VIA MEDICA

CASE REPORT

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# The search for causes of resistance to pembrolizumab in lung adenocarcinoma with PD-L1 expression — focus on intestinal microbiome

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## ABSTRACT

Anti-PD-1 or PD-L1 immunotherapy in some patients with non-small cell lung cancer (NSCLC) may not be effective, despite the high percentage of cancer cells with PD-L1 expression (≥ 50%). TMB (tumor mutation burden), smoking status and low intestinal microbiome diversity may be associated with lack of efficacy of immune checkpoints inhibitors treatment in NSCLC patients. The case presented here concerns a non-smoking female patient with lung adenocarcinoma, in whom, despite the high percentage of PD-L1 positive tumor cells (50%), pembrolizumab therapy was ineffective. Next generation sequencing (NGS) was performed using the FOCUS panel allowing the analysis of 52 genes whose damage is associated with various types of solid tumors, including lung cancer. Benign genetic changes clinically irrelevant for patients with non-small cell lung cancer have been observed. In the meantime, profiling of the patient's intestinal microbiome was performed, due to the fact that the composition of the intestinal microbiome may be a decisive factor in the lack of response to immunotherapy in patients with high PD-L1 expression and no driver mutations. Low diversity of bacteria in the intestines, with a noticeable dysbiosis (dysbacteriosis), was observed. The presence of bacteria *Akkermansia, Enterococcaceae, Bifidobacteriaceae* or *Coriobacteriaceae*, especially the presence of *Akkermansia mucinifila* seems to be a favourable factor of the possibility of obtaining response to immunotherapy and prolongation of progression-free survival (PFS). In the intestinal microbiome of the presented case, no bacteria from the *Verrucomicrobia phylum*, to which *A. mucinifila belongs*, were found. In addition, only 0.011% of *Enterococcaceae* were found. Studies on the intestinal microbiome in cancer patients receiving immunotherapy appear to be necessary to correctly understand the effect of microbiome composition on the effectiveness of this treatment method.

**Key words**: immunotherapy, intestinal microbiome, NGS, NSCLC

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## Case report

In August 2019, a 48-year-old patient came to the Department of Pneumonology, Oncology and Allergology in Lublin due to severely increased dyspnea, highly reduced exercise tolerance and a dry, persistent cough. She never smoked. Chest x-ray revealed a large amount of fluid in the left pleural cavity with atelectasis above the fluid level and left hilar enlargement. Thoracentesis was performed several times during hospitalization, however pathomorphological examination of pleural effusion did not allow for definitive diagnosis. Computed tomography (CT) scans revealed tumor in a left lung (13 × 10 cm), constricting the left upper lobe and lower

lobe bronchi, fluid in the left pleural cavity, and significant pleural thickening. In the preserved lower lobe of the left lung multiple small metastatic nodules were visible. In the abdominal cavity numerous enlarged hepatic hilar and periaortic lymph nodes were found. Magnetic resonance imaging of the spine also revealed numerous metastases with pathological vertebral compression fractures (Th3–Th4 and Th8–Th10). In September, the patient underwent bronchofiberoscopy with transbronchial biopsy of the mediastinal lymph nodes. Specimens were obtained from infiltrated carina and right main bronchus, transesophageal and transesophageal fine-needle aspiration of the tumor as well as left mediastinal lymph nodes (station 7) were also performed.

The tissue samples were preserved in formalin and embedded in paraffin wax blocks. Histological examination confirmed adenocarcinoma with thyroid transcription factor 1 (TTF1) expression on cancer cells. In the fixed cytological material all predictive factors for therapies registered in European Union countries were examined. The *EGFR* (epidermal growth factor receptor) and *BRAF* (B-Raf Proto-Oncogene) genes mutations were excluded using real-time polymerase chain reaction (RT-PCR), *ROS1* gene rearrangement was excluded using fluorescent in situ hybridization (FISH), and the expression of ALK (anaplastic lymphoma kinase) fusion protein using immunohistochemistry (IHC). Programmed cell-death ligand 1 (PD-L1, CD274) expression was also analyzed by IHC (antibody clone SP263). Surface PD-L1 expression was detected in 50% of tumor cells.

Based on the results of the aforementioned examinations and the clinical factors (stage IV lung adenocarcinoma) it was decided to use pembrolizumab in first-line treatment. Unfortunately, after two cycles of immunotherapy, the disease progressed and the patient's clinical condition worsened. The patient consistently refused chemotherapy. Therefore, only local treatment of the obstructive bronchus lesion with brachytherapy, radiotherapy and the best supportive care was used.

## Searching for the causes of resistance to immunotherapy

This is one of the examples when immunotherapy is not effective despite the high PD-L1 expression on cancer cells. The reason for this could be the occurrence of a single rare driver mutation that could not be detected by monogenic tests. Low tumor mutation burden (TMB) may result in ineffectiveness of immunotherapy. Low TMB is also affected by smoking history and the ability to repair damaged cellular DNA, determined by germinal or somatic mutations or polymorphisms of genes encoding DNA repair pathway proteins. Therefore, it was decided to perform next-generation sequencing (NGS) to look for driver mutations qualifying to molecularly targeted therapies. Sequencing was carried out with Ion Torrent technology in the S5 (Thermo Fisher Scientific) apparatus using the FOCUS Oncomine™ (Thermo Fisher Scientific) panel, which allows simultaneous analysis of single nucleotide polymorphisms (SNPs), copy number variation (CNV) , INDEL-type aberrations (insertions/deletions) in tumor DNA, as well as gene rearrangements in mRNA (including the rearrangement of *ALK*, *ROS1*, and *NTRK1-3* genes). The FOCUS panel allows the identification of abnormalities in selected 52 genes associated with various types of solid tumors including lung cancer. From a technical perspective, sequencing was successful. Genetic abnormalities with the status "benign" were detected, being currently of no clinical significance for patients with non-small cell lung cancer (NSCLC). No personalized therapies have been developed so far for patients with such genetic variation; on the other hand, it has not been proven that such genetic abnormalities can cause malignancies. There were substitutions in exon 29 of *ALK* gene: c.4587C>G (p.Asp1529Glu) and c.4381A>G (p.Ile1461Val), occurring outside the tyrosine kinase domain coding region. In addition, an aberration in exon 4 of *FGFR4* (fibroblast growth factor receptor 4) gene was found: c.407C>T (p.Pro136Leu). These abnormalities did not predispose to targeted molecular treatment registered in European Union countries or used in clinical trials.

In the meantime, profiling of the patient's intestinal microbiome was carried out as part of scientific research (consent of the Bioethics Committee of the Medical University of Lublin No. KE-0254/58/2019). The study was performed on the Illumina MiSeq apparatus (Illumina) using Nextera (Illumina) kits, dedicated to small, including bacterial genomes. The composition of the gut microbiome may be a decisive factor in the lack of response to immunotherapy in patients with high PD-L1 expression and no driver mutations. In our patient, we observed a low diversity of particular types of bacteria found in the intestines with a noticeable state of dysbiosis (dysbacteriosis). The majority of the gut microbiome of the examined stool sample (as much as 80.6%) was *Firmicutes* bacterium, including *Lactobacillus*, *Streptococcus*, *Clostridium*, *Veilonella*, *Enterococcus* and *Ruminicoccus* spp. [1]. In healthy people, this group of bacteria accounts for about 45–60% of microbiome bacteria 1]. *Firmicutes* and *Bacteroidetes* together should constitute about 90% of the intestinal microbiome [1]. In our patient it was 88.5%, however, a large disproportion between these two groups of bacteria was visible because *Bacteroidetes* constituted only 7.9%. In a normal biotic state, *Bacteroidetes* should account for 25–45% of the microbiome composition. *Bacteroidetes* include primarily *Bacteroides* and *Prevotella* [1–4]. Figure 1 presents graphically representation of the percentage microbiome composition (at *Phylum* level) in our patient compared to patients with disease control during immunotherapy.

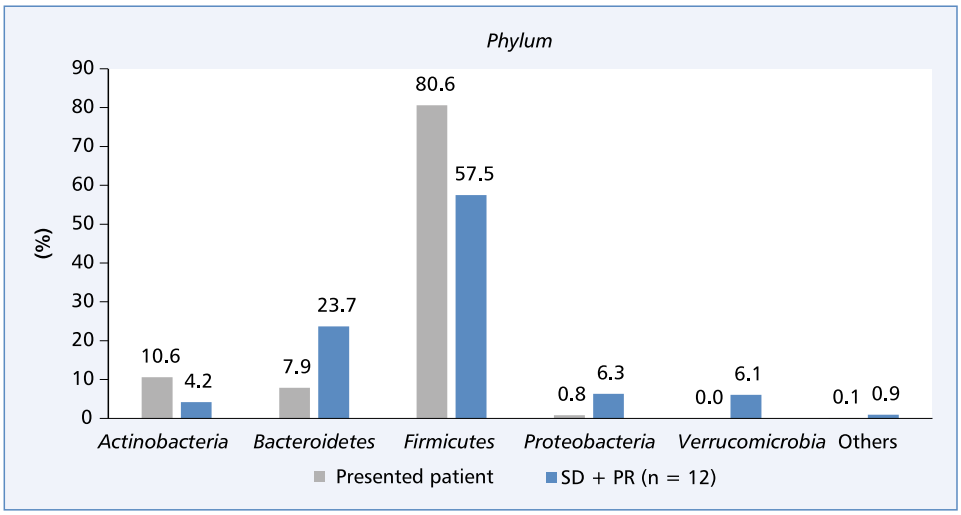


**Figure 1.** Percentage composition of the gut microbiome at *Phylum* level (bacterial type) in a patient with the progression of adenocarcinoma after two administrations of pembrolizumab and 12 NSCLC patients with disease stabilization during immunotherapy. A microbiome was examined in the specimen collected prior to immunotherapy. SD — stable disease; PR — partial response

## Discussion

Vetizou and Trinchieri point to several factors that affect the composition of the gut microbiome [5]: genetic factors, lifestyle, the state of the immune system or the use of antibiotics. According to their opinion, all these factors and the composition of the gut microbiome are connected with the possibility of obtaining a response to immunotherapy in cancer patients. The more diverse the microbiome and the higher the percentage of "beneficial" bacteria in the intestine, the more likely it is to achieve the response to immunotherapy associated with the higher percentage of CD8 + T cells infiltrating the tumor stroma [6]. The "beneficial" bacteria include *Akkermansia muciniphila* (*Verrucomicrobia*, *Akkermansia* spp.), *Enterococcus hirae* (*Firmicutes, Enterococcocae* spp.), *Bifidobacterium longum* (*Actinobacteria*, *Bifidobacteriaceae* spp.), *Collinsella aerofaciens* (*Actinobacteria*, *Coriobacteriaceae* spp.), *Enterococcus faecium* (*Firmicutes*, *Enterococcocae* spp.) [5, 6]. The presence of *Akkermansia mucinifila* seems to be an especially beneficial factor for the possibility of achieving a response to immunotherapy and improving progression-free survival (PFS), which is also indicated by Routy et al. [7]. We did not find *Verrucomicrobia* spp., to which *A. mucinifila* belongs, in the gut microbiome of our patient (0%). In addition, we found only 0.011% of *Enteroccocae* spp., to which *E. hirae* and *E. faecium* belong.

Gopalakrishnan et al. point to an unfavorable intestinal microbiome that may affect the ineffectiveness of anti-PD-1 immunotherapy in patients with skin melanoma [6]. First of all, they indicate a low diversity of intestinal bacteria as a negative predictor of response to anti-PD-1 treatment. They also state that a high percentage of *Bacterioidates* spp. may have an impact on impaired systemic and anti-tumor immune responses, with limited tumor infiltration by immune cells, and inhibited antigen presenting ability of antigen-presenting cells (APCs) [6]. The authors also indicate a positive correlation between the percentage of TCD8 + lymphocytes infiltrating the tumor stroma and the participation of bacteria from the *Ruminococcae* family in the gut microbiome [6]. In our patient's microbiome, we observed 26.1% of this type of microorganisms (Fig. 2), which could be a beneficial predictor for immunotherapy.

Further research on the gut microbiome in cancer patients receiving immunotherapy seems to be necessary to correctly understand the effect of microbiome composition on the effectiveness of this treatment method. It should be remembered that prior to immunotherapy our patient received antibiotics and steroid therapy with methylprednisolone, which has been described in the literature as a negative predictive factor for immunotherapy. Antibiotic therapy was probably responsible for dysbiosis of the gut microbiome, and steroid therapy could additionally inhibit the immune system. On the other hand, many other causes of resistance to immunotherapy cannot be excluded. One of them may be the transformation of commensal bacteria into pathogenic ones. In addition, despite advanced genetic testing, including NGS, low TMB cannot be excluded, and this genetic abnormality requires examination of several hundred genes, not several dozen. Such a study could confirm the existence of a very rare genetic abnormality leading to cancer development. The probability of such a mutation is high due to the young age of the patient and the fact that she does not smoke cigarettes. NSCLC patients with high TMB are mostly heavy smokers. The

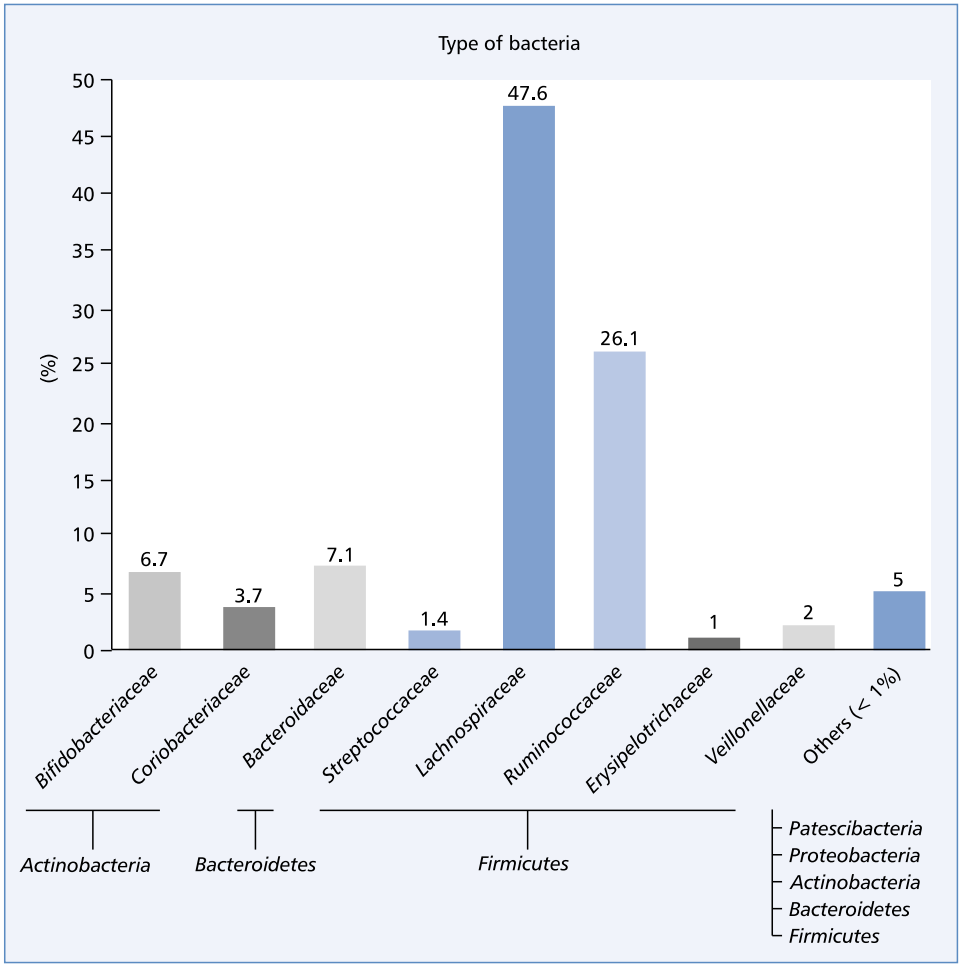


**Figure 2.** Percentage composition of the gut microbiome at the level of bacterial type (family) in a patient with the progression of adenocarcinoma after two administrations of pembrolizumab

carcinogenic effect of tobacco smoke promotes the formation of many somatic mutations in bronchial epithelial cells. A low TMB occurs in non-smokers and is associated with the occurrence of single driver mutations or rearrangements in such genes as *EGFR*, *ALK*, *ERBB2*, *ROS1*, *RET*, *MET*, *NTRK* [9, 10]. A response rate to PD-1 or PD-L1 inhibitors among NSCLC patients was higher in current or former smokers than in non-smokers [11–13]. Therefore, low TMB, smoking status and low diversity of the gut microbiome may be associated with a lack of effectiveness of treatment with immune checkpoints inhibitors in NSCLC patients.

The variety of potential causes of primary resistance to immunotherapy makes us realize how little we know about this method of treatment.

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