Article

# **Rapid Response to the Combination of Lenvatinib and Pembrolizumab in Patients with Advanced Carcinomas (Lung Adenocarcinoma and Malignant Pleural Mesothelioma)**

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**Simple Summary:**

**Abstract:** The new era of cancer treatments has made immune checkpoint inhibitors (ICIs) and emerging multikinase inhibitors (TKIs) the standards of care, thus drastically improving patient prognoses. Pembrolizumab is an antiprogrammed cell death-1 antibody drug, and lenvatinib is a TKI with a preferential antiangiogenic activity. We present, to our knowledge, the first reported series of cases consisting of patients with metastatic non–small cell lung cancer and malignant pleural mesothelioma who were treated with several types of combinations of chemotherapies and ICIs followed by disease progression. They were subsequently treated with combined immunotherapy and TKI treatment, resulting in a near complete response within a very short time. Clinical responses were supported by in vitro testing of each patient’s lymphocyte response to lenvatinib after exposure to pembrolizumab.

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**Keywords:** lung adenocarcinoma; NSCLC; malignant pleural mesothelioma; multikinase inhibitor; immune checkpoint inhibitor; lenvatinib; pembrolizumab

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in the United States and is a significant health care concern throughout the world [1]. Non–small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. The natural history of NSCLC is often insidious, with few if any symptoms until the disease is relatively widespread. Thus, most lung carcinomas are diagnosed at an advanced stage, resulting in a poor prognosis [2]. Malignant pleural mesothelioma (MPM) is one of the oncologic pathologies expected to be on the rise in the upcoming 10–20 years, secondary to asbestos exposure in factory workers in the mid to late 20th century [3]. Unfortunately, this diagnosis is not accompanied by a good prognosis [4]. Even with current treatment schemes, overall and median survival rates are not promising. Mutational cues and patient background characteristics have little influence in directing therapy and predicting outcomes [5].

Pembrolizumab is a humanized IgG4 monoclonal antibody directed against the programmed cell death receptor -1 (PD-1), a major immune checkpoint receptor that regulates T-cell response. Pembrolizumab blocks PD-1 activity, thereby enhancing antitumor T-cell activity [6]. Lenvatinib is a multitargeted tyrosine kinase inhibitor of vascular endothelial growth factor receptors 1, 2, and 3, fibroblast growth factor receptors 1–4, platelet-derived growth factor receptor α, RET, and KIT [7].

In a phase 2 trial, the combination of pembrolizumab and lenvatinib achieved encouraging antitumor results with a manageable safety profile in patients with selected advanced solid tumors including NSCLC [8]. The decision to combine these agents was based on the antitumor activity shown in preclinical data [9].

To show the potential efficacy of the combination of immune checkpoint inhibitors and multikinase inhibitors, we report a series of five cases, four of metastatic NSCLC and one of MPM, that responded dramatically to the combination treatment of pembrolizumab and lenvatinib after progression following prior lines of therapy. To support the clinical outcomes observed, we conducted in vitro laboratory tests for four of the five patients to test the CD8+ T cells' sensitivity to pembrolizumab after exposure to lenvatinib. This test was obtained by measurement of the interferon-gamma (IFN-γ) secretion of CD8+ T cells using the A549 cell line (carcinoma cell line) as target cells [10]. This technique was first used at Ben-Gurion University in collaboration with the Weizmann Institute of Science, using a syngeneic tumor target and CD8+ T cells.

We hope to further explore the combination of anti-PD-1 therapy and multikinase targeted therapy for different carcinoma treatments in the future.

2. Materials and Methods

2.1 Patient Selection

All five patients were selected to enter trials that were ongoing in our country. Unfortunately, they did not meet the inclusion criteria or had one of the exclusion criteria. Consequently, they received the treatments described from their health maintenances organization, private insurance, or donation. The trials are still ongoing, and all the required regulatory and ethical permits were provided.

2.2 Isolation and Culturing of the Patients’ Peripheral CD8+ T Cells

Isolation of peripheral blood mononuclear cells (PBMCs) from the peripheral blood of the patients was done according to a standard protocol using Lymphocyte Separation Medium (MP Biomedicals, SKU 0850494-CF). Then, PBMCs were cultured with 200U IL-2, and 2 days later, CD8+ T cells were isolated by using human CD8 Microbeads (Miltenyi Biotec, 130-045-201), LS column (Miltenyi Biotec, 130-042-401) and MidiMACS Separator (130-042-302) following the manufacturer’s protocol. RPMI containing 10% human male AB plasma (Sigma, H4522), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies), 200 IU/mL recombinant human IL-2 (200-02-500UG, PeproTech), and 50 ng/mL antihuman CD3 Antibody (BioLegend, 317302) were used for the first 48 h of culture. Then, culturing and passaging was done using complete media without antihuman CD3 antibody.

2.3 PDX Generation and Propagation

Patient-derived tumor tissue was implanted in male NOD/SCID subcutaneously as we described before [11]. The growth rate of implanted tumors varied from 1 to 6 months. Generation and propagation of the PDXs were done under the guidelines of the institutional animal care and use committee of Ben-Gurion University of the Negev. The animal ethical clearance protocol number used for this research was IL-29-05-2018(E).

2.4 Co-culture of A549 CD8+ T Cells

At 24 h before the experiment, A549 cells were seeded using normal complete DMEM media 10% FBS in 96 a Flat-bottom well plate with 60% confluency. The cells were washed and incubated with either lenvatinib (5 uM) in 200 uL of media or control media for 10 h, and 10 h later, cells were washed twice with 1X PBS; 100,000 cells/well CD8+ T cells were added to the washed wells along with pembrolizumab (20 ug/mL) or mock in complete RPMI media containing 20 U IL-2 and 10% human serum. After 18 h of incubation, the supernatant was collected from the wells and then assayed for standard IFN-γ ELISA assay (ELISA MAX, Biolegend) according to the manufacturer's instructions.

2.5 Immuno-Tumor Ex Vivo Analysis (i-TEVA)

Tumors from PDX mice were collected once they reached approximately 500 mm3. Then, tumors were cut into 2×2×2 mm3 tissue explants using a unique cutting tool. All explants were cultured in 48-well tissue culture plates using DMEM culture media (TEVA media) as described by Ghosh and colleagues [11]. Explants were incubated either with lenvatinib or TEVA media for 10 h. Then, explants were washed three times with PBS 1x and incubated for 18 h with 2×105 T cells in complete RPMI media containing 20 U of IL-2 (200-02-500UG, PeproTech).

**2.6 Tumor Tissue Explants Culture, Preparation of FFPE Blocks, and Tissue Microarray**

The PDXs were used for preparation of 2×2×2 mm3 ex vivo tumor tissue explants culture according to a previously published protocol [11]. Then FFPE blocks were prepared from the explants using an automated tissue processing machine (Leica Biosystems) as previously described [11–13]. Finally, tissue microarray (TMA) blocks were prepared from donor FFPE blocks using 3-mm T-SueTM punch needles (Simport); each block contained up to 24 tissue explants.

2.7 Immunohistochemistry Staining and Quantification

Immunohistochemical staining was done as previously described [11] (Ki67 [1:250, Vector laboratories, cat no- VP K451] and PD-L1 [1:500, abcam, cat no-ab205921]). A panoramic scanner (3DHISTECH) was used to take the TMA images. Images were analyzed by HistoQuantTM software (3DHISTECH). For both Ki67 and Pd-L1, the number of positive nuclei was calculated, and the value was expressed as object frequency (pcs/mm2).

3. Results

We have chosen to describe five cases of patients who had a significant response to combined treatment with pembrolizumab and lenvatinib. All patients’ clinical data and outcomes following treatment with pembrolizumab plus lenvatinib are presented in Table 1. In addition, we deepened our investigation of four of the five patients by testing their CD8+ T cells' sensitivity for pembrolizumab after exposure to lenvatinib treatment and explored the differences between the radiological and laboratory results.

**Table 1.** Summary of clinical characteristics and treatment outcomes of patients presented in cases 1–5.

3.1. Case Series

3.1.1. Case 1—Excellent response combined with disappearance of the lower left lung after treatment with lenvatinib and pembrolizumab

A 68-year-old man with cough and shortness of breath (Patient 1) was referred to the emergency department in November 2018 by a primary care physician. He was a smoker (30 pack-y over the previous 20 y) and was receiving treatment for type 2 diabetes mellitus, prostatic benign hyperplasia, and hyperlipidemia. There was no family history of cancer. Chest radiography showed a right upper lung (RUL) ground-glass opacity. He was admitted to the hospital for further evaluation. A computed tomography (CT) scan of the chest showed a 2.5-cm mass in the RUL. Positron emission tomography–computed tomography (PET-CT) showed hypermetabolic uptake in the RUL (the 2.5-cm mass) and hypermetabolic uptake in the left adrenal (3.5 cm in diameter) as well as in the retropancreatic region (2 cm in diameter).The pathologic stage was determined to be T1C N0 M1 (stage 4-C). CT of the head was done for further investigation, which showed no evidence of metastatic disease.

A biopsy from the RUL mass was taken under CT guidance, with the histopathologic finding of adenocarcinoma of lung origin. A molecular testing panel of the tumor tissue showed the presence of 21 mutations (none of them treatable), including MDM2 (mu-rine double minute 2), KRAS (Kirsten rat sarcoma) amplification, RB1 (retinoblastoma protein) amplification, and STK11 (Serine/Threonine Kinase 11).

 The patient underwent left adrenalectomy in November 2018 (histopathologic findings showed mucinous adenocarcinoma of lung origin) and 1 mo later underwent tumor resection from the lung (right upper lobectomy). The patient received systemic intravenous chemoimmunotherapy consisting of pemetrexed (500 mg/m2) plus carboplatin (dosed to AUC-5) and pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d, with a partial response. Pembrolizumab and pemetrexed were continued as maintenance. After 8 mo of maintenance therapy, PET-CT in October 2019 showed disease progression with hypermetabolic uptake in a right adrenal mass (4 cm in diameter) and a mass in the right pectoralis (1.4 cm in diameter) as well as a suspected metastasis to the sigmoid colon (2 cm in diameter). In addition, a hypermetabolic uptake (3 cm in diameter) was seen in the left lower lung (LLL).The patient received radiotherapy to the right adrenal (30 Gy, 3 fractions). One week after finishing radiotherapy treatment, he then underwent a right mastectomy and resection of the metastasis in the colon (both resections showed adenocarcinoma of lung origin).

A month after the last surgery, the patient received one dose of intravenous docetaxel (at a dosage of 75 mg/m2) in the context of a clinical trial. He suffered from severe adverse effects including grade 3 diarrhea, grade 2 neuropathy, and severe weakness, resulting in dropping out of the trial after the single dose.

In June 2020 (a month after docetaxel treatment), the patient resumed pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d with the addition of oral lenvatinib daily (at a dosage of 14 mg). Approximately 40 d later, a CT of the chest and abdomen showed an excellent response, with the disappearance of the LLL nodule and shrinking of the mass in the right adrenal; a PET-CT scan in November 2020 showed complete response (Figure 1).

**Figure 1.** A schematic illustration of Patient 1’s timeline, between November 2018 (diagnosis of the disease) and November 2020, including the course of treatment and radiological follow-up. Each rectangle represents 1 mo, and the response to treatment during each month is color-coded in green, red, or yellow, as described in the upper legend. Treatment administered at each timepoint is indicated by the blue rectangles. Radiological findings and additional landmarks are presented at relevant points throughout the timeline.

3.1.2. Case —A multiple radiological response followed by less metabolic uptake immediately after combination of lenvatinib and pembrolizumab

A 68-year-old man (Patient 2) was referred to the emergency department in March 2018 owing to fainting and loss of consciousness. He had a history of malignant melanoma (stage 1) resected from the chest wall, he was a smoker (60 pack-y over the previous 45 y), and he was receiving treatment for hypertension. There was no family history of cancer. A total body CT scan showed two nodules in the RUL (1.3 cm and 0.8 cm, respectively), and PET-CT showed hypermetabolic uptake in both nodules (RUL area). Magnetic resonance imaging (MRI) of the head showed no evidence of brain metastases. The pathologic stage was determined to be T1b N0 M0 (stage 1-B). The patient was admitted to the hospital for bisegmentectomy of the RUL. Histopathologic findings of both nodules were adenocarcinoma of lung origin, and resection margins were free of disease.

The patient remained in follow-up and free of disease until March 2020, when PET-CT showed hypermetabolic uptake in the left upper lung (LUL) (2.7 cm × 1.6 cm in diameter); an LLL nodule with a diameter of up to 4 mm, which was too small in size to show uptake on PET; hypermetabolic uptake in the spinal bones (C4, C6); and hypermetabolic uptake in the paraspinal muscle and in two major muscles in the left anterior thigh. MRI of the head showed no evidence of brain metastases. The new pathologic stage was determined to be T2 N0 M1c (stage 4-B). The patient received systemic intravenous chemoimmunotherapy consisting of pemetrexed (500 mg/m2) plus carboplatin (dosed to AUC-5) and pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d, and after 2 cycles of follow-up, a PET-CT showed progression of the disease. The known findings were stable, but new findings with hypermetabolic uptake were found in the left hilar lymph node and in the paravertebral area of the spinal bones (D3 and D5). For further investigations, new biopsies were taken from the patient’s paravertebral area.

After the fourth cycle, PET-CT in August 2020 showed stable disease, but only the two metastases in the major muscles of the left anterior thigh were reduced (by 30%). Later, we received the pathology results, which were negative for melanoma and positive for lung adenocarcinoma. Pemetrexed was stopped, but the patient continued with pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d, and daily oral lenvatinib (at a dosage of 20 mg) was started. PET-CT from September 2020 showed a significant radiological response in the LUL mass (0.2 cm in diameter) as well as in the metabolic uptake of the left hilar lymph node and in the paravertebral area. In addition, a significant radiological response (diameter and metabolic uptake) of the two major muscles in the left anterior thigh was seen (Figure 2).

**Figure 2.** A schematic illustration of Patient 2’s timeline, between March 2018 (diagnosis of the disease) and September 2020, including the course of treatment and radiological follow-up. Each rectangle represents 1 mo, and the response to treatment during each month is color-coded in green, red, or yellow, as described in the upper legend. Treatment administered in each timepoint is indicated by the blue rectangles. Radiological findings and additional landmarks are presented at relevant points throughout the timeline.

3.1.3. Case 3—Radiological improvement achieved after treatment with pembrolizumab and lenvatinib in a patient after hospitalization in an intensive care unit

A 64-year-old woman (Patient 3) was referred to the emergency department in February 2020 by a primary care physician owing to cough and progressive shortness of breath. She had a history of uterus papillary serous carcinoma (stage 1) in 2011, which was resected. She was an active smoker (40 pack-y during the previous 28 y) and was receiving treatment for diabetes mellitus type 2.

Chest radiography (CXR) showed a mass in the RUL (diameter of 6 cm) and a right pleural effusion. Draining of the pleural effusion was performed (6 L were drained), and the drainage was sent for a cytology test. Histopathologic findings showed adenocarcinoma of lung origin. PET-CT showed hypermetabolic uptake in the RUL mass (10.6 cm × 10.5 cm in diameter) that was penetrating into the mediastinum with right pleural effusion and several masses that were connected to the right internal pleura (diameter of 1.5 cm). MRI of the head showed no evidence of brain metastases. The pathologic stage was determined to be T4 N2 M1 (stage 4-B). The patient received systemic intravenous chemoimmunotherapy consisting of pemetrexed (500 mg/m2) plus carboplatin (dosed to AUC-5) and pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d. After three cycles, chest CT showed stable disease, and after the fifth cycle, PET-CT (May 2020) showed stable disease once again. Two weeks later, the patient was hospitalized in the intensive care unit owing to oxygen desaturation, sepsis, and acute renal failure. She was treated with artificial respiration and underwent insertion of a stent to the right lung, which opened the airway to the RUL. Four days later, she was extubated. There was a dilemma between BSC and between starting pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d and oral lenvatinib daily (at a dosage of 14 mg, by private insurance). In July 2020, treatment was started, and after 3 wk, PET-CT showed radiological improvement by reduction in the intensity of the absorption in the extensive hypermetabolic findings in the right lung, but hypermetabolic uptake was seen in the eighth and ninth left ribs (suspected of fracture) (Figure 3).

**Figure 3.** A schematic illustration of Patient 3’s timeline, between February 2020 (diagnosis of the disease) and November 2020, including the course of treatment and radiological follow-up. Each rectangle represents 1 mo, and the response to treatment during each month is color-coded in green, red, or yellow, as described in the upper legend. Treatment administered in each timepoint is indicated by the blue rectangles. Radiological findings and additional landmarks are presented at relevant points throughout the timeline.

3.1.4. Case 4—A significant response to pembrolizumab, lenvatinib, and gemcitabine in a patient with malignant pleural mesothelioma

A 50-year-old woman (Patient 4) was referred to the emergency department in January 2018 by a primary care physician owing to chest discomfort and shortness of breath. She was generally healthy with no chronic disease and was taking no chronic medications. She was a smoker (10 pack-y over the previous 15 y) and was receiving treatment for hypertension. Her father was diagnosed at age 70 with mesothelioma.

CXR showed a left pleural effusion. A drainage of the pleural effusion was performed and was sent for cytology testing. Histopathologic findings showed malignant pleural mesothelioma. For further investigation, she underwent PET-CT, which showed hypermetabolic uptake and thickening in the left circumferential pleura and bilateral hypermetabolic uptake and enlargement of lymph nodes (LNs) in the mediastinal area. The pathologic stage was determined to be T4 N2 M0 (stage 3-B). The patient received systemic intravenous chemoimmunotherapy therapy consisting of pemetrexed (500 mg/m2) plus carboplatin (dosed to AUC-5) and bevacizumab (15 mg/kg) on day 1 every 21 d for 5 cycles. PET-CT was performed for follow-up and showed good partial response in the thickening of the left circumferential pleura and the mediastinal LNs, and the patient had a left lung decortication. Carboplatin, pemetrexed, and bevacizumab were given again (at the same dosages) after 2 cycles owing to brain thrombosis; bevacizumab and carboplatin were discontinued, and the patient remained on pemetrexed for another cycle. The patient underwent PET-CT (April 2019) that showed disease progression with hypermetabolic uptake and enlargement of the right mediastinal LN (1.4 cm in diameter), left supraclavicular LN (1.7 cm in diameter), and left axilla LN (2.3 cm in diameter) and a hypermetabolic uptake in several retroperitoneal LN. The treatment was changed to pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d for 5 cycles. PET-CT showed partial response, and the left axillary LN was resected. Ipilimumab (1 mg/kg every 4 wk) was added to the pembrolizumab for 5 cycles; PET-CT showed significant partial response, and ipilimumab was stopped owing to liver injury (immunotherapy-induced grade 2 toxicity). Pembrolizumab was continued alone for 5 cycles. PET-CT (June 2020) showed disease progression with hypermetabolic uptake in the left pleural hemithorax and spinal bone (D6) and hypermetabolic uptake and enlargement in the left supraclavicular LN, left axillary LN, and retroperitoneal LN. Pembrolizumab was continued with the addition of oral lenvatinib daily (by personal insurance at a dosage of 20 mg) and gemcitabine (1000 mg/m2 on day 1 every 21 d). After 2 mo, PET-CT from August 2020 showed a significant radiological response—a decrease of hypermetabolic uptake and enlargement in the left supraclavicular LN, left axillary LN, left area of the pleural hemithorax, retroperitoneal LN, and spinal bone (D6) (Figure 4).

**Figure 4.** A schematic illustration of Patient 4’s timeline, between January 2019 (diagnosis of the disease) and September 2020, including the course of treatment and radiological follow-up. Each rectangle represents 1 mo, and the response to treatment during each month is color-coded in green, red, or yellow, as described in the upper legend. Treatment administered in each timepoint is indicated by the blue rectangles. Radiological findings and additional landmarks are presented at relevant points throughout the timeline.

3.1.5. Case 5—Combined treatment stabilized the patient’s widespread disease for a period of 10 mo

A 61-year-old man (Patient 5) was seen in August 2016 for cough and progressive shortness of breath (especially during effort) of three months’ duration. He had a history of hypertension and he was a smoker (35 pack-y over the previous 20 y), with no family history of cancer. CXR showed a ground-glass opacity in the LUL. Pneumonia was suspected, and he was treated with antibiotics. One month later he underwent follow-up CXR, which revealed a RML ground-glass opacity (the same opacity that was seen a month before). For further investigation, the patient underwent a CT scan that showed an LUL mass (5.5 cm × 4.6 cm). PET-CT showed hypermetabolic uptake in the LUL mass, hypermetabolic uptake and enlargement of the mediastinal and retroperitoneal LNs, and hypermetabolic uptake in the left femur and left pelvis (up to 1 cm). MRI of the head showed no evidence of brain metastases. Histopathologic findings of both nodules were adenocarcinoma of lung origin. A molecular testing panel of the tumor tissue was positive for KRAS, STK11, and PDL1<24%. The patient received systemic intravenous chemotherapy and vascular endothelial growth factor therapy consisting of pemetrexed (500 mg/m2), cisplatin (75 mg/m2), and bevacizumab (15 mg/kg), all given on day 1 every 21 d for 5 cycles followed by significant partial response. In March 2017, treatment was changed to combination of chemoimmunotherapy pemetrexed (same dosage) and pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d, with metabolic complete response. Six months later, in September 2017, pemetrexed was stopped and the patient continued treatment with pembrolizumab (same dosage) until March 2018 owing to weakness and fatigue. One month after stopping the treatment, the patient reported improvement of symptoms; however PET-CT showed a hypermetabolic left hilar and subpancreatic uptake. Thereafter, pembrolizumab (same dosage) was rechallenged for 3 cycles and led to further progression of the disease. The patient received systemic intravenous chemoimmunotherapy consisting of pemetrexed (500 mg/m2) plus carboplatin (dosed to AUC-5) and pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d for 4 cycles, which led to a significant response. In January 2019, mild local progression (in the lung) of the disease was seen. Therefore, hypofractionated radiation therapy to the left hilar and to the mediastinum was added (30 Gy in 10 fractions), and treatment with pemetrexed was continued. In May 2019, PET-CT showed progression of the disease in several sites, including the groin, iliac, mediastinum, and neck. It was decided to begin treatment with pembrolizumab and ipilimumab for 4 cycles; this was followed by further progression of the disease, exhibited by PET-CT. The patient went through dissection of the inguinal LN, which was confirmed by pathologic testing to be a TTF1-positive adenocarcinoma lesion. Next, chemotherapy treatment was renewed with cisplatin (75 mg/m2) and pemetrexed (500 mg/m2). After the fourth cycle, the patient showed stable disease; then, in January 2020, the patient was treated with pembrolizumab (at a dosage of 200 mg) on day 1 every 21 d and lenvatinib (at a dosage of 20 mg daily). One month later, significant regression of the lesions was seen with improvement in lung findings and a decrease in the diameter of LNs, and 3 mo afterward, the mediastinal lesion was decreased by half of its size. PET-CT from May 2020 presented preservation of the good response. At this stage, the lenvatinib dosage was decreased to 14 mg daily owing to aggravation of the mouth ulcers. The patient continued the combined treatment, which stabilized his disease with no adverse events for a period of 10 mo. A lingering cough then appeared and brought about sudden deterioration of the disease, which eventually led to the patient’s death (Figure 5).

**Figure 5.** A schematic illustration of Patient 5’s timeline, between August 2016 (diagnosis of the disease) and September 2020 (patient’s death), including the course of treatment and radiological follow-up. Each rectangle represents 1 mo, and the response to treatment during each month is color-coded in green, red, or yellow, as described in the upper legend. Treatment administered in each timepoint is indicated by the blue rectangles. Radiological findings and additional landmarks are presented at relevant points throughout the timeline.

3.2. Testing CD8+ T cell responses of four patients to lenvatinib in vitro

We further studied the importance of our results for human T-cell response on a more mechanistic level. We first withdrew blood from four patients (patients 1, 2, 4, and 5) and isolated the PBMCs using the FICOL gradient. Two days later, CD8+ T cells were isolated and cultured with 200 U IL-2 for a week. The four patients were clinically negative responders to pembrolizumab. In vitro treatment with anti-PD-1 with or without lenvatinib on coated 96-well plates with A549 (carcinoma cell line) showed a significant elevation secretion of IFN-γ by the primary CD8+ T cells of patients 4 and 5 in response to the combined treatments compared with IFN-γ levels secreted by these cells following treatment with anti-PD1 alone (Figure 7). Fully saturated secretion of IFN-γ was seen in the primary CD8+ T cells of patients 1 and 2 only by using the anti-PD-1 (Figure 6).

3.3. Lenvatinib blocks the upregulation of PD-L1 marker combined with low proliferation levels while using pembrolizumab (anti-PD-1)

The PDX and T cells from patient 4 were used to truly understand the link between cotreatment with lenvatinib and pembrolizumab. We performed the i-TEVA method using patient 4’s PDX and T cells and examined the outcomes of three main combinations: tumor with media, tumor with T cells, and tumor with T cells and pembrolizumab. Each group was tested with and without addition of lenvatinib to the media. Then, we stained the FFPE sections either with PD-L1 or Ki67 (proliferation marker) (Figure 7A and 7B). Figure 7A shows Ki67 staining; the group treated with lenvatinib and pembrolizumab and T cells had significantly lower proliferation than the untreated group. Figure 7B shows PD-L1 staining; upregulation blocking of PD-L1 after pretreating with lenvatinib was seen, in contrast to the untreated group. Figure 7C shows representative pictures of each group.

**Figure 6.** In vitro treatment with anti-PD-1 with or without lenvatinib for CD8+ T cells of patients 1, 2, 4, and 5. All four patients were tested in vitro for 12 different conditions in triplicates. This figure shows the IFN-γ values in pg units upon 12 different conditions. Mizoribine (red) and crizotinib (green) were used as positive and negative controls, respectively; the control (black) was untreated, and lenvatinib (blue) was the experimental test. A549 cells were first seeded on 96-well plates 24 h before adding the drugs. Then, drugs were added and cells were incubated for 10 h. Media with or without anti-PD-1 antibody (pembrolizumab) containing the T cells of different patients was added after performing three PBSX1 washes for the whole 96-well plate. Human IFN-γ was performed using the collected media from the experimental wells and measured using the enzyme-linked immunosorbent assay (ELISA) method at a wavelength of 650 nm. A two-way analysis of variance (ANOVA) multiple comparisons test was performed among the groups for each patient individually, with three biologically independent counts in each group. \*\*\**p* < 0.0001 for both the mizoribine- and the lenvatinib-treated groups in patients 4 and 5. All statistical data analysis was performed using GraphPad Prism, version 8.0.2.

**Figure 7.** Correlation between the multikinase inhibitor (lenvatinib) and the immune checkpoint inhibitor (pembrolizumab) treatments. A 3-dimensional (3D) ex vivo assay was performed as an i-TEVA assay on patient 4’s tumor and T cells. (A–B) Lenvatinib (blue) was the experimental test, and the control (black) was untreated. From the 3D ex vivo assay, 5-µm FFPE sections were stained either for Ki67 (proliferation marker) or PD-L1 expression. A two-way ANOVA multiple comparisons test was performed among the groups for each patient individually, with three biologically independent counts in each group. *\*p* *=* 0.0498; *\*\*\*p* *=* 0.0007. All statistical data analysis was performed using GraphPad Prism, version 8.0.2. (C) This figure shows representative pictures for each group from 1 to 6. Pictures were captured using CaseViewer 40× magnification at 300 dpi; scale bar is located at the left bottom corner, 100 µm (red).

 4. Discussion

We have described five patients with metastatic adenocarcinoma of the lung (stage IV on presentation). The first patient had progressive disease after two lines of chemoimmunotherapy, maintenance immunotherapy, and one dose of single-agent chemotherapy. The second patient had progressive disease after the first line of chemoimmunotherapy. The third patient was in a very dramatic situation, and it was decided not to treat her. The fourth patient had MPM and was treated with different treatments of chemotherapy and immunotherapy. The fifth patient had widespread disease and had received numerous previous immunotherapy and chemotherapy treatments. All five patients had progressed after receiving different types of treatments (as acceptable for the disease); however, after receiving the combination of immunotherapy and TKI, significant responses were seen among all patients. Patients 1, 2, and 5, all of whom had NSCLC, showed a significant radiological response followed by a near-complete response of the widespread metastatic disease shortly after administrating the combination of TKI and immunotherapy treatment. The third patient, who had aggressive and resistant disease, presented a partial radiological response less than a month after initiation of the combined treatment. The fourth patient, who had MPM, also had a significant radiological response of the widespread and aggressive metastatic disease.

To show the mechanistic base of the response to combined therapy with pembrolizumab and lenvatinib seen in all five cases, we performed in vitro tests to assess the response of CD8+ T cells to treatment in four of the five patients (patients 1, 2, 4, and 5). The results showed a significant elevation in IFN-γ secretion levels after exposure to anti-PD-1 combined with lenvatinib compared with anti-PD-1 only in patients 4 and 5. However, patients 1 and 2 had fully saturated IFN-γ levels at exposure to anti-PD-1 with no difference after the addition of lenvatinib. A reasonable explanation to these findings might be the status of the cells taken from each patient. In Figures 1 and 2, patients 1 and 2 were currently under clinical treatment of lenvatinib and pembrolizumab according to their treatment scheme, whereas patient 4 was 4 mo after treatment and patient 5 was before clinical treatment with lenvatinib and pembrolizumab. This fact can explain and support our results; we claim that CD8+ T cells responded rapidly to pembrolizumab after exposure to lenvatinib, meaning that the CD8+ T cells’ sensitivity to pembrolizumab after treatment with lenvatinib was improved at least for the short term. In Figure 7, we used a unique assay to investigate the role of lenvatinib in combination with pembrolizumab. It is known that PD-L1 acts as an immune checkpoint in many solid tumors, including NSCLC [14–20]; it also known that the vast majority of tumors successfully overcome the effect of any treatment by generating a new resistant mutation [21–24]. Figure 7B shows confirmation of both facts, but interestingly, once the tumor was pretreated with lenvatinib, lenvatinib seemed to block the PD-L1 upregulation levels, thus making the tumor visible for the immune system.

The main weakness of this study was the limited number of patients. We intend to expand our sample size in the future to confirm the findings. In addition, the response to lenvatinib alone was not tested in this study. Therefore, the responses observed may possibly be a result of the lenvatinib treatment and not necessarily of the combination with pembrolizumab. Nonetheless, promising preclinical data from recent ongoing trials has shown robust antitumor activity with a manageable safety profile in multiple solid tumors with limited treatment options, including lung cancer (ref LEAP).

5. Conclusions

According to the case series we have presented and the in vitro study we conducted to test the mechanistic basis of the outcomes, treatment with lenvatinib plus pembrolizumab may potentially provide a beneficial treatment option for patients with NSCLC and MPM, as previously suggested by recent clinical trials.

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