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**IDENTIFICATION OF ALTERNATIVE STRATEGIES IN ADVANCED NON-
SMALL CELL LUNG CANCER (NSCLC) PATIENTS RESISTANT TO IMMUNE
CHECKPOINT INHIBITORS (ICIs) OR WITH RARE HISTOLOGIES**

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ABSTRACT

Immune checkpoint inhibitors (ICIs), such as antibodies directed against PD-1/PD-L1, have led to a paradigm shift in the management of non-small cell lung cancer (NSCLC). However, the effectiveness of these drugs appears to be limited only to a minority of NSCLC patients, particularly to those cancers with a strong PD-L1 expression. Many of the knowledge on the molecular mechanisms of primary and acquired resistance, which may be related to the tumor or to the tumor or circulating microenvironment, during ICIs therapy come from studies conducted on other tumors; by contrast, few data are available in NSCLC. The PhD research project is part of a project that has received funding from the Ministry of Health's Finalized Research 2018 (GR-2018-12368031, P.I: dr. Francesco Gelsomino) and entitled "Understanding molecular mechanisms of resistance to immune checkpoint inhibitors (ICIs) in advanced non-small cell lung cancer (NSCLC)". The activity of the PhD project focused on two strands of research:

- a. the role of PD-L1 and interferon-gamma (IFN- γ) as regulators of tumor plasticity and ICIs resistance in cell models derived from patients with NSCLC and hyperprogressive disease (HPD) during ICIs;
- b. EGFR tyrosine kinase inhibitor (EGFR-TKI) activity in cell models derived from patients with advanced NSCLC and Class 3 BRAF mutation progressed on ICIS treatment.

In addition, during the PhD program I have been engaged in two further lines of research regarding the ICIs use in the clinic in two specific settings:

- c. patients with advanced NSCLC and PD-L1 $\geq 50\%$ with early progression disease on ICIs as first line therapy: the role of salvage chemo-immunotherapy;
- d. patients with advanced NSCLC and rare histology: atezolizumab activity.

PD-L1 AND IFN- γ AS REGULATORS OF NON-SMALL CELL LUNG CANCER (NSCLC) CELL PLASTICITY AND IMPLICATIONS FOR IMMUNOTHERAPY RESISTANCE

ABSTRACT

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide. Although immune checkpoint inhibitors (ICIs) have demonstrated outstanding clinical efficacy in the treatment of NSCLC patients, ICIs-based therapy has also been associated with a paradoxical acceleration of tumor growth defined as hyperprogressive disease (HPD). In this paper we evaluated distinct plasticity traits in a model of HPD-NSCLC, with the aim of clarifying the mechanisms contributing to ICI resistance that involve IFN- γ and PD-L1.

For this purpose, primary cell cultures were established from two samples obtained from one patient with stage IV NSCLC: one, prior to ICI initiation (NSCLC-B, baseline) and the other at the time of radiological evidence of HPD under ICI treatment (NSCLC-H, hyperprogression). Compared to NSCLC-B cells, NSCLC-H cells exhibited a more aggressive *in vivo* and *in vitro* behavior, higher MAPK activation and owned the ability to develop tumoroids. The 3500 differentially expressed transcripts, according to whole transcriptome analysis, well described the profound plastic evolution of cells from NSCLC-B to NSCLC-H culture, as well as the increase of CD44 in the NSCLC-H cell line model, in which transcripts of CD44 isoforms lacking the variant domain were abundant. Transcripts of genes involved in the cellular response to IFN- γ were down-regulated in NSCLC-H compared to NSCLC-B, together with CD274. Conversely, an up-regulation of genes related to inflammatory response, including IL1- β and IFNGR1, was found. *In vitro* response to IFN- γ was compromised in both NSCLC-B and NSCLC-H cells, since IFN- γ failed to exert its antiproliferative effect on these cells and to effectively induce PD-L1 expression in 2D-growth assays. Nevertheless, the cytokine induced the activation of both type I and type II IFN-pathway mediators. In addition, treatment with IFN- γ induced NSCLC-H traits in NSCLC-B cells, promoting a striking increase in the number of NSCLC-B 3D-soft agar colonies. Low IFN- γ doses or modulation of PD-L1

contributed to the evolution of NSCLC-B towards NSCLC-H phenotype through the augment of CD44 expression on cell membrane, cell morphological changes and increased cell growth or sphere formation ability.

In conclusion, we report a modulation of plasticity by PD-L1 regulation and IFN- γ in a NSCLC cell culture established from a treatment-naïve patient, who developed HPD under ICI therapy, suggesting an association between NSCLC plasticity and these main actors involved in ICI activity.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide [1,2]. Non-small cell lung cancer (NSCLC) represents the most common histological subtype of lung cancer, accounting for approximately 85% of total diagnoses [3]. The presence of nonspecific clinical symptoms and the paucity of effective screening programs lead to delayed NSCLC diagnosis and reduced survival rates for these patients [4]. The clinical management of NSCLC appears even more difficult if we consider that only about one third of this neoplasm harbors a targetable driver alteration [5]. The discovery and the following approval in clinical practice of monoclonal antibodies targeting immune checkpoint molecules, including cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) and its ligand (PD-L1), able to restore anti-tumor immunity, led to a paradigm shift in the treatment of non-oncogene-addicted advanced NSCLC [6]. Immune checkpoint inhibitors (ICIs) have indeed demonstrated unprecedented durable responses and significant improvement in patients' survival in a variety of tumor types, becoming the standard of care for the treatment of several malignancies, including NSCLC [7–9]. Nevertheless, despite the impressive breakthrough provided by ICIs, it has been reported that only 20-30% of NSCLC patients have a long-term survival benefit from these drugs because of both primary and acquired mechanisms of resistance [10,11]. In addition, the success of ICI therapy is hindered by the development of detrimental, atypical patterns of tumor response [12,13]. Between these atypical patterns of response, hyperprogressive disease (HPD) is the

most controversial and damaging one. The phenomenon of hyperprogression was first assessed in retrospective studies involving several patients affected by different tumor types, who showed a paradoxical acceleration of tumor growth, reduced survival, and early death under ICI therapy [14]. For what concerns NSCLC, evidence suggests an incidence rate of HPD ranging between 5 to 37% [15–17]. Even though several factors associated with hyperprogression have been pointed out, including older age and genomic alterations such as MDM2/MDM4 amplifications, effective predictive biomarkers for HPD occurrence are not yet available [18–20]. Similarly, despite some groups demonstrated a significant enrichment of specific immune cells in tumor microenvironment (TME) of HPD patients, including M2-like CD163+ macrophages and regulatory T cells (Treg) [21,22], the underlying immunological mechanisms of HPD remain unknown and whether these mechanisms are tumor-dependent or not has to be still clarified [14].

In this paper, we report our findings regarding the possible roles of IFN-gamma (IFN- γ) signaling pathway and PD-L1 modulation in the development of HPD under ICI therapy in NSCLC. For this purpose, we derived two NSCLC cell lines from tumor biopsies of an HPD patient, obtained before treatment initiation (NSCLC-B cell line) and after hyperprogression onset (NSCLC-H cell line). NSCLC-B and NSCLC-H cells showed deep phenotypic and genotypic differences, evidencing strong plasticity traits in the latter cell line. Additionally, we proved an involvement of IFN- γ and PD-L1 in the modulation of tumor plasticity, suggesting a possible, intriguing tumor-dependent mechanism of survival and “self-empowerment” in tumors of HPD patients, in presence of ICI therapy.

MATERIALS AND METHODS

Clinical setting and cell lines

A 64-year-old, former light smoker (10 packs/year) woman was diagnosed with stage IVA NSCLC. At the time of HPD under pembrolizumab (Keytruda, Merck) treatment, the tumor was classified as a poorly differentiated, stage IVB NSCLC. Tumor samples were collected from the patient before and after treatment initiation, at three different time points. Specifically, two samples were obtained before treatment initiation: one at the diagnosis (time of diagnosis, Td), the other one month later (time of baseline, Tb). The third sample was obtained three months after treatment initiation, at the time of radiological evidence of HPD (time of hyperprogression, Th).

NSCLC-B cell line (B: “baseline”) was derived from the pleural effusion of the treatment-naïve patient at the Tb point, while NSCLC-H cell line (H: “hyperprogression”) was established from a subcutaneous tumor biopsy collected at the Th point. NSCLC-B primary cell culture was established as previously described [23], while NSCLC-H primary cell culture was derived as follows: tumor biopsy was dissected through a sterile surgical lancet. The obtained fragments were then placed into a 25 cm² PRIMARIA tissue culture flask (Corning Life Science). Both NSCLC-B and NSCLC-H cell lines were established and cultured in RPMI medium (Thermo Fisher Scientific, Monza, Italy) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin and 10 µg/mL streptomycin (Thermo Fisher Scientific) and grown at 37°C in a humidified atmosphere at 5% CO₂.

The study was conducted in accordance with the Declaration of Helsinki. Human samples were collected after the patient gave her informed consent. Human samples and metadata including relevant clinical data were de-identified before being shared between laboratories involved in this study. The protocol was approved by the Ethics Committee Center Emilia-Romagna Region, Italy (GR-2018-12368031).

Patient-derived xenograft models

8–30-week-old female BALB/c Rag2^{-/-}; Il2rg^{-/-} (BRG) immunodeficient mice (breeders kindly provided by Drs T. Nomura and M. Ito, Central Institute for Experimental Animals, CIEA, Kawasaki,

Japan) [24] were bred under sterile condition in our animal facilities and were used for patient-derived xenograft (PDX) establishment. To generate NSCLC-B PDX, the pleural effusion of the patient was centrifuged at 250 g for 5 minutes to obtain cell sediment. Cells were counted and tested for viability using erythrosine. BRG mice received subcutaneous injection of 10⁷ cells, in the right hind leg. To generate NSCLC-H PDX, a fragment of tumor biopsy with a diameter of 3-4 mm was implanted in the left flank of a female anesthetized BRG mouse. NSCLC-B and NSCLC-H PDX tumors were subsequently propagated until the third in vivo passage by subcutaneous implantation of tumor fragments with a diameter of 3-4 mm in the left flank or at the level of trans-scapular brown fat of female anesthetized BRG mice as previously described for other PDX cancer models [25]. Mice were inspected weekly for tumor growth. Tumor diameter was measured with sterile calipers and volume was calculated using the formula $\pi[(a \times b)^3]/6$, where a=maximal tumor diameter and b=minor tumor diameter perpendicular to a. When tumors reached a volume of 1.5–1.9 cm³, animals were euthanized by CO₂ inhalation and cervical dislocation and an accurate necropsy was performed. All animal procedures were performed in accordance with European directive 2010/63/UE and Italian Law (No. DL26/2014); experimental protocols were reviewed and approved by the institutional animal care and use committee of the University of Bologna and by the Italian Ministry of Health with letter 32/2020-PR.

Immunohistochemical and molecular analyses on tumor specimens

From formalin-fixed paraffin-embedded (FFPE) tumor blocks collected at different time points (Td, Tb and Th), 3 µm-thick sections were cut. Hematoxylin and eosin (H&E) staining was performed on the FFPE sections, and the immunohistochemistry studies were conducted with automatic immunohistochemistry stainer instrument's, Benchmark Ultra (Ventana/Roche Group Tucson, AZ, USA). The following pre-diluted antibodies were used: PD-L1 TPS (clone SP263, Ventana/Roche), TTF1 (clone 8G7G3/1, Ventana/Roche), BerEP4 (clone BER-EP4, Ventana/Roche). The immunostaining for CD44 was performed using a polyclonal antibody (ab157107, AbCam plc,

Cambridge, UK) at dilution 1:1200 and was conducted with antigen retrieval Cell Conditioning 1 for 40 min at 99 °C. The revelation system used is OptiView DAB (12 minutes linker and 12 minutes HRP multimer) (Ventana/Roche).

Mutational profile was investigated on the sample obtained at the Td point, using OncoPrint Focus Assay (Thermo Fisher Scientific) able to identify 35 hotspot genes, including KRAS. Sequencing was performed using the Ion GeneStudio S5 (Thermo Fisher Scientific).

Whole transcriptome sequencing of NSCLC-B and NSCLC-H cell lines

Total RNA was extracted from three different in vitro passages (between 16th and 31st) of NSCLC-B and NSCLC-H 2D-cultured cells using GenUP Total RNA Kit (Biotechrabbit GmbH, Berlin, Germany), according to the manufacturer's instructions. Library preparation and sequencing were performed by IIGM - Italian Institute for Genomic Medicine (Turin, Italy) and the procedure is briefly reported below. RNA integrity was checked, for each sample, by an electrophoretic run on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US) loaded with an RNA 6000 Nano Chip. RNA concentration was assessed by a Qubit 4 fluorometer with a Qubit RNA BR Assay (Thermo Fisher Scientific), and RNA purity was assessed as 260/280 and 260/230 ratios with a NanoDrop 8000 Spectrometer (Thermo Fisher Scientific). Total RNA libraries were prepared with the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (Illumina Inc., San Diego, CA, USA), according to manufacturer's instructions with no protocol modifications. Libraries size and quality were assessed by electrophoretic run on 2100 Bioanalyzer with a High Sensitivity DNA Chip. After measuring individual library concentration with a High Sensitivity (HS) Assay on a Qubit 4 fluorometer, libraries were pooled, and the pool diluted at 1.4 nM. The pool with 1% PhiX spike-in was then sequenced on a NovaSeq6000 (Illumina Inc.) sequencer, using a SP v1.5 flow cell, with a pair-end 2x100 protocol. RNA integrity was assessed using the RNA 6000 Nano Kit on a Bioanalyzer (Agilent Technologies). All samples showed an RNA integrity number (RIN) > 8. RNA samples were quantified using the Qubit RNA BR Assay Kit (Thermo Fisher Scientific).

Whole transcriptome analysis

Quality of reads was assessed using the FastQC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Raw reads were trimmed with fastp (v.0.21.0) [26] with `--trim_poly_x` parameter, to remove adapters and low-quality bases with default parameters. Filtered reads were aligned to the Homo sapiens reference genome (Ensemble v.110) using STAR aligner (v2.7.9a) [27] with parameter `--peOverlapNbasesMin 5`. To identify differentially expressed isoforms, STAR was used with parameter `-peOverlapNbasesMin 5-twopassMode Basic`. Gene and isoform expression quantification has been performed using RSEM (<https://github.com/deweylab/RSEM>) (v1.3.1) and Salmon (v0.13.1) [28], respectively, based on the Homo sapiens Ensembl v110 annotation. Abundance estimated counts at gene or isoform level, respectively, were summarized into a matrix using the R package tximport (within DESeq2 package [29]) and subsequently the differential expression analysis was performed with DESeq2 (v1.30.1) [30]. To generate more accurate log2 fold change estimates for low expressed genes, the shrinkage of the Log2 FoldChange was performed using the apeglm method [30]. Gene Ontology (GO) enrichment analysis of DEGs with $\text{padj} < 0.01$ and $\text{Log2 FoldChange} > 1$ and $\text{Log2 FoldChange} < -1$ have been performed using ClusterProfiler (v 4.6.2) (10.18129/B9.bioc.clusterProfiler). Subsequently, the top 20 enriched Biological Processes GO and KEGG categories have been plotted. The function simplifies with similarity cutoff of 0.7 has been applied to remove redundancy of enriched GO terms. Unless otherwise stated, the default parameters were utilized when applying the different software.

Raw reads have been processed using the bioinformatics pipeline nf-core/rnasplice (<https://nf-co.re/rnasplice/dev>). Following quality control and trimming as described above, reads have been aligned with STAR [27]. To perform differential exon usage analysis, we have chosen to count the reads with HTSeq [31] and perform the differential analysis with DEXSeq [32]. To perform differential transcript usage analysis, we performed the counts with Salmon, refined the feature modelling using DRIMSeq [33] as recommended, and performed the differential analysis with the

package DEXSeq. To better investigate indication of potential novel transcripts, annotated during the DEXU analysis, previously STAR-aligned BAM files have been used to assemble RNA-seq alignments into potential transcripts, using StringTie [34], with default parameters.

Single nucleotide variants (SNVs) of each cell line have been identified from NSCLC-B and NSCLC-H conditions, independently, on pooled RNA-sequencing replicates (~100M fragments each). SNV calling, annotation, filtering and prioritization were performed based on the CTAT-Mutation Pipeline (<https://github.com/NCIP/ctat-mutations/wiki>) that integrates the GATK Best Practice workflow (<https://gatk.broadinstitute.org/hc/en-us/articles/360035531192-RNAseq-short-variant-discovery-SNPs-Indels->). Trimmed reads were aligned to the reference human genome sequence (GRCh38/hg38 GENCODE release 36) using STAR v 2.7.8a two-pass mode, to obtain improved alignments around novel splice junctions, and with the option `--peOverlapNbasesMin 12` to allow the re-alignment of overlapping mates as single ends. Duplicated reads were marked with PicardTools-MarkDuplicates, alignments that span introns were reformatted with SplitNCigarReads and Base Quality Recalibration was performed using GATK v4.1.9.0. Overlapping reads originated from the same fragments were soft clipped using BamUtil clipoverlap v1.0.14 to avoid counting reads originated from the same fragment. Variants were identified using GATK HaplotypeCaller v4.1.9.0 with a minimum phred-scaled confidence threshold of 20. For each identified variant, the allele frequency has been calculated as the percentage of reads supporting the variant divided by the overall coverage at that locus. Subsequently, variants have been annotated using SnpEff (v4.1k) and BCFtools (v1.9), exploiting the CTAT-mutation library version Jul272020. Likely RNA-editing events have been identified using the RADAR and RediPortal databases. Variants unique identifiers (rs ID) have been annotated using dbSNP database; variants frequency in the population has been annotated using gnomAD database and known cancer mutations based on the COSMIC database. Finally, we used Open-CRAVAT 2.0.1 to annotate variants according to their putative biological impact and relevance to cancer: clinically significant variants have been annotated based on ClinVar, pathogenicity scores have been predicted using VEST and CHASMplus. Variants with Fisher Strand > 30, quality by depth (QD) < 2, occurring

within 3 bp of splice junctions and clusters of at least three SNVs within a 35 base pair window were filtered out according to GATK RNA-seq Best Practices. Based on annotation, variants within HLA and immunoglobulin genes, RNA-editing sites, repetitive and homopolymeric regions, and with a variant allele frequency < 20% were excluded.

CRISPR/Cas9 genome editing

The silencing of PD-L1 in NSCLC-B cells was performed using PD-L1 (CD274) Human Gene non-homology mediated CRISPR knockout kit (Origene Cat. KN413071, Rockville, MD), according to the manufacturer's instructions. Kit contained two RNA guides and a linear donor DNA including gene for puromycin resistance and GFP. TransIT-X2® Dynamic Delivery System MIR 6004 (Mirus Bio, Madison, WI, USA) was used for transfection. Cells were selected and maintained in 1 µg/ml puromycin, obtaining the two cell line clones NSCLC-B CL1 and NSCLC-B CL2 (CL: "clone") derived by NSCLC-B cells transfected with guide 1 and guide 2, respectively. The expression levels of PD-L1 and CD44 on these clones were measured by flow cytometry as described below.

2D-growth and clonogenic assay

Cells were seeded in a 24-well plate at 20,000 cells/well in RPMI+10% FBS (for NSCLC-B and NSCLC-H cells) or RPMI+10% FBS + 1 µg/ml puromycin (Thermo Fisher Scientific) (for NSCLC-B CL1 and NSCLC-B CL2 cells). Cell viability was evaluated at different time points by the Neubauer hemocytometer. Clonogenic assay was performed by seeding 400, 800 or 1,600 cells/well in 6-well plates. All cells were seeded in RPMI+10% FBS. After 7 days, cultures were fixed in 96% ethanol and stained with 0.05% crystal violet. Colonies, defined as groups with more than 10 cells, were counted under an inverted microscope (Olympus CK2).

To assess cell sensitivity to IFN-γ treatment and PD-L1 inhibition, NSCLC-B and NSCLC-H cell lines were seeded at 0.5×10^6 cells/25 cm² cell culture flasks in RPMI+10% FBS. After 24 hours,

cells were treated with recombinant human IFN- γ (kindly provided by G. Garotta, F. Hoffmann-La Roche & Co., Basel, Switzerland) or anti-PD-L1 monoclonal antibody (mAb) atezolizumab (Tecentriq, Genentech), whose final concentrations were reported in the figures or in figure legends. Cell growth and immunological modulation of MHC class I complex, PD-L1 and CD44 were assessed 72 hours later by vital counting and by cytofluorimetric analysis, respectively.

Soft-agar colony formation assay

For 3D-soft agar clonogenicity evaluation, NSCLC-B and NSCLC-H cells were seeded at 200 cells/well in 24-well plate in semisolid medium consisting of RPMI+10% FBS + 0.33% agar (Sea-Plaque Agarose, Lonza, Switzerland), with a 0.5% agarose underlay. For the assessment of cell line sensitivity to IFN- γ treatment and PD-L1 modulation in 3D culture conditions, NSCLC-B cells were seeded at 2,000 cells/well in 24-well plate in semisolid medium consisting of RPMI+10% FBS + 0.33% agar, containing recombinant human IFN- γ atezolizumab or the only medium, with a 0.5% agarose underlay. Colonies (diameter > 90 μ m) were counted 2-3 weeks later under an inverted microscope in dark-field, as previously described [35–37]. NSCLC-B soft-agar colonies, grown in presence of the previously described treatments or only medium, were picked-up through a sterile pipette tip and subcultured in the same adherent conditions described for NSCLC-B and NSCLC-H cell lines. The derived cell lines were defined as “bulk agar” cell lines, and the treatment of the 3D soft-agar original well was also reported.

Sphere-forming assay

For the assessment of NSCLC-B and NSCLC-H sphere formation ability, cells were seeded at 10,000 cells in 4 mL complete MammoCult medium (STEMCELL Technologies, Vancouver, Canada) without serum in 6-well Ultra-Low adherence plate (Corning Life Sciences), according to the MammoCult Human Medium Kit protocol. For the assessment of cell line sensitivity to IFN- γ treatment and PD-L1 modulation in sphere-formation assay, NSCLC-B cells were seeded at 500, 5,000 or 10,000 cells in 4 mL complete MammoCult medium without serum in 6-well Ultra-Low

adherence plates, according to the MammoCult Human Medium Kit protocol. Recombinant human IFN- γ or atezolizumab were added to the medium at the concentrations reported in the figures or in figure legends. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere for a week. Spheres, multi-cell structures, with a diameter larger than 90 μ m were counted about 7 days after the seeding under an inverted microscope in dark field [23].

Establishment of organoids

Approximately 30,000 cells were resuspended in Geltrex LDEV-Free Reduced Growth Factor (RGF) Basement Membrane Extract (BME) (Thermo Fisher Scientific), seeded in low adhesion 48-well plates (GreinerBio), and after domes polymerization an expansion medium was added. The expansion medium was composed of Advanced DMEM/F-12 (Thermo Fisher Scientific) with 1% penicillin/streptomycin, 1% Glutamax (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific) with the addition of 25 ng/ml fibroblast growth factor 7 (FGF7) (Proteintech), 100 ng/ml FGF10 (QKine), 100 ng/ml Noggin (Proteintech), 500 nM A83-01 (Tocris), 10 μ M ROCK inhibitor (Y-27632) (Tocris), 0.5 μ M SB202190 (Merck), 1.25 mM N-acetyl-L-cysteine (Merck), 10 mM nicotinamide (Merck), 10% R-Spondin1 conditioned medium (Merck), 50 ng/ml epidermal growth factor (EGF) (Proteintech) and B-27 (1:50) (Thermo Fisher Scientific). Organoids were passed by mechanical dissociation and incubation in cold PBS for 1 hour on ice, every 14 days.

Organoid immunofluorescence characterization

Before being used for an immunofluorescence staining, organoids were gently removed from their matrix by incubation in cold PBS for 1 hour and then were fixed in cold 4% paraformaldehyde (Merck) for 40 minutes at 4°C. Subsequently, to complete the permeabilization phase, cell structures were resuspended in PBS containing 0.1% Tween20 (Appllichem) for 10 minutes at 4°C and then were blocked for 1 hour at room temperature in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and 2% Bovine Serum Albumin (Pan Biotech) solution to minimize background non-specific staining. Then, primary antibodies CD44 (clone IM7, 1:250, Biolegend) and Ki67 (clone SP6, 1:250,

Thermo Fisher Scientific) diluted in PBS solution containing 0.1% Triton X-100 and 0.5% BSA were incubated overnight at 4°C on a shaker. After washing, 1 hour incubation with secondary antibodies anti-rat IgGAF555 (for CD44) and anti-rabbit AF647 (for Ki-67) was performed (1:500, Thermo Fisher Scientific). Hoechst 33342 (Thermo Fisher Scientific) (1:8000) incubated for 20 minutes at room temperature in a dark room was used to counterstain nuclei. Confocal images were captured on a Leica SP8 inverted confocal microscope (Leica).

Cytofluorimetric analysis

Harvested cells were analyzed by immunofluorescence and cytofluorimetric analysis as previously described [35]. The following antibodies were used for direct and indirect immunofluorescence: anti-human PD-L1 (100 µg/ml, atezolizumab, Tecentriq, Roche or atezolizumab, Selleck Chemicals, Houston, Texas, USA), anti-human MHC class I (clone W6/32, diluted 1:80, Sera-Lab), anti-mouse CD44PE or BV711 (clone IM7, diluted 1:10, BioLegend), goat anti-human IgG secondary antibody FITC or AF674 (diluted 1:20, Thermo Fisher Scientific) and anti-mouse IgGAF488 (diluted 1:100, Thermo Fisher Scientific). Cell cycle analysis was performed using the Phase-FlowT Alexa Fluor 647 BrdU Kit (BioLegend), according to the manufacturer's instructions performing only staining with 7-AAD. Data were acquired using CyFlow Space (Sysmex Partec, Germany) and BD FACS Lyric cytometer and analyzed using FCS Express (De Novo Software, Glendale, CA, USA).

Western blot analysis

Protein extraction, quantification and Western blotting were performed as previously reported [38]. The effect of IFN-γ (Proteintech) or anti-PD-L1 mAb (atezolizumab, Selleck Chemicals) on NSCLC-B and NSCLC-H cells was evaluated, exposing cells to the treatment for 6 hours. Treatment was added the day after seeding and an untreated sample ran in parallel. A list of used primary antibodies was reported in Supplementary Table 3 (all by Cell Signaling Technology). Mouse anti-actin antibodies (clone 8H10D10, 1:3000, Cell Signaling Technologies or clone AC-40, 1:3000, Merck) or rabbit anti-GAPDH antibody (clone D16H11, 1:1000, Cell Signaling Technologies) were used to

detect reference proteins. Membranes were either incubated with polyclonal horse-radish-peroxidase (HRP) conjugated anti-rabbit and anti-mouse IgG antibodies (Bio-Rad Laboratories). Proteins were detected by chemiluminescent reactions visualized using the digital imaging system Azure C600 (Azure Biosystems). Protein abundance was defined through densitometric analysis of bands by Azure Spot software (Azure Biosystems).

Statistical analysis

Experimental in vitro conditions analyzed with statistical measures were repeated two times, at least. The number of samples and replicates was reported in figure legends. The two-tailed unpaired Student's t-test or One sample t and Wilcoxon test were performed according to assumptions of the tests and the variance between the compared groups. Statistical analyses were performed through Prism 10 software (GraphPad software, La Jolla, CA, USA).

RESULTS

Patient clinical history and molecular data

A 64-year-old, former light smoker (10 packs/year) woman was diagnosed with stage IVA (pleura and pericardium) adenocarcinoma of the lung. Tumor cells, derived from pleural effusion at the diagnosis (Td), were positive for PD-L1 (tumor proportion score, TPS: 70%) and CD44 (31% of tumor cells were 2+ positive). KRAS^{G12V} mutation was detected from the tumor sample obtained at the time of diagnosis (Td) through next generation sequencing (NGS) analysis. A second sample collected before treatment (baseline, Tb), consisting of pleural effusion and parietal tissue, was analyzed. On this second sampling obtained at the Tb point, CD44 expression was not identified in tumor cells but only in stromal components, while PD-L1 TPS staining was confirmed to be 70%. Additionally, tumor cells at both Td and Tb points were positive for TTF1 and BerEP4 (data not shown). A first-line treatment with pembrolizumab was administered for 5 cycles. Radiological tumor

assessment through computed tomography scans showed evidence of progressive disease, with metastatic tumor dissemination in thoracic wall, soft tissue, and abdominal and thoracic lymph nodes (**Figure 1A**). At this time, tumor cells collected from a new subcutaneous biopsy (Th) maintained the expression of TTF1 and BerEP4 (data not shown), together with PD-L1 (TPS: 65%) and CD44 (58% of tumor cells were 2+ positive) (**Figure 1B**).

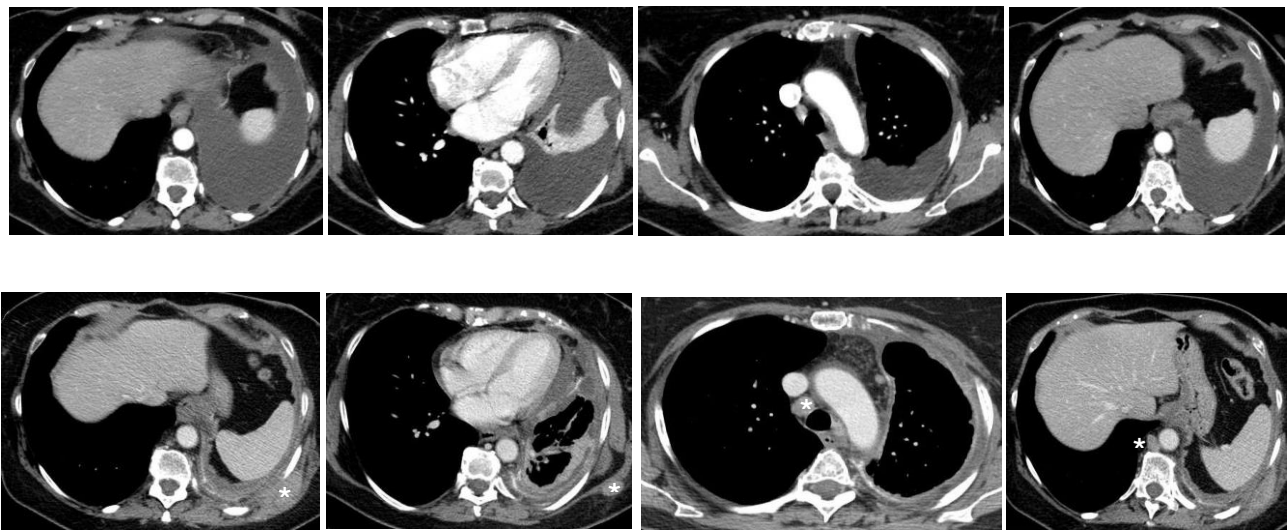


Figure 1A. CT scan imaging at baseline (upper scans) and at the time of progressive disease under ICI (lower scans).

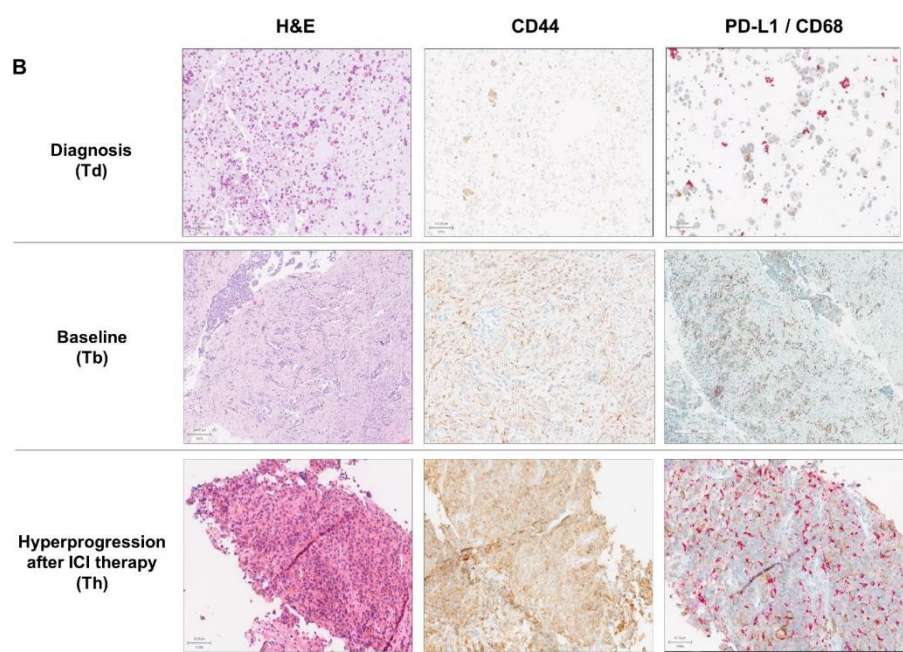


Figure 1B. Hematoxylin and eosin (H&E) staining and CD44 and PD-L1 immunohistochemical staining of tumor samples: Td, pleural effusion; Tb, pleural biopsy; Th, subcutaneous lesion. Left: H&E; middle: membrane CD44 expression on neoplastic cells; right: PD-L1 expression on tumor cells by double stain for PDL1 and CD68 (PD-L1: brown, CD68: red).

Second-line treatment consisting of pembrolizumab, carboplatin and pemetrexed was administered, with no clinical improvement. Third-line treatment consisting in docetaxel administration was initiated, but unfortunately the patient died under the treatment.

Establishment and characterization of NSCLC-B and NSCLC-H cell lines

Two primary cell cultures were established from tumor samples, obtained before ICI-based treatment initiation at the Tb point (NSCLC-B cell line) and at the time of radiological evidence of HPD under ICI treatment, i.e. Th point (NSCLC-H cell line). KRAS^{G12V} mutation, previously identified in the tumor at the Td point, was also confirmed in both cell lines by Real-Time PCR (data not shown) and Whole Transcriptome Sequencing (shared raw data).

In adherent culture conditions, cell lines showed different morphology and behavior: while NSCLC-B formed a compact homogeneous monolayer of cells with polygonal morphology (**Figure 2A, left**), NSCLC-H showed a stratified layer of cells not so well organized as the former (**Figure 2A, right**). Interestingly, NSCLC-H cells showed the ability to generate organoids which, on the contrary, was not observed in NSCLC-B cell line (**Figure 2B-C**).

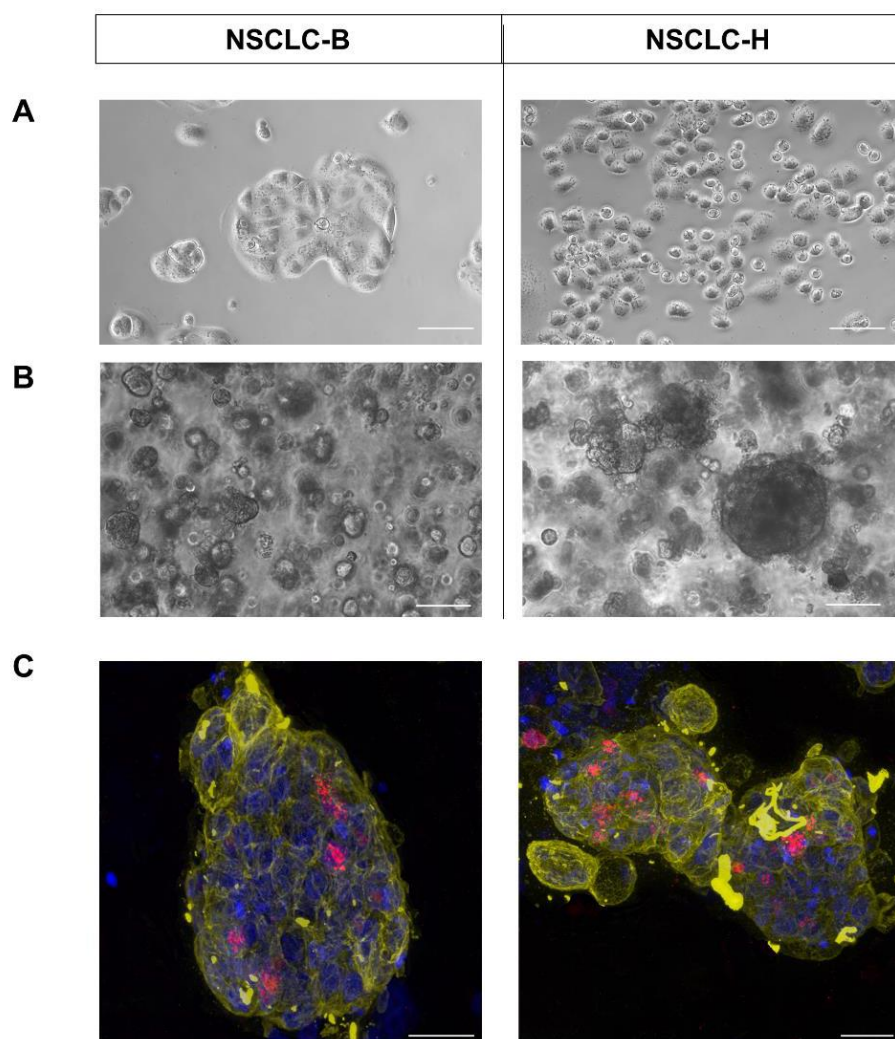


Figure 2. Morphological, phenotypical and functional characterization of NSCLC-B and NSCLC-H cell lines. **(A)** Morphology of NSCLC-B and NSCLC-H cells cultured in 2D conditions, observed through an inverted microscope. White line corresponds to 100 μ m. **(B)** Structures observed two weeks after seeding of NSCLC-B and NSCLC-H cells in matrigel with appropriate organoid medium. White line corresponds to 100 μ m. **(C)** Immunostaining of an organoid derived from NSCLC-H cell line performed using DAPI (blue) together with anti-Ki-67 (red) and anti-CD44 (yellow) antibodies. White line corresponds to 20 μ m. Leica TCS SP8 Microscope, HC PL APO CS2 40x/1.30 OIL.

The behavior of NSCLC-B and NSCLC-H cell lines was furtherly evaluated for comparison, both *in vitro* and *in vivo*. In general, NSCLC-H cells exhibited a significant higher 2D and 3D-soft agar clonogenicity and sphere forming ability (**Table 1**). Furthermore, NSCLC-H cell line was characterized by faster 2D-growth capacity, showing a significant decrease of cells in the G1 phase of the cell cycle compared to NSCLC-B cells (**Table 1**). Finally, patient-derived xenografts (PDX) derived from the tumor biopsy at Th showed a faster growth compared to the PDX established from the tumor sample at the Td point (**Table 1**).

	NSCLC-B	NSCLC-H	p-value
2D-growth (doubling time-hours)	79.16±6.58	41.90±2.84	< 0.01
2D-clonogenicity (%)	1±1%	29±1%	< 0.001
3D-soft agar clonogenicity (%)	0.15±0.05%	18.15±0.50%	< 0.001
Sphere formation ability (number of spheres)	51±2	82±5	< 0.05
PDX tumor growth rate (doubling time – weeks)	1.430±0.154	0.562±0.104	< 0.05
Cells in the G1 phase of the cell cycle (%)	63±3%	47±2%	< 0.01

Table 1. Growth-related features of NSCLC-B and NSCLC-H cell lines. Statistical analysis: Student's t-test.

Transcriptome analysis showed profound differences between NSCLC-H and NSCLC-B cells (Supplementary Figure 1A, PC1 96% variance), evidencing 3525 genes differentially expressed between the two cell lines (p-adjust < 0.01 and absolute Log2FC > 1 or < -1), of which 1732 up-regulated and 1793 down-regulated in NSCLC-H cell line compared to NSCLC-B (**Figure 3A** and Supplementary Figure 1B). According to overrepresentation analysis, up-regulated genes were involved in a plethora of biological processes (GO terms BP). Specifically, the top-20 GO terms BP evidenced enrichment in pathways of different cell types/tissues, including neural, epithelial, bone, muscle, and connective tissues, suggesting that NSCLC-H tumor cells acquired a plasticity or a less differentiated status (**Figure 3B, upper left**). On the other hand, down-regulated genes in NSCLC-H cell line enriched in GO terms BP were associated with the differentiative status of epithelial cells, supporting evidence of a partial epithelial-to-mesenchymal transition (EMT) from NSCLC-B to NSCLC-H phenotype (**Figure 3B, upper right**). Up-regulated genes were enriched in molecular

functions (GO terms MF), of which the top-20 included channel activity and ion transportation, insulin-like growth factor binding and, more in general, growth factor activity, carbohydrate and glycosaminoglycan binding (**Figure 3B, lower left**). Down-regulated genes were enriched in molecular functions associated with cell-cell interactions, cytoskeletal binding, including actin filaments, lamin binding and DNA binding (**Figure 3B, lower right**). GSEA analysis of curated gene lists revealed clusters of genes, mainly up-regulated in the NSCLC-H cell line, involved in plasticity, WNT pathway, cell cycle and invasiveness (**Figure 3C**). Of note, the up-regulated genes in NSCLC-H cell line, with a less stringent absolute Log2FC threshold (i.e., < 0.58), were also enriched in BP associated with chromosome rearrangements and DNA remodeling, as shown by the top-20 GO terms and as also suggested by GSEA analysis (Supplementary Figure 1C). Furtherly, GSEA analysis of chromosome regions revealed an enrichment of genes in regions localized on chromosome 2 and 7, responsible for translational and post-translational processes. (Supplementary Figure 1D). Of note, the 7q21 enrichment core included genes involved in cell plasticity (i.e., HGF, COL1A2, BET1, CDK14, SEM3A and FZD1).

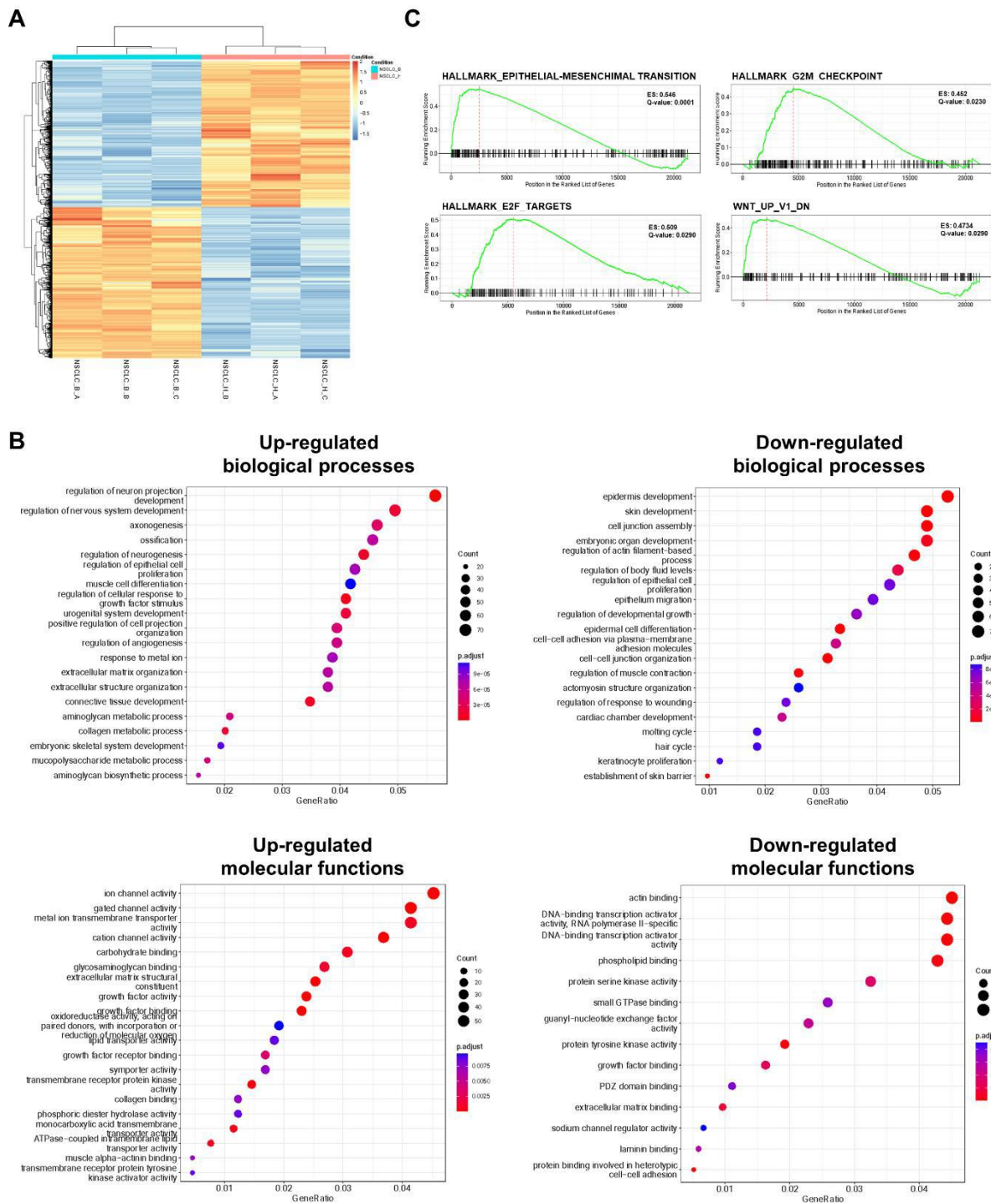


Figure 3. Transcriptome analysis of NSCLC-H vs NSCLC-B cells. **(A)** Heatmap showing the z-score of normalized expression values (RLOG) of differentially expressed genes (DEG, $p\text{-adj} < 0.01$ and absolute $\text{Log}_2\text{FC} > 1$) in NSCLC-H vs NSCLC-B cells. **(B) Up:** Top-20 GO term Biological Processes enriched in up-regulated (**left**) and down-regulated (**right**) genes, identified by over representation analysis of DEG; **bottom:** Top-20 GO term Molecular Functions enriched in up-regulated (**left**) and down-regulated (**right**) genes, identified by over representation analysis of DEG. **(C)** GSEA enrichment plots of curated lists related to plasticity, WNT pathway, cell cycle and invasiveness.

CD44 transcripts

CD44 is a transmembrane glycoprotein with pleiotropic functions associated with cancer progression, invasiveness, and plasticity [39]. Interestingly, CD44 was more expressed by NSCLC-H cells compared to NSCLC-B cells (**Figure 4A**). Given that together with the standard form of CD44 (CD44s) multiple isoforms of CD44 have been reported in both normal and malignant cells [40], we specifically investigated the transcriptional profile of both cell lines for CD44 variants. CD44 transcript isoforms differentially identified in NSCLC-H vs NSCLC-B cell line are shown in **Figure 4B-C** and Supplementary Table 1. NSCLC-H cell line mainly showed increased levels of the short transcript isoforms CD44-201 (CD44s), CD44-205 and CD44-209 (**Figure 4C**). Other transcript isoforms more abundant in NSCLC-H cells compared to NSCLC-B cells were also found (CD44-224, CD44-204, CD44-211 and CD44-220). In addition, in NSCLC-H cell line we also identified increased levels of two transcripts with a premature translation termination codon (CD44-207 and CD44-210), three transcripts unable to be translated due to intron retaining (CD44-225, CD44-232 and CD44-215) and higher levels of two short transcript isoforms (CD44-222 and CD44-230) (**Figure 4C**). Interestingly, although total CD44 transmembrane protein was more expressed in NSCLC-H cells compared to NSCLC-B cells, longer transcripts of the protein, such as CD44-206 (also known as V6 variant) and CD44-208, were more abundant in NSCLC-B cell line (**Figure 4C**).

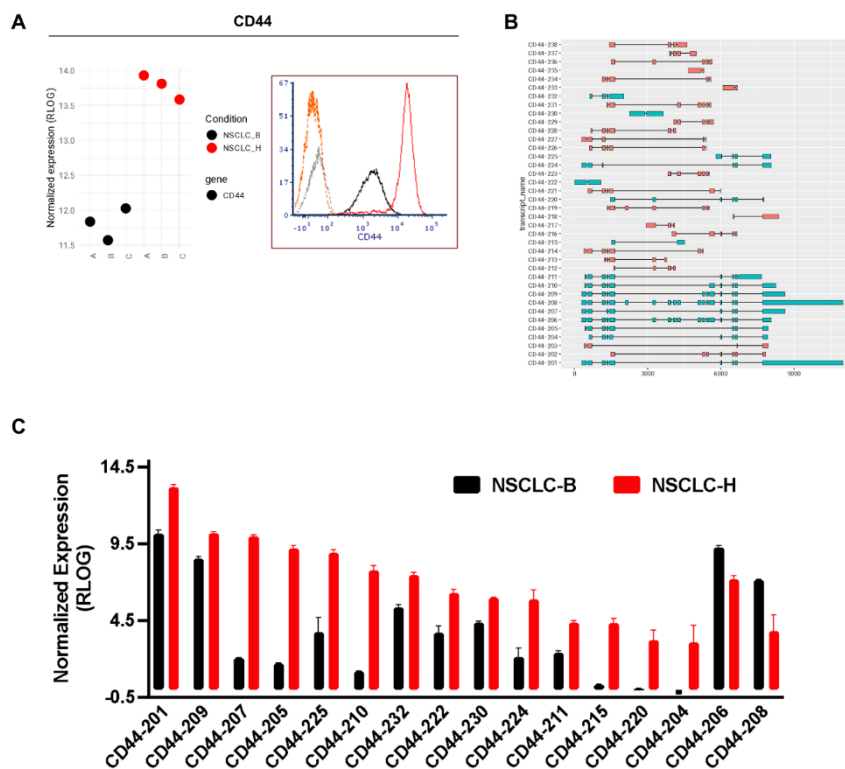


Figure 4. CD44 gene and CD44 transcript isoforms expression in NSCLC-B and NSCLC-H cell lines. (A) **Left:** normalized expression (RLOG) of the CD44 gene (Log2FC 2.882, p-adj 6.20E-49), as identified by RNAseq analysis; **right:** CD44 expression quantified by flow-cytometry (NSCLC-B: unstained in grey, stained in black; NSCLC-H: unstained in orange, stained in red). (B) Graphical representation of CD44 transcript isoforms. Transcript isoforms in green-water were found in at least one cell line, while transcript isoforms in red were not detected. (C) Normalized expression (RLOG) of

CD44 transcript isoforms, as identified by RNAseq analysis. Log2FC and p-adj of each transcript isoform in NSCLC-H vs NSCLC-B are reported in Supplementary Table 1.

Cellular response to immune components

PD-L1 is a transmembrane glycoprotein involved in immune response inhibition and tumor immune evasion, representing one of the most common targets for immune checkpoint blockade therapy [41]. On the other hand, IFNGR1 constitutes the ligand-binding chain of the receptor of IFN- γ , which is the main effector cytokine involved in anti-tumor T-cell-mediated responses [42,43]. Of note, preliminary variant cell analysis revealed alterations of genes involved in IFN- γ and PD-L1 signaling in both NSCLC-B and NSCLC-H cells (Supplementary Table 2). Despite this observation, the expression of PD-L1 (**Figure 5A**) and IFNGR1 (**Figure 5B**) genes, was respectively down and up-modulated in NSCLC-H cell line, compared to NSCLC-B. Consistently, GSEA analysis showed that the “inflammatory response” Hallmark gene set was significantly up-regulated in the NSCLC-H model, compared to the NSCLC-B one (**Figure 5C**). Furthermore, a subgroup of enriched score genes associated to GSEA analysis and related to the cellular response to IFN- γ and IFN-alpha (IFN- α) was down-regulated in NSCLC-H cells compared to NSCLC-B cells (**Figure 5D** and Supplementary

Figure 1E). On the contrary, genes associated with IFN-beta (IFN- β) response were up-regulated in NSCLC-H cells, together with genes involved in IL-6/JAK/STAT3 signaling (Figure 5D, Supplementary Figure 1E).

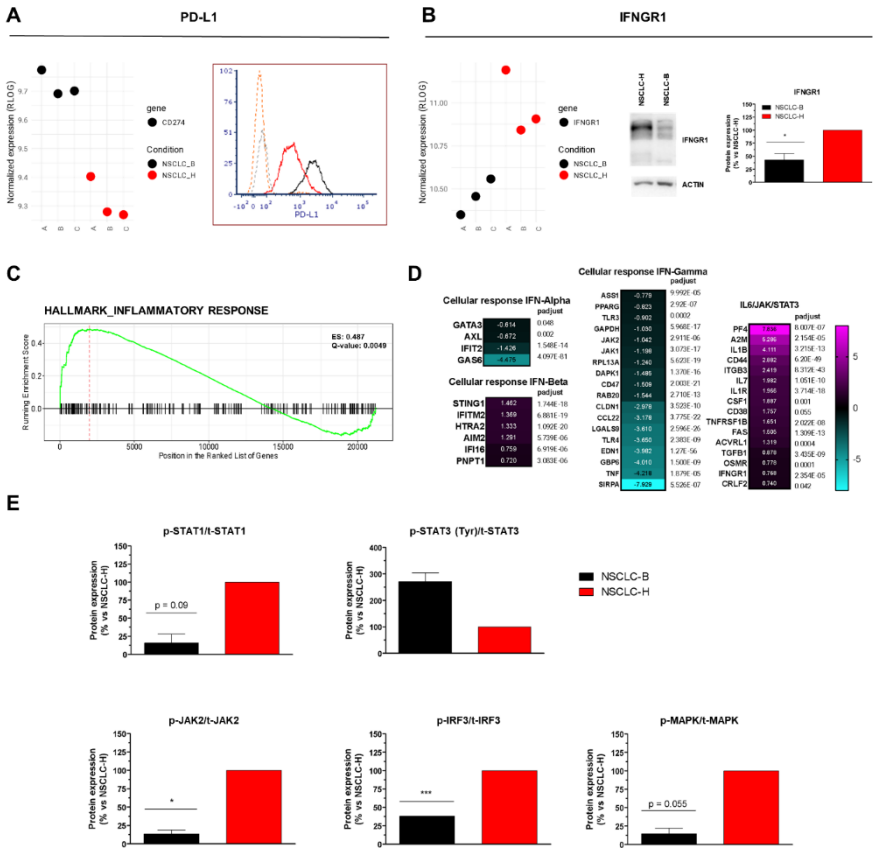


Figure 5. Comparison of immune-related genes and proteins between NSCLC-B and NSCLC-H cell lines. (A) Normalized expression (RLOG) of CD274 gene (PD-L1), as identified by RNAseq analysis (Log2FC -0.589, p-adj 0.0003), and validation by flow cytometry (NSCLC-B: unstained in grey, stained in black; NSCLC-H: unstained in orange, stained in red). (B) Normalized expression (RLOG) of IFNGR1 gene (Log2FC 0.768, padj 2.3535E-05), as identified by RNAseq analysis and validation by western blotting. **Left:** gene expression; **middle:** western blotting representative picture; **right:** densitometric quantification of IFNGR protein

normalized on actin. Values are reported as percentage of NSCLC-H protein expression. Bar represents mean and SEM (n=3). Statistical analysis: One Sample t and Wilcoxon test. (C) GSEA analysis of curated list related to inflammatory response. (D) Heatmap showing the Log2FC of a subgroup of enriched score genes associated to GSEA analysis reported in Supplementary Figure 1E and involved in immune-related cellular responses. Log2FC of differentially expressed genes in NSCLC-H compared to NSCLC-B is reported in the box and p-adj is reported on the right. (E) Western blot analysis for proteins involved in IFN- γ signaling (n=2). *, p<0.05, ***, p<0.001 by One Sample t and Wilcoxon test. Each bar represents mean with SEM.

Additionally, two molecules that have been associated with reduced T cell responsiveness, namely NDRG1 and ADORA1, resulted to be significantly overexpressed by NSCLC-H cell line, compared to NSCLC-B cells (Supplementary Figure 1F) [44,45]. Investigating the activation of molecular pathways associated with response to inflammatory stimuli and cell proliferation, we observed an increased activation of JAK2, STAT1 and IRF3 in NSCLC-H compared to NSCLC-B cells, while, on the contrary, a reduced tyrosine phosphorylation of STAT3 was observed in NSCLC-H cell line.

Interestingly, the ERK/MAPK pathway was more activated in the HPD cell line compared to the baseline one (**Figure 5E**, Supplementary Figure 2).

***In vitro* response of NSCLC-B and NSCLC-H cell lines to IFN- γ**

The mechanism of action of ICIs is based on the enhancement of T-cell activation and reversal of T-cell exhaustion through the targeting of immune checkpoints. The antitumor effect of cytotoxic T-cell populations, reactivated by ICI therapy, is predominantly mediated by the release of IFN- γ , which exerts antiproliferative, cytostatic, pro-apoptotic and immunogenic effects on tumor cells [43,46]. Thus, we evaluated the effect of IFN- γ on NSCLC-B cells, mimicking the interaction between tumor cells at baseline and IFN- γ released by reactivated cytotoxic T cells. In adherent culture conditions, NSCLC-B cells were resistant to the antiproliferative effect of IFN- γ (**Figure 6A**). Furthermore, IFN- γ failed in up-regulating the expression of PD-L1 (**Figure 6B**), while maintaining its capability of inducing MHC class I expression (**Figure 6C**). Similar effects were also observed on NSCLC-H cells in presence of IFN- γ (Supplementary Figure 3A-C). Of note, a pro-growth effect was observed on NSCLC-B cells seeded at low density in presence of IFN- γ (Supplementary Figure 3D). Western blot analysis on NSCLC-B cell line grown in adherent conditions revealed, in presence of IFN- γ , an increased activation of IRF9, MAPK, JAK1, STAT1 and STAT2 and a decrease of IFNGR1 (**Figure 6D**, Supplementary Figure 2).

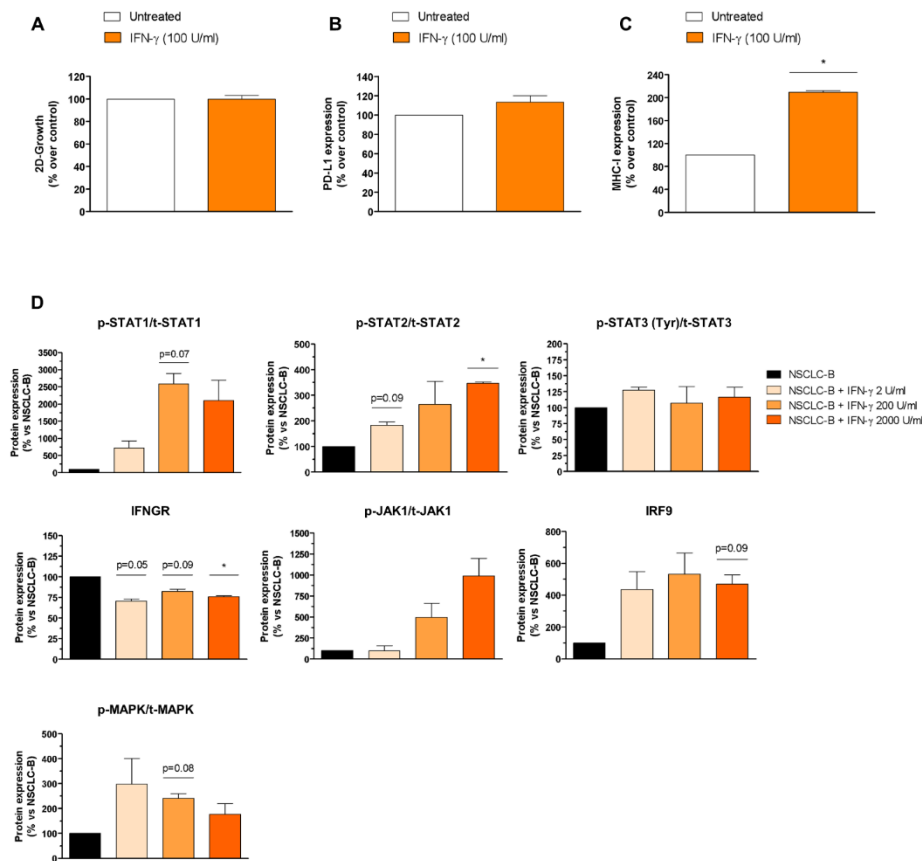


Figure 6. Response of NSCLC-B cell line to IFN- γ in 2D culture conditions. **(A)** Sensitivity of NSCLC-B cell line to the antiproliferative effect of IFN- γ 100 U/ml (n=2). Bar represents mean with SEM. **(B)** Percentage of induction of PD-L1 expression on NSCLC-B cells (n=2). Bar represents mean with SEM. **(C)** Percentage of induction of MHC class I complex expression on NSCLC-B cells (n=2). *, p<0.05 by One Sample t and Wilcoxon test. Bar represents mean with SEM. **(D)** Western blot analysis for proteins involved in IFN- γ signaling and in cell growth on NSCLC-B cells treated with different doses of IFN- γ in 2D-adherent culture conditions (n=2). *, p<0.05 by One Sample t and Wilcoxon test. Each bar represents mean with SEM.

The effect of the treatment with increasing doses of IFN- γ was evident only on JAK1 activation, in which it was possible to observe a dose-response effect. STAT1 activation was also modulated by IFN- γ , although no differences were observed treating cells with medium or higher IFN- γ doses. In 3D culture conditions, IFN- γ surprisingly exerted a dose-independent, pro-growth effect on NSCLC-B cells, inducing a significant increase in the number of colonies in soft agar (p<0.05 by One sample t and Wilcoxon test, **Figure 7A**). “Bulk agar” cell lines were established from 2D-subcultured soft-agar colonies (**Figure 7B**). Interestingly, only “bulk agar” cells derived from colonies grown in presence of low-dose IFN- γ showed increased CD44 expression, which was also accompanied by a partial change in cell morphology (**Figure 7B**, top, second and third line and bottom). Accordingly, sphere formation assay showed a significant increase of sphere production only in presence of a low dose of IFN- γ (**Figure 7C**).

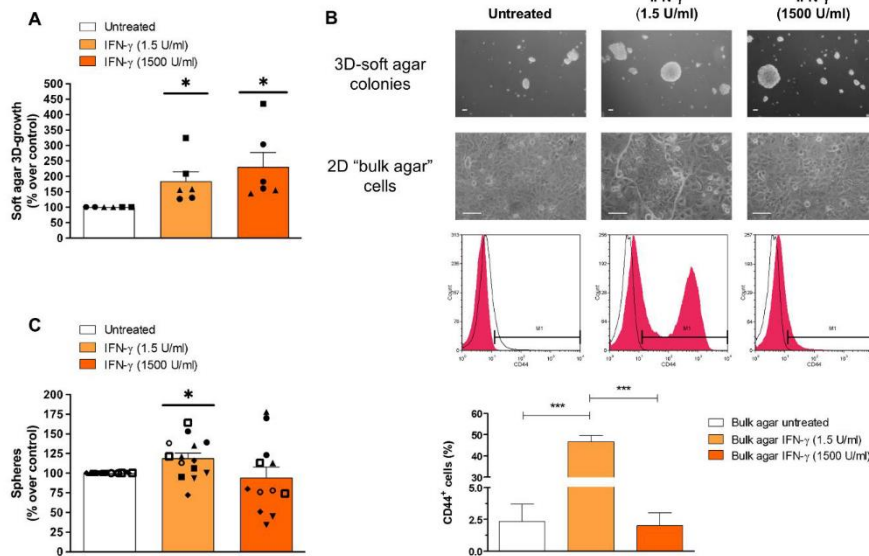


Figure 7. Response of NSCLC-B cell line to different doses of IFN- γ in 3D culture conditions. **(A)** NSCLC-B soft-agar colony formation in presence of low (1.5 U/ml) and high (1500 U/ml) doses of IFN- γ (n=6, three experiments -circle, triangle, square- each one with two technical replicates). *, p<0.05 by One sample t and Wilcoxon test. Each bar represents mean with SEM. **(B)** Morphological and phenotypical characterization of NSCLC-B "bulk agar" cell lines obtained by 2D-

subcultured agar colonies grown in presence of IFN- γ . **First line:** representative pictures of NSCLC-B soft-agar colonies grown without any treatment or in presence of IFN- γ , observed through an inverted microscope in dark-field; **second line:** morphologies of NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, observed through an inverted microscope. White line corresponds to 100 μ m. To improve the visual appearance of the images, photos were converted into grayscale. For pictures in the second line, contrast and brightness were also enhanced (+40% and +10%, respectively); **third line:** expression of CD44 on NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, measured by cytofluorimetric analysis (M1: marker); **bottom:** percentage of CD44⁺ cells on NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, measured by cytofluorimetric analysis (n=3). ***, p<0.001 by Student's t-test. Each bar represents mean with SEM. **(C)** NSCLC-B sphere formation in presence of low (1.5 U/ml) and high (1500 U/ml) doses of IFN- γ (untreated and IFN- γ 1.5 U/ml: n=14, seven experiments -different symbols-, each one with two technical replicates; IFN- γ 1500 U/ml: n=12, six experiments, each one with two technical replicates). *, p<0.05 by One sample t and Wilcoxon test. Each bar represents mean with SEM. Each dot represents a replicate.

PD-L1 modulation on NSCLC-B cell line

PD-L1 is known to have pro-oncogenic functions, which are not necessarily dependent on its interaction with PD-1 receptor [47,48]. Several studies have indeed demonstrated the existence of a PD-1-independent intracellular signaling of PD-L1 in tumor cells [49–53]. Hence, we evaluated the effect of PD-L1 modulation on the NSCLC-B cell line. First, PD-L1 was targeted by the monoclonal antibody (mAb) atezolizumab in 3D culture conditions. The blockade of PD-L1 showed no significant effect on NSCLC-B growth in 3D-soft agar assay (**Figure 8A**). Despite this, the inhibition of PD-L1 not only promoted a change in cell morphology in colonies-derived "bulk agar" cells, but it also surprisingly increased CD44 expression, compared to control (p<0.01 by Student's t test, **Figure 8B**,

top, second line and third line and bottom). Interestingly, atezolizumab also significantly increased the number of NSCLC-B spheres ($p < 0.001$ by One sample t and Wilcoxon test, **Figure 8C**).

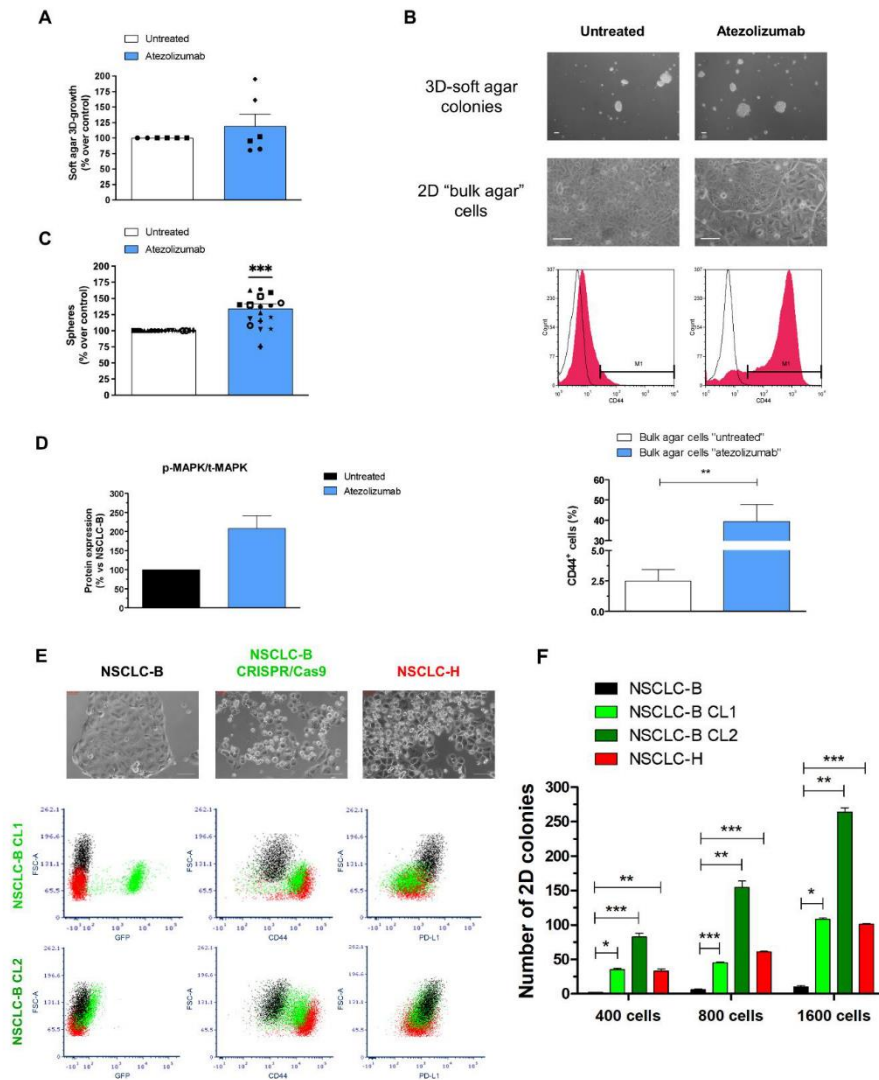


Figure 8. Response of NSCLC-B cell

line to PD-L1 modulation in 2D and 3D culture conditions. **(A)** NSCLC-B soft-agar colony formation in presence of atezolizumab 10 $\mu\text{g/ml}$ ($n=6$, three experiments-circle, triangle, square-, each one with two technical replicates). Each bar represents mean with SEM. Each dot represents a replicate. **(B)** Morphological and phenotypical characterization of NSCLC-B "bulk agar" cell lines obtained by 2D-subcultured soft-agar colonies grown in presence of atezolizumab 10 $\mu\text{g/ml}$.

First line: representative pictures of NSCLC-B soft-agar colonies grown without any treatment or in presence of atezolizumab, observed through an inverted microscope in dark-field; **second line:** morphologies of NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, observed through an inverted microscope. White

line corresponds to 100 μm . To improve the visual appearance of the images, photos were converted into grayscale. For pictures in the second line, contrast and brightness were also enhanced (+40% and +10%, respectively); **third line:** expression of CD44 on NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, measured by cytofluorimetric analysis (M1: marker); **bottom:** percentage of CD44+ cells on NSCLC-B "bulk agar" cell lines cultured under 2D-adherent conditions, measured by cytofluorimetric analysis ($n=4$). **, $p < 0.01$ by Student's t-test. Each bar represents mean with SEM. **(C)** NSCLC-B sphere formation in presence of atezolizumab 10 $\mu\text{g/ml}$ ($n=18$, nine experiments-different symbols-, each one with two technical replicates). ***, $p < 0.001$ by One sample t and Wilcoxon test. Each bar represents mean with SEM. Each dot represents a replicate. **(D)** Western blot analysis for MAPK activation in NSCLC-B cells treated with atezolizumab 5 $\mu\text{g/ml}$ in 2D-adherent culture conditions ($n=2$). Each bar represents mean with SEM. **(E)** Comparison of morphological and phenotypical features between NSCLC-B CRISPR-engineered cell lines (NSCLC-B CL1 and NSCLC-B CL2), NSCLC-B and NSCLC-H cell lines. **First line:** representative photos of NSCLC-B, NSCLC-B CL1 and NSCLC-H cell lines, observed through an inverted microscope in dark-field. White line corresponds to 100 μm ; **second line:** expression of GFP, CD44 and PD-L1 on NSCLC-B, NSCLC-B CL1 and NSCLC-H cell lines cultured under 2D-adherent conditions, measured by cytofluorimetric analysis (**black:** NSCLC-B, **green:** NSCLC-B CL1, **red:** NSCLC-H); **third line:** expression of GFP, CD44 and PD-L1 on NSCLC-B, NSCLC-B CL2 and NSCLC-H cell lines cultured

under 2D-adherent conditions, measured by cytofluorimetric analysis (**black**: NSCLC-B, **green**: NSCLC-B CL2, **red**: NSCLC-H. (F) Comparison of 2D-clonal efficiency between NSCLC-B, NSCLC-B CL1, NSCLC-B CL2 and NSCLC-H cell lines (n=2). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ by Student's t-test. Each bar represents mean with SEM. The horizontal axis reports the number of seeded cells per well.

Preliminary investigations on intracellular pathways influenced by PD-L1 blockade in NSCLC-B cells were carried out through western blot analyses, highlighting increased activation of MAPK in presence of atezolizumab (**Figure 8D**, Supplementary Figure 2). To further explore the role of PD-L1 modulation in cell behavior, we isolated two clones (NSCLC-B CL1 and NSCLC-B CL2) from NSCLC-B cells, previously transfected with a CRISPR/Cas9 guide designed to silence PD-L1 encoding gene (CD274). Both clones showed a decrease of PD-L1 expression compared to NSCLC-B parental cell line (**Figure 8E**). Surprisingly, not only decreased expression of PD-L1 resulted in increased expression of CD44, leading to an expression profile that was like the one of NSCLC-H cells, but a change in cell morphology, which became more similar to the one of NSCLC-H cells, was also observed in the clones (**Figure 8E**). In addition, NSCLC-B CL1 and CL2 cells also showed an evident increase in in vitro growth rate, which was comparable or even higher than the one of NSCLC-H cells (**Figure 8F** and Supplementary Figure 4A-B). Increased expression of IFNGR1 was also observed in the clone compared to NSCLC-B cells (Supplementary Figure 4C).

DISCUSSION

Even these agents directed against PD-1 or PD-L1 have led to unprecedented long-lasting clinical responses and significant improvement in patients' survival, ICI therapy has also been associated with primary and secondary mechanisms of resistance, many of which are not yet understood [54]. Looking deep into patients with NSCLC who develop fast progression due to primary resistance to ICI, 10% of them show hyperprogressive disease (HPD), consisting in a paradoxical acceleration of tumor growth induced by ICI-based treatment [55]. This is a phenomenon whose molecular mechanisms and predictive biomarkers are still unknown, and that heavily hinders the clinical management of these patients.

We described two cell line models derived from two distinct NSCLC samples of the same patient, who developed HPD on ICI-based therapy. The cell lines we presented here are representative models of the NSCLC tumor evolution under ICI therapy, since samples from which they were established were collected before initiation of any treatment (baseline, NSCLC-B cell line) or during progression under ICI therapy (hyperprogression, NSCLC-H cell line), offering the opportunity to study the result of the ICI treatment and the selective pressure exerted by immune system on cancer cells.

In general, NSCLC-H cells showed stemness-like and pro-growth features, as demonstrated by their ability to generate organoids, even after 50 in vitro passages, faster in vivo tumor growth and increased in vitro clonogenicity, compared to NSCLC-B cell line. The higher aggressivity of the NSCLC-H cell model was well described by whole transcriptome analysis, which revealed a completely different gene expression profile, with almost 3000 differentially expressed transcripts, between NSCLC-H and NSCLC-B cell lines. Several regulatory pathways associated with neural and muscle development, ossification and extracellular matrix remodeling were enriched in NSCLC-H overexpressed transcripts and were also associated with chromatin remodeling. These data suggest the acquisition of a genetic and phenotypic plasticity phenotype in the process of tumor evolution from NSCLC-B to NSCLC-H model, driven by a strong transcriptional reprogramming, which could be responsible for the lack of tumor response to immunotherapy and hyperprogression development [56,57]. Additionally, NSCLC-H cells showed a markedly increased expression of CD44 compared to NSCLC-B cells. CD44 is a pleiotropic transmembrane protein which acts not only as a receptor for hyaluronic acid (HA) and many other extracellular matrix (ECM) components, but also as a coreceptor for growth factors and cytokines. More importantly, CD44 has also been considered a marker of cancer stem cells in many solid malignancies, including NSCLC [39,58,59]. Besides CD44 expression, the presence of several CD44 isoforms has been well documented in cancer, although role, function and structure of these distinct molecules is not well defined, as well as their impact on therapeutic response [39]. NSCLC-B and NSCLC-H cell lines showed a distinct transcriptional profile for CD44 isoforms: NSCLC-H cell model indeed showed an increase of transcripts lacking

several exons of the variable region and a decrease of transcripts of variants CD44-206 and CD44-208. The significance of this different expression of CD44 transcript isoforms will need further investigations, and it will be essential to identify the isoforms modulated by IFN- γ and PD-L1 inhibition in our experiments.

Together with a plastic phenotype, NSCLC-H cells also showed a down-regulation of genes involved in the cellular response to IFN- γ , although some mediators of this pathway, including STAT1, JAK2 and IRF3, resulted to be more activated in NSCLC-H cell line compared to NSCLC-B cells. IFNGR1 expression was also higher in NSCLC-H than NSCLC-B cell line. This apparent discrepancy between down-regulated IFN- γ -related genes and overactivated IFN- γ pathway-related proteins in the NSCLC-H model could be justified by the strong interplay between IFN- γ and IFN-type I signaling pathways, which have been reported to share common mediators, including STAT1 and JAK2 [60,61]. In support of this, IFN- γ gene expression analysis revealed an increased expression of genes involved in cellular response to IFN- β signaling pathway in NSCLC-H cell line, compared to NSCLC-B cells, suggesting a possible role of this pathway in NSCLC-H cell line phenotype and in the activation of IFN-related proteins. Interestingly, NSCLC-H cell line also showed a stronger activation of IRF3, which not only regulates IFN- γ release by T cells, but it is also a key regulator of type I IFN gene expression [62–64]. In support of these observations, alterations of both type I and type II IFNs signaling pathways have been previously reported in a few cases of NSCLC patients who developed ICI resistance [65].

The lack of response to immunotherapy and the development of HPD observed in the patient, encouraged us to investigate the presence of mutations able to modulate IFN- γ and ICI therapy susceptibility in our cell line models. Hence, alterations shared between NSCLC-B and NSCLC-H cell lines on genes involved in IFN and PD-L1 pathways, were identified. In this regard, preliminary analysis of variants revealed alterations in ISG15, a post-translational modifier of PD-L1 involved in

the response to immunotherapy [66] and IFITM1 and IFITM2 [67,68], which have a controversial role in cancer and were also overexpressed in NSCLC-H cell line, compared to NSCLC-B cell model.

The role played by IFN- γ in the response to ICI therapy and the evidence of alterations of genes involved in interferon and PD-L1 pathways encouraged us to investigate the response of both NSCLC-B and NSCLC-H cell lines to IFN- γ *in vitro* and, the behavior of NSCLC-B cells in response to PD-L1 modulation. Surprisingly, both NSCLC-B and NSCLC-H cell lines showed no inhibition in cell growth and no induction of PD-L1 expression when treated with a similar dose of IFN- γ *in vitro*, suggesting a possible deficiency of the IFN- γ intracellular pathway in both cell lines. On the other hand, MHC class I complex was induced in NSCLC-B cell line in presence of IFN- γ . The evaluation of the activation of IFN pathway-related proteins in NSCLC-B cells treated with IFN- γ revealed the activation of STAT 1 and STAT2 proteins, JAK1 and increased expression of IRF9, but no increase in JAK2 activation was observed, suggesting a preferential induction of type I IFN pathway, rather than the canonical IFN- γ pathway [69–71]. Interestingly, in presence of IFN- γ treatment, NSCLC-B cells also showed a reduction of IFNGR1 expression. This observation further supports the hypothesis of the activation of a type I IFN-related pathway. It has indeed been reported that type I IFN treatment is able to reduce the expression of cell surface IFNGR1 in mouse and human myeloid cells through the silencing of *Ifngr1* gene transcription and epigenetic modifications, resulting in reduced responsiveness to IFN- γ [72–74]. This unusual pattern of type I IFN pathway activation could be explained by the documented capacity of IFN- γ to induce non-canonical transcriptional complexes that are overlapped with the ones induced by type I IFNs [75]. Of note, exposure of NSCLC-B cells to IFN- γ also significantly activated the proliferative MAPK pathway, which has been reported to be triggered by both IFN- γ and type I IFNs [76–78].

Surprisingly, IFN- γ treatment exerted a pro-growth effect on NSCLC-B cells in both 2D and 3D cell culture assays, in which we observed an increase of colonies and sphere formation ability in presence of the cytokine. Interestingly, only low doses of IFN- γ resulted in increased number of NSCLC-B

spheres, while high doses of the cytokine did not affect NSCLC-B sphere formation ability. In our experiments we observed that treatment of 3D-cultured NSCLC-B cells with low doses of IFN- γ resulted in the emergence of a CD44-positive cell subpopulation with a distinct cell morphology, suggesting an alternative signaling pathway able to induce cancer plasticity. The reason for the lack of CD44 increase in presence of high doses of IFN- γ should be further investigated, but it could be due to the activation of different signaling pathways in presence of low or high doses of IFN- γ [79]. Speaking of stemness induction, it is worth to mention the work of Beziaud and colleagues, who recently proved that relatively low doses of IFN- γ (10 U/ml) were able to induce CD90+ cancer stem cell phenotypes in tumor cells through the activation of BCAT1, leading to immunotherapy resistance [80]. In this regard, we also observed the up-regulation of BCAT1 gene in NSCLC-H compared to NSCLC-B cells. In relation to the paradoxical effect on tumor cell growth exerted by IFN- γ in our cell line model, Memon and colleagues recently reported a persistent activation of IFN- γ transcriptome programme in tumor samples of patients who developed resistance to ICI [81]. In addition, the chronic in vitro IFN- γ treatment of murine lung cancer cells was able to induce in vivo resistance to ICI [81].

Interestingly, NSCLC-B cells, when treated with anti-PD-L1 mAb atezolizumab, showed a response in terms of sphere formation ability and induction of CD44+ cell subpopulation that was identical to the one observed in presence of low doses of IFN- γ . Additionally, the modulation of PD-L1 expression in NSCLC-B cells not only increased CD44 cell surface expression, but it also resulted in alterations in cellular morphology and increased in vitro growth properties, leading to a switch of NSCLC-B traits towards the ones of NSCLC-H cells. This evidence confirmed a direct involvement of PD-L1 in CD44 cell surface expression and in cell growth regulation. PD-L1 transcript resulted to be more abundant in NSCLC-B compared to NSCLC-H cell line and a difference was also observed in terms of protein expression. In accordance with our findings, the involvement of PD-L1 in plasticity of macrophages and cancer cells was recently documented and associated with epigenetic changes [82–84]. In this regard, we described how NSCLC-H cell line showed a differential

expression of genes associated with chromatin remodeling, suggesting the existence of a relation between PD-L1, cell plasticity and epigenetic changes in our model.

Based on our results, we not only increase the evidence of the paradoxical role of IFN- γ and PD-L1 signaling pathway on cancer survival, which have been widely documented [43,85–88], but we also demonstrate the existence of a molecular circuit involving low doses of IFN- γ , PD-L1 and CD44, implicated in the regulation of tumor stemness and proliferation in a cellular model of hyperprogressive NSCLC.

In the clinical setting, it can be hypothesized that tumors resistant to ICI-based therapy that go through hyperprogression may be characterized by the activation of a non-canonical signaling pathway in response to IFN- γ , which not only may activate genes that overlap with type I IFN molecular pathway, as suggested by our results, but may also induce tumor plasticity in presence of low levels of the cytokine.

CONCLUSION

Our work suggests that immunotherapy may not only affect the plasticity of the immune populations localized in the tumor microenvironment, but it may also profoundly modify cancer cell phenotype and genomics, leading to plasticity and stemness tumoral features that could be responsible for the development of resistance to ICI therapy and HPD. Nevertheless, we demonstrated the existence of intrinsic tumoral responses not only to the exposure of cancer cells to IFN- γ , but also to the modulation of PD-L1 expression, which both converge to the modulation of tumoral CD44 expression, tumoral plasticity and cell growth alterations. These findings pave the way for the understanding of the intrinsic tumoral mechanisms underlying the phenomenon of HPD and for the identification of novel drug targets to prevent cancer progression in ICI-resistant patients.

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SUPPLEMENTARY MATERIAL

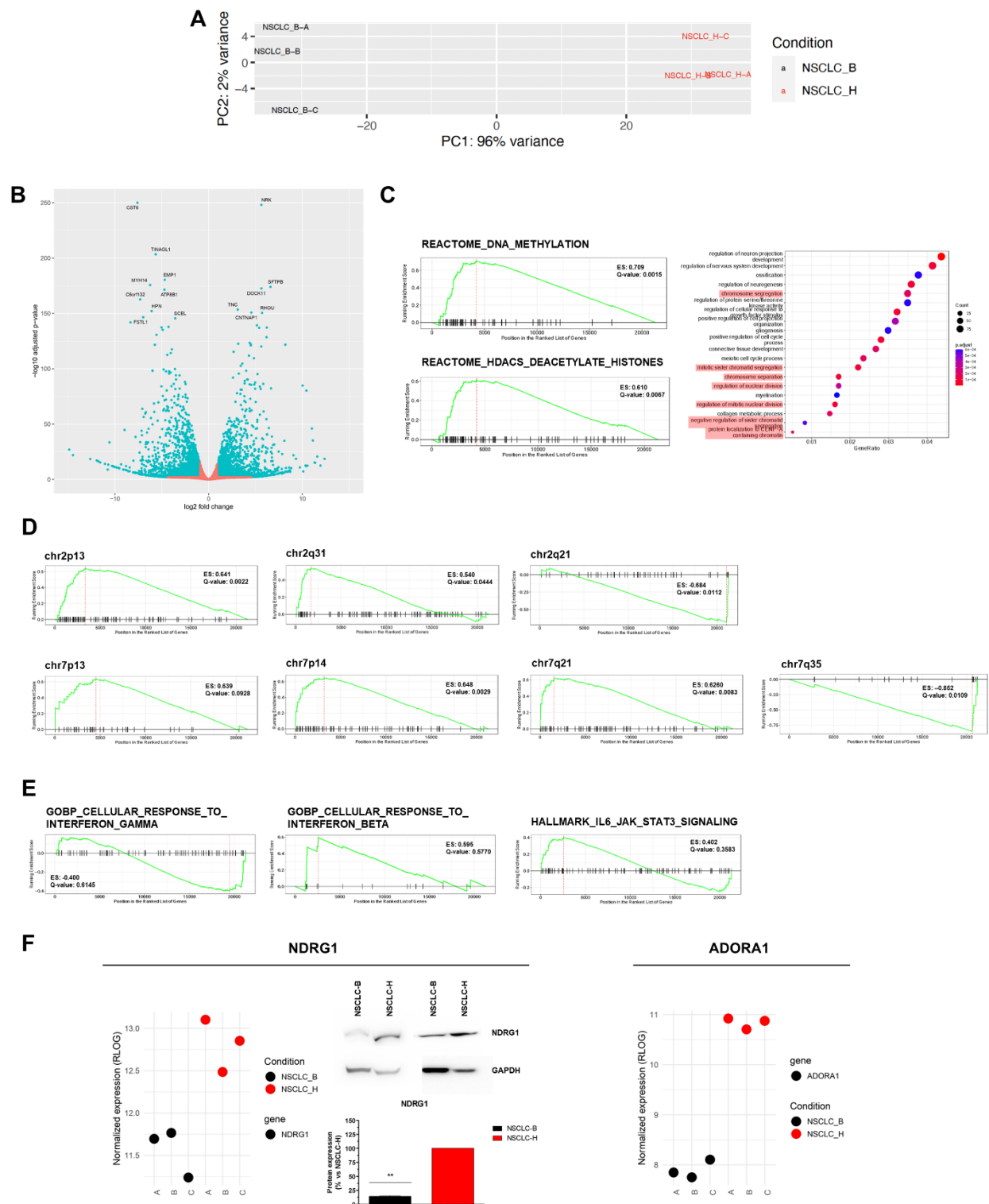


Figure S1. Transcriptomic and molecular characterization of NSCLC-B and NSCLC-H cell lines. (A) Scatter plots showing the combined projections of the first two components of a principal component analysis (PCA) considering the whole transcriptome expression of NSCLC-H and NSCLC-B cells. (B) Volcano plots showing the Log2Fold Change vs $-\log_{10}$ p-adj of genes in the comparison NSCLC-H vs NSCLC-B. Genes with p-adj < 0.01 and absolute Log2FC > 1 are highlighted in green-water. (C). Left: GSEA

enrichment plots of curated lists related to DNA changes; right: Top-20 GO Terms Biological Processes enriched in up-regulated genes with an absolute $\text{Log}_2\text{FC} > 0.58$ and $p\text{-adj} < 0.01$ (NSCLC-H vs NSCLC-B). Several pathways involving DNA modifications were identified (pink). (D) GSEA enrichment plots of curated lists associate to human chromosome cytogenetic bands. (E) GSEA enrichment plots of curated lists associate to IFN response and IL6-JAK-STAT pathway. (F) NDRG1 and ADORA1 transcript and protein expression in NSCLC-B and NSCLC-H cell lines. Left: normalized expression of NDRG1 in NSCLC-H and NSCLC-B cells, as identified by RNAseq analysis (Log_2FC 1.803, $p\text{adj}$ 5.016E-14); middle: western blotting picture of two distinct biological replicates and densitometric quantification of NDRG1 protein normalized on GAPDH. Expression is reported as percentage of NSCLC-H protein expression. Bar represents mean and SEM. **, $p < 0.01$ by One Sample t and Wilcoxon test; right: normalized expression of ADORA1 in NSCLC-H and NSCLC-B cells, as identified by RNAseq analysis (Log_2FC 4.503, $p\text{adj}$ 8.214E-122).

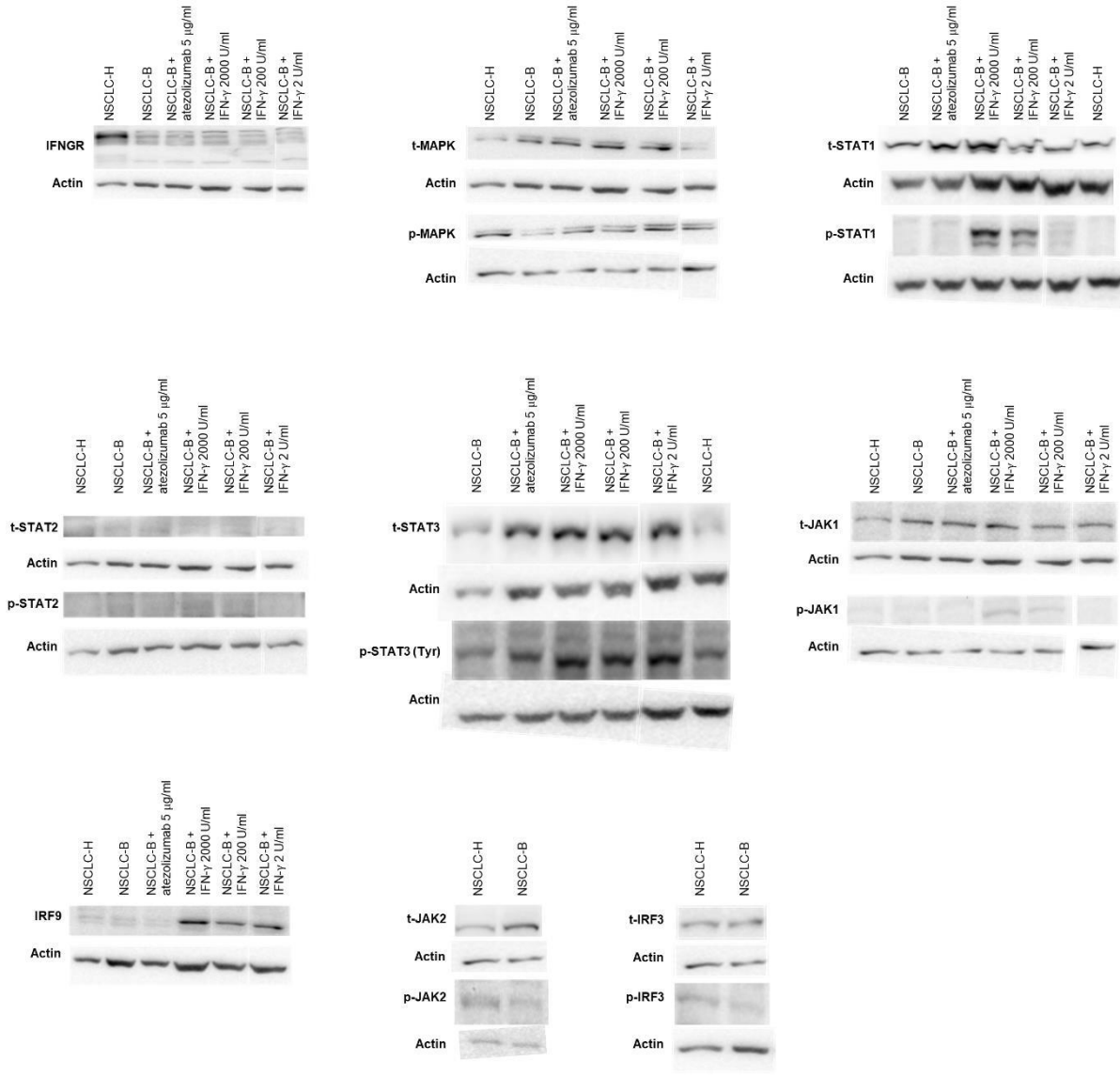


Figure S2. Levels of total and phosphorylated proteins involved in cell growth and IFN- γ signaling in NSCLC-B and NSCLC-H cells, in presence or not of IFN- γ or atezolizumab treatment, detected by Western blot analysis.

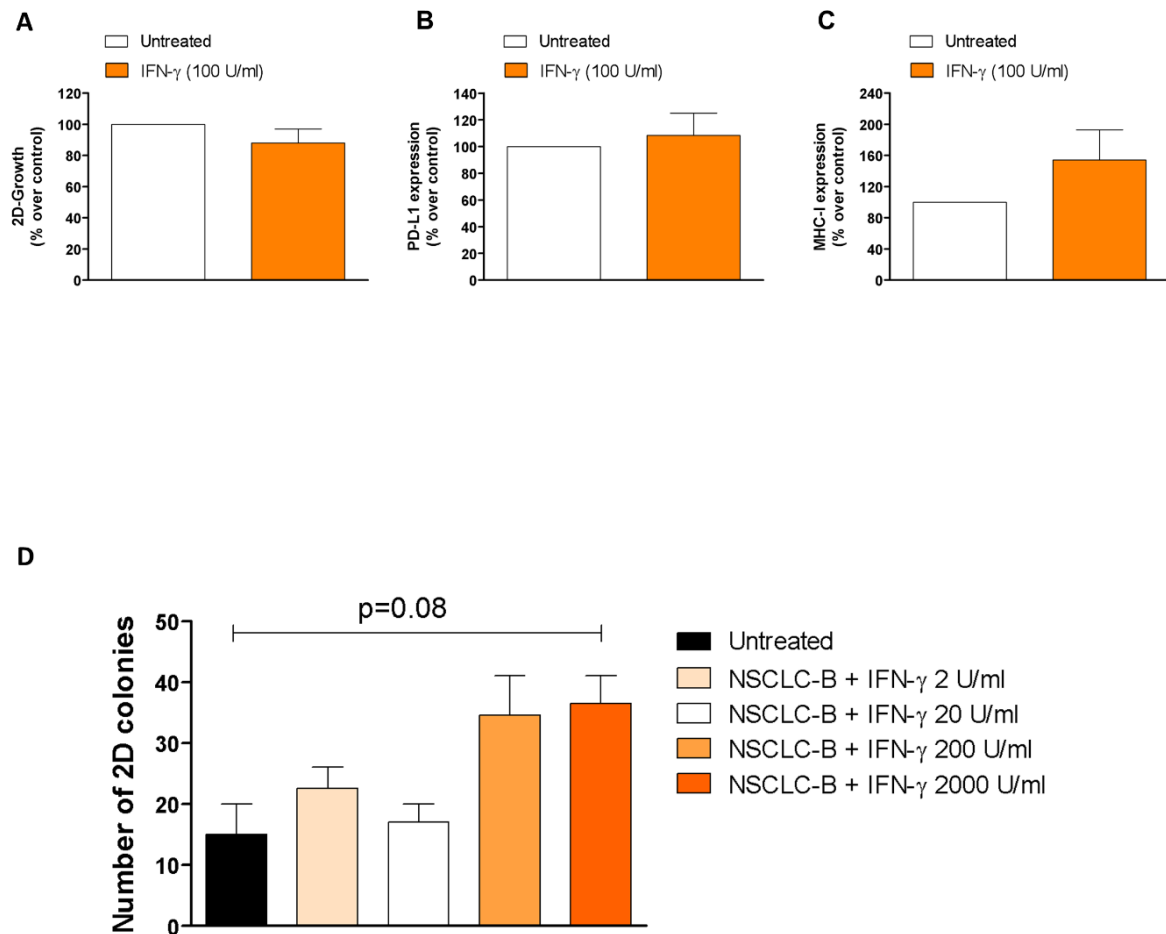


Figure S3. Response of NSCLC-H and NSCLC-B cell lines to IFN- γ in 2D culture conditions. (A) Sensitivity of NSCLC-H cells to the antiproliferative effect of IFN- γ (100 U/ml, n=2). Bar represents mean with SEM. (B) Percentage of induction of PD-L1 expression on NSCLC-H cells in presence of IFN- γ (n=2). Bar represents mean with SEM. (C) Percentage of induction of MHC class I complex expression on NSCLC-H cells in presence of IFN- γ (n=2). Bar represents mean with SEM. (D) 2D-growth of low density seeded-NSCLC-B cells treated with different doses of IFN- γ (n=2). Each bar represents mean with SEM.

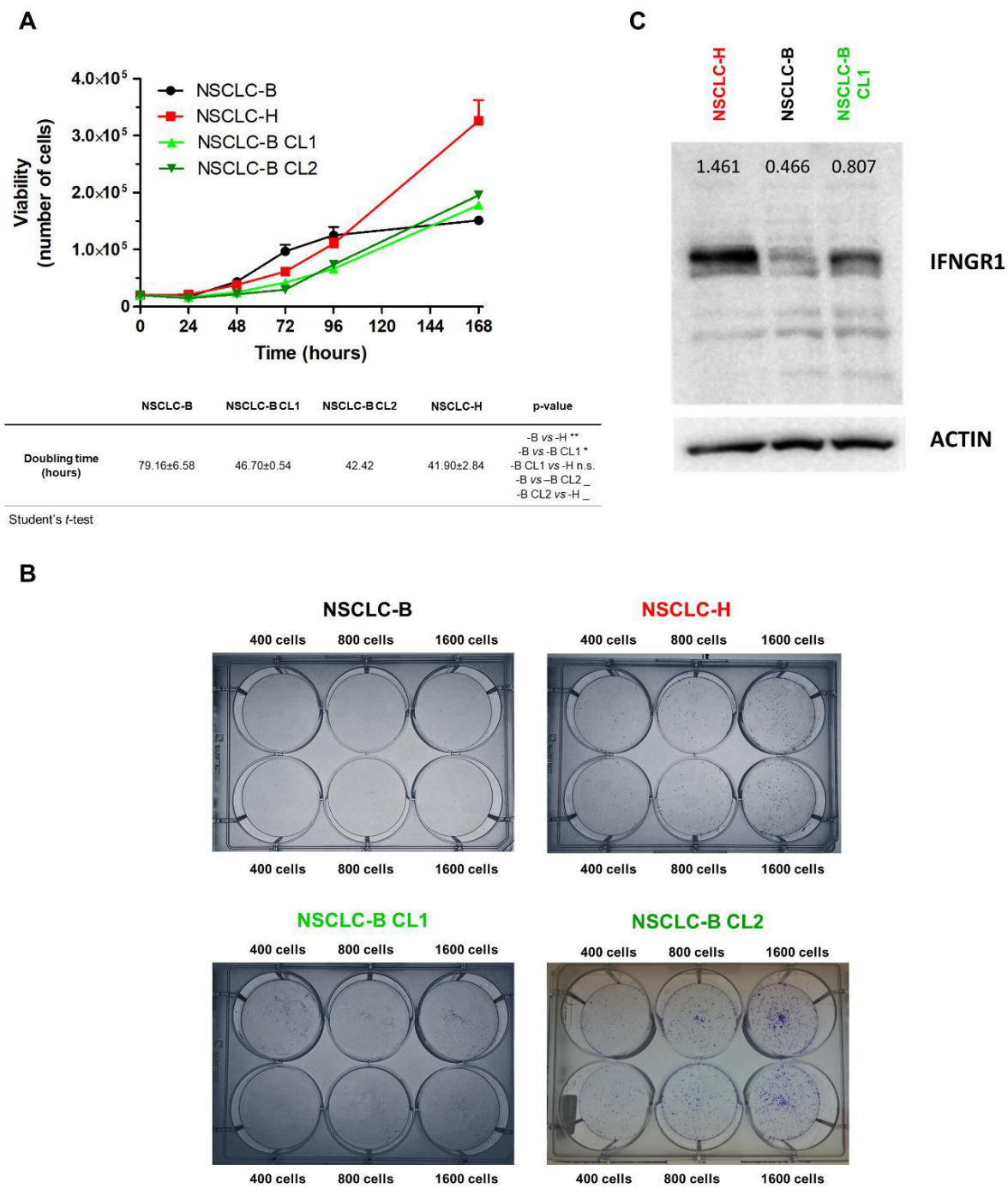


Figure S4. Comparison of cell behavior in 2D culture conditions between NSCLC-B, NSCLC-B CL1, NSCLC-B CL2 and NSCLC-H cell lines. (A) Comparison of cell growth curves (NSCLC-B and NSCLC-H n=3, NSCLC-B CL1 n=2, NSCLC CL2 n=1). Bars represent mean with SEM. Doubling time of culture in 2D-growth adherent condition was reported in the table. (B) IFNGR1 expression detected by Western Blot analysis (numbers: densitometric data). (C) Comparative images of cells' 2D-clonogenicity. To improve the visual appearance of the images, photos were converted into grayscale.

Gene	Transcript	Short name	UNIPROT ID	Description	Log2 Fold Change	padj
ENSG00000026508	ENST00000263398	CD44-201	P16070-12	Merged Havana/Ensembl protein coding	3.840943	6.07E-42
ENSG00000026508	ENST00000425428	CD44-207	Q86UZ1	Merged Havana/Ensembl non-sense-mediated decay	14.71797	1.25E-26
ENSG00000026508	ENST00000433892	CD44-209	P16070-10	Merged Havana/Ensembl protein coding	2.100291	5.02E-18
ENSG00000026508	ENST00000352818	CD44-205	P16070-18	Merged Havana/Ensembl protein coding	13.8054	2.91E-22
ENSG00000026508	ENST00000415148	CD44-206	P16070-4	Merged Havana/Ensembl protein coding	-2.57714	1.69E-12
ENSG00000026508	ENST00000527326	CD44-225	--	processed transcript retained intron	4.65833	0.00324
ENSG00000026508	ENST00000434472	CD44-210	P16070-11	Merged Havana/Ensembl protein coding	12.22925	2.21E-16
ENSG00000026508	ENST00000531118	CD44-232	--	processed transcript retained intron	2.673561	7.40E-10
ENSG00000026508	ENST00000428726	CD44-208	P16070-1	Merged Havana/Ensembl protein coding	-2.2409	0.03343
ENSG00000026508	ENST00000526025	CD44-222	E9PKC6	Ensembl protein coding	3.126152	0.0000163
ENSG00000026508	ENST00000526669	CD44-224	HOYD13	Ensembl protein coding	4.827026	0.0000372
ENSG00000026508	ENST00000528922	CD44-230	--	protein coding CDS not defined	2.085657	0.00000336
ENSG00000026508	ENST00000525241	CD44-215	--	processed transcript retained intron	7.948969	0.0000251
ENSG00000026508	ENST00000279452	CD44-204	HOY2P0	Ensembl protein coding	0.456276	0.006981
ENSG00000026508	ENST00000442151	CD44-211	HOYSE4	Merged Havana/Ensembl protein coding	2.701999	0.001073
ENSG00000026508	ENST00000525688	CD44-220	HOYF08	Ensembl protein coding	6.325978	0.005413

Table S1. CD44 isoform analysis. Detected isoforms, NSCLC-H vs NSCLC-B: log2 Fold Change and significance were reported.

Gene	Variant Status	Variant reference
IFITM1	Homozygous	rs9667990
IFITM2	Heterozygous	rs10398
	Heterozygous	rs1058873
	Homozygous	rs1058900
	Homozygous	rs1058935
	Heterozygous	rs1059091
	Homozygous	rs14408
ISG15	Homozygous	rs15842
	Homozygous	rs8997
IRF3	Homozygous	rs2304205
SETD1B	Homozygous	rs3741593
MED24	Heterozygous	rs755993594
TRAPPC12	Heterozygous	rs10176767
CST3	Homozygous	rs6138024

Table S2. Variants identified in genes involved in IFN- γ pathway. Variants were detected in both NSCLC-B and NSCLC-H

Antibody (target)	Clone	Molecular weight (KDa)	Dilution
IFNGR1	E444	45-90	1:1000
JAK1	6G4	130	1:1000
phospho-JAK1 (Tyr 1034/1035)	D7N4Z	130	1:1000
JAK2	DEE12	125	1:1000
phospho-JAK2 (Tyr 1007/1008)	C80C3	125	1:1000
STAT1	D1K9Y	84, 91	1:1000
phospho-STAT1 (Tyr 701)	D4A7	84, 91	1:1000
STAT2	D9J7L	97, 113	1:1000
phospho-STAT2 (Tyr 690)	D3P2P	97, 113	1:500
STAT3	D1B2J	79, 86	1:1000
phospho-STAT3 (Tyr 705)	D3A7	79, 86	1:1000
phospho-STAT3 (Ser 727)	Polyclonal	79, 86	1:1000
IRF9	D2T8M	48	1:1000
IRF3	D6I4C	50-55	1:1000
phospo-IRF3 (Ser 386)	E7J8G	50-55	1:1000
pMAPK42/44 (Erk1/2)	137F5	42-44	1:1000
phospho-pMAPK 42/44 (Erk1/2) (Thr202/Tyr204)	D13.14.4E	42-44	1:500
NDRG1	D8G9	46	1:1000

Table S3. List of primary antibodies used for Western Blot analyses

PRECLINICAL AND CLINICAL ACTIVITY OF EGFR INHIBITORS IN NON-SMALL CELL LUNG CANCER (NSCLC) PATIENTS HARBORING BRAF CLASS 3 MUTATIONS

ABSTRACT

BRAF^{non-V600} class III alterations are reported in one third of non-small cell lung cancers (NSCLCs). This class of mutations is currently orphan of targeted therapies and thus treated as non-oncogene addicted, representing an unmet clinical need. In our Institution, one patient (patient #1) with metastatic NSCLC harboring a BRAF class 3 mutation has achieved an ongoing complete tumor remission since the initiation of third line erlotinib in 2011. We also describe the case of patient #2, with advanced NSCLC and a BRAF class 3 mutations, who received erlotinib as second-line therapy. To investigate the underlying mechanisms of this response, we derived cell lines from patient-derived xenograft (PDX) of patient #2 and from tumor biopsy of patient #3, diagnosed with NSCLC patient harboring BRAF p.G466V class 3 mutation and progressing on immunotherapy, to test in-vitro tumor sensitivity to EGFR. The behavior of ADK-14 and ADK-36 cell lines were compared to ADK-17 cell line, derived from a NSCLC at the diagnosis and harboring KRAS p.G12V mutation and wild-type BRAF. Patient #1, a 60-year-old male with metastatic NSCLC carrying a BRAF p.D594N mutation, received erlotinib after the exhaustion of standard treatments, obtaining a complete tumor remission that is still ongoing after 12 years. Patient #2, a 60-year-old female with metastatic NSCLC carrying a BRAF p.D594G mutation, progressed after first-line chemoimmunotherapy and received erlotinib as second-line therapy, achieving an objective partial response after 1 month of treatment. Cell lines derived from PDX of patient #2 (PDX-ADK-36) and tumor biopsy of patient#3 (ADK-14) showed higher 2D-growth sensitivity to erlotinib than ADK-17 cell line (PDX-ADK-36, IC₅₀ 2.182 μ M; ADK-14, IC₅₀ 2.366 μ M; ADK-17, 10.14 μ M. 3D-growth of BRAF-class III cell lines, unlike ADK-17, was also affected by erlotinib (1 μ M) both in 3D-soft agar assay (ADK-14 growth inhibition >50%) and sphere formation assay (ADK-14 and PDX-ADK-36 sphere formation inhibition >50%). Western blot analysis confirmed erlotinib-mediated inhibition of EGFR activity in BRAF class III

ADK-14 cell line. NSCLC patients harboring BRAF class 3 mutations might be sensitive to EGFR inhibition, warranting investigation in clinical studies.

INTRODUCTION

The RAS/RAF/MEK/ERK signal transduction cascade is one of the main oncogenic pathways in cancer. The activation of RAS effectors, including ARAF, BRAF, and CRAF, three members of the RAF protein kinase family, promotes cell proliferation [1]. RAS-GTP binds to RAF family members and causes their homo- and heterodimerization and activation of their kinase activity [2]. These enzymes catalyze the phosphorylation and activation of MEK1 and MEK2, which in turn activate ERK1 and ERK2. The activation of the MAPK/ERK pathway is involved in many cellular processes, including cell proliferation, differentiation, transcriptional regulation, and survival. Activating mutations of members of the RAF, MEK, and ERK families have all been identified in cancers, with BRAF mutations being by far the most common [3-6]. BRAF is a gene that encodes the serine/threonine protein kinase BRAF. There are approximately 300 BRAF mutations. BRAF mutations can be found in 6% of all patients with cancer [7-9] including 3% of Non-Small Cell Lung Cancer (NSCLC) [10,11]. BRAF mutations are generally classified into three functional classes: class 1 (V600*) mutations, characterized by strong activity of BRAF kinase domain and constitutive activation of the MAPK pathway; class 2 mutations, with intermediate to high activity of BRAF kinase domain, activating Ras independent signaling as dimers; class 3 consists of kinase-inactivating RAS-dependent heterodimers with wild-type BRAF or CRAF requiring additional upstream signaling (e.g. G596R, D594G, N581, G466V, D287Y) [12,13]. The inactive kinase domain prevents autophosphorylation of inactivating residues, and thus prolongs the active conformation state. Furthermore, the mutants bind more tightly than wild-type BRAF to RAS-GTP, and their binding to and activation of wild-type CRAF is enhanced, leading to increased ERK signaling [13,14]. Of note, BRAF-inactivating mutations can modulate the intensity and duration of MAPK activity leading to

different effects on cells, including tumor cells: whereas excess MAPK activity induced DNA damage-mediated cellular toxicity, insufficient MAPK signaling cannot sustain tumor growth [14]. TP53, EGFR, KRAS, and NF1 were among the most frequently co-altered genes in BRAFV600 mutant NSCLC [15]. For those class 3 BRAF tumors without coexisting RAS or NF1 alterations, upstream signaling, including EGFR and SHP23, may be targeted. PTPN11 and CBL are potentially targetable if their alterations are present [16]. In clinical practice, the combination of BRAF and MEK inhibitors became the standard of care for BRAFV600 mutant advanced NSCLC patients [17-19]. However, about a half of BRAF mutations in NSCLC are non-V600 and for these cases the discovery of targeted therapies remains an unmet clinical need. Preclinical studies proved that different cell lines harboring class 3 BRAF mutations, including three NSCLC cell lines, had high RAS-GTP level and ERK signaling activation. Ras activation was mediated by tyrosine kinase receptors, particularly EGFR in NSCLC cell lines. The growth of cell lines with class 3 BRAF mutations was inhibited by the exposure to cetuximab [13]. A multicenter pooled analysis of 40 metastatic colorectal cancer patients with BRAF non-V600 mutations showed that class 3 BRAF mutants had a significantly higher response rate to anti-EGFR antibody therapy when compared to class 2 BRAF ones [20]. More recently, concomitant inhibition of BRAF/MEK and SHP2 proved synergistic therapeutic effects in class 3 BRAF mutant cells [21], providing the bases for clinical evaluation (NCT03284502). Moreover, inhibitors directed at the heterodimer interface between class 3 BRAF mutants and CRAF might be an encouraging strategy for innovative anticancer drugs [22]. Lastly, new approaches, coming from BRAF-targeting in colorectal cancers (CRC), could be envisioned also for non-V600 BRAF+ lung cancer. The triplet co-administration of BRAFi, MEKi and anti-EGFR antibody (encorafenib, binimetinib and cetuximab), which demonstrated to prolong OS in V600E pre-treated colorectal cancer patients [23], is now under evaluation also in non-V600E ones [24]. This strategy, only experimentally investigated in lung cancer [25], might represent a powerful option targeting class 3 BRAF-mutant NSCLC. Although these combinatorial strategies might be promising, the risk of significant toxicities derived from the combination of different tyrosine kinase inhibitors is not

negligible. An activation of RAS signaling by receptor tyrosine kinases (RTK), especially EGFR, was observed in NSCLC harboring class 3 BRAF mutations [13].

Erlotinib is a first-generation EGFR tyrosine kinase inhibitor (TKI) approved for pretreated advanced NSCLC patients regardless of EGFR mutational status that inhibits EGFR autophosphorylation [26,27]. Whether an anti-EGFR therapy is effective in BRAF mutated NSCLC patients has not been previously explored. The aims of the present study were to investigate the clinical activity of EGFR inhibitors in patients with NSCLC and BRAF class 3 mutations; and to evaluate in vitro the activity of different EGFR inhibitors using cell cultures and patient-derived xenografts (PDX) of NSCLC harboring BRAF class 3 alterations.

MATERIALS AND METHODS

We retrospectively reviewed clinicopathologic and outcomes data of two patients with metastatic lung adenocarcinoma and a BRAF class 3 mutation without concurring alterations in EGFR or other molecular drivers who were treated with erlotinib 150 mg daily in our Institution. In both cases, erlotinib was administered after failure of standard treatments, including 1L platinum-based chemotherapy and 2L docetaxel (before the approval of PD-(L)1 inhibitors) for one patient, and 1L platinum-based chemotherapy plus pembrolizumab for the other patient. We then produced a patient-derived xenograft (PDX) from one of the two erlotinib-treated patients with available tumor tissue (PDX-ADK-36) before receiving the EGFR-inhibitor, and a cell line from a third patient with NSCLC and a BRAF class 3 mutation who was treated in our Institution but did not receive erlotinib (ADK-14). Lastly, we produced a cell line (ADK-17) derived from a treatment-naïve NSCLC patient, carrying a KRAS^{G12V} mutation without BRAF alterations as control. The ADK-14 cell culture was derived from a biopsy of a lymph node metastasis of patient (#3) at the time of disease progression to 1L pembrolizumab. The PDX-ADK-36 mouse model was established from a biopsy of a lymph node metastasis of patient #2. We derived PDX-ADK-36 cell culture from the tumor of a PDX-ADK-

36 mouse at the second *in vivo* passage. ADK-14 cells were cultured in MammoCult medium (STEMCELL Technologies, Vancouver, Canada) supplemented with 1% fetal bovine serum (FBS, Thermo Fisher Scientific, Monza, Italy), 100 U/ml penicillin and 10 µg/ml streptomycin (Thermo Fisher Scientific) at 37°C in a humidified atmosphere at 5% CO₂. PDX-ADK-36 and ADK-17 were cultured in RPMI+10%FBS and antibiotics.

To externally validate our findings, we searched the Cancer Cell Line Encyclopedia, a dataset available online on cBioPortal.org, for cell lines of NSCLC harboring BRAF class 3 alterations without other concurring driver alteration having available treatment data (IC₅₀) [28-30]. The sensitivity (IC₅₀) of NSCLC cell lines harboring BRAF class 3 alterations to EGFR inhibitors (afatinib and gefinitib), and other agents (trametinib, dabrafenib, cabozantinib and doxorubicin) was evaluated and compared to that of NSCLC cell lines harboring BRAF class 2 alterations, EGFR, or KRAS driver mutations.

2D-growth sensitivity assay

Sensitivity to erlotinib and other EGFR tyrosine kinase inhibitors (dacomitinib, afatinib, and osimertinib), and to the combination of erlotinib plus trametinib (Selleck Chemicals, Houston, TX) was determined using the WST-1 reagent (Roche, Merck, Milan, Italy), according to the manufacturer's instructions. Cells were seeded at 15.625×10^3 cells/cm² in 96-well culture plate. Treatment was performed 24 hours after the seeding. Drug sensitivity was evaluated 72 h after treatment.

3D-growth in soft agar

ADK-14, ADK-17 and PDX-ADK-36 cells were seeded at 500 cells/well in 24-well plate in semisolid medium—MammoCult + 1% FBS + 0.33% agar (Sea-Plaque Agarose, Lonza, Switzerland), containing erlotinib (0.1 and 1 µM), and dacomitinib (0.01, 0.1 and 1 µM) with a 0.5% agarose

underlay. Colonies (diameter >90 μm) were counted 2–4 weeks later under an inverted microscope in dark-field, as previously described [31,32].

Sphere formation assay

Cells were seeded at 4,000 cells in 4 mL complete MammoCult medium without serum in 6-well Ultra-Low adherence plate (Corning Life Sciences), according to the MammoCult Human Medium Kit protocol. Drugs and vehicle were added to the medium at different doses. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for a week. Spheres, multi-cell structures, with a diameter larger than 90 μm were counted about 7 days after the seeding [33].

Western Blot analysis

Protein extraction and Western blotting were performed as reported previously [34]. EGFR/HER1, BRAF, MAPK, and AKT The following primary antibodies were used: anti-EGFR (clone LA22, Sigma-Aldrich, Merck), anti-BRAF (clone D9T6S, Cell Signaling), anti-MAPK (clone p44/42, diluted 1:1000, Cell Signaling), anti-pAKT (ser473, diluted 1:1000, Cell Signaling) Membranes were either incubated with polyclonal horse-radish-peroxidase conjugated anti-rabbit IgG antibody (diluted 1:3000, Bio-Rad Laboratories), or anti-mouse IgG antibody (diluted 1:1000, Santa Cruz Biotechnology). Protein presence was detected by chemiluminescent reaction (Bio-Rad Laboratories) before film exposure.

Statistical analysis

Comparisons of continuous variables were computed using the Mann-Whitney U test, the t-test, or the Kruskal-Wallis test, when appropriate. All P-values are two-sided and confidence intervals are at the 95% level, with significance pre-defined to be at $P < 0.05$. Statistical analyses were performed using R version 3.6.3.

RESULTS

Clinical cases

Patient #1 is a male, former smoker, who was diagnosed at the age of 60 with stage IVB lung adenocarcinoma for the presence of metastasis in lymph nodes and brain. A first line treatment with chemotherapy, consisting of carboplatin AUC5 day 1 plus gemcitabine 1000 mg/m² days 1,8 was administered for 4 cycles, and stereotactic radiation therapy was effectively performed on the single brain metastasis. Following evidence of intrathoracic nodal progressive disease at the first radiographic tumor reassessment after initiation of chemotherapy, a second-line treatment with docetaxel 75 mg/m² was administered for a total of 10 cycles, obtaining stable disease as best response. Disease progression was subsequently evidenced on mediastinal lymph nodes, for which a third-line treatment with erlotinib 150 mg daily was initiated and led to a complete response that is still ongoing after 12 years (**Figure 1**). A next generation sequencing (NGS) panel (Oncomine Focus Assay – ThermoFisher Scientific, Kit RUO) performed in 2016 on archival tissue collected before the initiation of erlotinib documented the presence of the class 3 BRAF (p.D594N) mutation together with the CTNNB1 (p.S37C) mutation.

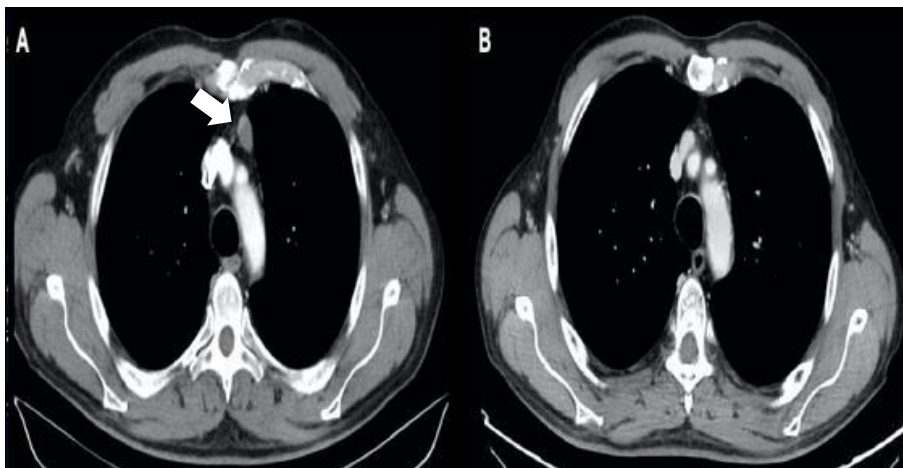


Figure 1. Radiographic assessment of erlotinib activity in patient #1. Computed tomography scans performed before the initiation of the treatment with erlotinib (**A**) and after 3 months from the start of the EGFR inhibitor (**B**) showing complete response on the mediastinal lymphadenopathy (white arrow) which is still ongoing after 12 years.

Patient #2 was a 60-year-old female, current heavy smoker at diagnosis, who was diagnosed in 2021 with stage IVB adenocarcinoma of the lung for the presence of pleural, bone and extensive lymph

node metastases. The NGS (Oncomine Focus Assay – ThermoFisher Scientific, Kit RUO) panel showed a BRAF class 3 mutation (p.D594G). PD-L1 tumor proportion score was 0% (clone SP263, Ventana). A first line treatment with carboplatin AUC5, pemetrexed 500 mg/m² and pembrolizumab 200 mg flat dose every 3 weeks was administered for 3 cycles before a documented clinical and radiographic evidence of progressive disease, mainly in the intrathoracic metastatic sites. Given the primary resistance to the combination of chemotherapy and immunotherapy, and considering our experience with patient #1, a second line treatment with erlotinib 150 mg daily was started. After 1 month of treatment, the radiographic reassessment performed through computed tomography scans showed objective partial response (**Figure 2**). Unfortunately, the patient died a few days after tumor reassessment at home, probably due to an acute cardiovascular event although the exact cause of death could not be documented.

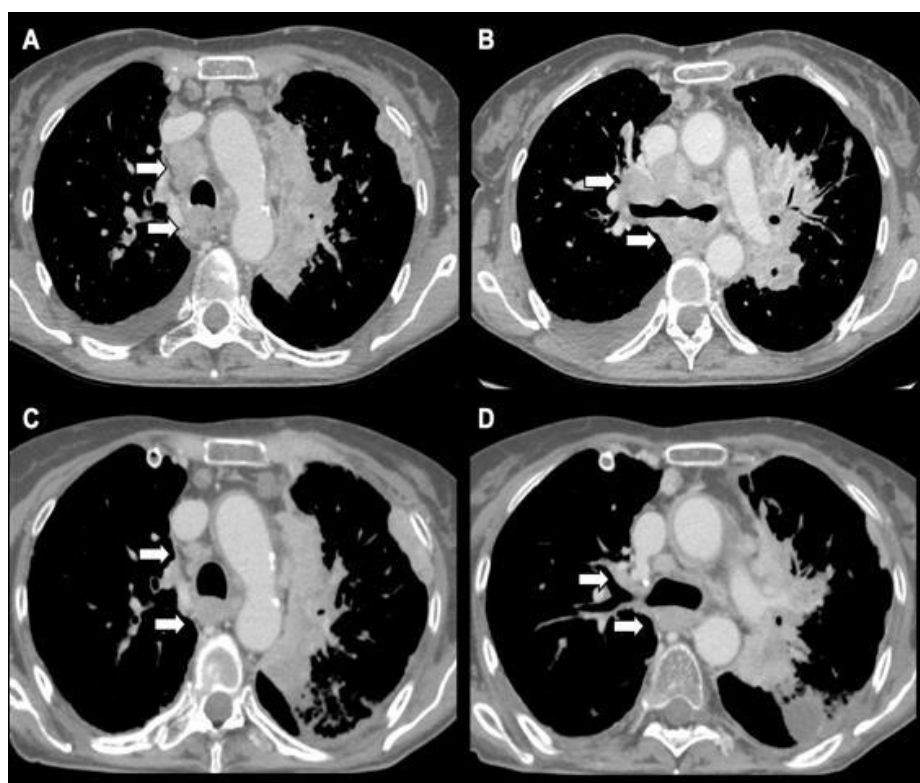


Figure 2. Radiographic assessment of erlotinib activity in patient #2. Computed tomography scans performed before (A, B) and after 1 month (C, D) from the initiation of the treatment with erlotinib, showing a partial response (white arrows).

2D growth activity of EGFR TKIs on BRAF class 3-mutated cell lines

To investigate the activity of EGFR inhibition in NSCLC harboring BRAF class 3 mutations, we derived cell lines from the tumor of a patient-derived xenograft (PDX) mouse established from patient

#2 (PDX-ADK-36) and from a biopsy of a lymph node metastasis from a third patient (patient #3, ADK-14), diagnosed with advanced NSCLC harboring a BRAF p.G466V class 3 mutation without other concurrent genomic drivers and progressing on pembrolizumab, to test *in vitro* tumor sensitivity to EGFR-TKIs. The behavior of ADK-14 and ADK-36 cell lines was compared to that of the ADK-17 cell line, which was derived at diagnosis from a patient with NSCLC harboring a KRAS p.G12V mutation, wild-type for BRAF alterations and without other genomic drivers.

We explored *in vitro* the sensitivity of ADK-14 and PDX-ADK-36 cell lines to EGFR inhibition (Figure 3).

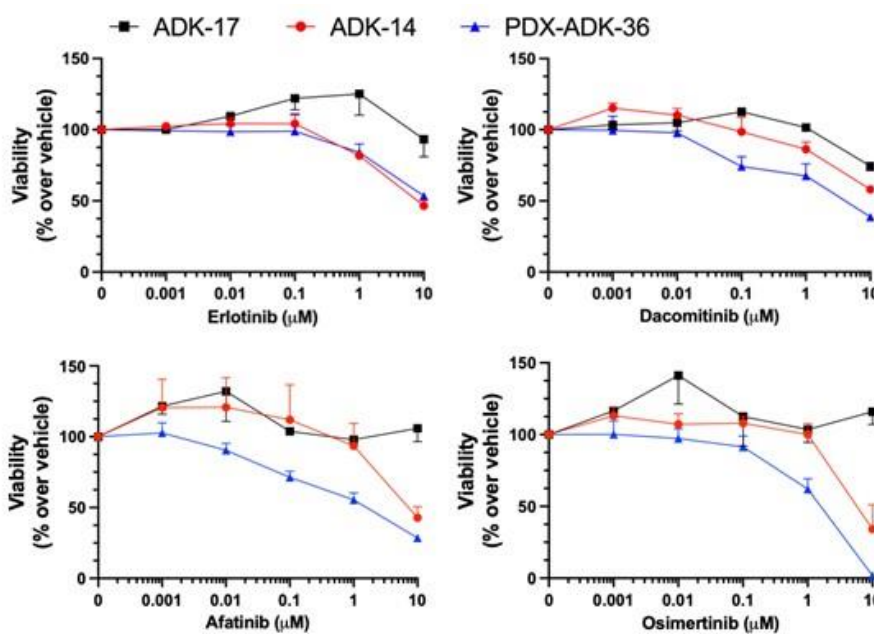


Figure 3. In vitro 2D-growth of ADK-14, PDXADK-36 and ADK-17 cells in presence of EGFR TKI. Each point represents mean and SEM. **Erlotinib:** ADK-17 vs ADK-14 at 1 and 10 μM $p < 0.05$, at least; ADK-17 vs PDX-ADK-36 at 10 μM $p < 0.05$. **Dacomitinib:** ADK-17 vs ADK-14 at 10 μM $p = 0.0557$, at 0.1 μM $p < 0.05$; ADK-17 vs PDX-ADK-36 at 0.1 μM $p < 0.05$, at 1 μM $p = 0.054$, at 10 μM $p < 0.05$. **Afatinib:** ADK-17 vs ADK-14 at 10 μM $p < 0.05$; ADK-17 vs PDX-ADK-36 from 0.1 μM $p < 0.01$. **Osimertinib:** ADK-17

vs ADK-14 at 10 μM $p < 0.05$; ADK-17 vs PDX-ADK-36 at 1 μM $p < 0.05$, at 10 μM $p < 0.001$.

2D cell cultures of ADK-14 and PDX-ADK-36 cell lines showed higher sensitivity to EGFR TKI than ADK-17 cell line: ADK-14 (IC₅₀ erlotinib 7.856 μM, IC₅₀ dacomitinib 12.89 μM, IC₅₀ afatinib 8.276 μM, IC₅₀ osimertinib 6.847 μM); PDX-ADK-36 (IC₅₀ erlotinib 10.45 μM, IC₅₀ dacomitinib 2.784 μM, IC₅₀ afatinib 1.297 μM, IC₅₀ osimertinib 1.340 μM); ADK-17 (IC₅₀ erlotinib >200 μM, IC₅₀ dacomitinib 30.68 μM, IC₅₀ afatinib >200 μM, IC₅₀ osimertinib >200 μM).

Erlotinib, at doses 1 and 10 μM, inhibited the growth of both ADK-14 ($p < 0.001$, EC₅₀ 2.370 μM, IC₅₀ 7.856 μM) and PDX-ADK-36 cell lines ($p = 0.05$ at 1 μM and $p < 0.001$ at 10 μM, EC₅₀ 2.815

μM , IC_{50} 10.450 μM) while no effect was observed on ADK-17 cells (EC_{50} not estimable, IC_{50} >200 μM). Dacomitinib also partially inhibited the growth of PDX-ADK-36 cells ($p<0.05$ at 0.1-1 μM , and $p<0.001$ at 10 μM , EC_{50} 0.140 μM , IC_{50} 2.784 μM) and ADK-14 cells ($p<0.01$ at 10 μM , EC_{50} 1.556 μM , IC_{50} 12.890 μM) although at 10 μM also ADK-17 cells were partially inhibited ($p<0.05$ at 10 μM , EC_{50} not estimable, IC_{50} 30.680 μM).

Intriguingly, we found that growth inhibition was significantly enhanced by combining erlotinib with trametinib compared to their use as single agents (not shown) on ADK-14 and PDX-ADK-36 cell lines ($p<0.05$), but not on the ADK-17 cell line (**Figure 4**).

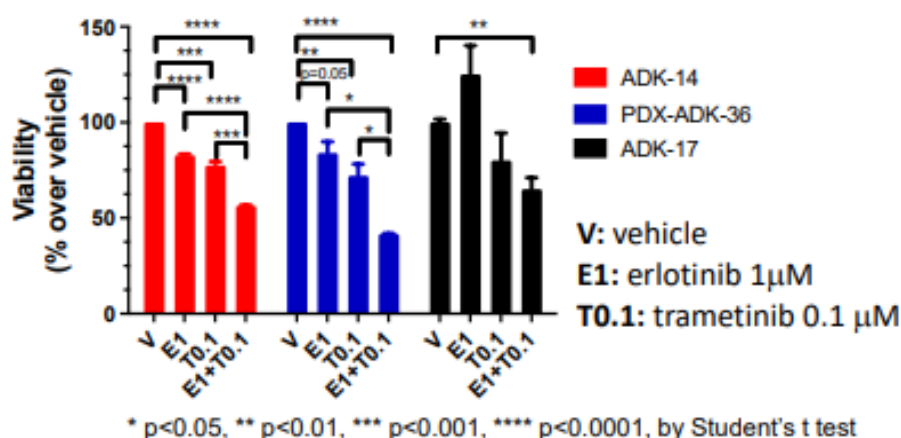


Figure 4. Combining erlotinib and trametinib (MEK inhibitor) showed a synergistic effect in inhibiting ADK-14 and PDX-ADK-36 cell growth. No synergistic effect with the combination of the two drugs was observed in ADK-17 cell line compared to their use of as single agents.

3D growth activity of EGFR TKIs on BRAF class 3-mutated cell lines

3D models allow a better interpretation of TKI activity. Therefore, consistently with our previous analyses [35], we tested the activity of erlotinib, and dacomitinib on 3D-soft agar cultures and sphere formation assay of ADK-14, PDX-ADK-36, and ADK-17 lines (**Figure 5**). In 3D-soft agar cultures, erlotinib at the dose of 1 μM reduced the growth of ADK-14 colonies by about 50% ($p<0.05$) and the growth of PDX-ADK-36 colonies by about 90% ($p<0.001$) (**Figure 5A**). Similarly, dacomitinib determined a strong inhibition of the growth of PDX-ADK-36 at both doses of 0.1 μM ($p<0.05$) and 1 μM ($p<0.001$), while showing a weaker but still significant inhibition of the growth of ADK-14 at the doses of 0.01 and 1 μM ($p<0.05$) (**Figure 5B**). In contrast, both EGFR-TKIs showed no effect on the inhibition of the growth of ADK-17 cells. Consistent with previous findings, also sphere

formation was reduced by erlotinib in both ADK-14 (1 μ M, $p < 0.01$) and PDX-ADK-36 cells (1 μ M, $p < 0.05$) (**Figure 5C**), while dacomitinib seemed to inhibit sphere formation only on ADK-14 cells ($p < 0.001$) (**Figure 5D**). Again, no effect of erlotinib nor dacomitinib was observed on ADK-17 cells.

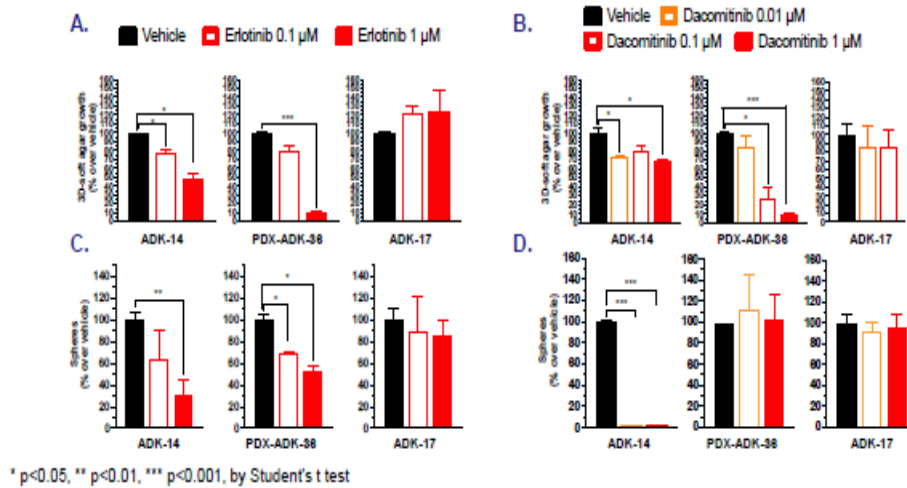
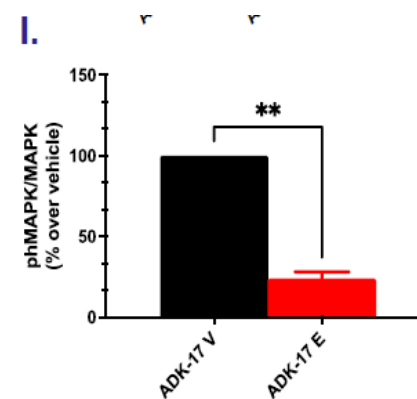
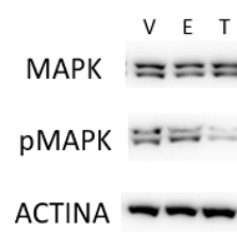
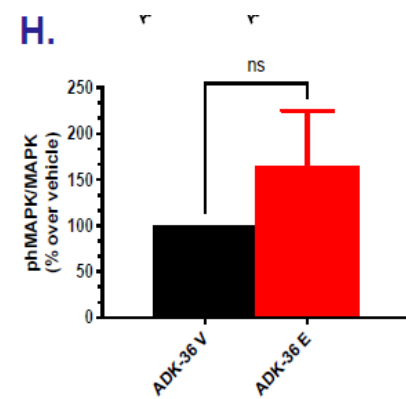
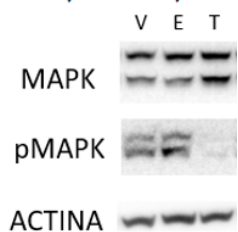
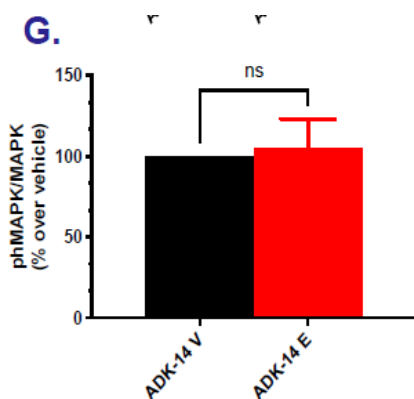
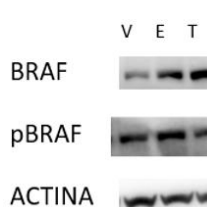
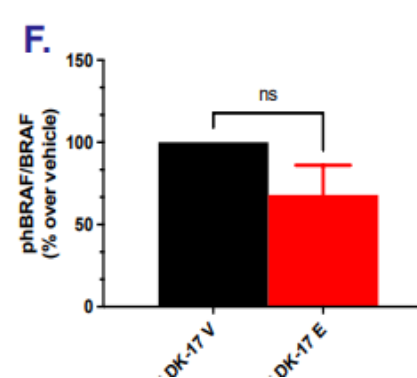
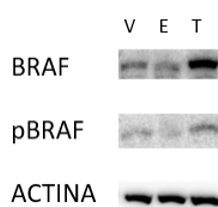
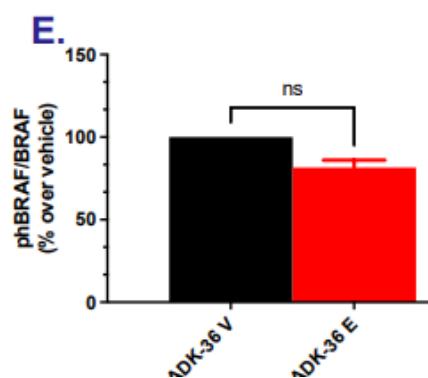
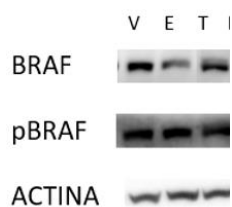
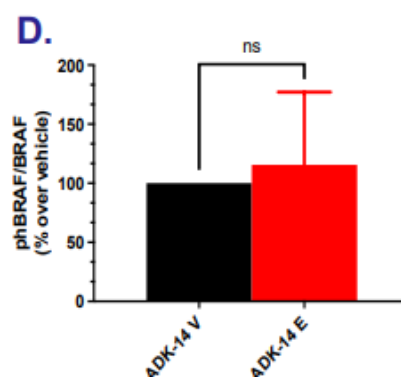
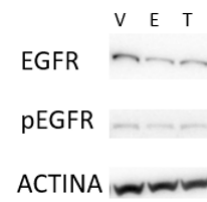
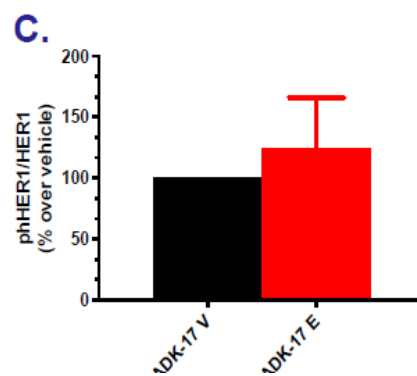
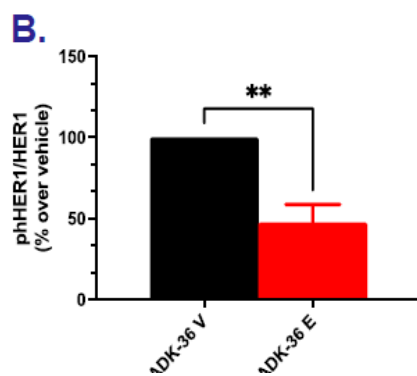
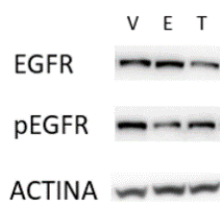
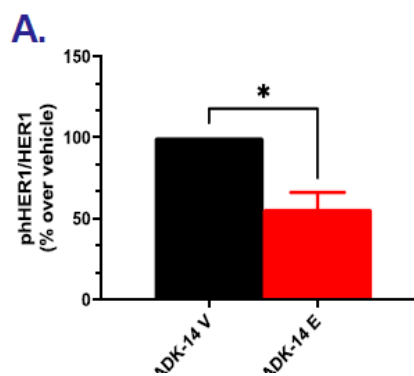


Figure 5. Erlotinib inhibited the growth of agar colonies and reduced sphere formation of both ADK-14 and PDX-ADK-36 cell lines (**A,C**). Dacomitinib showed a similar effect on ADK-14 cells and PDX-ADK-36 in 3D-soft agar assay (**B**), while in sphere formation assay it only inhibited ADK-14 sphere formation (**D**). Both EGFR-TKIs showed no effect on 3D-growth of ADK-17 cell line. Each bar represents mean and SEM

Erlotinib effect on pathways activation in BRAF class 3-mutated cell lines

Next, we investigated the inhibition of erlotinib on the activation of EGFR/HER1, BRAF, MAPK, and AKT by evaluating the ratio between their phosphorylated and non-phosphorylated isoforms. Erlotinib significantly reduced EGFR activation in both ADK-14 ($p < 0.05$) and PDX-ADK-36 ($p < 0.01$) cells, but not in ADK-17 cells (**Figure 6A-C**). No effect was evidenced on BRAF phosphorylation (**Figure 6D-F**). Erlotinib modulated MAPK pathway only on ADK-17 treated cells ($p < 0.01$), but not in both ADK-14 and PDX-ADK-36 (**Figure 6G-I**), while it did not produce any effect on AKT pathway in all three cell line models (**Figure 6J-L**).



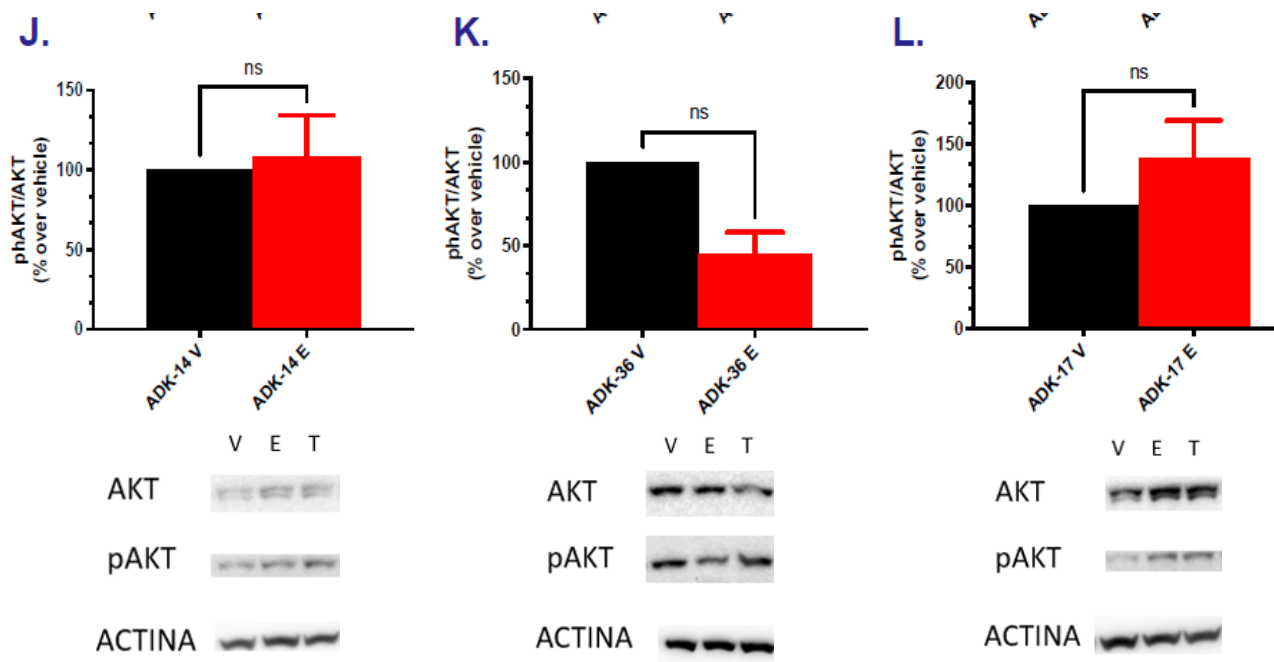


Figure 6. Protein extraction and Western blotting. Inhibition of erlotinib on the activation of EGFR/HER1 (A-C), BRAF (D-F), MAPK (G-I), and AKT (J-L) calculated as ratio between their phosphorylated and non-phosphorylated isoforms. **Black columns:** vehicle. **Red columns:** erlotinib. * $p < 0.05$, ** $p < 0.01$, by Student's t test

External validation of in vitro results: comparative sensitivity of BRAF class 3 cell lines to multiple agents in the Cancer Cell Line Encyclopedia

The Cancer Cell Line Encyclopedia was interrogated for BRAF class 3 mutations to provide an external validation for our findings. Two cell lines of non-squamous NSCLC harboring BRAF class 3 mutations without any other concurrent driver alteration and available treatment data were found (**Figure 7**). To provide control groups for our analyses and allow comparisons, we also identified cell lines of lung adenocarcinoma harboring BRAF class 2 alterations (N=4), EGFR (N=5) or KRAS (N=34) driver mutations. We then evaluated and compared the sensitivity of these cells, expressed as IC50, to multiple agents, including EGFR inhibitors of first-generation (gefitinib) and second-generation (afatinib), a MEK inhibitor (trametinib), a BRAF inhibitor (dabrafenib) a multi-TKI (cabozantinib) and chemotherapy (doxorubicin). Cell lines harboring BRAF class 3 mutations were significantly more sensitive to EGFR-TKIs than cell lines harboring BRAF class 2 mutations ($p = 0.008$) or KRAS mutations ($p = 0.06$), showing no statistically significant difference when their

sensitivity was compared to that of EGFR-driven cell lines ($p = 0.66$). Notably, included EGFR cell lines only harbored EGFR exon 19 deletion or exon 21 L858R mutations, which are known to respond to first- and second-generation EGFR-TKIs, and lacked resistance mutations such as T90M or C797X. Confirming these results, no differences were observed in the sensitivity to other agents (trametinib, alectinib, cabozantinib and doxorubicin).

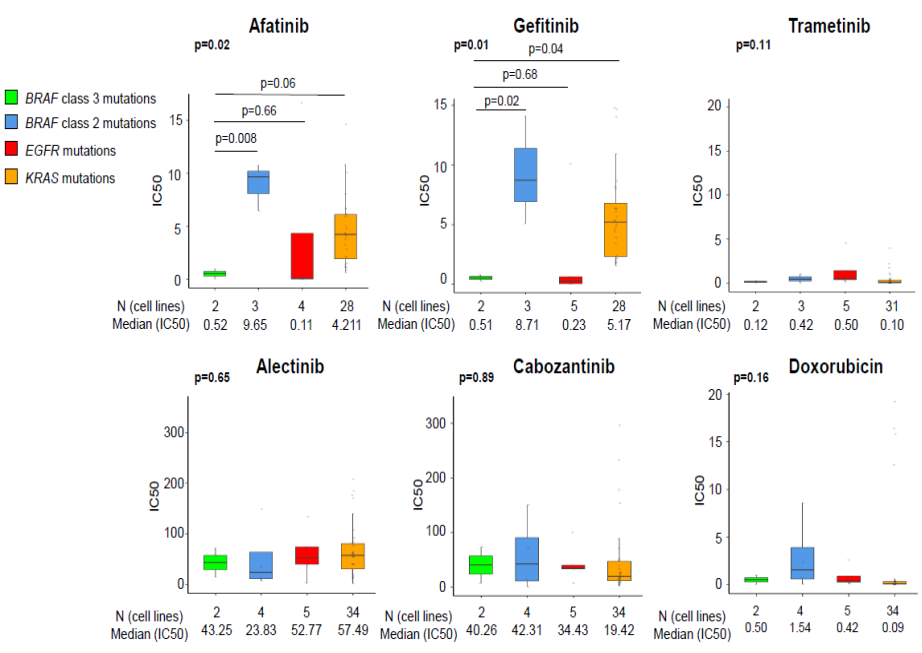


Figure 7. Cancer Cell Line Encyclopedia (cBioPortal.org). Cell lines harboring BRAF class 3 mutations were significantly more sensitive to afatinib and gefitinib (EGFR TKIs) than cell lines harboring BRAF class 2 mutations ($p=0.008$ for afatinib and 0.02 for gefitinib) or KRAS mutations ($p=0.06$ for afatinib and 0.04 for gefitinib), showing no statistically significant difference when their sensitivity was compared to that of EGFR-driven cell lines ($p=0.66$ for afatinib and 0.68 for gefitinib).

DISCUSSION

BRAF mutations can be found in 6% of all patients with cancer [7-9] including in 3% of Non-Small Cell Lung Cancer (NSCLC) [10,11]. In clinical practice the combination of BRAF and MEK inhibitors became the standard of care for BRAFV600 mutant advanced NSCLC patients [17-19]. However, about a half of BRAF mutations in NSCLC are non-V600 and for these cases the discovery of targeted therapies remains an unmet clinical need. Preclinical studies proved that different cell lines harboring class 3 BRAF mutations, including three NSCLC cell lines, had high RAS-GTP level and ERK signaling activation. Ras activation was mediated by tyrosine kinase receptors, particularly EGFR in NSCLC cell lines. The growth of cell lines with class 3 BRAF mutations was inhibited by

the exposure to cetuximab [13]. A multicenter pooled analysis of 40 metastatic colorectal cancer patients with BRAF non-V600 mutations showed that class 3 BRAF mutants had a significantly higher response rate to anti-EGFR antibody therapy when compared to class 2 BRAF ones [20].

Although RAS signaling could be activate by receptor tyrosine kinases (RTK), especially EGFR, in NSCLC harboring class 3 BRAF mutations [13], whether an anti-EGFR therapy is effective in BRAF mutated NSCLC patients has not been previously explored.

In the current study, we show that patients with NSCLC harboring BRAF class 3 alterations may be sensitive to EGFR-TKIs. Starting from the clinical observation of a patient who achieved benefit from erlotinib after failure of all previous standard therapies, we confirmed the presence of clinical activity by observing a partial response in a patient with primary resistance to chemo-immunotherapy and high burden of disease. Then, to have an in vitro validation of our findings, we established cell lines with BRAF class 3 alterations and observed that these cells show sensitivity to EGFR-TKI, which was confirmed in an external validation performed on a large publicly available dataset. we hypothesize that, in presence of a class 3 mutation, the mutant BRAF protein can amplify RAS signaling downstream when RAS is already partially activated by other mechanisms, such as an upstream activation coming from an hyperactivated wild-type EGFR or another tyrosine kinase receptor. In this condition, the mutant BRAF determine a stronger RAS signaling that is capable of driving proliferation and survival of cancer cells, but that would not be sufficient in absence of the mutant BRAF [13]. Therefore, blocking the EGFR receptor may prevent RAS overstimulation even in presence of the BRAF class 3 mutation.

CONCLUSION

Patients with NSCLC harboring BRAF class 3 alterations may be sensitive to EGFR inhibition and thus respond to EGFR-TKIs. These findings warrant validation in prospective clinical studies, as

BRAF class 3 alterations might identify a new population of NSCLC patients that benefit from targeted agents.

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PD-L1 $\geq$ 50% LUNG CANCER REFRACTORY TO PD-1 INHIBITION: THE ROLE OF SALVAGE CHEMO-IMMUNOTHERAPY COMBINATION

ABSTRACT

In Europe, single-agent immune-checkpoint inhibitors (ICIs) represent the standard of care for non-oncogene addicted, advanced, non-small cell lung cancer (NSCLC) with PD-L1 $\geq$ 50%. However, 30-40% of patients have a primary resistant disease.

We conducted an observational study to describe the clinical efficacy of the addition of histology-driven chemotherapy (CT) to upfront ICI for NSCLC with PD-L1 $\geq$ 50% as a rescue treatment of primary resistance (PD as the best response or stable disease $<$ 6 months). Overall survival (OS) was defined as the time from ICI start to death for any cause.

134 patients received upfront pembrolizumab at our Institution. Overall, the median OS was 16.9 months (95% CI, 12.9-24.1), and the median progression-free survival (PFS) was 6.3 months (95% CI, 3.9-11.9). 68.6% of patients had a PD at any time, and 47% had a primary resistance. Among 35 patients who received a second-line, 28.5% were treated with platinum-based doublets, 5.7% with a single-agent CT, 5.7% with targeted therapies, and 60% with the addition of CT to pembrolizumab upon PD (CT-ICI cohort). Within the CT-ICI cohort, most were male (66.7%), with a nonsquamous histology (90.5%), and an ECOG PS of 0-1 (90.5%). Among them, the median PFS2 was 12.5 months (95% CI, 11.5-NR), and the median OS was 18.0 months (95% CI, 13.4- NR). The objective response rate (ORR) for CT-ICI was 75% (95% CI, 53-89). Considering patients with primary resistance, 15 received CT-ICI (71.4%), while 6 received another therapy (4 platinum-doublet, 1 single agent CT, 1 sotorasib). The median OS was 18.0 months (95% CI, 13.4-NR) for CT-ICI and 12.3 months (6.6-NR) for others ($p=0.18$). The PFS2 was 12.5 months (95% CI, 11.9-NR) versus 8.3 (95%CI, 5.2-NR) ($p=0.29$). The ORR was 80% (95% CI, 55-94) for CT-ICI and 33.3% (95% CI, 15-55) for other treatments ($p=0.02$).

The addition of histology-driven CT may be a promising strategy to rescue patients with advanced NSCLC showing early PD to upfront pembrolizumab who potentially still derive clinical benefit by continuing the ICI. Larger studies confirming these results are warranted.

INTRODUCTION

First-line treatment approaches in EGFR and ALK wild-type advanced non-small-cell lung cancer (NSCLC) have dramatically evolved in the last few years. The improvement of the most relevant clinical outcomes, such as progression-free and overall survival (PFS and OS) produced by using different treatment strategies incorporating immune checkpoint inhibitors (ICIs) led to the novel therapeutic paradigms that have been introduced in clinical practice. According to the significant OS benefit obtained in KEYNOTE-024 and KEYNOTE-042 studies for metastatic PD-L1 high-positive (tumor proportion score [TPS] $\geq 50\%$) NSCLC populations, pembrolizumab became the standard-of-care in first-line setting [1,2]. The KEYNOTE-189 was a double-blind, placebo-controlled, Phase III study that compared first-line pembrolizumab plus platinum-based chemotherapy with chemotherapy alone in nonsquamous NSCLC [3]. In this trial, OS and PFS were significantly longer for the combination regimen arm, regardless of PD-L1 status, although the highest benefit was among the PD-L1 strong positive NSCLC patients. Similar results were obtained for squamous histology in the Phase III KEYNOTE-407 study [4]. Based on these data and considering the 5-year OS results from KEYNOTE-001, -024, -189 and -407 studies [5-8], a relevant issue in the current clinical practice is the best treatment strategy for PD-L1 TPS $\geq 50\%$ NSCLC patients, as both clinical trials and regulatory agencies suggest that either pembrolizumab monotherapy or pembrolizumab-chemotherapy combinations may be first-line valid options.

About 30% of metastatic NSCLC patients with PD-L1 TPS $\geq 50\%$ progress or die on first line pembrolizumab during the first 3 months of treatment [1]. To date, no head-to-head study was designed to compare the efficacy of pembrolizumab single agent to that of pembrolizumab-

chemotherapy combination in this setting. Cross trial comparisons suggest that in this population the use of combination strategy could increase objective response rates and prevent a proportion (~20%) of early failures [9].

Clinical, pathological, and molecular features suggesting which patients can benefit from PD-1 inhibition only, or on the contrary need the addition of chemotherapy, are eagerly awaited, considering the risk of over-treatment and toxicity [10].

So far, no trials have been published that have identified the most suitable treatment for patients with advanced NSCLC and PD-L1 $\geq 50\%$ progressed on ICI or ICI-CT. In patients who progress on single-agent immunotherapy, the standard treatment is histology-driven platinum-based chemotherapy (pemetrexed and cis/carboplatin for non-squamous histology or paclitaxel, gemcitabine or vinorelbine in combination with cis/carboplatin for squamous histology).

When rapid cytoreduction is needed, as in the case of patients with important symptoms due to high tumor burden or hyperprogressive disease, adding chemotherapy to immunotherapy in patients with PD-L1 $\geq 50\%$ could ensure early disease control [11,12].

The objective of the present study is to evaluate the effectiveness of rescue treatment with chemo-immunotherapy in advanced NSCLC patients with PD-L1 $\geq 50\%$ progressed on first line pembrolizumab at 3-6 months from the beginning of treatment.

MATERIALS AND METHODS

Patient population and collected data

This is a retrospective, observational study carried out at Medical Oncology Unit of the IRCCS Azienda Ospedaliero-Universitaria of Bologna.

All of the following inclusion criteria had to be met: age > 18 years; metastatic NSCLC; PD-L1 TPS $\geq 50\%$ (evaluated by IHC on sample taken within six months of the start of first line treatment); clinical-radiological progression within 3-6 months from the beginning of single-agent immunotherapy in first line setting (6-month cut-off was chosen taking into account the lower limit of the median PFS confidence interval in the KEYNOTE-024 study); addition of chemotherapy as rescue therapy to single-agent immunotherapy following clinical-radiological progression within 6 months from the start of first-line treatment; available and searchable clinical data.

The exclusion criteria were as follows: presence of mutations in TKI target oncogenes; patient refused to participate in the study.

Clinical, biological, pathological, and radiological data from all patients included in this study have been recorded. Dates of start and end of each treatment, last disease reassessment, disease progression, death and last follow-up have been reported.

The information was collected following consultation of medical records (both in paper and digital format) and GALILEO software (Digital Order Management). All information was obtained and used in accordance with the Helsinki Declaration. The study was approved by the local Ethics Committee (EC Code 404/2019/Oss/AOUBo).

Statistical analysis

OS was calculated from the time of commencement of first-line immunotherapy treatment to death for any cause. PFS1 was calculated from the beginning of immunotherapy to the first evidence of progressive disease (PD). PFS2 was calculated from the date of the start of treatment following PD to the first line until the progression of disease or death for any cause. OS, PFS1 and PFS2 were calculated using the Kaplan-Meier method. Cox regressions were performed for univariate analysis for OS and PFS2.

RESULTS

Patients' characteristics

From November 2019 to June 2023, 134 patients received upfront pembrolizumab at our Institution. Overall, the median OS was 16.9 months (95% CI, 12.9-24.1), and the median progression-free survival (PFS) was 6.3 months (95% CI, 3.9-11.9). 92 (68.6%) patients had a PD at any time, and overall, 63 (47%) patients had a primary resistance. Among 35 (26.1%) patients who received a second-line, 10 (28.5%) patients were treated with platinum-based doublets, 2 (5.7%) patients with a single-agent CT, 2 (5.7%) with targeted therapies, and 21 (60%) patients with the addition of CT to pembrolizumab upon PD (CT-ICI cohort).

CT-IT overall population cohort

Within the CT-ICI overall population cohort, 14 (66.7%) patients were males, 19 (90.5%) patients had ECOG PS 0-1 (at the time of diagnosis) and nonsquamous histology. Mean ($\pm$ SD) age at diagnosis was 64.24 ($\pm$ 9.65) years and median age was 64 (range, 40-82) years. 5 (23.8%) patients had no history of smoking habit. Median PD-L1 TPS was 80 (IQR, 70-87.5). Genomic alterations were detected for 16 (76.2%) cases: KRAS 11 cases (G12C, G12D, and G12V 3 cases each; G12A, 2 cases), MET ex14 skipping (1 case), BRAF non-V600 (1 case), PIK3CA (1 case), others (2 cases). Median (range) number of metastatic sites at diagnosis was 2 (1-9). The most frequent metastatic sites (at diagnosis) were lymph nodes (61.9%), bone (42.8%), lung and pleura (38.1% each), and brain (28.3%). Pembrolizumab was the drug used as single agent in first line in 20 (95.2%) patients and in one patient (4.8%) a bispecific anti-PD-L1/anti-TGFbeta antibody was administered for one cycle only. The median number of immunotherapy single agent doses was 4 (range, 1-22). Seven (33.3%) patients received a number of cycles of immunotherapy single agent $\leq$ 2; 6 (28.6%) patients received a number of courses of 3-5; and another 7 (33.3%) patients received more than 8 courses of pembrolizumab single agent. The median PFS to immunotherapy single agent (PFS1) was 2.30 (95% CI, 1.55 - 3.26). 15 (71.4%) patients had progressive disease (PD) as best response under

immunotherapy single agent and 7 (33.3%) of them after only 2 cycles. The most frequent sites of progression on immunotherapy single agent were lung (61.9%), lymph nodes (57.1%), bone (38.1%), pleura (23.8%), liver and adrenal gland (19.0% each).

At the time of PD on first line immunotherapy, histology-driven platinum-based chemotherapy for up to 4 cycles were added to pembrolizumab (200 mg flat dose) every 3 weeks, followed by maintenance pembrolizumab (plus pemetrexed for nonsquamous histology) every 3 weeks. At the date of the last follow-up, the median number of salvage chemo-immunotherapy (CT-IT) courses was 5 (range, 1-31). The objective response rate (ORR) for CT-IT was 75% (95% CI, 53-89). After a median follow-up of 11.8 (range, 3.5-41.1) months, 11 (52.4%) patients died. The median PFS2 was 12.5 months (95% CI, 11.5-NR), and the median OS was 18.0 months (95% CI, 13.4- NR).

CT-IT population with primary resistance to immunotherapy single agent

Out of 63 advanced NSCLC patients with primary resistance to immunotherapy single agent in first line setting, 21 (33.3%) of them started a second-line treatment. For 15 (71.4%) patients histology-driven platinum-based chemotherapy was added to pembrolizumab for up to 4 cycles every 3 weeks, followed by maintenance with pembrolizumab (plus pemetrexed for nonsquamous histology) every 3 weeks. The remaining 6 (28.6%) patients received other therapy (4 platinum-based chemotherapy, 1 gemcitabine single agent, 1 sotorasib). Within this patients' population, 9 (42.8%) patients were males, 18 (85.7%) patients had ECOG PS 0-1 (at the time of diagnosis) and nonsquamous histology, while squamous histology was reported for 3 (14.3%) patients (**Table 1**). Mean ($\pm$ SD) age at diagnosis was 64.29 ($\pm$ 8.75) years and median age was 65 (range, 40-77) years. 4 (19.0%) patients had no history of smoking habit. Median PD-L1 TPS was 75 (IQR, 62.5-87.5). Genomic alterations were detected for 14 (66.7%) cases: 12 KRAS, 1 MET ex14 skipping, 1 other. Median (range) number of metastatic sites at diagnosis was 2 (1-5). The most frequent metastatic sites (at diagnosis) were lymph nodes (66.7%), bone (38.1%), lung (42.8%), pleura (38.1%), and brain (23.8%).

		Other treatment	ICI plus CT	Total (%)	P value
age	>65	4 (66.7)	6 (40.0)	10 (47.6)	0.534
	≤65	2 (33.3)	9 (60.0)	11 (52.4)	
sex	Female	5 (83.3)	7 (46.7)	12 (57.1)	0.296
	Male	1 (16.7)	8 (53.3)	9 (42.9)	
ps_stat	0-1	5 (83.3)	14 (93.3)	19 (90.5)	1.000
	= 2	1 (16.7)	1 (6.7)	2 (9.5)	
smoke	ever smoker	6 (100.0)	10 (76.9)	16 (84.2)	0.545
	never smoker		3 (23.1)	3 (15.8)	
histo	nonsquamous	5 (83.3)	13 (86.7)	18 (85.7)	1.000
	squamous	1 (16.7)	2 (13.3)	3 (14.3)	
sites	<3	5 (83.3)	11 (73.3)	16 (76.2)	1.000
	≥3	1 (16.7)	4 (26.7)	5 (23.8)	
brain	no	5 (83.3)	11 (73.3)	16 (76.2)	1.000
	yes	1 (16.7)	4 (26.7)	5 (23.8)	
pdl1	<90	5 (83.3)	11 (73.3)	16 (76.2)	1.000
	≥90	1 (16.7)	4 (26.7)	5 (23.8)	
type	chemo	1 (16.7)		1 (4.8)	<0.001
	other	1 (16.7)		1 (4.8)	
	platinum-doublet	4 (66.7)		4 (19.0)	
	triplet		15 (100.0)	15 (71.4)	
best2	PD	4 (66.7)	1 (6.7)	5 (23.8)	0.013
	PR	2 (33.3)	12 (80.0)	14 (66.7)	
	SD		2 (13.3)	2 (9.5)	
response2	no	5 (83.3)	3 (20.0)	8 (38.1)	0.028
	yes	1 (16.7)	12 (80.0)	13 (61.9)	

Table 1. Baseline characteristics in the patients' cohort with primary resistance to immunotherapy single agent

Pembrolizumab was the drug used as single agent in first line in 20 (95.2%) patients and in one patient (4.8%) a bispecific anti-PD-L1/anti-TGFβ antibody was administered for one cycle only. The median number of immunotherapy single agent doses was 3 (range, 1-10). Seven (33.3%) patients received a number of cycles of immunotherapy single agent ≤2; 11 (52.4%) patients received a number of courses of 3-5; 2 (9.5%) patients received a number of courses of 6-7 and one patient received a total number of 10 courses. The median PFS to immunotherapy single agent (PFS1) was 1.71 (95% CI, 1.48 - 2.17). 20 (95.2%) patients had progressive disease (PD) as best response under immunotherapy single agent and 7 (33.3%) of them after only 2 cycles. Overall, 16 (76.2%) patients

progressed within 3 months of starting immunotherapy single agent in first line setting. The most frequent sites of progression on immunotherapy single agent, among the 15 candidates to receive salvage chemo-immunotherapy, were lymph nodes (66.7%), lung (60%), bone (46.7%), pleura and adrenal gland (26.7% each), liver and brain (20% each).

At the date of the last follow-up, the median number of salvage chemo-immunotherapy (CT-IT) courses was 11 (range, 2-31). The objective response rate (ORR) for CT-IT was 80% (95% CI, 55-94) for salvage CT-ICI group compared with 33.3% (95% CI, 15-55) for other treatments (p=0.02). After a median follow-up of 11.9 (range, 3.6-41.1) months, 13 (61.9%) patients died. The PFS2 was 12.5 months (95% CI, 11.9-NR) versus 8.3 (95%CI, 5.2-NR) (p=0.29) (**Figure 1**). The median OS was 18.0 months (95% CI, 13.4-NR) for CT-ICI and 12.3 months (6.6-NR) for others (p=0.18) (**Figure 2**).

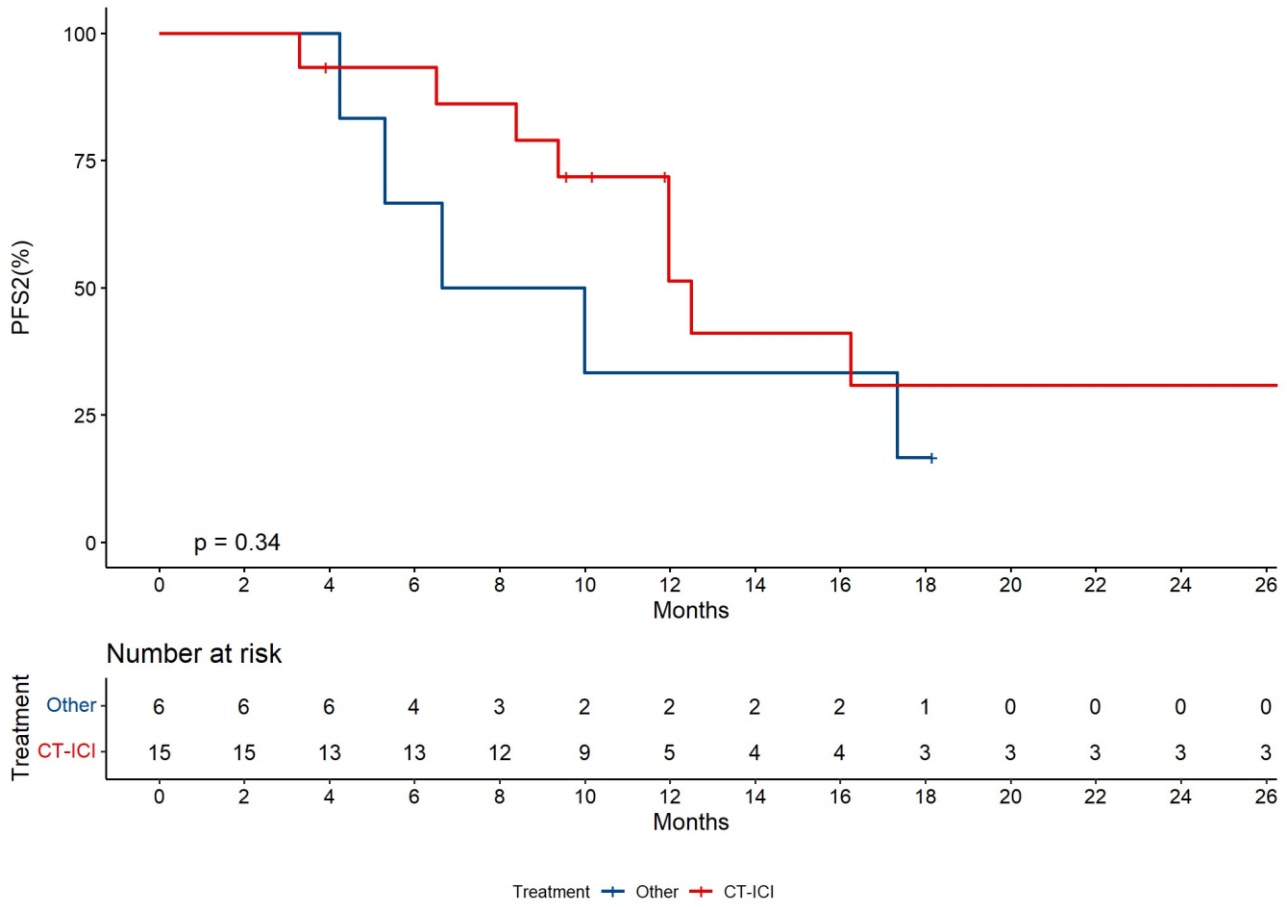


Figure 1. PFS2 curves among patients with primary resistance to immunotherapy single agent and treated with salvage CT-IT versus other treatment

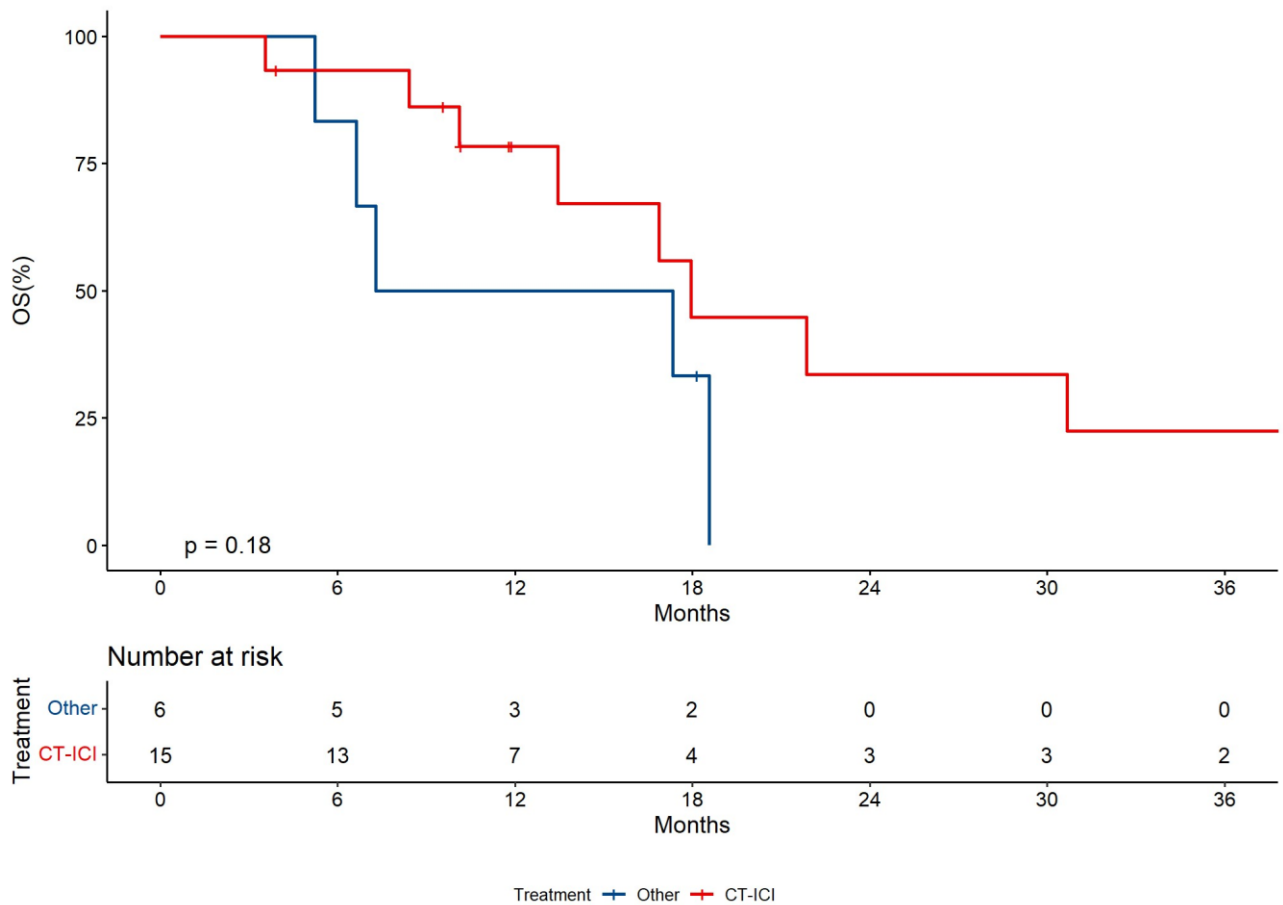


Figure 2. OS curves among patients with primary resistance to immunotherapy single agent and treated with salvage CT-IT versus other treatment

DISCUSSION

Our study evaluated the effectiveness of combining immunotherapy and chemotherapy in metastatic NSCLC patients with PD-L1 $\geq 50\%$ progressed on single agent immunotherapy in first line setting. Considering the study cohort progressed within 6 months from the beginning of 1L immunotherapy (primary resistant group), PD occurred within 3 months of beginning first line immunotherapy for 76.2% of the study patients. Of note, 33.3% of these patients progressed after only 2 courses of single agent immunotherapy. Among the 15 patients who started salvage CT-IT, 93.3% of them obtained a disease control (PR + SD) from this strategy, associated with an ORR of 80%. Although our study is limited by the retrospective nature and small sample size, there is a remarkable difference between salvage CT-IT group and other treatment group in terms of activity (80% vs 33.3%, $p = 0.02$). The

median PFS1 was 1.71 (95% CI, 1.48 - 2.17), median PFS2 was 12.5 months (95% CI, 11.9-NR) and median OS was 18.0 months (95% CI, 13.4-NR). To date, the only prospective data on the potential effectiveness of chemo-immunotherapy in patients progressed on 1L immunotherapy concern the subgroup analyses of the EMPOWER-Lung 01 study [13]. This randomized phase III trial demonstrated the effectiveness of cemiplimab, an anti-PD-1 antibody, in metastatic NSCLC patients with PD-L1 $\geq$ 50%, as 1L treatment as compared to standard platinum-based chemotherapy [14]. The trial was designed to allow, at the Investigator discretion, the addition of 4 cycles of chemotherapy (based on histology) to cemiplimab in case of PD. 64 patients progressed on cemiplimab continued to receive the same agent with the addition of chemotherapy as a 2L treatment; median PFS2 was 6.6 months (95%CI, 6.1-9.3) and 6-month PFS rate was 66.2%. Median OS was 27.4 months (95%CI, 23.0-31.8) with a median OS2 of 15.1 (95%CI, 11.3-18.7). It is not known whether patients who have been offered the combination of chemo-immunotherapy have experienced disease progression within 6 months of the start of 1L cemiplimab, unlike the population enrolled and analyzed in our study. However, in our CT-IT overall population cohort (also including patients who progressed on single agent immunotherapy >6 months), the median PFS2 was 12.5 months (95% CI, 11.5-NR), and the median OS was 18.0 months (95% CI, 13.4- NR). in the study cohort

From the analyses carried out on the population enrolled in our study it is not possible to draw certain conclusions on the effectiveness of salvage chemo-immunotherapy after early immunotherapy failure given the retrospective nature of the same study and the number of patients enrolled.

It would be desirable to be able to identify factors that allow to select with greater accuracy the optimal treatment for patients with PD-L1 strong-positive NSCLC. In selected patients, a chemo-immunotherapy regimen, administered in 1L setting or at the time of PD to 1L may prevent premature death or rapid progression of disease.

CONCLUSION

The results of our retrospective study, although prospective data and comparative studies lack, suggest that the addition of histology-driven CT to immunotherapy may be a promising strategy to rescue patients with advanced NSCLC showing early PD to upfront single agent immunotherapy who potentially still derive clinical benefit by continuing IT. Larger studies confirming these results are warranted.

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PHASE II, OPEN-LABEL STUDY OF ATEZOLIZUMAB IN A COHORT OF PRETREATED, ADVANCED NON-SMALL CELL LUNG CANCER (NSCLC) PATIENTS WITH RARE HISTOLOGICAL SUBTYPES (CHANCE TRIAL)

ABSTRACT

Despite a widening use of immunotherapy in different tumor types, its role for NSCLCs with uncommon histologies remains unclear. The aim of this study is to explore the antitumor activity and the safety profile of atezolizumab in pretreated advanced NSCLC patients with rare histological subtypes. CHANCE (NCT03976518) was an open-label, single-arm, multicenter, phase II study designated for evaluating the activity and safety of atezolizumab in a cohort of pretreated, advanced NSCLC patients with rare histological subtypes. The primary endpoint of the study was disease control rate. Eligible patients will receive intravenous infusion of atezolizumab (flat dosing of 1200 mg) once every 3 weeks. Treatment was continued until progressive disease, intolerable toxicity, patient refusal or Investigator's decision. 47 patients from 12 Italian centers were screened for the study. Of them, 43 patients received at least one course of atezolizumab (modified intention to treat, ITT, population). The study did not meet its primary endpoint. DCR was 41.9%. No new safety signals were reported. Survival outcomes are currently under evaluation. CHANCE is the first prospective study to evaluate the efficacy and safety of immune checkpoint inhibitor in advanced pretreated NSCLC with uncommon histology. The study did not meet its primary objective. However, one third of these patients benefitted from study drug. Larger prospective and translational studies are warranted.

INTRODUCTION

In the last decade, the advent of immune checkpoint inhibitors (ICIs) has substantially changed the treatment of many tumors, including thoracic malignancies. Despite a widening use of immunotherapy in different tumor types and settings of disease, its role in immunotherapy for NSCLCs with uncommon histologies remains unclear, as these tumors are generally excluded from clinical trials and most of the available data comes from case series or small retrospective studies. Nowadays, a wide and heterogeneous spectrum of lung malignancies is included in the definition of NSCLC with rare histologies, characterized by different histopathological and clinical features, prognoses and treatment responses [1]. According to the 2015 WHO Classification of Lung Tumors, the main rare histological entities are pulmonary sarcomatoid carcinoma (PSC), large-cell carcinoma (LCC), lymphoepithelioma-like carcinoma (LELC), large-cell neuroendocrine carcinoma (LCNEC), nuclear protein of testis (NUT) carcinoma, salivary gland-type tumors, some variants of adenocarcinoma (mucinous, colloid, fetal, enteric-types) and mixed histologies (adenosquamous type) [2]. Among them, PSC [3,4] and LCNEC [5] have high PD-L1 expression and tumor mutational burden (TMB), providing rationale for the use of immunotherapeutic agents [6].

The aim of this study is to explore the antitumor activity and the safety profile of atezolizumab in pretreated advanced NSCLC patients with rare histological subtypes.

METHODS

Study design and endpoints

CHANCE (NCT03976518) was an open-label, single-arm, multicenter, phase II study designated for evaluating the activity and safety of atezolizumab in a cohort of pretreated, advanced NSCLC patients with rare histological subtypes. Approximately 43 subjects in 12 national centers were planned to be recruited. Male and female patients aged ≥ 18 years with Eastern Cooperative Oncology Group

(ECOG) performance status (PS) of 0 to 2 who have locally advanced, relapsed, or metastatic NSCLC with histologically proven rare subtypes and who experienced disease progression during or after at least one previous standard chemotherapy line were eligible (**Figure 1**).

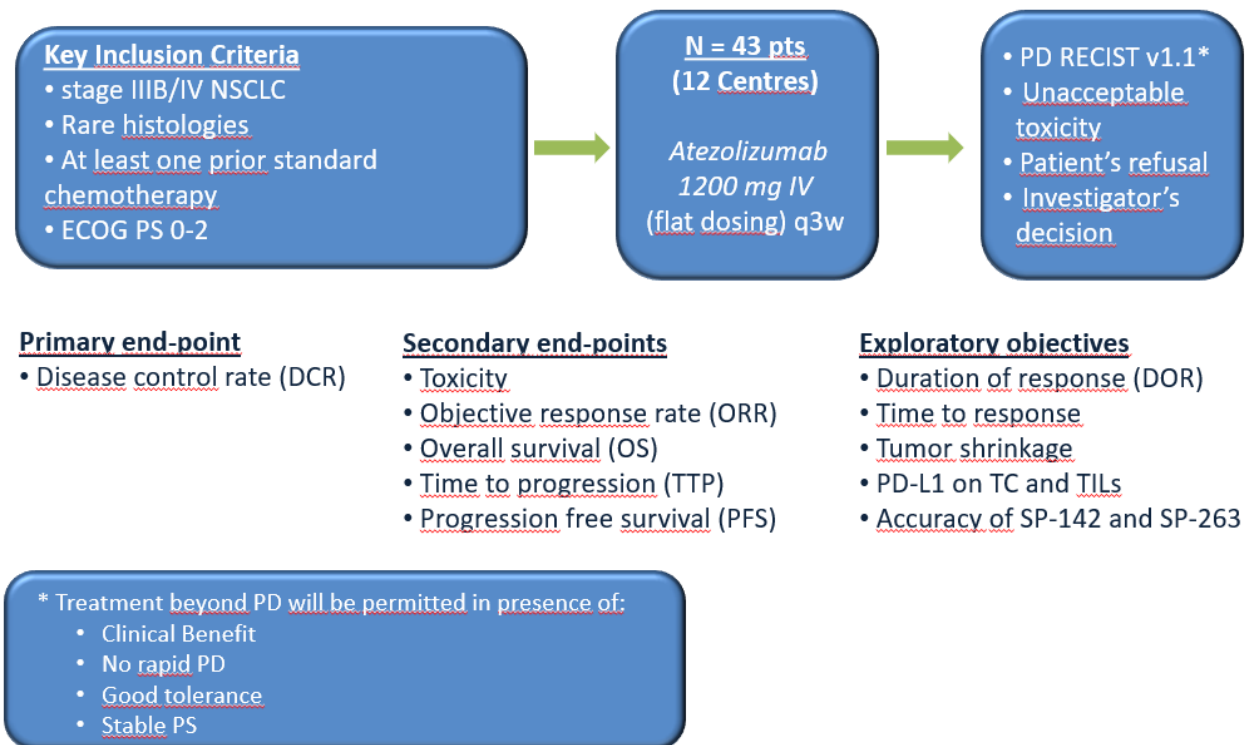


Figure 1. Study design of the CHANCE trial. ECOG PS, Eastern Cooperative Oncology Group performance status; PD, progressive disease; RECIST, Response Evaluation Criteria in Solid Tumors.

Central pathology revision was planned during the study. Availability of tumor tissue for assessing PD-L1 expression was mandatory. The assessment of PD-L1 expression was performed by local laboratory as a part of the screening process. The study enrolled all patients whose tissue was evaluable for expression testing by local laboratory, regardless of PD-L1 expression status. Subsequently, tumor specimens from enrolled patients were tested for PD-L1 expression, using both SP-142 and SP-263 antibody assays, by a central laboratory.

Eligible patients will be registered to receive IV infusion of atezolizumab (flat dosing of 1200 mg over 1 hour) once every 3 weeks. Subjects attended clinic visits at regular intervals to receive trial

treatment and for efficacy and safety assessments. All subjects were monitored continuously for any AE while on study treatment. Treatment was continued until progressive disease, intolerable toxicity, patient refusal or Investigator's decision, or any criterion for withdrawal from the trial or trial drug was fulfilled. Radiological assessment was performed by computed tomography (CT) scan every 6 weeks ($\pm$ 7 days) until 1 year to determine response to treatment that will be assessed by the Investigator using RECIST v1.1. Tumor assessments was conducted every 8 weeks ($\pm$ 1 week) thereafter. At the time of first tumor assessment, subjects treated with atezolizumab were permitted to continue investigational treatment beyond initial RECIST v 1.1 defined progressive disease (PD), assessed by the Investigator, in presence of the following criteria: Investigator-assessed clinical benefit; no rapid disease progression; good tolerance of study drug; stable performance status. For the subjects who continued atezolizumab study therapy beyond progression, further progression was defined as an additional 10% increase in tumor burden volume from time of initial PD. If PD was confirmed, atezolizumab treatment was permanently discontinued. A blinded independent radiological committee (BIRC) review will be performed retrospectively to confirm the type of response as per Investigator assessment.

The study was conducted in accordance with the requirements of the Italian regulatory authority and International Conference on Harmonization (ICH) Good Clinical Practice (GCP) Guidelines. The protocol and all amendments were approved by institutional review board or local ethics committee. All patients provided written informed consent.

The primary objective was to evaluate the therapeutic activity of atezolizumab in this selected population. Disease control rate (DCR), considered as the sum of complete responses (CRs), partial responses (PRs) and stable disease (SD), based on the Investigator's assessment according to standard RECIST 1.1 criteria, was the primary end point to measure the antitumor activity of atezolizumab. Secondary objectives were to evaluate safety and efficacy of atezolizumab in the same population,

measured as toxicity, objective response rate (ORR), overall survival (OS), time to progression (TTP) and progression free survival (PFS).

Statistical analysis

According to the Simon's two stage design, the null hypothesis that the true disease control rate is $\leq 50\%$ will be tested against a one-sided alternative. In the first stage, 15 patients will be accrued. If there are 8 or fewer responses (OR+SD) in these 15 patients, the study will be stopped early for futility. Otherwise, 28 additional patients will be accrued for a total of 43. The null hypothesis will be rejected if 27 or more responses (OR+SD) are observed in 43 patients. This design yields a type I error rate of 0.050 and power of 0.804 when the true disease control rate is 0.70.

For this cohort, we assume an accrual rate of approximately 20 subjects/year, a screening failure rate of 20% and a rate of patients lost to follow up equal to 10%.

At the time of the analysis of the primary endpoint from the first 15 patients, the accrual will not stop if there will be other potential patients to be enrolled, in order to avoid both losing patients who could benefit from the treatment and slowing down the subsequent enrolment.

For the primary endpoint and all secondary endpoints, the modified intention to treat population will be analyzed. Descriptive tables will be produced for the tumor response rate and the best overall response. Exact binomial method will be used to estimate the disease control rate (CR+PR+SD) and its 95% confidence interval.

Probabilities of progression-free survival and probabilities of overall survival will be estimated according to the Kaplan and Meier product-limit method.

Toxicity descriptive tables will be produced which provide the worst degree of toxicity measured over all cycles according to the NCI-CTCAE version 4.03.

As this is a phase II trial, no statistical comparisons will be made between the groups and no p values will be provided.

RESULTS

Patients

Between May 07, 2019, and June 28, 2022, 47 patients from 12 Italian centers were screened for the study. Of them, 43 patients received at least one course of atezolizumab (modified intention to treat, ITT, population) (**Figure 2**).

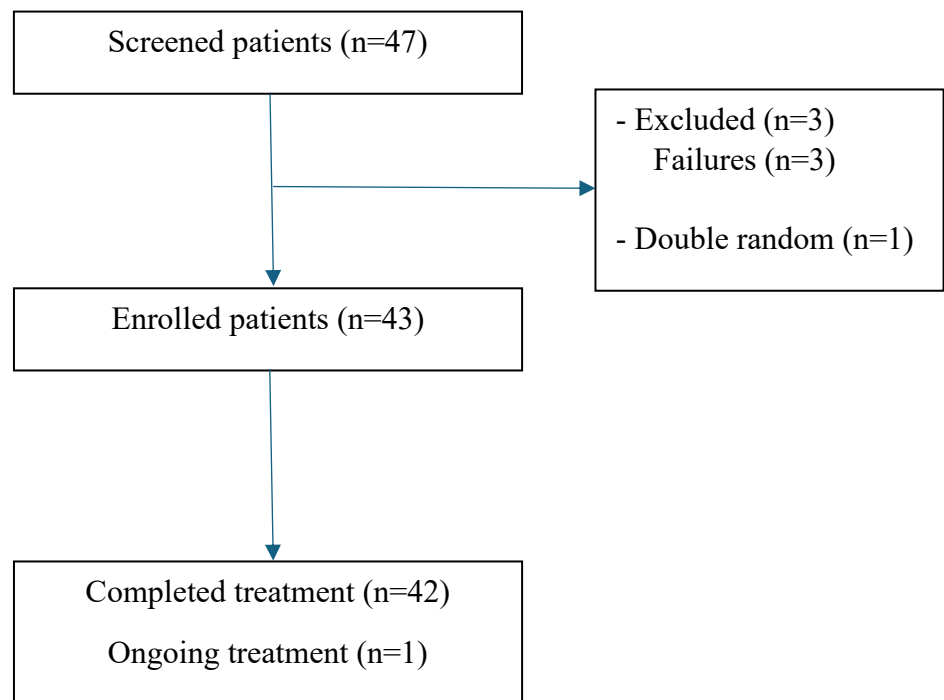


Figure 2. CONSORT diagram

In the modified ITT population (n=43), the median age was 71 (range, 45-87). Among the enrolled patients, 28 (65.1%) were males, 34 (79.1%) ever smokers, 41 (95.3%) had an ECOG performance status of 0-1, 33 (76.7%) patients had stage IV at diagnosis (70%), and 40 (93.0%) of them had received at least one prior standard treatment. Histological subtypes were reported as follow: 16 (37.2%) LCNEC, 11 (25.6%) sarcomatoid carcinoma, 6 (14%) adenoidocystic carcinoma, 2 (4.6%) LCC, 2 (4.6%) enteric, 6 (14%) others (1 pleomorphic carcinoma, 1 plasmacytoid/rhabdoid, 1 pulmonary carcinosarcoma, 1 undifferentiated NSCLC with pleomorphic aspects, 1 primary mucoepidermoid carcinoma, 1 NUT carcinoma). Patients' characteristics are listed in **Table 1**.

Variable		N.	(%)
Total n. of patients		43	100
Age	Median (range)	71	45-87
Sex	Male	28	65.1
	Female	15	34.9
Smoking status	Current/former	34	79.1
	Never	9	20.9
ECOG PS	0-1	41	95.3
	2	2	4.7
Histology	LCNEC	16	37.2
	Sarcomatoid	11	25.6
	Adenoid cystic	6	14.0
	LCC	2	4.6
	Enteric	2	4.6
	Others	6	14.0
Stage (at diagnosis)	IIIA/IIIB	6	14.0
	IVA	9	20.9
	IVB	24	55.8
	Unknown	4	9.3
Prior therapy (line)	0	3	7.0
	1	32	74.4
	≥ 2	8	18.6
Prior RT	Yes	17	39.5
	No	22	51.2
	Unknown	4	9.3

ECOG PS, Eastern Cooperative Oncology Group performance status; LCNEC, large cell neuroendocrine carcinoma; LCC, large cell carcinoma; RT, radiotherapy

Table 1. Baseline patients' characteristics

Treatment

At the data cut-off date (December 31, 2023), 39 (90.7%) patients died, 2 were still alive and the remaining 2 patients lost to follow-up. The study was still ongoing and survival data do not yet report. During treatment, 42 (97.7%) patients had PD, 7 (16.3%) patients died without any radiological tumor reassessment, and one patient is still on treatment.

Among the 36 (83.7%) patients with available radiological tumor evaluation, best response was stable disease (SD) in 18 (41.9%) patients, PD in 18 (41.9%), while the 7 patients with no radiological revaluation were considered treatment failures. No partial or complete response was reported.

At the time of data cut off, a total of 328 courses of atezolizumab was administered, with a median of 4 cycles (interquartile range [IQR], 2-11). 18 (41.9%) patients received no more than 3 courses of atezolizumab, 11 (25.6%) patients received 4-7 courses, 3 (6.9%) received 8-10 courses and 11 patients (25.6%) received > 11 courses. Patients (18, 41.9%) who rapidly progressed under atezolizumab had the following histological subtypes: LCNEC (10 cases), sarcomatoid carcinoma (4 cases), enteric/NUT/plasmacytoid-rhabdoid/primary mucoepidermoid (one each). Among the 14 (32.6%) patients who benefitted from study drug for at least 6 months, there were 6 adenoid cystic cases (receiving 9, 11, 21, 23, 23 and 35 courses), 4 sarcomatoid (receiving 9, 13, 17 and 31 courses) and 1 pleomorphic case (8 cycles), and 3 LCNEC cases (receiving 11, 12 and 14 cycles).

Safety

Safety data were available for all patients (n. 43) included in the ITT population who received 328 courses of atezolizumab. The study treatment was well tolerated, and no new safety signals were registered. The most frequently reported adverse events (of any grade) were fatigue (9.7%), fever and

anemia (8.4% each), GGT elevation (7.7%), arthralgia (7.1%) and loss of appetite (5.8%), while the most commonly reported grade ≥ 3 were GGT elevation (41.7%), ALT/AST elevation (16.7/8.3%), hyperglycemia and rash (8.3% each). No grade 5 adverse events were reported (**Table 2**). No patient discontinued study treatment due to adverse events. Serious adverse events were reported in 10 cases, of which 1 was reported as treatment-related. Among the 3 adverse events of special interest (AESI), including cardiac disorder, hyperglycemia and infusion-related reaction, the last two have been reported as treatment-related.

Adverse event	Any grade (%) (N=155)	Grade 1 (%) (N=111)	Grade 2 (%) (N=32)	Grade ≥ 3 (%) (N=12)
Fatigue	15 (9.7)	10 (9.0)	5 (15.6)	0 (0)
Fever	13 (8.4)	10 (9.0)	3 (9.4)	0 (0)
Anemia	13 (8.4)	9 (8.1)	4 (12.5)	0 (0)
Increased GGT	12 (7.7)	6 (5.4)	1 (3.1)	5 (41.7)
Arthralgia	11 (7.1)	9 (8.1)	2 (6.2)	0 (0)
Loss of appetite	9 (5.8)	7 (6.3)	2 (6.2)	0 (0)
Pruritus	7 (4.5)	6 (5.4)	1 (3.1)	0 (0)
Vomiting	6 (3.9)	5 (4.5)	1 (3.1)	0 (0)
Hyperglycemia	6 (3.9)	4 (3.6)	1 (3.1)	1 (8.3)
Edema	5 (3.2)	5 (4.5)	0 (0)	0 (0)
Increased ALT	5 (3.2)	3 (2.7)	0 (0)	2 (16.7)
Increased AST	4 (2.6)	3 (2.7)	0 (0)	1 (8.3)
Diarrhea	4 (2.6)	4 (3.6)	0 (0)	0 (0)
Dysgeusia	4 (2.6)	3 (2.7)	1 (3.1)	0 (0)
Rash	4 (2.6)	2 (1.8)	1 (3.1)	1 (8.3)
Nausea	4 (2.6)	4 (3.6)	(0)	0 (0)

Hypotension	3 (1.9)	2 (1.8)	1 (3.1)	0 (0)
Creatinine elevation	3 (1.9)	3 (2.7)	0 (0)	0 (0)
TSH elevation	3 (1.9)	1 (0.9)	2 (6.2)	0 (0)
GGT, gamma-glutamyl-transferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TSH, thyroid stimulating hormone				

Table 2. Adverse events (at least 2%) reported among the ITT study population (N=43)

DISCUSSION

To the best of our knowledge, our study is the first prospective trial designed to evaluate the efficacy and safety of an immune checkpoint inhibitor in NSCLC patients with uncommon histology. The primary objective was to evaluate the therapeutic activity of atezolizumab in this selected population. Disease control rate (DCR), considered as the sum of complete responses (CRs), partial responses (PRs) and stable disease (SD) was the primary end point to measure the antitumor activity of atezolizumab. According to the Simon's two stage design, 15 patients had to be enrolled and at least 9 patients should have had a disease control to allow us of enrolling additional 28 patients. The goal of the first stage was reached and second stage started. During the second stage, because of slow accrual, the study protocol was amended and LCNEC histology included. Overall, the primary endpoint of the study did not meet. DCR was only 42%. Survival outcome estimates are currently under evaluation.

In our opinion, different observations could be explained, at least in part, the negative results obtained from our study. As expected, the study population was very heterogeneous, including histological subtypes with different biological features and prognosis. This population heterogeneity may have influenced the results of the study. In particular, more than 75% of study population had tumors with highly aggressive behaviors, poor prognosis (i.e LCNEC, sarcomatoid carcinoma) and very limited

data on potential efficacy of single agent immunotherapy. 42% of patients (10/16 LCNEC and 4/11 sarcomatoid carcinoma) did not receive more than 3 cycles due to rapid progressive disease and 16% of patients died without having undergone the first radiological tumor assessment. Conversely, 33% of patients benefitted from study drug for at least 6 months (6/6 adenoid cystic, 4/11 sarcomatoid carcinoma, 3/16 LCNEC).

Thus, our data suggest that previously treated patients for tumors with indolent evolution, poor and heavy treatment options, such as adenoid cystic carcinoma of the lung, could benefit from single agent immunotherapy. For highly aggressive tumors, such as LCNEC, sarcomatoid/pleomorphic carcinoma, NUT carcinoma and others, we need to explore and discovery predictive biomarkers of response to immunotherapy aimed at selecting the patients who could benefitted from immune checkpoint inhibitors and avoiding the use of expensive and potentially harmful drugs to those who do not. A previous pooled analysis including 90 patients with advanced pulmonary sarcomatoid carcinoma or pleomorphic carcinoma showed that PD-L1 expression was significantly associated with higher response rate and improved survival outcomes after immune checkpoint inhibitor [6]. In another retrospective study 44 patients with NSCLC with uncommon histology were included and matched with 44 patients with common histology for analyses. In multivariate analysis, good ECOG-PS and PD-L1 expression were predictive for efficacy of ICIs in patients with uncommon histology [7]. Beyond PD-L1 expression, tumor mutational burden could be another potential predictive biomarker of effectiveness from immunotherapy in sarcomatoid carcinoma [8]. Nevertheless, whole exome sequencing analyses have characterized in sarcomatoid carcinoma at least two mutational signatures comprising different mutational processes, gene alterations, and PD-L1 expression that could pave the way to the use of tailored therapies for specific clusters and immunotherapy for others [9]. The exploratory objectives of our study included also the central evaluation of PD-L1 expression on both tumor cells (TCs) and tumor infiltrating lymphocytes (TILs) by SP-142 and SP-263 aimed at correlating its expression and clinical outcomes (ORR, PFS, and OS) and accuracy of both antibodies. Unfortunately, these exploratory analyses are currently not available.

CONCLUSION

CHANCE is the first prospective study to evaluate the efficacy and safety of immune checkpoint inhibitor in advanced pretreated NSCLC with uncommon histology. The study did not meet its primary objective. However, one third of these patients benefitted from study drug. Larger prospective and translational studies are warranted.

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