Supplementary data

Material and Methods

Generation of ex vivo cultures from NSCLC patient

We developed a protocol for *ex vivo* 3D cultures from patient tumor sample[1,2,3]. Tumor was firstly removed from the patient and the fresh tissue sample was kept on ice and processed in sterile conditions on the day of collection. Tissue fragments are obtained by manual dissecting with a scalpel and then a digestion buffer was added (Collagenase and Hyaluronidase Solution) in a 37°C shaker at low to moderate speed (e.g. 200 rpm) for incubation time between 12-18 hours and cells were separated with serial centrifugation. For 3D cultures, cells were seeded in matrigel in order to preserve 3D structure.

Immunohistochemistry

A standard protocol was used for the immunostaining of the paraffin embedded samples with clone SP263 Ventana antibody to evaluate the expression of PD-L1. An appropriate external positive control tissue was used for each staining procedure; the negative control consisted of performing the entire IHC procedure on an adjacent section in the absence of the primary antibody. Briefly, paraffin slides (4 µm) were cut, deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed with slides heated in citrate buffer (pH 6) in a bath for 20 min at 97 °C. The endogenous peroxidase was inactivated with 3% hydrogen peroxide and then the protein block (BSA 5% in PBS 1×) was performed. The sections and spheroids were incubated with the primary antibodies according to the specific conditions tested: dilution 1:100, incubated at 4°C overnight. The sections were rinsed in TBS and incubated for 20 min with Novocastra Biotinylated Secondary Antibody (RE7103), a biotin-conjugated secondary antibody formulation that recognized mouse and rabbit immunoglobulins. Then the sections were rinsed in TBS and incubated for 20 min with Novocastra Streptavidin-HRP (RE7104) and then peroxidase reactivity was visualized using a 3,3'-diaminobenzidine (DAB). Slides were observed under a light microscope (Leica LEITSDMRB; Leica Microsystem, Milan, Italy) and images were captured using a high-resolution digital camera (Leica MC 170 HD, Software Leica Application Suite 4.3). Immunohistochemistry score was assessed based on the percentage of positive stained cells.

Immunofluorescence

To determine PD-L1, Vimentin and CD45 localization in patient derived tumor spheroids, the samples were processed as described by Venditti et al[4]. Briefly, the samples were firstly fixed in 4% paraformaldehyde in PBS, and then washed in phosphate buffer (0.01 M PBS, pH 7.4). The slides were incubated with 0.1% (v/v) Triton X-100 in PBS for 10 min. Later, nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in PBS containing 5% (w/v) BSA before the addition of the primary antibody (PDL1 CASA and Vimentin (Elabscience Biotechnology, Wuhan, China), diluted 1:100 for overnight incubation at 4° C. After washing in PBS, slides were incubated for 1 hour with the appropriate secondary antibody (Anti-Rabbit Alexa Fluor 488, Invitrogen; FITC-Jackson, ImmunoResearch; Anti-Mouse IgG 568, Sigma–Aldrich) diluted 1:500 in the blocking mixture. The slides were mounted with Vectashield + DAPI (Vector Laboratories) for nuclear staining, then observed under the optical microscope (Leica DM 5000 B + CTR 5000) with UV lamp, and images where viewed and saved with IM 1000.

Cell proliferation assays

Cell proliferation was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay the MTT assay. For 3D cultures, cells were extracted from matrigel with cold PBS-EDTA solution after the coloration with MTT and then lysed according to protocol instructions, as previously described[5]. Results represent the median of three separate experiments, each performed in quadruplicate.

Radiological assessment

Radiological assessment was performed every 9 weeks with a total body multiphasic multidetector computed tomography (MDCT) examination using a standardized technique, which involved the acquisition of a baseline scan and post-contrastographic phases, timed by bolus tracking technique with intravenous injection of nonionic iodinated contrast. In addition, at every evaluation assessment, a positron emission tomography with 2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with computed tomography (^{18}F-FDG PET/CT) was performed[6]. Response to treatment was interpreted according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1)[7].

Spectratyping on PBMCs and TILs

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of the patient collected at time of surgery by density gradient separation through Ficoll-Paque Plus (GE Healthcare). TILs were isolated from lung surgery specimen using CD3 microbeads (MiltenyiBiotec) and passed over MS columns. Purity was achieved through FACS analysis.

The analysis of TCR repertoire was conducted using Spectratyping technique. This is a PCR based technique that allows to amplify the CDR3 region of the different families of the repertoire; BV families were examined. PCR was composed of: denaturation step at 94 °C for 5 min; 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72°C for 30 s; and a final extension at 72 °C for 7 min. In brief, 20 ng of cDNA were used as a template for 27 TCR βV specific forward primers; as reverse was used a common Cβ primer, recognizing the constant region, labeled at the 5' end with 6-carboxyfluorescein. After the amplification, it was conducted a fluorescence based capillary electrophoresis where 1 uL of PCR-labeled product was mixed with 11 uL of formamide and 0.3 uL of LizSize Marker (Applied Biosystems), heated at 92°C for 2 min and run on a 3130 Genetic analyzer sequencer (Applied Biosystems). The TCR-CDR3 length analysis was evaluated using the GeneMapper software (Applied Biosystems).

Next generation sequencing

DNA extraction from cells derived from spheroids culture was obtained using the QIAamp® DNA FFPE Tissue (Qiagen), according to the manufacturer's instructions. Extracted DNA was eluted in 30 μL of elution buffer and then DNA was quantified by a Qubit® 2.0 Fluorometer (Life Technologies) using the Qubit® dsDNA HS Assay kit, according to the manufacturer's recommendations. The extracted DNA was stored at -20°C. Ten ng of DNA were used to prepare the sequencing libraries. The libraries were prepared with the IonAmpliSeq™ Library kit 2.0 (Thermo Fisher Scientific) and with a primer pool (IonAmpliSeq Colon and LungCancerResearch Panel v2 - Thermo Fisher Scientific) to analyze 504 mutational hotspots and targeted regions in 22 genes commonly implicated in colon/lung cancer development and treatment resistance[8]: AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, and TP53. Amplified

products were purified with AgencourtAMPure XP beads (BeckmanCoulterGenomics, High Wycombe, UK). Concentrations of amplified and bar-coded libraries were measured using the Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay kit. DNA libraries were stored at -20°C. The libraries were clonally amplified on IonSphere™ particles after dilution of the libraries to 100 pM. Template preparation was performed with the IonOneTouch™ 2 System (Thermo Fisher Scientific), an automated system for emulsion PCR, recovery of Ion Sphere™ Particles, and enrichment of template-positive particles. The Ion Sphere™ particles coated with template were applied to the semiconductor chip. A short centrifugation step was conducted to allow the spherical particles to be deposited into the chip wells. Finally, sequencing was carried out using Ion 318™ chips on the Ion Personal Genome Machine System (PGM™, Thermo Fisher Scientific) using the Ion PGM™ Hi-Q viewSequencingkit v2. The Torrent Suite Software v.4.0.2 (Life Technologies) was used to assess run performance and data analysis. Integrative Genomics Viewer (IGV v 2.2, BroadInstitute) was used for visual inspection of the aligned reads. Sequencing data were analyzed using Ion Reporter software (https://ionreporter.lifetechnologies.com/) and further filtered through quality checking. We selected all SNVs in the studied genes resulting in a non-synonymous amino-acid change, or a premature stop codon, and all short in-dels resulting in either a frameshift or insertion/ deletion of amino-acids. All SNVs were analyzed for previously reported hotspot mutations (somatic mutations reported in COSMIC database) and novel variations, i.e. new mutations detected by NGS but not reported in either COSMIC or dbSNP databases.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software version 6.0 (Graphpad Software Inc., San Diego, CA, USA). Data were compared with One-way ANOVA statistical test followed by Tukey's test.

List of abbreviations

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

Multiphasic multidetector computed tomography (MDCT)

2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with computed tomography (^{18}F-FDG PET/CT)

Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1)

Peripheral blood mononuclear cells (PBMCs)

References

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