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ORIGINAL ARTICLE

PD-L1 gene copy number and promoter polymorphisms regulate PD-L1 expression in tumor cells of non-small cell lung cancer patients

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Abstract

Most drugs targeting PD-1 or PD-L1 are more effective when cancer cells of non-small cell lung cancer (NSCLC) patients express PD-L1 protein. The polymorphisms of *PD-L1* gene and *PD-L1* gene copy number could be responsible for PD-L1 mRNA and protein expression.

We analyzed PD-L1 protein expression using two IHC assays, mRNA (PD-L1) expression by qRT-PCR, *PD-L1* gene promoter region polymorphisms (rs822335 and rs822336) by qPCR and *PD-L1* gene copy number by fluorescence in situ hybridization method.

Patients with CC genotype in rs822335 had significantly ($p = 0.043$) higher percentage of tumor cells with PD-L1 expression (test with 22C3 antibody) than patients with CT or TT genotypes. *PD-L1* gene copy number significantly positively correlated with percentage of tumor cells with PD-L1 expression detected in tests with 22C3 antibody ($p = 0.005$, $R = +0.442$) and with SP142 antibody ($p = 0.021$, $R = +0.369$). *PD-L1* gene copy number did not correlate with PD-L1 mRNA expression. Patients with PD-L1 expression tested with 22C3 antibody had significantly higher expression of PD-L1 mRNA ($p = 0.023$), number of chromsosme 9 centromeres ($p = 0.023$) and *PD-L1* gene copy number ($p = 0.003$) than patients without PD-L1 expression on tumor cells

PD-L1 gene polymorphisms and *PD-L1* gene copy number may be a predictor for PD-L1 protein expression on tumor cells.

Keywords PD-L1 in NSCLC, Single nucleotide polymorphisms, Gene copy number.

Introduction

Immunotherapy with immune-checkpoint inhibitors is a promising treatment method for locally advanced or advanced non-small cell lung cancer (NSCLC) patients. Monoclonal antibodies against programmed death 1 (PD-1) and against its

Abbreviations: PD-1, programmed death 1; PD-L1, programmed death ligand 1; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry; SNPs, single nucleotide polymorphisms; FFPE, formalin-fixed paraffin-embedded; NOS, not-otherwise specified; CNS, central nervous system; FISH, fluorescence in situ hybridization; SqCC, squamous cell cancer; 3′UTR, 3′untranslated region; PFS, progression free survival; CNA, copy number alteration; TMB, tumor mutation burden; IFN-γ, Interferon Gamma; IDO1, Indoleamine 2,3-Dioxygenase 1.

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ligand (PD-L1) are used in several indications. Nivolumab (anty-PD-1 antibody) and atezolizumab (anti-PD-L1 antibody) were approved in second line therapy in a wide group of NSCLC patients [1,2]. While pembrolizumab (anti-PD-1 antibody), in monotherapy, could be used in first or second line treatment of NSCLC patients with PD-L1 expression on tumor cells. Pembrolizumab is also applied in first line treatment, in combination with chemotherapy, in tretament-naïve patients [3–5]. Durvalumab (anti-PD-L1 antibody) was approved for the treatment of patients with locally advanced, unresectable stage III NSCLC, who have not progressed following chemoradiotherapy [6].

Immunotherapy is designed to activate the cytotoxic function of T lymphocytes in the tumor environment. The coupling of PD-L1 on tumor or antigen presenting cells with PD-1 on lymphocytes causes functional exhaustion of lymphocytes (negative "immunological synapse"). This is despite the fact that lymphocytes recognize cancer antigens and they are activated by co-stimulatory molecules (e.g. coupling B7-1 or B7-2 molecules with CD 28 molecule – positive "immunological synapse"). Usage of monoclonal antibodies against PD-1 or PD-L1, reactivates the cytotoxic function of lymphocytes [7]. It seems that immune-checkpoints inhibitors should be effective only in patients with PD-L1 expression on tumor cells. Indeed, the response to anti-PD-1 or anti-PD-L1 antibodies is observed more commonly in patients with PD-L1 expression on tumor cells. In fact, the expression of PD-L1 on tumor cells is the only recognized predictive factor for immunotherapy in NSCLC patients, however the response to immunotherapy is also possible in patients without PD-L1 expression on tumor cells [1,2,5,6]. Moreover, the reliability of immunohistochemistry (IHC) assessing PD-L1 expression on tumor and immune cells is insufficient. The result of the IHC examination depends on the type of monoclonal antibodies used for diagnosis (22C3, SP142, SP263, 28-8, 73-10 monoclonal antibody clones), type of diagnosed material, time of sample collection (archival material) and pathologists' subjective assessment [8,9]. Therefore, other predictors for immunotherapy should be discovered (intensive research on tumor mutation burden and microsatellite instability as predictive factors for immunotherapy are underway). In addition, the molecular background of differences in PD-L1 expression on tumor cell in particular patients should be investigated.

Our study, in which we analyzed the effect of single nucleotide polymorphisms (SNPs) in the promoter region of *PD-L1* gene on PD-L1 protein and mRNA expression in NSCLC tumor cells, is unique worldwide. We studied two polymorphisms: rs822335 and rs822336 in promoter of *PD-L1* gene. Simulations, based on appropriate databases, indicated that these polymorphisms can affect the activity of the *PD-L1* gene promoter. Moreover, we examined the correlation between *PD-L1* gene copy number and PD-L1 protein and mRNA expression.

Materials and methods

Patients

We enrolled 47 NSCLC patients (median age: 65 $\pm$ 7.6 years). Tissue samples (formalin-fixed paraffin-embedded, FFPE) from surgically resected primary tumors, neurosurgically resected central nervous system (CNS) metastases and bronchoscopy biopsies (cellblocks) were available. Adenocarcinoma was diagnosed in 30 patients, squamous cell carcinoma in 13 and NSCLC NOS (not-otherwise specified) in 4 patients. All patients were radiotherapy and chemotherapy naïve. Detailed characteristic of our study group was described in Table 1.

Immunohistochemistry (IHC)

Immunohistochemical analyses of PD-L1 protein expression were carried out on paraffin embedded tissue cut into 3 µm sections and fixed on Thermo Scientific Superfrost Plus™ glass slides. All glass slides with tissue sections were preheated in 59 °C on hotplate, prior to IHC staining, for at least 3 h.

PD-L1 protein IHC staining was carried out using two different antibody clones – Ventana SP142 and Dako 22C3. The IHC staining procedure using Ventana antibody was carried out on Ventana Benchmark GX equipment, using CE-IVD approved Ventana PD-L1 (SP142) Assay, utilizing OptiView Amplification Kit and OptiView DAB IHC Detection Kit as a detection system. Counterstaining, using hematoxylin (Ventana Medical System, Tucson, AZ, USA), was included in the staining protocol. Rabbit monoclonal negative control immunoglobulin (Ventana Medical System, Tucson, AZ, USA) was used as a negative control.

Deparaffinization and antigen retrieval was carried out prior to the staining procedure on Dako PT Link equipment. The IHC staining procedure using Dako (Denmark) antibody was carried out on Dako Autostainer Link 48 equipment, utilizing CE-IVD approved PD-L1 IHC 22C3 PharmDx kit, using EnVision FLEX visualization system and counterstaining with hematoxylin, as a part of the staining protocol. As previously described [9].

After staining all glass slides were washed and dehydrated in a series of two 96% ethanol and two xylene washing steps, and then covererslipped. Pathomorphological analysis was performed if at least 100 tumor cells outside the necrosis area were present in the slides. In this experiment, PD-L1 expression was only evaluated on tumor cells.

The cut of points for the assessment of cancer cell percentages with and without PD-L1 expression ($<$50% and $\geq$50% of tumor cells with PD-L1 expression) were adopted from the KEYNOTE-024 clinical trial, which compared the efficacy of pembrolizumab and first line chemotherapy based on platinum compounds. In this study, the expression of PD-L1 was assessed with IHC assay using 22C3 antibody clone [3]. In our experiment, patients were divided as positive ($\geq$50% of tumor cells with PD-L1 expression) and negative ($<$50% of tumor cells with PD-L1 expression) in terms of PD-L1 expression. However, in all patients, the percentage of tumor cells with PD-L1 expression was also precisely determined. The percentage of tumor cells expressing PD-L1 was subsequently correlated with the occurrence of genetic factors.

Fluorescence in situ hybridization (FISH)

The ZytoLight SPEC CD274, PDCD1LG2/CEN9 Dual Color Probe (CE-IVD marked, Zytovision, Germany) was used to

Table 1 Characteristics of NSCLC patient according to frequency of genotypes of analysed polymorphisms in *PD-L1* gene.

Characteristic	rs822335			p, χ^2	rs822336			p, χ^2
	CC	CT *n* (%)	TT		CC	CG *n* (%)	GG	
Age								
< 65	12 (54.5)	10 (45.5)	0 (0)	0.162	5 (22.7)	10 (45.5)	7 (31.8)	0.442
≥ 65	9 (36)	13 (52)	3 (12)	3.643	7 (28)	14 (56)	4 (16)	1.633
Gender								
Male	10 (50)	9 (45)	1 (5)	0.805	5 (25)	8 (40)	7 (35)	0.242
Female	11 (40.7)	14 (51.9)	2 (7.4)	0.435	7 (25.9)	16 (59.3)	4 (14.8)	2.839
Histology								
SqCC	5 (38.5)	7 (53.8)	1 (7,7)	0.864	1 (7.7)	8 (61.5)	4 (30.8)	0.218
Other	16 (47.1)	16 (47.1)	2 (5.8)	0.292	11 (32.3)	16 (47.1)	7 (20.6)	3.043
***EGFR, ALK* genes status**								
Wild-type	18 (50)	16 (44,4)	2 (5.6)	0.412	9 (25)	19 (52.8)	8 (22.2)	0.906
Mutated	3 (27.3)	7 (63.6)	1 (9.1)	1.773	3 (27.3)	5 (45.5)	3 (27.3)	0.197
PD-L1 expression (22C3)								
Negative	9 (34.6)	15 (57,7)	2 (7.7)	0.303	5 (19,2)	15 (57.7)	6 (23.1)	0.495
Positive	12 (57.1)	8 (38.1)	1 (4.8)	2.387	7 (33.3)	9 (42.9)	5 (23.8)	1.408
PD-L1 expression (SP142)								
Negative	14 (38.9)	20 (55.5)	2 (5.6)	0.26	8 (22.2)	19 (52.8)	9 (25)	0.632
Positive	7 (63.6)	3 (27.3)	1 (9.1)	2.697	4 (36.4)	5 (45.4)	2 (18.2)	0.916
***PD-L1* gene copy number**								
< 2.86	8 (36.4)	12 (54.5)	2 (9.1)	0.501	3 (13.7)	14 (63.6)	5 (22.7)	0.167
≥ 2.86	13 (52)	11 (44)	1 (4)	1.381	9 (36)	10 (40)	6 (24)	3.581
PD-L1 mRNA expression								
< median	16 (51.6)	13 (41.9)	2 (6.5)	0.388	8 (25.8)	14 (45.2)	9 (29)	0.395
≥ median	5 (31.25)	10 (62.5)	1 (6.25)	1.892	4 (25)	10 (62.5)	2 (12.5)	1.856

detect *PD-L1* gene copy number by fluorescence in situ hybridization technique. Additionally, ZytoLight FISH-Tissue Implementation Kit (Zytovision, Germany) was also used for prestaining procedure. At least 60 non-overlapping nuclei was evaluated for each sample, in 3 different regions of interest.

The paraffin sections of 3–5 µm thick were cut and mounted on positively-charged glass slides. The unstained specimen was baked for 10 min. at 70 °C. Afterwards, the slides were immersed twice in xylen for 10 min and dehydrated two times in 100% ethanol for 5 min and in 90% and 70% ethanol for 5 min each, at ambient temperature. In sequence, the slides were washed twice in deionized water for 2 min, and were immersed for 15 min in pre-warmed Heat Pretreatment Solution Citric at 98 °C. Then, the slides were purified twice in deionized water for 2 min. After removing excess water from slides, the appropriate amount of pepsin solution was applied on the slides and they were incubated for 12 min at 37 °C in a humidity chamber. Then, the slides were washed in Wash Buffer for 5 min and were dehydrated in 70%, 90% and 100% ethanol for one minute each and allowed to dry. The slides were placed in dark room, 10 µl of probe mixture was applied to a slide and immediately coverslipped and sealed with rubber cement. The slides were placed for 10 min on hotplate at 75 °C and then at 37 °C for overnight hybridization. At the end of the hybridization period, the rubber cement was removed from the slides and they were placed in Wash Buffer at ambient temperature to allow the coverslips to float off the slides. Afterwards, the slides were immersed twice for 5 min in Wash Buffer previously warmed to 37 °C and were dehydrated in 70%, 90% and 100% ethanol for one minute each and allowed to dry in dark room. 10 µl of DAPI counterstain was applied to the target area, then coverslipped and the specimens were enumerated under a fluorescence microscope (Nicon Eclipse 55i, Japan).

The SPEC CD274, PDCD1LG2/CEN 9 Dual Color probe is a mixture of a green fluorochrome direct-labeled probe, specific for *CD274* (also called *PD-L1*) and *PDCD1LG2* (also designated as *CD273* or *PD-L2*) genes on chromosome 9 (9p24.1), and orange fluorochrome direct-labeled probe specific for the classical satellite III region of chromosome 9 centromere. In a normal nucleus, two orange and two green signals are expected. In a cell with polysomy or amplification of *CD274* or *PDCD1LG2* genes, multiple copies of the green signal or large green signal clusters were observed.

The dual color probe in the FISH method does not allow to evaluate the whole gene copy number as the molecular probe only covers the fragment of examined gene. Therefore, the term "gene copy number" used in this article is not precise. We should rather describe the number of duplications of red signal from the probe attached to *PD-L1* gene fragment so the results should be described as lack of amplification, low level of amplification or high level of amplification of *PD-L1* gene. Such nomenclature complicates the results, hence wherever we described the gene copy number, we meant the number of signals from the molecular probe used in the FISH technique.

RNA isolation

Total RNA was isolated from FFPE tissues using the miRNeasy FFPE Kit (Qiagen Inc., Germany) according to the

manufacturers' instructions. RNA was stored at −80 °C until synthesis of complementary DNA (cDNA).

Quantification of PD-L1 mRNA expression

We scored PD-L1 mRNA relative expression in reference to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression as an internal control. Reverse transcription PCR (RT-PCR) was conducted using High-Capacity RNA-to-cDNA Kit (Life Technologies, USA) according to the manufactures' instructions.

Real-time PCR was performed using TaqMan Fast Advanced Master Mix (Life Technologies, USA) with standard protocol, on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA). The 20 µl of PCR mixture contained: 10 µl of TaqMan Fast Advanced Master Mix, 1 µl of TaqMan Gene Expression Assay and 5 µl of RNaze free water, as well as 4 µl of cDNA. Reaction was conducted in subsequent conditions: 95 °C for 20 s (activation of enzyme) and 40 cycles: 95 °C for 3 s, 60 °C for 30 s. Ct values were obtained for cDNA from *PD-L1* mRNA and for cDNA from GAPDH mRNA. Analysis was performed using $2^{-\Delta Ct}$ method.

DNA extraction

Genomic DNA was isolated from formalin-fixed paraffin-embedded tissues using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany) according to the manufactures' instruction. The yield and purity of DNA were analyzed using a BioPhotometer UV/Vis Spectrophotometer (Eppendorf, Germany).

Genotyping of *PD-L1* gene promoter polymorphisms

We studied two polymorphisms (rs822335 and rs822336) in promoter of *PD-L1* gene by quantitative real-time PCR method. PCR reaction was performed using 5.5 µl of Genotyping MasterMix (Life Technologies, USA), 4 µl of DNA (5 ng/ul), 0.5 µl of TaqMan SNP Genotyping Assay (Life Technologies, USA). Protocol of real-time PCR was performed on Illumina Eco Real-Time PCR System (Illumina Inc, San Diego, USA) in following conditions: initial denaturation and enzyme activating: 95 °C for 10 min, and 40 cycles: 95 °C for 15 s, 62 °C for 90 s.

Statistical analysis

Statistical analysis was carried out using Statistica 13.1 software (TIBCO Software Inc, USA). The Spearman test was used to score the correlation between *PD-L1* gene copy number and PD-L1 mRNA and protein expression. U-Mann–Whitney test was used to analyze *PD-L1* gene copy number and PD-L1 expression on tumor cells in groups of patients with different demographic, clinical and genetic factors. Associations between genotypes of *PD-L1* gene and clinical factors, as well as PD-L1 mRNA and protein expression were examined using the Fisher Chi square test. *P* values below 0.05 were considered significant.

The study was approved by the Ethics Committee of the Medical University of Lublin, Poland (No. KE-0254/95/2018).

Results

Our results of examination of PD-L1 expression on tumor and immune cells using immunohistochemistry tests with 22C3 and SP142 antibody clones have been previously described [9]. In this study, we focused on the molecular background of differences in PD-L1 expression.

Freequency of *PD-L1* gene polymorphisms

rs822335 and rs822336 polymorphisms in promoter of *PD-L1* gene were analyzed by quantitative real-time PCR method. 21 patients were carriers of the CC genotype, 23 patients – CT genotype and 3 patients – TT genotype at rs822335 polymorphic site of *PD-L1* gene, while the distribution of genotypes at rs822336 polymorphic site was as follows: CC genotype was present in 12 patients, CG genotype – in 24 patients and GG genotype – in 11 patients.

The frequency of studied genotypes did not significantly depend on gender, age, pathomorophologic diagnosis and the presence of *EGFR* gene mutations or *ALK* gene rearrangement (Table 1). However, G allele in rs822336 was insignificantly less frequent ($p = 0.083$, $\chi^2 = 3.008$) in squamous cell cancer (SqCC) patients (7.7% of SqCC patients) than in patients with other NSCLC types (32.4% of such patients).

The relationship between *PD-L1* gene polymorphisms and PD-L1 expression

The frequency of particular genotypes was similar in patients with and without expression of PD-L1 on more than 50% of tumor cells (PD-L1 expression assesed by IHC tests using 22C3 and SP142 monoclonal antibodies, Table 1). However, T allele presence in rs822335 was insignificantly less frequent in patients with PD-L1 expression compared to patients witohout PD-L1 expression ($p = 0.122$, χ^2=2.385 for test with 22C3 monoclonal antibody and $p = 0.148$, χ^2=2.088 for test with SP142 monclonal antibody).

The distribution of the examined genotypes was similar in the groups of patients with high and low PD-L1 mRNA expression tested with quantitative real-timie PCR with reverse transcription (Table 1). The frequency of described genotypes was similar in patients with high and low *PD-L1* gene copy number, assessed by fluorescence in situ hybridization technique (Table 1). G allele in rs822336 was insignificantly less frequent ($p = 0.079$, χ^2=3.078) in patients with low copy numebr of *PD-L1* gene (64% of such patients) than in patients with high *PD-L1* gene copy number (85.7% such patients).

Patients with CC genotype in rs822335 had significantly ($p = 0.043$) higher percentage of tumor cells with PD-L1 expression (test with 22C3 antibody) than patients with CT or TT genotypes (median and standrad deviation: 60 ± 39.869% vs. 8.5 ± 39.078, Fig. 1). Moreover, patients with CC genotype in rs822336 had slightly higher percentage of tumor cells with

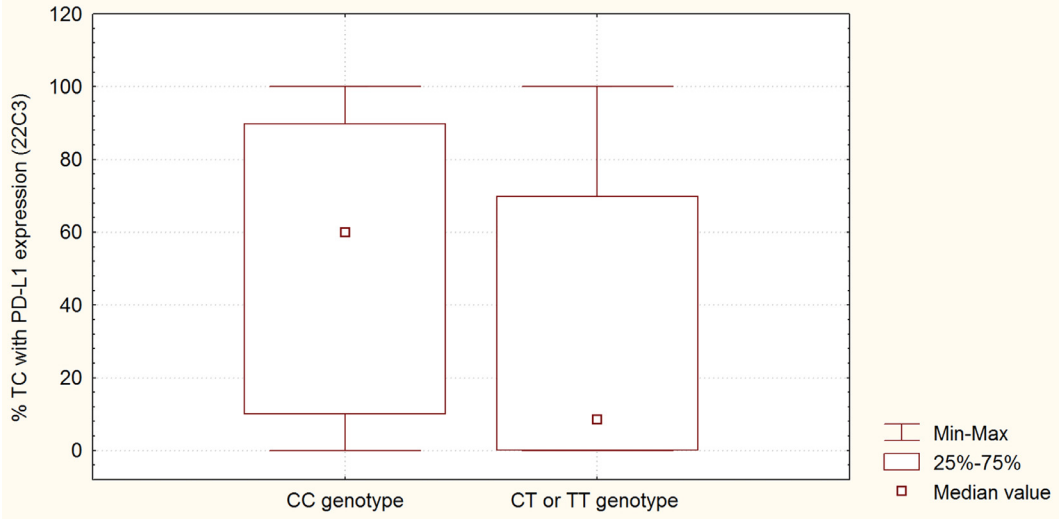

Fig. 1 Percentage of tumor cells with expression of PD-L1 (IHC test with 22C3 antibody) in patients with different genotypes in rs822335 of *PD-L1* gene.

expression of PD-L1 (test with 22C3 antibody) than patients with CG or GG genotypes ($p = 0.105$).

Examined polymorphisms did not affect PD-L1 mRNA expression and *PD-L1* gene copy number.

PD-L1 gene copy number in analysed NSCLC patients

In tumor cells, median of *PD-L1* gene copy number was 2.86 ($\pm$ 1.05) and median number of chromosome 9 centromeres was 3.05 ($\pm$ 1.58). Therefore, median ratio of *PD-L1* gene copy number to chromosome 9 centromeres number was 0.94 ($\pm$ 0.61). These parameters did not depend on sex, age and status of *EGFR* and *ALK* genes. However, patients with SqCC had significantly higher *PD-L1* gene copy number (median and standard deviation: 3.75 $\pm$ 1.47, $p = 0.032$) and ratio of *PD-L1* gene copy number to number of chromosome 9 centomeres (median and standard deviation: 1.055 $\pm$ 0.789, $p = 0.022$) in comparison to patients with other pathomorphological diagnosis (median and standard deviation: 2.45 $\pm$ 0.668 and 0.85 $\pm$ 0.471, respectively).

Correlation between *PD-L1* gene copy number and PD-L1 mRNA as well as protein expression

PD-L1 gene copy number significantly positively correlated with percentage of tumor cells with PD-L1 expression detected in tests with 22C3 antibody ($p = 0.005$, $R = +0.442$) and with SP142 antibody ($p = 0.021$, $R = +0.369$) (Figs. 2 and 3). *PD-L1* gene copy number did not correlate with PD-L1 mRNA expression.

Patients with PD-L1 expression tested with 22C3 antibody had significantly higher expression of PD-L1 mRNA ($p = 0.023$), number of chromosome 9 centromeres ($p = 0.023$) and *PD-L1* gene copy number ($p = 0.003$) than patients without PD-L1 expression on tumor cells (Fig. 4). Patients with PD-L1 expression in test with SP142 antibody had insigficantly higher expression of PD-L1 mRNA ($p = 0.069$), number of chromososme 9 centromeres ($p = 0.096$) and significantly higher *PD-L1* gene copy number ($p = 0.027$) compared to patients without PD-L1 expression on tumor cells (Fig. 5).

Discussion

IHC method in assessment of PD-L1 expression, as a predictor for immunotherapy, is simple and available in many laboratories. However, PD-L1 expression on tumor cells is not a good predictive factor for immunotherapy: response to treatment can be observed in patients without PD-L1 expression on tumor cells, while rapid progression during immunotherapy may appear in patients with high expression of PD-L1 on tumor cells. Because of such cases it is necessary to search for new predictors for immunotherapy. Genetic factors such as tumor mutation burden (TMB) have a potentially predictive character in qualification of patients to immunotherapy, but TMB assessment is difficult and long-lasting, and requires good quality DNA and validation process. It seems advisable to discover simple genetic predictive factors for immunotherapy. Such genetic factors could be determined within comprehensive gene sequencing (CGS) with the next generation sequencing (NGS) technique. Then, simultaneous examination of immunotherapy and molecularly targeted therapies predictors (driver mutations and gene rearrangements) becomes possible.

In our study, we have proven that *PD-L1* gene promoter polymorphisms and *PD-L1* gene copy number may be predictors for PD-L1 protein expression on tumor cells in NSCLC Caucasian patients (most of the studies regarding SNPs of *PD-L1* gene were conducted in Asian patients). For the first time, we have shown that cancer patients with CC genotype in rs822335 were predisposed to higher expression of PD-L1 protein on tumor cells. Most previous studies have focused on the ability of microRNAs binding to polymorphic forms of promoter or 3′untranslated region (3′UTR) of PD-L1 mRNA

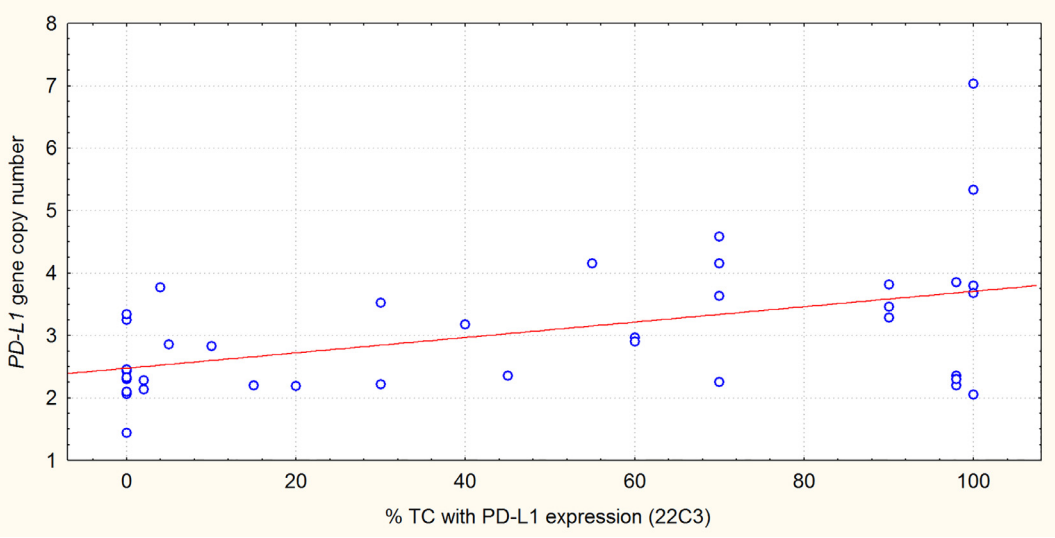

Fig. 2 Correlation between *PD-L1* gene copy number and percentage of tumor cells with PD-L1 expression (IHC test with 22C3 antibody).

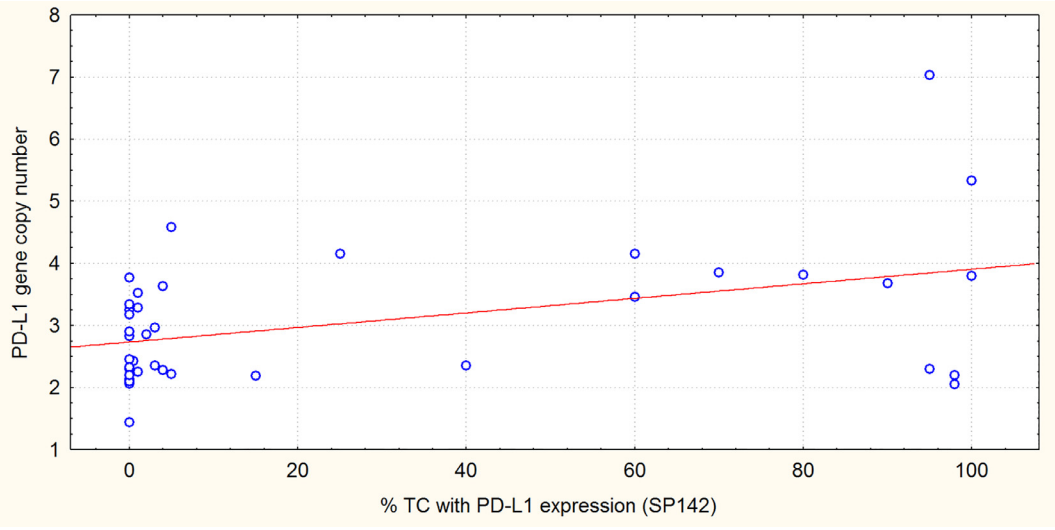

Fig. 3 Correlation between *PD-L1* gene copy number and percentage of tumor cells with PD-L1 expression (IHC test with SP142 antibody).

(luciferase assays), which only indirectly suggests the influence of polymorphisms on PD-L1 expression. Moreover, we have found that the high *PD-L1* gene copy number is significantly associated with the high expression of PD-L1 protein, which can be a useful predictor in the qualification for immunotherapy. Thus, we have proven that abnormalities in the DNA of tumor cells have the leading effect on PD-L1 expression on tumor cells.

The largest studies on the influence of *PD-L1* gene polymorphisms on PD-L1 expression and prognosis in NSCLC patients were made by Lee and co-workers. These authors examined 12 SNPs of *PD-L1* gene in 354 early stage NSCLC patients who underwent curative surgical resection. Presence of rare alleles in rs822336, rs822337 and rs4143815 (C, A and G alleles, respectively) was associated with worse overall survival of NSCLC patients. Moreover, lower expression of *PD-L1* mRNA was a negative prognostic factor for NSCLC patients. In luciferase assay, authors detected that presence of G allele in rs4143815 as well as C and A alleles in rs822336 and in rs822337 decreased transcription and promoter activity of *PD-L1* gene. However, there was no significant correlation between relative PD-L1 expression and the genotypes in rs4143815, rs822336 and rs822337 [10]. These results are consistent with our observations that rs822336 polymorphism has little effect on PD-L1 expression on tumor cells [10].

In another study, Lee et al. analyzed correlation between 12 SNPs in *PD-L1* gene and response to first line paclitaxel-cisplatin chemotherapy, and overall survival in 379 advanced NSCLC patients. The authors ascertained that presence of C allele in rs2297136 and G allele in rs4143815 were responsible for better response to chemotherapy and longer overall

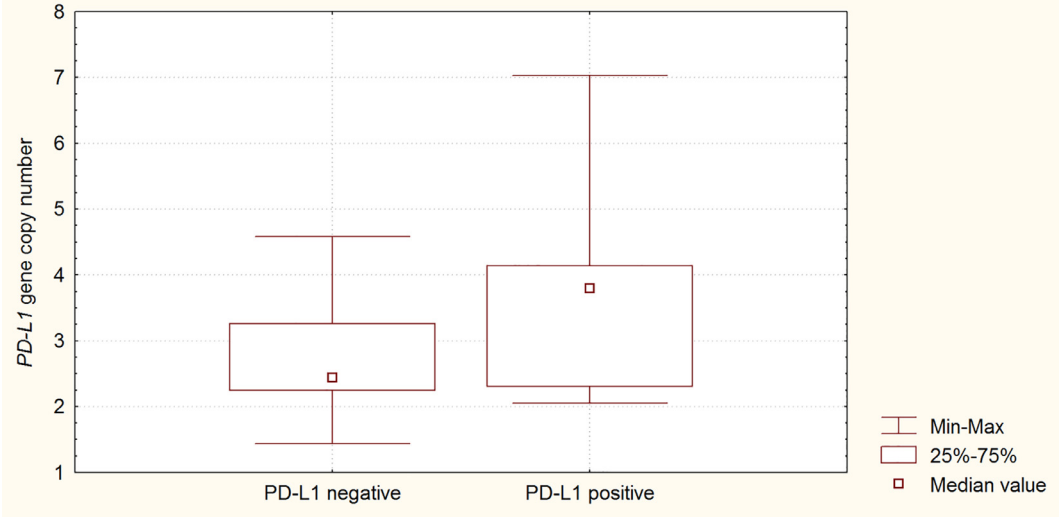

Fig. 4 *PD-L1* gene copy number in patients with and without PD-L1 expression on tumor cells (IHC test with 22C3 antibody).

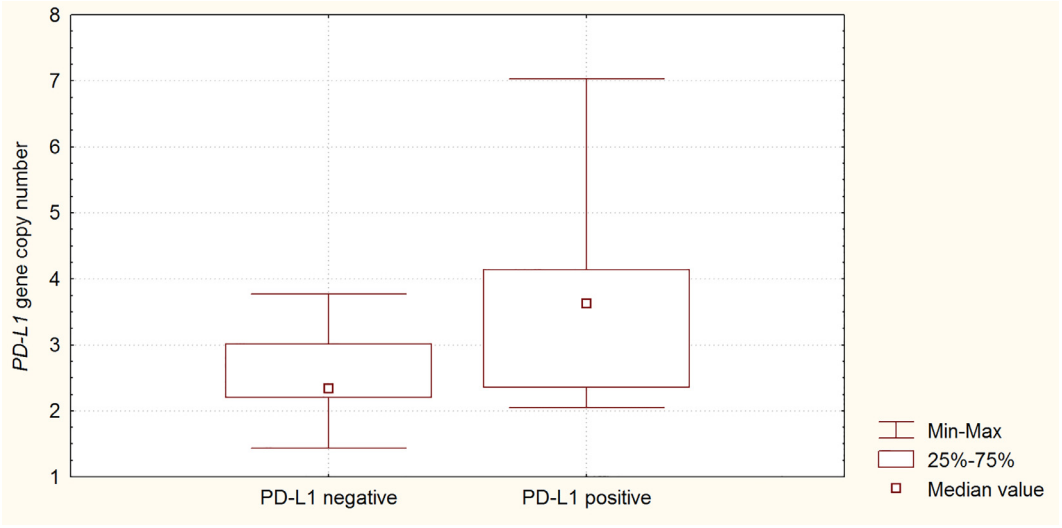

Fig. 5 *PD-L1* gene copy number in patients with and without PD-L1 expression on tumor cells (IHC test with SP142 antibody).

survival of NSCLC patients. Moreover, the authors suggested that presence of these alleles may affect PD-L1 expression by altered microRNA binding to 3′UTR region of PD-L1 mRNA [11].

Yeo et al. studied prognostic role of three SNPs in *PD-L1* gene (rs4143815, rs822336 and rs822337) in surgically resected NSCLC patients (84 adenocarcinoma and 63 squamous cell carcinoma). In adenocarcinoma patients, PD-L1 expression correlated with shorter disease-free survival and higher frequency of lymph nodes metastases. Moreover, the authors found, that patients with adenocarcinoma and with GG genotype in rs4143815 showed a trend of shorter overall survival and disease-free survival than patients with other genotypes of this SNP. Whereas, CC genotype in rs4143815 was statistically associated with positive expression of PD-L1 tested with 22C3 monoclonal antibody (cutoff at 50% of tumor cells). The authors did not find relationships between genotypes in rs822336 and in rs822337 and PD-L1 expression [12].

Du et al. showed that rs2297136 and rs4742098 polymorphisms in 3′untranslated region of *PD-L1* gene are associated with risk of NSCLC, metastases development, tumor infiltration and stage of disease. Luciferase assay showed that rs2297136 and rs4742098 in *PD-L1* 3′UTR contributed to the occurrence of NSCLC through disruption of the interaction between miR-296-5p, miR-138 and PD-L1 mRNA [13].

Cheng et al. examined SNP in intron 4 of *PD-L1* gene (rs2890658) and level of soluble PD-L1 (sPD-L1) in blood plasma of 288 NSCLC patients and 300 healthy persons. Authors found that NSCLC patients showed increased plasma levels of sPD-L1 compared to controls. Furthermore, lung adenocarcinoma patients had higher sPD-L1 levels than patients with squamous cell carcinoma. However, no association was observed between the different genetic variants of *PD-L1* gene and sPD-L1 plasma concentrations, as well as risk of lung cancer [14]. The same polymorphism was studied by Ma and co-workers and Chen and colleagues regarding the risk of lung cancer in large Chinese population (821 NSCLC patients

and 893 healthy person, all together). Analysis revealed that prevalence of C allele in rs2890658 was significantly higher in NSCLC patients than in healthy controls and in smoking NSCLC patients (especially in heavy smokers) than in non-smoking NSCLC patients [15,16].

Finally, Nomizo et al. examined clinical impact of SNPs in *PD-L1* gene (rs1411262, rs2282055, rs4143815, rs2890658, rs822339, rs2227981, rs2227982) on nivolumab effectiveness in advanced non-small cell lung cancer patients. They found that two of seven SNPs showed association with response rate and progression free survival (PFS) in nivolumab treated patients. Response rate was 25%, 15%, and 0% for the GG, GT and TT genotypes in rs2282055, respectively. G allele presence in rs2282055 was significantly associated with better clinical response compared with T allele presence. The median PFS was significantly higher in carriers of GG and GT genotypes (2.6 months) than in patients with TT genotype (1.8 months). Moreover, CC and CG genotypes in rs4143815 (in 3′UTR) were significantly associated with better response rate and longer PFS than GG genotype in NSCLC patients treated with nivolumab [17].

There is evidence for a strong relationship between PD-L1 mRNA expression and PD-L1 protein expression on tumor cells [18]. However, there are only few studies in which *PD-L1* gene copy number was correlated with PD-L1 expression on tumor cells in NSCLC patients (there are several such studies in other cancers). Ikeda et al. investigated copy number alteration (CNA) in *PD-L1* gene by real-time PCR in 94 surgically resected lung cancer samples and found possible associations between *PD-L1* CNA and lung cancer biology (the authors did not examine PD-L1 expression on tumor cells in NSCLC patients). In this study, only five samples had *PD-L1* gene CNA (much less than in our study). The patients with *PD-L1* gene CNA had worse prognoses. Flow cytometry analysis revealed that the lung cancer cell line HCC4006 with *PD-L1* gene amplification had higher PD-L1 protein expression than other NSCLC cell lines [19].

Inoue et al. observed prognostic implications of PD-L1 gene copy number gains, assessed by FISH method, in 654 patients with resected NSCLC. PD-L1 amplification and polysomy were observed in 3.1% and 13.2% of patients and were independently associated with PD-L1 protein overexpression. In our study, at least three *PD-L1* gene copies, in most of analyzed tumor nuclei, were observed in 42% of NSCLC patients (more than eight *PD-L1* gene copies were observed in 2 patients), and the relationship between *PD-L1* gene copy number and PD-L1 protein expression was evident. Inoue et al. showed that PD-L1 gene copy number gains were more commonly observed in smokers and in patients without *EGFR* gene mutations or *ALK* gene rearrangement (this could not be demonstrated in our study). Furthermore, authors indicated that tumor PD-L1 copy number status detected by FISH was more consistent and reproducible than tumor PD-L1 protein expression detected by IHC when primary tumors and synchronous regional lymph node metastases were comparatively analyzed (such an analysis was not carried out in our study due to low number of patients) [20].

Buczies et al. employed The Cancer Genome Atlas (TCGA) datasets to comprehensively analyze 22 major cancer types for *PD-L1* gene copy number alterations, mRNA expression and tumor mutation burden. *PD-L1* gene copy number gains most frequently occurred in ovarian, head and neck, bladder, colorectal and cervical cancers as well as in sarcomas (they were not so frequent in NSCLC). *PD-L1* CNA correlated significantly with PD-L1 mRNA expression in many cancer types, and tumors with *PD-L1* gene copy gains harbored significantly higher TMB compared to non-amplified cases (78 non-synonymous mutations vs. 40 non-synonymous mutation). Authors identified 75 top genes that were strongly and recurrently up-regulated in tumor cells with chromosome 9 gains: one implicated in cell cycle regulation and others implicated in modulation of the immune system (cytokines, chemokines, and other immunoregulatory genes such as *IFN-γ* and *IDO1*) Moreover, authors observed that *PD-L1* gene CNA was associated with poor prognosis of cancer [21,22].

Cited data and our results indicate that genetic polymorphisms and *PD-L1* gene copy number alterations could modify PD-L1 protein expression, immunotherapy effectiveness, cancer susceptibility and prognosis. In conclusion, *PD-L1* gene copy number gains, represent rather frequent genetic alteration in NSCLC patients and have influence on PD-L1 expression, and may be explored as a predictive biomarker for immunotherapy regimens. Although SNPs in *PD-L1* gene have lower impact on *PD-L1* gene expression (e.g. promoter region transcriptional activity), they can affect the ability to microRNAs binding to PD-L1 mRNA. Nevertheless, our study has some limitations (in particular, small group of patients and heterogeneous type of material for PD-L1 expression assessment: primary vs. metastatic tumor, small biopsies vs. histological material), we strongly believe that there are merits of this retrospective study that deem it worthy of publication. It should be noted that our study has an initial character. It is the first study to correlate the presence of selected polymorphisms of *PD-L1* gene with PD-L1 protein expression on tumor cells. Our results highlight the need to determine associations between the various genetic factors and PD-L1 protein expression for discovery of new predictive factors for immunotherapy. Further studies on the regulation of PD-L1 expression in tumor cells are necessary.

Conflicts of interest

The authors declare that they have no competing interests.

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