RESEARCH PAPER

Neurologia i Neurochirurgia Polska
Polish Journal of Neurology and Neurosurgery
2019, Volume 53
DOI: 10.5603/PJNNS.a2019.0025

ISSN 0028–3843

Autoimmune response in lung cancer patients with neurological paraneoplastic syndromes

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Abstract

Aim of the study. The aim of this study was to evaluate granzyme B, perforin and FasL expression in peripheral blood mononuclear cells (PBMCs) in lung cancer patients and in paraneoplastic neurological syndromes (PNS).

Clinical rationale for the study. Cellular immune response is activated as part of anti-tumour reaction of the malignancy-bearing host. Paraneoplastic neurological syndromes (PNS) are defined as indirect effects of cancer on the nervous system and are considered immune-mediated. Such stimulation of the immune system may limit the aggressiveness of cancer and the development of metastasis, and thereby improve survival. Granzyme B and perforin pathway, and Fas ligand (FasL) – Fas receptor interaction play an important role in cytotoxic response.

Materials and Methods. Fifty-two patients were included in the study: 28 subjects with PNS and 24 subjects with lung cancer. PNS cases were diagnosed according to the Graus criteria. The presence of onconeural antibodies (anti-Hu/anti-Ri/anti-Yo/anti-Ma/Ta/anti-CV2/anti-amphiphysin/anti-myelin/anti-neuroendothelium/anti-MAG/anti-GAD) was detected with indirect immunofluorescence and confirmed with Line Blotting. The expression of granzyme B, perforin and FasL was detected in PBMCs with ELISA.

Results. PPBMC-FasL expression was increased in lung cancer compared to other patient groups. The granzyme to FasL ratio was significantly higher in lung cancer patients with peripheral than with central PNS involvement. In a multiple regression model, sex was an independent factor influencing PBMC expression of granzyme and perforin.

Conclusions. FasL expression in PBMCs is up-regulated in lung cancer patients. The interplay between granzyme B and FasL may be involved in the development of PNS at the level of the peripheral and the central nervous systems in different manners. Gender is associated with PBMC expression of granzyme B and perforin in lung cancer patients.

Clinical Implications. The novel findings that we report broaden the current knowledge on PNS pathomechanism, with aspects that have not been previously explored. Our findings provide a rationale for further exploration of the granzyme B/FasL pathway with regards to its potential diagnostic value. However, our study is preliminary and needs further research, especially in the context of the prognostic value of the proposed markers.

Key words: lung cancer, paraneoplastic neurological syndromes, cytotoxicity, onconeural antibodies, Fas ligand, granzyme B, perforin

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Introduction

Paraneoplastic neurological syndromes (PNS) are defined as the pathology of the nervous system observed in the course of a neoplastic disease, but unrelated to the direct effects of the tumour mass, such as infiltration, compression or metastasis [1]. PNS diagnosis is based on detailed criteria that consider both the clinical manifestation and the presence of onconeural antibodies [1]. In general, PNS may affect central (encephalitis, limbic encephalitis, brain stem encephalitis, paraneoplastic cerebellar degeneration and opsoclonus/myoclonus) and peripheral (polyneuropathies) nervous systems, as well as the neuromuscular junction (myasthenic syndrome) and skeletal muscles (dermatomyositis). Among the most common malignancies associated with PNS are pulmonary pathologies, particularly small cell lung carcinoma (SCLC) [1, 2]. The clinical significance of PNS stems mainly from the fact that the diagnosis of the neurological pathology often precedes neoplastic manifestation. Patients affected with PNS undergo detailed oncological diagnostic procedures that frequently result in detection of the tumour at an early stage of development. It has been suggested that neoplasms associated with these syndromes are less advanced, that metastases are rare, and that the overall outcome is favourable [3]. Cases of SCLC regression have been reported [4]. Some studies have suggested that in the course of PNS an effective anti-tumour response may develop [3]. Nonetheless, neither the origin of PNS nor the pathophysiological basis of a potentially more favourable outcome of the neoplastic disease is known, and this remains a hypothesis.

The prevailing view on the pathogenesis of PNS is based on the assertion that the immune system attacks the tumour and nervous tissue cells that share common antigens. The humoral response, however, has an unclear role in the pathomechanism of the disease. In some cases in animal models, the passive transfer of antibodies did not lead to nervous system pathology [5]. It appears, in turn, that lymphocytes withdrawn from blood samples of PNS patients were active against the same antigens as the antibodies used in diagnostic procedures e.g. onconeural antibodies. Mononuclear infiltrates have been observed in neuropathological studies of affected nervous tissue [6]. Immunohistopathologically it has been defined that these infiltrates consist mainly of lymphocytes $CD3^+$ and $CD8^+$ as well as less numerous $CD4^+$ cells [7]. Cerebrospinal fluid analysis in PNS patients has revealed an increase of white blood cell count, including elevated T lymphocytes $CD8^+$ and $CD4^+$, natural killer cells (NK) and especially B cells [8]. Interestingly, patients afflicted with SCLC-associated anti-Hu syndrome with high pleocytosis have turned out to have a better overall survival rate [8]. The above-mentioned studies imply that a cell-mediated immune response takes part in the pathogenesis of PNS. One needs to recognise that the type of immune response may depend on the localisation of the antigens under attack. In classical paraneoplastic syndromes, where intracellular antigens (i.e. Hu, Ri, amphiphysin) are targeted by onconeural antibodies, the cellular response seems to play a key role. However, in cases of surface antigens (i.e. NMDA, GABA, AMPA), the humoral response, with antibody-mediated receptor dysfunction, may be of more significance. Apoptosis related to cytotoxic effect is mediated by Tumour Necrosis Factor (TNF) receptor, through FasL receptor (Fas) activation or by means of granzyme B containing granule excretion and perforin involvement. These molecules play a role in autoimmune disorders, but also in anti-tumour reaction.

Granzymes are serine proteases that launch cell death in a number of mechanisms. When released to the extracellular space, they promote an inflammatory reaction [9]. The proapoptotic function appears to be dependent on the presence of perforin that is a prerequisite to their entrance into the cell [10]. Granzymes may induce cytochrome c release, directly activate caspases or interfere with cell membrane integrity by cleavage of laminin B [11]. The most significant molecule in this class is granzyme B. The expression of both granzyme B and perforin turns out to play an important role in the reaction against tumour cells. Their concentration is closely related to survival in patients with neoplastic disease.

Perforin is a Ca^{+2}-dependent pore-forming protein with lytic activity. It leads to apoptosis through up-regulation of caspase-3 activity, release of apoptosis inducing factor (AIF), and cytochrome c from the mitochondria [12].

Fas Ligand (FasL) belongs to the TNF superfamily. It is a membrane protein that induces apoptosis by the specific receptor CD95 on a target cell. FasL is mainly expressed by cytotoxic lymphocytes and natural killer cells, but also by regulatory T lymphocytes. Fas activation induces two major mechanisms of cell death, depending on the cell type. It may either cause activation of mitochondria and release of cytochrome C, or induce direct caspase cascade [13]. Fas/FasL interaction is part of a mechanism to prevent autoimmune reaction. It is the pathway to eliminate overactive or abundant lymphocytes [14]. Improper or insufficient expression of FasL has been proven to trigger autoimmune conditions in mice and in humans [15]. Fas is also involved in the interaction between the emerging tumour and the host immune system. On one hand, neoplastic cells escape lymphocyte cytotoxicity by low expression of Fas protein. On the other hand, tumour cells up-regulate FasL to eliminate immune cells that form a tumour counterattack [16]. The expression of FasL has been revealed at the surface of human lung cancer cells [17].

For the complex interactions between cytotoxic immune cells and target cells expressing well-characterised onconeural antibodies, see Figure 1.

Clinical rationale for the study

In the present study, in order to determine the role of humoral and cytotoxic reactions in lung cancer patients, serum

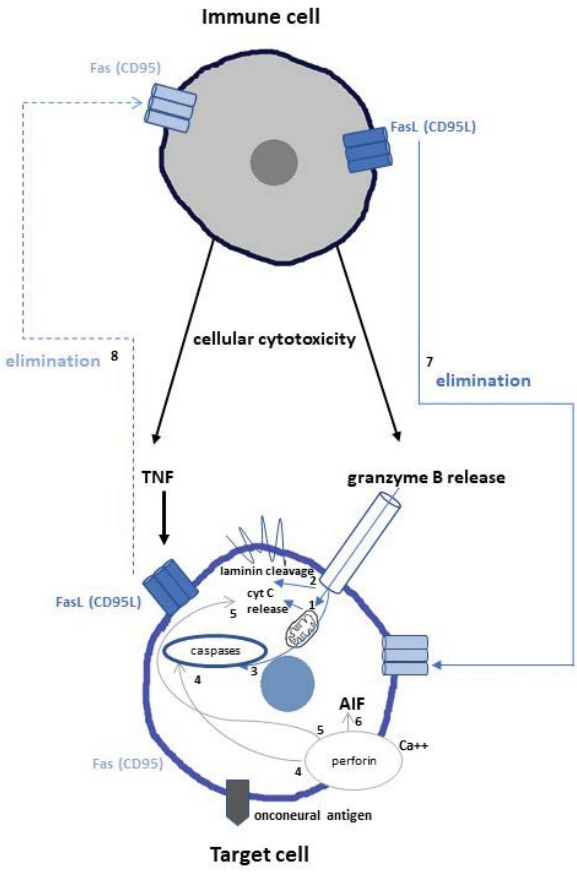

Figure 1. Schematic presentation of the interactions between cytotoxic immune cell and target cell (neoplasm or normal cell expressing onconeural antigen): 1 – granzyme B from immune cell releases cytochrome C from target cell mitochondria; 2 – granzyme B-mediated laminin cleavage; 3 – granzyme B-mediated caspase activation; 4 – perforin-mediated caspase activation; 5 – perforin-mediated cytochrome C release from target cell mitochondria; 6 –perforin-mediated AIF release; 7 – elimination of target cell by immune cell via Fas/FasL interaction; 8 – elimination of immune cell by FasL-expressing tumour cells. AIF – apoptosis inducing factor; cytC – cytochrome C; Ca – calcium; TNF – Tumour Necrosis Factor; FasL – Fas ligand

autoantibodies, as well as the expression of granzyme B, perforin and FasL in peripheral blood mononuclear cells (PBMC), were investigated in the context of PNS. The immunological pathomechanism of paraneoplastic syndrome remains poorly described. A better understanding in this area could contribute to new diagnostic and therapeutic developments.

Material and methods

Fifty-two patients were recruited for the study: 28 subjects with suspected PNS who were hospitalised in the Department of Neurology, Poznan University of Medical Sciences, and 24 subjects with lung cancer who were admitted to the Department of Pulmonology, Allergology and Respiratory Oncology, Poznan University of Medical Sciences. The study protocol was accepted by the Ethical Review Board of Poznan University of Medical Sciences, and each recruited participant gave written informed consent. PNS were diagnosed according to the 2004 Graus criteria [1]. The presence of well-defined onconeural antibodies (anti-Hu, anti-Ri, anti-Yo, anti-Ma/Ta, anti-CV2, anti-amphiphysin) was detected by means of indirect immunofluorescence. If positive, it was confirmed with a Line Blot (Euroimmun, Germany). Indirect immunofluorescence was also used for the detection of other antibodies: anti-myelin, anti-neuroendothelium, anti-myelin associated glycoprotein (anti-MAG) and anti-glutamic acid decarboxylase (anti-GAD). The patterns of positive reactions were evaluated under a fluorescent microscope (EUROStar, Zeiss). Each series of samples was preceded by testing the positive and negative control sera.

PBMCs were isolated from heparinised blood samples by concentration gradient centrifugation (Ficoll Paque Plus, Healthcare) and frozen at -70°C until further analysis. PBMC lysis was carried out with the use of lysis buffer containing a cocktail of proteinase inhibitors (Sigma-Aldrich, USA). In the next step, the expression of cytotoxicity markers: granzyme B (Abcam), perforin (Abcam), FasL (Enzo Life Sciences) was detected in the lysates with the use of ELISA. Protein concentration was estimated by means of the Lowry method [18]. The expression of cytotoxicity markers in PBMCs was expressed in pg/mg protein.

Statistical analysis was performed with the use of licensed MedCalc software version 12.3.0.0. First, the distribution of results was tested using the d'Agostino-Pearson test. The results with Gaussian distribution were expressed as means ± standard deviation (SD) and analysed using the Student's t-test. The results with a non-Gaussian distribution were expressed as median and interquartile range, and analysed using a non-parametric Mann-Whitney test.

Results

Having included all PNS and lung cancer patients, in the final study group there were 31 (60%) lung cancer patients, nine (17%) ovarian cancer, two (4%) prostate cancer, one (2%) with other malignancies, and nine (17%) cases without an identified malignancy.

Of the 31 lung cancer patients, 24 were referred from the Department of Pulmonology, Allergology and Respiratory Oncology, and the other seven were diagnosed in the Department of Neurology where they were admitted for suspected PNS.

Clinically, 20 (64.5%) patients manifested peripheral nervous system involvement (polyneuropathy/neuropathy, myasthenic syndrome, myopathy) and 11 (35.5%) had central nervous system involvement (cerebellar syndrome, motor neurone disease, extrapyramidal syndrome).

Table 1. The expression of FasL in peripheral blood mononuclear cells

Studied group	Studied subgroup	FasL [pg/mg of protein]	p
Lung cancer patients (n = 31)	Total (n = 31)	7.39 0.02–11.87	p = 0.0171*
	PNS (n = 17)	0.11 0.02–12.07	p = 0.9816
	No PNS (n = 14)	8.55 0.02–11.80	
	Well-defined antibodies (n = 8)	0.07 0.03–4.87	p = 0.1043
	Seronegative (n = 15)	11.38 6.25–12.06	
Other cancer patients (n = 12)	Total (n = 12)	0.027 0.01–5.73	p = 0.9955
	PNS (n = 8)	0.05 0.02–8.49	
	No PSN (n = 4)	0.05 0.02–8.11	
	Well-defined antibodies (n = 5)	0.06 0.02–11.60	p = 0.3209
	Seronegative (n = 6)	0.03 0.02–7.03	
All patients (n = 52)	PNS manifestation (n = 31)	0.06 0.02–11.85	p = 0.7052
	Without PNS symptoms (n = 21)	0.06 0.02–9.25	
Antibodies	1. Well-defined onconeural antibodies (n = 15)	0.09 0.02–8.09	1 vs 3: p = 0.7952 2 vs 3: p = 0.9088 1 vs 2: p = 0.5841 (1+2) vs 3: p = 0.8146
	2. Other autoantibodies (n = 15)	0.06 0.02–10.56	
	3. Seronegative (n = 26)	4.11 0.01–11.48	

*Statistically significant difference

In 15 (29%) patients, the presence of well-defined onconeural antibodies (anti-Hu, anti-amphiphysin, anti-Ri, anti-Yo, anti-Ma/Ta) was detected, and in 34 subjects (65%) other autoantibodies (anti-Tr, anti-MAG, anti-myelin) were found. In the lung cancer patients, eight (27%) had well-defined onconeural antibodies (anti-Hu, anti-amphiphysin, anti-Ri, anti-Yo, anti-Ma/Ta) and eight (27%) were seropositive for other autoantibodies (anti-Tr, anti-MAG, anti-myelin).

Coexisting autoantibodies were detected in 17% of lung cancer patients. The coexistence profile included: anti-Hu with anti-Ma/Ta, anti-Hu with anti-amphiphysin, anti-Hu with anti-myelin, and anti-Ri with anti-myelin. In 17% of other neoplasms, the coexistence of onconeural antibodies was detected (anti-Ri with anti-amphiphysin and anti-Ma/Ta with anti-amphiphysin and with anti-Tr).

The expression of FasL in PBMCs was increased in lung cancer patients compared to other groups of patients (Tab. 1).

No differences in granzyme B expression in PBMCs were observed between the studied subgroups of patients (Tab. 2). The Granzyme B to FasL ratio differed (P = 0.0180) between lung cancer patients with peripheral (11,589; 166-58,242) and central PNS (73; 34-112).

The expression of perforin in PBMCs of lung cancer patients with PNS was lower than in asymptomatic subjects (Tab. 3). And we observed a trend for down-regulation of perforin in lung cancer patients compared to other neoplasms (Tab. 3).

In multiple regression analysis with gender, the presence of small cell lung cancer, onconeural antibodies and diagnosis of PNS included in the model, gender was an independent factor influencing PBMC expression of granzyme (b = 1,536; p = 0.0126) and perforin (b = 30,925; p = 0.0174).

For the detailed characteristics of each individual patient with PNS in the study cohort, see supplementary Table 4.

Discussion

In the present study, lung cancer has been identified as a systemic malignancy that is associated with both the humoral response directed against neural antigens, as well as

Table 2. The expression of Granzyme B in peripheral blood mononuclear cells

Studied group	Studied subgroup	Granzyme B [pg/mg of protein]	p
Lung cancer patients (n = 31)	Total (n = 31)	1,839 1,150–3,213	p = 0.8965
	PNS (n = 17)	1,824 1,265–2,640	p = 0.7297
	No PNS (n = 14)	1,958 1,001–3,740	
	Well-defined antibodies (n = 8)	1,580 930–2,640	p = 0.8430
	Seronegative (n = 15)	1,708 1,177–2,184	
Other cancer patients (n=12)	Total (n = 12)	1,961 1,432–2,576	p = 0.8124
	PNS (n = 8)	1,959 1,284–2,741	
	No PNS (n = 4)	1,895 1,332–2,628	
	Well-defined antibodies (n = 5)	1,936 1,302–2,766	p = 0.8344
	Seronegative (n = 6)	1,961 1,268–2,716	
All patients (n=52)	PNS manifestation (n = 31)	1,895 1,304–2,606	p = 0.8481
	Without PNS symptoms (n = 21)	1,958 1,116–2,922	
Antibodies	1. Well-defined onconeural antibodies (n = 15)	1,936 1,268–2,594	1 vs 2: p = 0.6639 1 vs 3: p = 0.7328 2 vs 3: p = 0.5929 (1+2) vs 3: p = 0.9299
	2. Other autoantibodies (n = 15)	1,942 1,167–3,612	
	3. Seronegative (n = 26)	1,839 1,292–2,791	

*Statistically significant difference

with changes of cytotoxicity markers expression on peripheral blood mononuclear cells. Onconeural antibodies are currently considered a diagnostic tool for the definitive recognition of PNS in general [1], and lung cancer patients in particular [2], but their role in PNS pathogenesis remains unclear. While for many years onconeural antibodies were not considered pathogenic [5], the discovery of clearly pathogenic antibodies directed against surface receptors (i.e. anti-NMDAR) has changed this perspective.

In this study, we observed well-defined onconeural antibodies, and even their coexistence in lung cancer patients. However, the expression of granzyme B, perforin and FasL in PBMCs did not differ between seropositive and seronegative patients. Such an observation suggests an independence of the humoral and the cellular immune response in lung cancer patients. The cytotoxic effects in host-tumour interactions that lead to PNS play a particularly important role in the remote effects on the nervous system. Apoptosis and neurodegeneration triggered by mechanisms associated with cytotoxicity may be mediated by granzyme B, perforin and/or FasL. Mononuclear cells infiltrating dentate nucleus in paraneoplastic cerebellar degeneration express granzyme B [7].

Recently, an immunopathological study on autoimmune encephalitis, including cases of paraneoplastic origin, showed a higher CD8/CD3 ratio and a substantial collocation of neurons and T lymphocytes expressing granzyme B [19]. Based on the observed differences between immune reactions against intracellular antigens, Bien et al. suggested the crucial role of cytotoxic reaction mediated by T lymphocytes in cases where response against intracellular antigens was developed, while in autoimmune reaction against surface antigens the involvement of antibodies and complement takes place [19]. Cell specificity analysis revealed the existence of cytotoxic T lymphocytes aggressive towards HuD protein [20], which is an antigen expressed on both small cell lung cancer cells and neurons. One of the most common PNSs is Hu-syndrome

Table 3. The expression of Perforin in peripheral blood mononuclear cells

Studied group	Studied subgroup	Perforin [pg/mg of protein]	p
Lung cancer patients (n = 31)	total (n = 31)	13365 3634–38240	p = 0.0606
	PNS (n = 17)	12633 3968–38949	p = 0.0404*
	no PNS (n = 14)	23480 3639–35426	
	well-defined antibodies (n = 8)	14097 9608–38578	p = 0.9788
	seronegative (n = 15)	11138 3220–37822	
Other cancer patients (n = 12)	total (n = 12)	29663 20336–35881	p = 0.7311
	PNS (n = 8)	27307 16753–35664	
	no PNS (n = 4)	23135 11625–35893	
	well-defined antibodies (n = 5)	24986 6997–35447	p = 0.7490
	seronegative (n = 6)	26587 12633–35881	
All patients (n = 52)	PNS manifestation (n = 31)	22707 7592–33451	p = 0.4669
	without PNS symptoms (n = 21)	24105 11078–37065	
Antibodies	1. well-defined onconeural antibodies (n = 15)	26620 12633–33143	1 *vs* 3: p = 0.7952 2 *vs* 3: p = 0.7892 1 *vs* 2: p = 0.9128 (1+2) *vs* 3: p = 0.7491
	2. other autoantibodies (n = 15)	23480 8925–39325	
	3. seronegative (n = 26)	21736 4754–35012	

*Statistically significant difference

that coexists with SLCC and typically manifests as an encephalomyelitis or sensory neuronopathy associated with anti-Hu antibodies. Tumour infiltrating lymphocytes have also been shown to react specifically with HuD antigen [21]. However, in Hu-syndrome, two distinct functional forms of cytotoxic lymphocytes have been described. It appears that in the acute phase of paraneoplastic syndrome, specific immune cells release type 1 cytokines (tumour necrosis factor-alpha, interleukin-6, interleukin-17), which promote a cell-mediated cytotoxic response. On the other hand, in the chronic phase of the disease, lymphocytes instead produce type 2 cytokines (interleukin-4, interleukin-5, interleukin-13) that abate an anti-tumour cytotoxic reaction. This functional transition was thought to be related to cytokines released by the SCLC [22]. In experimental conditions, paraneoplastic cerebellar degeneration was associated with interplay between tumour necrosis factor-alpha and macrophage chemoattractant protein-1 [23]. Thus, it can be hypothesised that the initially aggressive cell-mediated immune response that triggered paraneoplastic syndrome was distorted by secretions released by the growing tumour.

Our study is in concordance with the above-mentioned observations, because in our cohort of lung cancer patients we have found both antibodies against intracellular antigens (anti-Hu, anti-Ri, anti-amphiphysin, anti-Yo, anti-Ma/Ta) and up-regulated markers of cytotoxicity in PBMCs (namely FasL). However, in our cohort the spectrum of cytotoxicity markers differs from some other studies. Bernal et al. (2002) did not find Fas and FasL-positive cells in the infiltrates in anti-Hu-associated paraneoplastic encephalomyelitis [24]. Similarly, Tüzün et al. (2009) observed only rare cells with granzyme B, perforin and Fas/Fas ligand expression in ovarian teratoma patients with NMDA-encephalitis [25]. One possible explanation for such discrepancies could be that the patient groups differed in terms of the underlying malignancy, clinical manifestation, and associated antibodies. This may confirm the hypothesis of different pathomechanisms involved in PNS depending on target antigens in autoimmune response.

Table 4. Clinical and immunological characteristics of each individual patient with paraneoplastic neurological syndrome in the study cohort

	Sex	Age	Type of malignancy	Neurological syndrome	PNS (1 - yes, 0 - no)	Onconeural antibody	well characte-rised (1 - yes, 0 - no)	FasL [pg/mg of protein]	Granzyme [pg/mg of protein]	Perforin [pg/mg of protein]
1	F	56	SCLC	Polyneuropathy	1	Anti-Ri, anti-myelin	1	0.115	1,242.580	12,633.078
2	F	46	SCLC	None	0	Anti-myelin	0	0.021	3,627.713	23,480.211
3	F	60	SCLC	Sensory Polyneuropathy	1	None	0	0.023	1,332.100	23,135.136
4	M	65	NSCLC, adenocarcinoma	None	0	Anti-myelin	0	0.021	4,077.149	27,306.679
5	M	64	NSCLC, adenocarcinoma	None	0	Anti-MAG	0	0.010	5,604.870	84,852.121
6	F	55	NSCLC, adenocarcinoma	Sensory Polyneuropathy	1	Anti-MAG	0	11.732	1,941.736	8,186.737
7	F	66	NSCLC, adenocarcinoma	None	0	None	0	13.307	2,861.381	32,612.500
8	M	73	NSCLC, squamous cell lung carcinoma	Sensory Polyneuropathy	1	Anti-Ma/Ta, Anti-Hu	1	0.010	4,053.088	22,706.826
9	M	60	NSCLC, squamous cell lung carcinoma	None	0	Anti-Tr	1	0.046	1,579.724	43,868.208
10	M	68	NSCLC, squamous cell lung carcinoma	Polyneuropathy	1	Anti-myelin	0	0.061	3,564.162	69,232.846
11	M	71	NSCLC	Cerebellar syndrome (PCD)	1	None	0	0.028	4,055.793	53,452.626
12	M	67	NSCLC	None	0	None	0	12.012	4,962.908	100,975.176
13	F	56	NSCLC (adenocarcinoma. bronchiolo--alveolar carcinoma)	None	0	None	0	8.321	1,957.548	24,104.858
14	M	65	SCLC	Polyneuropathy	1	Anti-Hu, anti--amphiphysin	1	13.905	2,628.593	44,220.834
15	F	79	SCLC	None	0	None	0	11.376	832.156	10,895.106
16	F	46	SCLC	None	0	Anti-myelin	0	20.166	1,056.799	11,138.486
17	M	54	NSCLC, large cell lung carcinoma	None	0	Anti-MAG	0	0.021	2,174.901	3,912.751
18	M	71	NSCLC	Cerebellar syndrome (PCD)	1	None	0	11.139	1,853.636	5,807.010
19	F	74	NSCLC, squamous cell lung carcinoma	None	0	None	0	11.732	84.882	1,647.467
20	M	63	Squamous cell lung carcinoma	Polyneuropathy	1	None	0	22.325	1,824.257	1,935.924
21	F	57	Lung adenocarcinoma	MND	1	Anti-MAG	0	13.307	776.313	498.359
22	F	40	Lung adenocarcinoma	None	0	None	0	8.549	293.349	687.356
23	M	61	NSCLC	None	0	None	0	11.480	1,291.789	2,818.571
24	M	65	NSCLC	None	1	Anti-Ri	1	x	x	x
25	F	57	No malignancy identified	Polyneuropathy	1	Anti-Yo	1	16.459	1,936.289	28,916.707
26	M	73	No malignancy identified	Myasthenic syndrome	1	Anti-Ma/Ta, anti-Ri, anti-amphiphysin, anti-myelin	1	5.728	1,276.509	27,372.811

Table 4. cd. Clinical and immunological characteristics of each individual patient with paraneoplastic neurological syndrome in the study cohort

	Sex	Age	Type of malignancy	Neurological syndrome	PNS (1 - yes, 0 - no)	Onconeural antibody	well characterised (1 - yes, 0 - no)	FasL [pg/mg of protein]	Granzyme [pg/mg of protein]	Perforin [pg/mg of protein]
27	M	82	NSCLC, squamous cell lung carcinoma	Polyneuropathy	1	None	0	12.188	1,707.761	70,424.531
28	F	54	Ovarian cancer	Cerebellar syndrome (PCD)	1	Anti-Tr, anti--amphiphysin	1	0.025	x	59,777.031
29	F	59	Ovarian cancer	None	1	Anti-Ri, anti-amphiphysin, anti-Ma/Ta	1	0.016	2,546.217	30,409.217
30	F	60	Ovarian cancer	Polyneuropathy	1	None	0	0.017	2,410.973	22,623.438
31	F	58	No malignancy identified	Polyneuropathy	0	None	0	8.193	2,554.212	20,303.411
32	F	74	Unidentified middle ear carcinoma	Movement disorder	0	Anti-myelin	0	0.021	1,498.024	40,472.876
33	F	53	Ovarian cancer	Cerebellar syndrome (PCD)	1	None	0	0.007	2,690.271	34,374.682
34	F	59	Ovarian cancer	None	0	Ma	1	8.087	1,409.857	5,455.177
35	M	71	No malignancy identified	None	0	Anti-myelin	0	7.026	1,767.929	18,473.667
36	F	56	Ovarian cancer	None	0	None	0	0.014	3,269.178	35,012.063
37	F	56	Ovarian cancer	None	0	None	0	0.006	2,942.212	32,237.117
38	F	72	Uncharacterized lung carcinoma	Polyneuropathy	1	Anti-Hu	1	6.457	571.380	14,097.272
39	F	74	No malignancy identified	Extrapyramidal syndrome	0	None	0	0.005	1,961.048	20,336.313
40	M	76	Prostate cancer	MND	1	None	0	0.035	1,649.618	16,178.975
41	F	52	No malignancy identified	Polyneuropathy	0	None	0	0.046	x	22,308.114
42	F	47	Ovarian cancer	Polyneuropathy	1	Amphiphysin	1	16.459	2,583.096	33,143.388
43	F	62	Ovarian cancer	Cerebellar syndrome	1	Anti-Yo	1	0.024	2,344.540	25,866.552
44	M	49	Lung carcinoma	None	0	None	0	0.007	2,791.262	35,928.829
45	F	51	Lung carcinoma	Myasthenic syndrome	1	Anti-Hu, anti-myelin	1	0.067	825.835	8,600.255
46	F	51	Lung carcinoma	Myasthenic syndrome	1	Anti-Hu, anti-myelin	1	0.023	2,644.582	0.000
47	F	77	No malignancy identified	Neuropathy	0	Anti-myelin	0	0.060	671.414	35,881.237
48	F	54	No malignancy identified	None	0	None	0	0.029	848.222	102,962.118
49	M	86	Prostate cancer	Polyneuropathy	1	None	0	0.013	1,034.246	4,753.848
50	F	54	Carcinoma solidum of the lung	Cerebellar syndrome	1	None	0	0.017	1,640.127	3,354.361
51	F	35	No malignancy identified	Myopathy	0	None	0	0.089	x	41,252.860
52	F	66	NSCLC, squamous cell lung carcinoma	Polyneuropathy	1	None	0	x	x	x

SCLC – small cell lung carcinoma; NSCLC – non-small cell lung carcinoma; PNS – paraneoplastic neurological syndrome; F – female; M – male

Interestingly, we confirmed that gender has an influence on PBMC expression of granzyme B and perforin in lung cancer patients. This observation requires further investigation and confirmation on a larger sample.

We expected to find increased levels of immune cytotoxicity markers in lung cancer patients with PNS, compared to those without neurological involvement. Neuronal destruction observed in the course of paraneoplastic syndromes could be driven by direct cytotoxic effects of immune cells activated in response to the tumour. In this case, the overexpression of immune cell-FasL, which enhances target (nervous system) cell elimination via interaction with Fas, and overexpression of membranolytic proteins (granzyme B and perforin), could reflect the role of cellular response in PNS pathophysiology. We did not manage to confirm this hypothesis. However, we must acknowledge that our study is limited by a relatively small sample size and the marked heterogeneity of our study cohort. It would be worthwhile to assess and compare cytotoxicity markers expression in larger and more focused cohorts, for example between cohorts with different antibody profiles (ant-Hu, anti-amphiphysin etc.) or larger samples of different malignancies.

It should be emphasised that in nine patients who were included in the study cohort, no malignancies were identified. However, according to the 2004 Graus criteria [1], when malignancy is not detected, it is still possible to diagnose a paraneoplastic syndrome. In fact, for definitive PNS, a neurological syndrome (classical or not) with well characterised onconeural antibodies (anti-Hu, Yo, CV2, Ri, Ma2, or amphiphysin) and no cancer, is considered paraneoplastic. For possible PNS, the criteria name two situations: a classical syndrome with no onconeural antibodies, no cancer, but at high risk of having an underlying tumour; or a neurological syndrome (classical or not) with partially characterised onconeural antibodies and no cancer.

In summary, ours is a preliminary study showing that lung cancer patients manifested a broader spectrum of coexisting autoantibodies than other patients with PNS in our cohort, namely patients with other malignancies and without an identified malignancy. FasL expression in PBMC is up-regulated in lung cancer patients. The interplay between granzyme B and FasL may be involved in the development of PNS at the level of the peripheral and central nervous systems in different manners.

Clinical implications and future directions

The novel findings that we report broaden the current knowledge regarding PNS pathomechanism with aspects that have not been previously explored. However, our study is preliminary and needs further research. Firstly, it would be valuable to verify protein expression by other methods, i.e. PCR or flow cytometry. Secondly, measurement of protein levels in particular lymphocytic populations, and not just in total PBMCs, could aid in the interpretation of the results. Importantly, in this study we did not include data with regards to treatment and response to therapy, so we could not analyse the prognostic aspect of our findings.

However, the purpose of this article was to provide an insight into the pathophysiology of PNS and to assess the potential diagnostic value of the proposed markers. For future studies, it would be interesting to investigate the prognostic value of the proposed markers as well.

Conflict of interest statement: *The authors declare no conflict of interest.*

Funding: *Not applicable (no external funding was provided for the study).*

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