)** box-plot of the PD-L1 (A), CTLA4 (B) expression values and mean expression of 26 immune gene sets (C) in patients with high and low level expression of a 22 genes signature MYC dependent (Ganci et al.) in HNSCC datasets from TCGA. Statistical significance between distributions was assessed by Wilcoxon rank-sum test. Multivariate regression models were built to adjust the differences of the genes between patients with...
high and low MYC signature. The models include T status, TP53 mutation, gender, smoking status and, HPV status. High and low expression of the MYC signature were evaluated by positive and negative z-scores of the mean gene expression, respectively. D) box-plot of the mean expression of 26 immune gene sets in 28 pre-treated HNSCC patients with high and low level expression of a 22 genes signature MYC dependent in GSE195832 dataset from GEO. Statistical significance between distributions was assessed by Wilcoxon rank-sum test. E-F) qRT-PCR analysis of PD-L1 (E) and CTLA4 (F) in Cal27 treated with JQ-1. Bars indicate the average of at least three independent experiments. Statistics (t-test): * p < 0.01, ** p<0.005.
Figure 4

Validation of immune gene sets and 22 genes MYC dependent signature in a cohort of HNSCC patients treated with PDL1-inhibitors. A) Overall survival (left panel) and Progression free survival (right panel) of 102 patients treated with PDL1 inhibitors from GEO database (GSE159067). Patients were split basing on the Immune Score. The high/low levels of Immune Score were obtained considering the positive and negative z-scores of the average expression of the 26 immune gene sets, respectively. Differences
between curves were evaluated by logrank test. The multivariate Cox Hazard regression analysis was adjusted for gender and HPV status. **B-C)** Average expression of the 26 immune gene sets and MYC signature distribution in 102 patients treated with PDL1 inhibitors (GSE159067, panel B and C, respectively). The immune gene sets expression was evaluated in patients with complete or partial response and patients with stable disease or progression disease after treatment (B). The MYC signature expression was evaluated according to the phenotype classification (“COLD” and “HOT” patients) obtained from Foy JP and colleagues (C). Differences were evaluated by Wilcoxon test. **D)** Significantly modulated cell type marker genes in 102 patients from GEO database (GSE159067) among the 7 cell types previously identified in the deconvolution analysis of TCGA HNSCC data. Statistical significance between patients with complete or partial response and patients with stable or progression disease after treatment with anti-PDL1 was evaluated by Wilcoxon test.
Figure 5

Association of the immune signature and mutational status with the aneuploidy score. **A)** The Spearman's correlation coefficient reveals a negative association between aneuploidy score and immune signature. **B)** Distributions of the aneuploidy scores among TP53 mutated patients, WT patients and co-mutated patients. Co-mutated patients show lower aneuploidy than TP53 mutated patients. Statistical significance was evaluated by Wilcoxon test. **C)** Multivariate regression model to assess the
weights in the immune gene sets prediction of the aneuploidy score and the TP53 mutational status. The variables resulted to be independent predictors of the immune signature. **D)** Multivariate regression model to assess the weights in the immune gene sets prediction of the aneuploidy score and the TP53 co-mutational status. The variables resulted to be independent predictors of the immune signature.

**Supplementary Files**

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