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Tumour necrosis factor, interferon-gamma and interleukins as predictive markers of antiprogrammed cell-death protein-1 treatment in advanced non-small cell lung cancer: a pragmatic approach in clinical practice

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Abstract

Background: The emergence of novel antiprogrammed cell death protein-1 (PD-1) inhibitors in non-small cell lung cancers (NSCLC) has revolutionized the therapeutic landscape of this disease. Although overall survival (OS) has improved in the first- and second-line therapy settings for advanced NSCLC, the benefit is not universal. In a climate of global scrutiny for healthcare costs and potential for toxicities related to immunotherapy, appropriate patient selection is crucial. The aim of this study was to evaluate potential prognostic and predictive biomarkers interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and a panel of interleukins (ILs) in the peripheral blood, and assess any correlation with response to anti-PD-1 inhibition, progression-free survival and OS in NSCLC patients.

Methods: We prospectively studied 26 NSCLC patients that received immunotherapy (either pembrolizumab or nivolumab). IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12 were analyzed by flow cytometry at the time of diagnosis and at 3 months after initiation of anti-PD-1 inhibition.

Results: Increased cytokine values (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-6 and IL-8) at the time of diagnosis and at 3 months after initiation of treatment were significantly correlated with improved response to immunotherapy and prolonged OS. There was no correlation between cytokine levels and programmed cell death ligand-1 (PD-L1) expression.

Conclusions: Increased IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12 levels resulted in better response to NSCLC anti-PD-1 inhibition and longer survival, and this could potentially play an important role in selecting patients that would benefit from anti-PD-1 inhibitors.

Keywords: anti-PD-1 treatment, interferon-γ, interleukins, non-small lung cancer, predictive markers, tumour necrosis factor

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Introduction

Immune checkpoint inhibitors have revolutionized the therapeutic landscape of advanced non-small cell lung cancer (NSCLC). Clinical trials have reported favourable responses using programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1) blockade as monotherapy or in combination with chemotherapy.[1–4] On the basis of these favourable results, the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved nivolumab and pembrolizumab as second-line therapies in advanced NSCLC.[5,6] The FDA has also recently approved

atezolizumab for the management of previously treated patients with advanced NSCLC.[7]

Despite prolonged overall survival (OS) in advanced NSCLC, the benefit of immunotherapy is not universal.[8] Currently, PD-L1 is the only biomarker available in clinical practice to identify patients that would be eligible for anti-PD-1 inhibition.[9]

Although several studies have addressed the prognostic role of PD-L1 expression in NSCLC,[10] the results remain controversial.[11]

In a climate of global scrutiny for healthcare costs and potential for toxicities related to immunotherapy, appropriate patient selection is crucial for maximizing clinical benefit and cost effectiveness from this novel treatment. Therefore, identification of prognostic biomarkers for patient selection in pragmatic clinical settings would be of great value in optimizing and personalizing immunotherapy; their use would facilitate identification of patients most likely to benefit from nivolumab and pembrolizumab in terms of clinical response, progression-free survival (PFS) and OS.

In recent years, the role of interleukins (ILs), interferons (IFNs) and tumour necrosis factor-alpha (TNF-α) has been extensively explored in immune regulation in tumour development, autoimmune diseases, allergy and asthma. IL involvement in lung cancer pathogenesis and progression has been thoroughly explored.[12]

The aim of our study was to investigate whether a defined cytokine panel (IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12) can play a prognostic or predictive role in NSCLC patients treated with anti-PD-1 inhibition, with the view to assess any potential correlation between their serum levels and response to treatment response/disease progression.

Materials and methods

This was a prospective study approved by the ethics committee of the G. Papanikolaou Hospital in Thessaloniki, Greece (registration number 93/17) and was conducted in accordance with Good Clinical Practice (GCP) guidelines. All patients provided written informed consent before inclusion in the study.

Patients and samples

We prospectively enrolled patients that met the following criteria: age ⩾18 years, histologically confirmed diagnosis of advanced or metastatic NSCLC, PD-L1 expression detected by immunohistochemistry, Eastern Cooperative Oncology Group performance status 0–1 (inclusive), adequate organ function and capacity to make an informed decision. All patients were negative for epidermal growth factor receptor (*EGFR*) mutation or anaplastic lymphoma kinase (*ALK*) translocation. Patients with a previous history of interstitial lung disease, systemic immunosuppressive therapy or active autoimmune disease were excluded.

Enrolled patients received either pembrolizumab as monotherapy (200 mg for chemotherapy-naïve patients, or 2 mg/kg for patients previously treated with chemotherapy every 3 weeks), or nivolumab administered intravenously at a dose of 3 mg/kg every 2 weeks. Agent choice was based on PD-L1 status and patients' previous treatment history (first- or second-line setting).

PD-L1 immunohistochemistry

Detection of PD-L1 expression was performed by 22C3 pharma DxTM Dako test, in formalin-fixed, paraffin-embedded (FFPE) patients tissue samples, using Autostainer Link 48 (Dako Denmark A/S). The Tumour Proportion Score (TPS) was calculated by evaluating the percentage of PD-L1-positive tumour cells relative to all viable tumour cells present in the specimen.

Tumour PD-L1 expression was confirmed when staining of the tumour cell membrane (at any intensity) was observed at prespecified expression levels of 1% or higher and 50% or higher in a section that included at least 100 tumour cells that could be evaluated.

Grading of toxicity

Toxic effects were graded with the use of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0. Scheduled computed tomography or magnetic resonance imaging was performed every 9 weeks. Treatment continued until confirmed disease progression by investigator-assessed immune-related response criteria, unacceptable toxicity, or withdrawal of consent. Although immune-related

response criteria were evaluated,[13] the primary radiographic assessment was assessed by Response Evaluation Criteria in Solid Tumours (RECIST), version 1.1.[14]

Cytokine testing in blood by flow cytometry

Peripheral blood samples were tested for immune response at the following time points: (a) before the initiation of anti-PD-1 inhibition treatment; (b) after 3 months of anti-PD-1 inhibition or when radiologically confirmed disease progression was reported.

The 3-month follow-up period was decided on the basis of the essential time required for the immune system to convert from innate to adaptive response.[15]

Blood samples were collected from all patients before starting anti-PD-1 inhibitors and at 3 months after initiation of anti-PD-1 inhibitors or at radiologically confirmed disease progression. Serum samples were collected and processed using the same standardized protocol. Briefly, blood samples were left to coagulate at room temperature for 30 min, centrifuged at 3000 g for 20 min (4°C), and the supernatants were collected, made to aliquots, and stored in −80°C until assayed. Time interval between processing and freezing was no more than 2 h for each sample. None of the samples were thawed more than twice before analysis.

The following cytokines were measured in blood samples: IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12. Serum levels of IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and IL-12 were determined using a panel kit (AimPlex Biosciences, Pomona, USA) tested by flow cytometry analysis, using a BD FACS Calibur system (BD Biosciences, San Jose, CA, USA), according to the manufacturer's recommendations and instructions. Specifically, 45 μl of serum sample and 45 μl beads of the panel kit were mixed and then incubated for 1 h in room temperature. After incubation, 0.5 ml of wash buffer was added, and the samples were centrifuged for 5 min. Samples were incubated first with biotin-conjugated monoclonal antibody (30 min) and then subsequently incubated with streptavidin-conjugated monoclonal antibody (20 min). Finally, wash reading buffer was added in all samples.

Statistical analysis

The response duration was defined as the time from first documented evidence of response until progression, according to RECIST. PFS and OS were defined as the time from the first dose of pembrolizumab/nivolumab to progression, according to RECIST, or death (for PFS) or death alone (for OS). We used the Kaplan–Meier method to calculate median values for the duration of response, PFS and OS. We performed Cox regression analysis to examine correlation of biomarker values with PFS and OS, while correlation of these values with patients' response was performed by one-way ANOVA (analysis of variance) statistical test (IBM Statistical Package for Social Sciences version 20, Armonk, New York, United States).

Results

Patients' characteristics

A total of 26 patients were enrolled in the study. Median age was 66.5 years (age range 55–74 years). At the time of data cut off, the median duration of follow up was 6 months, and 16 patients continued to receive anti-PD-1 inhibitors.

Pembrolizumab was offered to 8/26 (31%) patients; 3/8 (37.5%) patients as first-line treatment, and 5/8 (62.5%) patients as second-line treatment. The remaining 18/26 (69%) patients received nivolumab as a second-line treatment. PD-L1 expression was ⩾1% in 13/26 patients (50%). Among them, 10/13 or 10/26 (38.5%) patients had PD-L1 expression in ⩾50% of tumour cells and they were incorporated in the PD-L1 ⩾1 group. Most patients were men and were current or former smokers. The clinical characteristics of patients included in this study are shown in Table 1. A median of six doses of nivolumab and four doses of pembrolizumab were administered.

The rate of confirmed objective response was 31% for all patients who received either nivolumab or pembrolizumab.

At the time of analysis, median PFS for all patients was 4.2 months (95% CI, 2.9–4.8) and OS was 17 months (95% CI, 12.5–23). Patients who expressed PD-L1 ⩾1% had better PFS compared with those with PD-L1 <1% [3.3 months (range 3.0–3.6) *versus* 5.6 months (range 5.1–6.0), respectively, $p < 0.001$]. Furthermore, patients

Table 1. Patients' characteristics.

	Pembrolizumab	Nivolumab
	***n* = 8**	***n* = 18**
Age (years)		
Median	70	63
Sex		
Male	8 (100%)	17 (94%)
Female	0 (0%)	1 (6%)
Smoking status		
Current or former smoker	8 (100%)	18 (100%)
Never smoked	—	—
Histology		
Adenocarcinoma	6 (75%)	6 (33%)
Squamous	2 (25%)	12 (67%)
PD-L1 expression		
0%	0 (0%)	13 (72%)
⩾1%	8 (100%)	5 (28%)
Response		
Progressive disease	2 (25%)	7 (39%)
Stable disease	0 (0%)	9 (50%)
Partial response	6 (75%)	2 (11%)

PD-L1, programmed cell death ligand-1.

with PD-L1 ⩾1% had a better response to treatment.

The most frequently reported treatment-related adverse events were low in severity and included fatigue (in 15% of patients) and hypothyroidism (in 8% of patients). Importantly, they did not lead to discontinuation of treatment or patients' withdrawal from the study.

Cytokines results and clinical outcome to anti-PD-1 inhibition

Levels of IFN-γ, TNF-α and ILs in the peripheral blood of patients were significantly associated with improved treatment response and survival at 3 months after initiation of anti-PD-1 inhibition (Table 2). Cytokine measurements were performed in all patients enrolled in the study, irrespective of their response to treatment. Fluctuations in the value of each biomarker (difference in the first and second measurement) were strongly associated with patients' response and survival (Table 3). Increased cytokine values have been associated with improved response and prolonged survival. Levels of IFN-γ, TNF-α (Figure 1) and ILs (Figure 2) before anti-PD-1 inhibition, after 3 months, and their difference are shown in Figures 1 and 2 in a more understandable way.

There was no correlation between IFN-γ, TNF-α, cytokine levels and PFS.

Moreover, statistical analysis revealed that PD-L1 expression correlated significantly with the difference in IFN-α levels, but not with the other cytokines ($p = 0.03$).

Table 2. First and second measurements in IFN-γ, TNF-α, cytokine levels, their mean values, standard deviation, mean lower and upper bound (95% confidence interval) and their statistical correlation with patient response.

	Mean (pg/ml)	Standard deviation (pg/ml)	Standard error (pg/ml)	95% confidence interval for mean (pg/ml)		*p* value (correlation with response)
				Lower bound	Upper bound	
IFN-γ $_{1st}$	1.23	0.430	0.084	1.06	1.40	0.195
IFN-γ $_{2nd}$	12.38	23.257	4.561	2.99	21.78	0.000
TNF-α $_{1st}$	1.215	0.3663	0.0718	1.067	1.363	0.275
TNF-α $_{2nd}$	1.638	1.0632	0.2085	1.209	2.068	0.12
IL-1β $_{1st}$	1.462	0.6469	0.1269	1.200	1.723	0.265
IL-1β $_{2nd}$	1.615	1.3587	0.2665	1.067	2.164	0.087
IL-2 $_{1st}$	1.231	0.4297	0.0843	1.057	1.404	0.193
IL-2 $_{2nd}$	1.846	1.1897	0.2333	1.366	2.327	0.017
IL-4 $_{1st}$	2.3846	2.48317	0.48699	1.3816	3.3876	0.029
IL-4 $_{2nd}$	4.7692	9.04348	1.77357	1.1165	8.4220	0.04
IL-5 $_{1st}$	1.3846	0.63730	0.12499	1.1272	1.6420	0.379
IL-5 $_{2nd}$	1.0769	0.27175	0.05329	0.9672	1.1867	0.193
IL-6 $_{1st}$	32.5385	33.03239	6.47818	19.1964	45.8805	0.084
IL-6 $_{2nd}$	27.8462	33.46364	6.56276	14.3299	41.3624	0.401
IL-8 $_{1st}$	2.9231	3.65429	0.71667	1.4471	4.3991	0.066
IL-8 $_{2nd}$	67.6923	204.34183	40.07473	−14.8431	150.2278	0.009
IL-10 $_{1st}$	2.3077	2.05464	0.40295	1.4778	3.1376	0.058
IL-10 $_{2nd}$	2.9615	1.85969	0.36472	2.2104	3.7127	0.495
IL-12 $_{1st}$	1.2000	0.33466	0.06563	1.0648	1.3352	0.093
IL-12 $_{2nd}$	1.2769	0.37556	0.07365	1.1252	1.4286	0.726

IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Cytokine levels initially increased before the first administration of anti-PD-1 inhibition and then decreased 3 months later.

Discussion

In this study, we evaluated a small series of patients with a confirmed diagnosis of NSCLC that received anti PD-1 inhibitors nivolumab or pembrolizumab; our sample included patients that had already received first-line chemotherapy as well as chemo-naïve patients.

Most clinical trials measure a single immune system parameter in a single tissue compartment (mostly IFN-γ-secreting T cells in the peripheral blood) and this may insufficiently capture the immunological signature correlating with the development of an appropriate antitumour response. More data suggest that multiple functions of antigen-specific T cells, rather than T-cell numbers *per se*, correlate with clinical outcome. This has been described in studies of protective immune response to microbial infections in both mice and humans.

Table 3. Differences in first and second measurements in IFN-γ, TNF-α, cytokine levels, their mean values and their statistical correlation with patient response and overall survival.

	Mean values (pg/ml)	*p* value (correlation with response)	*p* value (correlation with survival)
DIFN-γ	11.15	0.000	0.002
DTNF-α	0.423	0.006	0.009
DIL-1β	0.154	0.038	0.003
DIL-2	0.62	0.011	0.002
DIL-4	2.38	0.018	0.138
DIL-5	−0.31	0.090	0.018
DIL-6	−4.69	0.014	0.027
DIL-8	64.7	0.008	0.015
DIL-10	0.65	0.081	0.004
DIL-12	0.7	0.482	0.001

DIFN-γ, difference in first and second measurements of IFN-γ; DTNF-α, difference in first and second measurements of TNF-α; DIL, difference in first and second measurements of interleukin; IFN-γ, interferon-gamma; IL, interleukin; TNF-α, tumor necrosis factor-alpha..

Early studies supporting the existence of cancer immune editing revealed an important function for IFN-γ in suppressing tumour development in models of both tumour transplantation and primary tumour induction.[16] In our study, IFN-γ levels have been associated with response to anti-PD-1 inhibitors, supporting its potential in suppressing tumour development.

Overall, IFNs are potent immunomodulators that shape patients' immunity through direct actions on innate and adaptive lymphocytes, enhancement of natural killer cell cytotoxicity and augmentation of dendritic cell function, which are central to the initiation of adaptive immune responses that suppress tumour development.[17]

Soluble TNF-α, along with other proinflammatory factors, recruits and activates neutrophils, macrophages and lymphocytes at the sites of damage and infection.[18]

Certain ILs, including IL-4, IL-1β and IL-6 are involved in key mechanisms of tumourigenesis, reported to be a promising pathway towards cancer immunotherapy.[19] The majority of ILs are secreted by CD4+ T-helper lymphocytes, as well as by monocytes, macrophages and endothelial

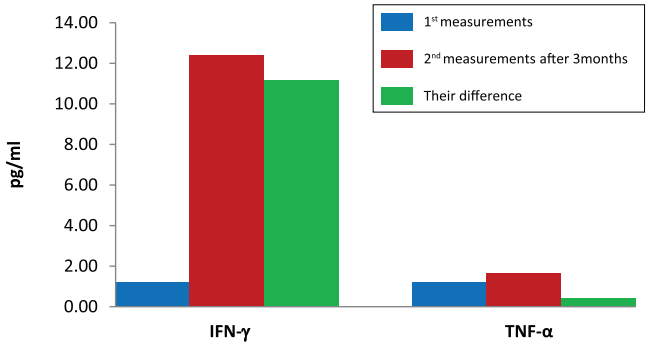

Figure 1. Levels of interferon-γ and tumour necrosis factor-α before immunotherapy, after 3 months, and their difference.
IFN-γ, interferon-gamma; TNF-α, tumour necrosis factor-alpha.

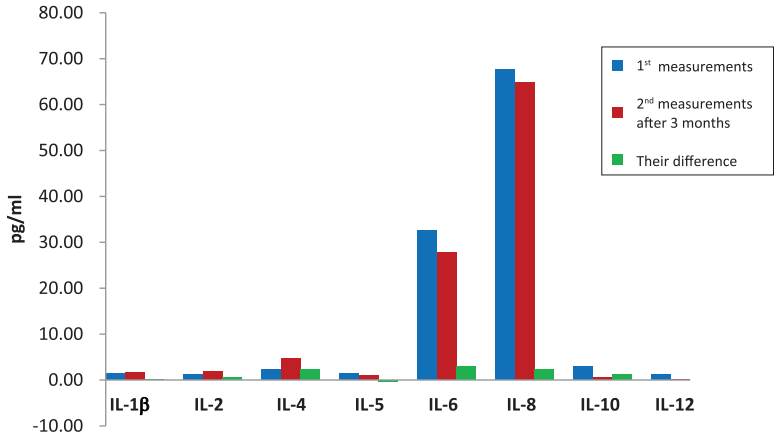

Figure 2. Levels of interleukins before immunotherapy, after 3 months, and their difference.

cells. IL-12 can induce cell cycle arrest, apoptosis in human hepatocellular carcinoma cells, and effectively shift the tumour microenvironment from pro-oncogenic to antitumour through recruitment of immune cells and inhibiting stromal cell growth.[20]

Cytokine concentrations seem to be correlated with PFS. Tran and colleagues reported increased IL-8 concentrations were correlated with shorter PFS in patients with renal cell carcinoma treated with pazopanib.[21] In our study, we found no correlation between cytokine levels and PFS, but this may be attributed to our small sample of patients. This is one of the limiting factors, as well as the fact that some of our patients have received first-line chemotherapy prior to anti-PD-1 inhibitors. The impact of conventional chemotherapy on cytokine levels on a background of second-line anti-PD-1 inhibition remains vague. Unfortunately, large phase III studies have been performed without taking this into account, as biomarkers have not been included in them. The recent negative trials on talactoferrin and anti-MUC-1 are examples of this lack of patient selection beforehand.[22–24]

Conclusion

Immune-checkpoint inhibition clearly has changed the therapeutic approach in NSCLC. Although intratumoural PD-L1 expression appears to identify patients eligible for anti-PD-1 treatment, it is unclear whether it will translate into a stand-alone and clinically meaningful biomarker predictive of clinical response. Cytokine serum levels could provide prognostic information and constitute predictive markers of immunotherapy benefit in patients with advanced NSCLC. Further studies of the predictive effects of these markers in bigger populations are warranted. Overall, the optimal predictive biomarker used in everyday clinical practice should be easily applicable in busy clinical settings, cost effective and provide an accurate prediction of a patient's clinical response. New knowledge obtained from ongoing and future research will refine our approach for the meaningful clinical application of biomarker research.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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