Original paper

Association of PD-1 and PD-L1 protein expression with selected clinical and morphological parameters in colorectal cancers

Paulina Poter[1,2,3], Sylwia Jankowska-Szabłowska[3], Tomasz Kolenda[4,5], Ewa Dobak[3], Elżbieta Urasińska[3], Katarzyna Karpińska-Łukaszewicz[3]

[1]Department of Tumor Pathology, Greater Poland Cancer Centre, Poznań, Poland
[2]Department of Oncologic Pathology and Prophylaxis, University of Medical Sciences, Poznań, Poland
[3]Department of Pathology, Pomeranian Medical University, Szczecin, Poland
[4]Laboratory of Cancer Genetics, Greater Poland Cancer Centre, Poznań, Poland
[5]Research and Implementation Unit, Greater Poland Cancer Centre, Poznań, Poland

Colorectal cancer is the third most common cancer worldwide and the second cause of death from malignant tumors. Colorectal cancers are treated with surgery, chemotherapy, gene therapy and immunotherapy. PD-1 and PD-L1 proteins have recently been considered as potential targets of anticancer therapy in colorectal cancer. The aim of this study was to evaluate the association of immunohistochemical expression of PD-1 and PD-L1 proteins in colorectal cancer patients with selected clinical and morphological parameters and their survival. Ninety-eight cases of colorectal cancer were studied.
Immunohistochemistry was used to evaluate the expression of PD-1 and PD-L1 proteins.
Correlations were found between the expression of PD-L1 protein in lymphocytes and lack of lymph node metastases and a lower clinical stage.
There was also a correlation between PD-L1 protein expression in cancer cells and a higher grade of histological malignancy.

Key words: colorectal cancer, PD-1, PD-L1.

Introduction

Colorectal cancer is the third most common cancer worldwide and the second cause of death from malignant tumors [1–3]. Colorectal cancers, depending on their clinical stage, are treated with surgery, chemotherapy and personalized therapy – gene therapy and immunotherapy [4–6]. Cancer cells have developed mechanisms that allow them to avoid the body's defense reaction [7, 8]. It is possible through a number of mechanisms, including the use of inhibitory pathways at key immune points. One such point is the PD-1/PD-L1 pathway [9, 10]. In the treatment of many types of cancer the use of the PD-1/PDL1 pathway is a therapeutic breakthrough [6]. PD-1 (programmed cell death protein 1), also known as CD279 [11], is a membrane protein found on the surface of T lymphocytes, involved in the process of cell apoptosis [12, 13]. PD-L1 is a ligand of the PD-1 protein – a key immunoregulatory molecule that suppresses the cytotoxic immune response of CD8-positive lymphocytes [14, 15]. Cancer cells and antigen-presenting cells express the PD-L1 ligand, as well as the second ligand of the PD-1 receptor, which is the PD-L2 protein [9, 16, 17]. The PD-1 and PD-L1 proteins are critical

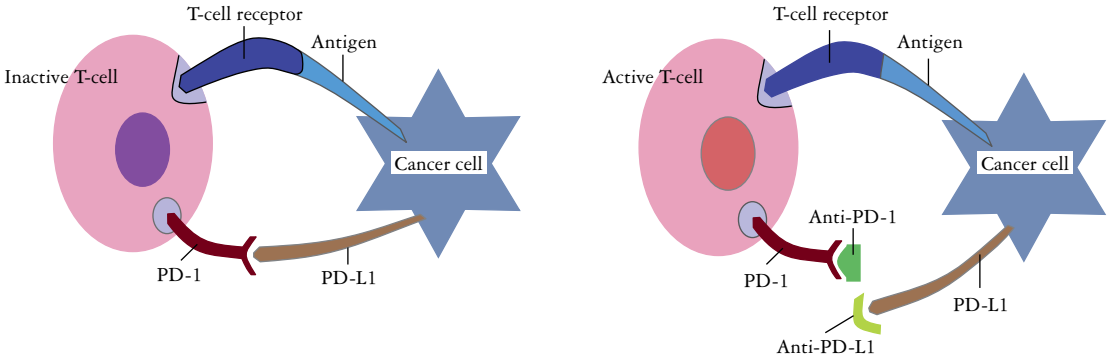


Fig. 1. Targeted therapy with monoclonal antibodies that block PD-1 and PD-L1 proteins

immune checkpoint proteins that are responsible for the negative regulation of the integrity and stability of the immune function of T lymphocytes [18–20].

The expression of PD-L1 ligand leads to the escape of tumor cells from immune control systems [21–23]. Anti-PD-1 and anti-PD-L1 monoclonal antibodies block the binding of PD-1 protein to its ligand (Fig. 1). Studies have shown that blockade of the PD-1/PD-L1 checkpoint using specific antibodies restores the anti-cancer activity of cytotoxic T-cells specific for the cancer antigen, which allows for therapeutic actions in patients with colorectal cancer [24]. The aim of this study was to evaluate the association of immunohistochemical expression of PD-1 and PD-L1 proteins with selected clinical and morphological parameters in patients with colorectal cancer and their survival.

Material and methods

The study included a group of 98 patients with a histopathologically confirmed diagnosis of colorectal cancer. Histological examinations of patients operated on in 2017 at the Department of General and Oncological Surgery and the Department of General and Gastroenterological Surgery of the Pomeranian Medical University in Szczecin were performed at the Department of Pathomorphology of the Pomeranian Medical University in Szczecin. The study design was approved by the local ethics committee of the university hospital. The survival data were obtained from the death register by the Systems Management Department of the Ministry of Digitization of the Republic of Poland. The clinical and morphological characteristics of the study group are presented in Table 1.

The technique of tissue microarray (TMA) made of paraffin blocks containing material collected for routine histopathological examination from a postoperative preparation fixed in 10% formalin and embedded in paraffin was used in the study [25–27]. Representative regions from the primary histopathological specimen stained with hematoxylin and eosin were taken. Then the manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA) was used to prepare TMA. The histological preparations of intestinal tumors were also evaluated for the presence of tumor budding and the presence of lymphocytes penetrating the tumor tissue and infiltrating the tumor area (TIL) in 1 mm^2. In the case of TIL, "+" was defined as present (> 30%) and "–" as absence of TIL (< 30%) [28], while the presence of tumor budding was defined as "+" when there were single tumor cells or small clusters of up to 5 cells in the stroma at the periphery of the tumor and "–" as the absence of this phenomenon [29]. In addition, in 28 patients whose *KRAS* gene status was assessed for diagnostic purposes, the relationship between *KRAS* gene status and the expression of PD-1 and PD-L1 proteins in cancer cells and in TIL was examined. To evaluate the expression of PD-1 and PD-L1 proteins, immunohistochemical staining was performed using monoclonal antibodies against the mentioned proteins. A mouse monoclonal antibody (NAT105; Roche Diagnostics Poland; catalog no. 07099029001) was used to assess PD-1 receptor expression. For the evaluation of PD-L1 ligand expression, a rabbit monoclonal antibody against PD-L1 (VENTANA SP263; Roche Diagnostics Polska; catalog no. 07419821001) was used. Those antibodies were optimally diluted to a form compatible with VENTANA detection kits and BenchMark devices (Roche Diagnostics Polska).

The presence of PD-1 protein expression in lymphocytes and PD-L1 in cancer cells and/or lymphocytes in stained immunohistochemical slides was first evaluated by an experienced pathomorphologist, and then virtual image analysis was used to standardize and unify the results. The slides were scanned using an APERIO CS scanner (Aperio Technologies Inc. California, USA). For image analysis, software that applies staining evaluation algorithms directly to the scanned image (Image Scope Version 11.2.0.780; Aperio Technologies, Inc. 2003–2012) was used.

In the case of a cytoplasmic reaction, the cytoplasmic v2 algorithm allows for assessment of the per-

Table 1. Characteristics of the study group

Parameters		Value, $n = 98$(%)	
Gender			
Female		41 (41.84)	
Male		57 (58.16)	
Age			
Range		37–92	
Mean	69	≥ 69	50 (51.02)
		< 69	48 (48.98)
Median		70	
Tumor location			
Right-sided		37 (37.76)	
Left-sided		61 (62.24)	
Clinical stage			
I		13 (13.27)	
II		32 (32.65)	
III		49 (50.00)	
IV		4 (4.08)	
Infiltration depth (T)			
T2		17 (17.35)	
T3		57 (58.16)	
T4		24 (24.49)	
Histological malignancy grade (G)			
G1		3 (3.06)	
G2		89 (90.82)	
G3		6 (6.12)	
Status of surrounding lymph nodes (N)			
N0		45 (45.92%)	
N1 + N2		53 (54.08)	
Tumor budding			
0		38 (38.77)	
1		60 (61.22)	
Lymphocytic infiltration			
In the tumor	0		71 (72.44)
	1		27 (27.55)
In the tumor area	0		63 (64.29)
	1		35 (35.71)
KRAS gene status, $n = 28$ (%)			
Mut		14 (50.00)	
wt		14 (50.00)	

0 = absent, 1 = present, mut – presence of mutation, wt – absence of mutation

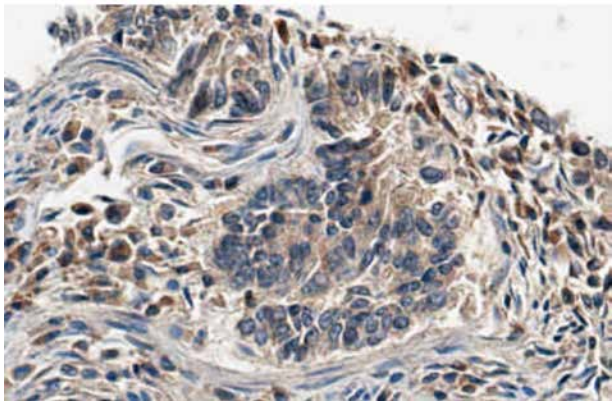


Fig. 2. PD-1 protein expression in the cytoplasm of lymphocytes infiltrating the tumor tissue (IHC staining, 20× magnification)

centage of cells expressing a given protein, as well as for assessing the intensity of staining of these cells (lack of reaction (0), weak reaction (1+), medium intensity reaction (2+), strong reaction (3+)). In the case of the PD-1 protein, the reaction was evaluated in lymphocytes infiltrating the tumor tissue (Fig. 2). The expression of a given protein was evaluated according to the H-score [30, 31]:

H-score = 1 × (% of 1+ cells) + 2 × (% of 2+ cells) + 3 × (% of 3+ cells).

The score has a range of 0–300.

For the membrane reaction, we used the membrane v9 algorithm designed to assess HER2 receptor status, which is also recommended to assess the expression of other proteins in the cell membrane. This algorithm, like the previous one, allows for assessment of the percentage of cells expressing a given protein, as well as for assessing the intensity of staining of these cells (0, 1+, 2+, 3+). In the case of PD-L1 protein, expression was assessed in the membrane of cancer cells (Fig. 3A) and lymphocytes infiltrating the tumor tissue (Fig. 3B). For further analysis, the H-score formula described earlier was used.

The normality of the distributions of all variables was checked by the Shapiro-Wilk test. These variables were described by means, standard deviations, medians, and minimum and maximum values. The statistical differences between the two groups were checked with the Mann-Whitney *U* test. The Kruskal-Wallis test was used to compare more than two groups. Spearman's rank correlation was used to test the correlation between variables. The results were described by the correlation coefficient r and the probability of p. Survival analysis was prepared using the log-rank (Mantel-Cox) test (p^a) and Gehan-Breslow-Wilcoxon test (p^b). All tests were performed using GraphPad Prism 8 software. The statistically significant differences in all the tests performed were those for which the probability was lower than 0.05.

Results

Immunohistochemical expression of PD-1 and PD-L1 proteins

Expression of PD-1 protein, which was studied in lymphocytes accompanying tumor infiltration in the intestinal wall, was found in 29 (29.59%) of the cases studied. PD-L1 ligand expression was studied in lymphocytes, as well as in colon cancer cells. PD-L1 protein expression was found in lymphocytes in 43 (43.88%) cases, and in cancer cells in 7 (7.14%) cases (Table 2).

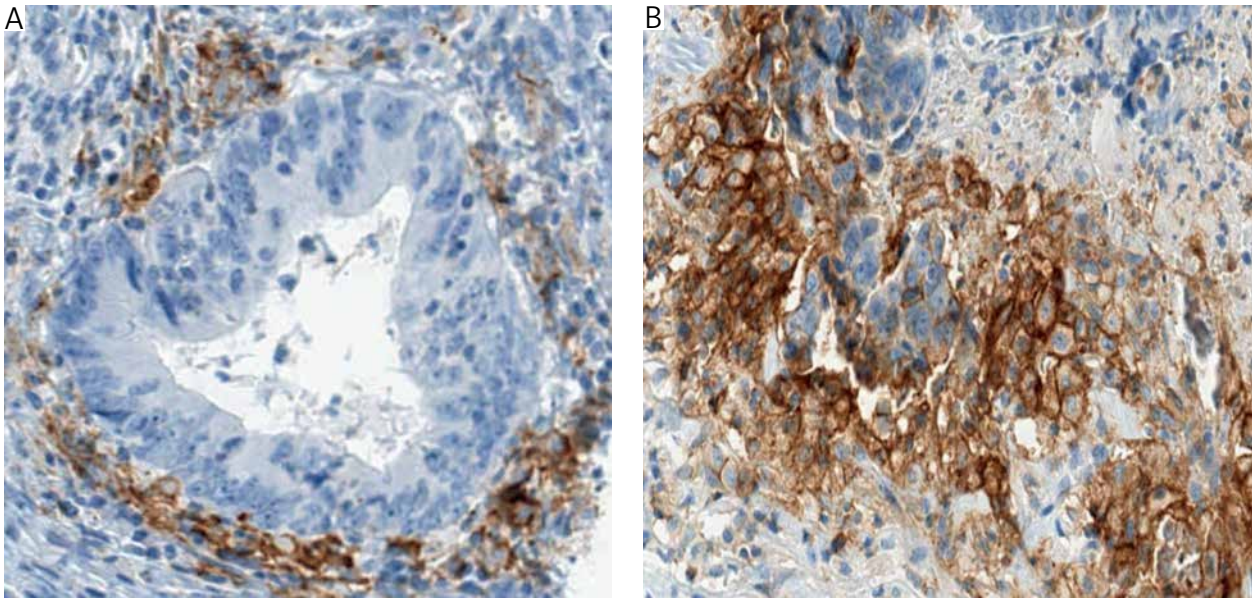


Fig. 3. PD-L1 protein expression. **A**) In the cell membrane of cancer cells. **B**) In the cell membrane of lymphocytes surrounding the cancer tubule (IHC staining, 20× magnification)

Table 2. Distribution of PD-1 protein and PD-L1 protein expression ($n = 98$)

Parameters	N(+)	N(–)	Min.	Max.	Mean	Median	SD
PD-1	29	69	0.00	108.95	6.29	0.00	16.84
PD-L1 tumor cells	7	91	0.00	227.15	9.62	0.00	37.81
PD-L1 lymphocytes	43	55	0.00	131.22	36.98	0.00	45.02

N(+) – number of cases with positive expression of the test protein, N(–) – number of cases with no expression of the test protein

Immunohistochemical expression of PD-1 and PD-L1 proteins and selected clinical and morphological parameters

The results are shown in Tables 3 and 4.

Immunohistochemical expression of PD-1 and PD-L1 proteins and patient survival

It was found that the expression of the PD-1 protein had a statistically significant impact on the patient survival in the first year after the diagnosis – 100% survival in patients with expression of the PD-1 protein ($p = 0.018$). However, there was no statistically significant relationship between PD-1 protein expression and patient survival at 5 years after diagnosis. There was no statistically significant relationship between patient survival and PD-L1 protein expression in either cancer cells or lymphocytes. These data are presented in Figures 4 and 5.

Discussion

The interactions of PD-1 and PD-L1 involve immunosuppressive processes in the tumor growth environment. In the last decade, monoclonal antibody therapy (anti-PD-1/anti-PD-L1) has been introduced to block the pro-tumor activity of this checkpoint. The use of PD-1/PD-L1 inhibitors is expected to block the anti-tumor activity of this complex [32, 33]. The expression of PD-L1 protein on the surface of cancer cells and PD-1 protein in lymphocytes accompanying the cancer infiltrate is an important predictive indicator for anti-PD-1 and anti-PD-L1 antibody therapy [34]. However, a clinical response to anti-PD-1/PD-L1 therapy is observed in only a minority of patients. Targeting DNA synthesis and replication through chemotherapy results in the elimination of cancer cells, while blocking the PD-1/PD-L1 checkpoint stimulates tumor-specific T-cells. This is because cell death is followed by an increase in the presence of tumor antigens, which stimulates lymphocytes [35]. Thus, taking this process into account, it seems that an appropriate combination of chemotherapeutic treatment with PD-1/PD-L1 inhibitors may lead to an increase in treatment efficacy especially in patients with less immunogenic and chemotherapy-sensitive tumors [18]. In addition, studies indicate that the use of PD-1 and PD-L1 inhibitors combined with radiotherapy in patients with advanced non-small cell lung cancer (NSCLC) improves both overall survival and recurrence-free survival. Geng *et al*. found that the use of PD-1/PD-L1 inhibitors after radiotherapy was more beneficial than concurrent PD-1/PD-L1 inhibitor therapy with radiotherapy or the use of radiotherapy after PD-1/PD-L1 inhibitors [36]. This result is in line with the theory that radiotherapy causes double-stranded DNA breaks and increases CD8+ T-cell infiltration, which in turn increases PD-L1 protein expression [36–39].

The aim of the present study was to compare the expression of PD-1 and PD-L1 proteins in patients with colorectal cancer with clinical and morphological parameters, *KRAS* gene status, tumor budding, and patient's survival. An immunohistochemical method was used to evaluate the expression of PD-1 and PD-L1 proteins.

A similar study related to PD-L1 protein expression in colorectal cancer was conducted by Shan *et al.*, demonstrating immunohistochemically the presence of expression in 42.5% of colorectal cancer cases studied. The authors found a positive correlation of PD-L1 expression with the occurrence of lymph node metastasis, distant metastasis, and the depth of tumor infiltration. However, there was no correlation with gender or the degree of histological differentiation of the tumor [40], which in turn were found in the present study. On the other hand, Masugi and his team analyzed the occurrence of PD-L1 protein expression in both tumor cells and the stroma, where 89% and 5% of cases, respectively, showed expression [41]. That is the opposite of the present study, where PD-L1 protein expression in cancer cells was observed in a lower percentage of cases than in lymphocytes, i.e. 7.1% of cases with PD-L1 expression in cancer cells and 43.9% of cases with expression in lymphocytes. Masugi studied the correlation of PD-L1 protein expression with the density of FOXP3 cells [41], which are regulatory T-cells that play an important role in inhibiting the anti-tumor response [42]. This relationship was found to be inversely related to PD-L1 protein expression in colorectal cancers, which may suggest an effect of PD-L1-expressing cancer cells on regulatory T-cells in the tumor microenvironment [41]. Zhao and his team also found a statistically significant correlation between regulatory T-cell infiltration and PD-L1 protein expression in cancer cells (expression was observed in 48.21% of cases, while PD-L1 expression was not observed in tumor infiltrat-

Table 3. Relationship between PD-1 and PD-L1 protein expression and selected clinical and morphological parameters

Parameters		Number of cases, *n* = 98(%)	PD-1	PD-L1 tumor cells	PD-L1 lymphocytes
			p-value		
Gender					
Female		41 (41.84)	0.5051	0.018	0.5532
Male		57 (58.16)			
Age					
< 69		48 (48.98)	0.0036	0.9918	0.4101
≥ 69		50 (51.02)			
Tumor location					
Right-sided		37 (37.76)	0.9926	0.1002	0.0963
Left-sided		61 (62.24)			
Clinical stage					
I		13 (13.27)	0.0112	0.9049	0.0428
II		32 (32.65)			
III		49 (50.00)			
IV		4 (4.08)			
Infiltration depth (T)					
T2		17 (17.35)	0.2677	0.5349	0.6153
T3		57 (58.16)			
T4		24 (24.49)			
Histological malignancy grade (G)					
G1		3 (3.06)	0.876	0.0457	0.2369
G2		89 (90.82)			
G3		6 (6.12)			
Status of surrounding lymph nodes (N)					
N0		45 (45.92)	0.0073	0.6492	0.0045
N1 + N2		53 (54.08)			
KRAS gene status (*n* = 28)					
Mut		14 (50.00)	0.0019	–	0.3083
wt		14 (50.00%)			
Lymphocytic infiltration					
In the tumor	0	71 (72.44%	0.0001	0.2681	0.7142
	1	27 (27.55)			
In the tumor area	0	63 (64.29)	0.8078	0.7340	0.5418
	1	35 (35.71)			
Tumor budding					
0		38 (38.77)	0.6854	0.5023	0.8596
1		60 (61.22)			

Mut – presence of mutation, wt – absence of mutation

ing cells). An association of PD-L1 protein expression in cancer cells with the depth of tumor infiltration and the presence of lymph node metastasis was also found [42]. In the present study, PD-L1 protein expression in lymphocytes was demonstrated in 43.9% of cases. PD-L1 protein expression in lymphocytes was found to be related to clinical stage and the presence of lymph node metastasis. The higher the clinical stage was, the less frequent was the expression of PD-L1 protein in lymphocytes. Moreover, PD-L1

Table 4. Correlation of PD-1 and PD-L1 protein expression with selected clinical and morphological parameters ($n = 98$)

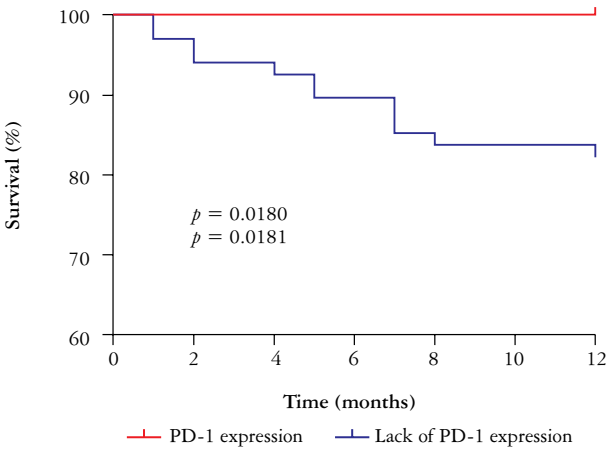
Parameters	PD-1		PD-L1 tumor cells		PD-L1 lymphocytes	
	Spearman R	*P*-value	Spearman R	*P*-value	Spearman R	*P*-value
Gender	–0.0684	0.5032	–0.2432	0.0158	–0.0609	0.5517
Age	–0.2924	0.0035	–0.0259	0.8005	–0.0843	0.4092
Clinical stage	–0.2600	0.0097	–0.0623	0.5425	–0.2581	0.0103
Histological malignancy grade (G)	–0.0442	0.6660	0.2280	0.0239	–0.1661	0.1021
Status of surrounding lymph nodes (N)	–0.2705	0.0071	–0.0592	0.5627	–0.2868	0.0042
KRAS gene status ($n = 28$)	–0.6209	0.0004	–	–	–0.2604	0.1808
Lymphocytic infiltration						
In the tumor	0.3658	0.0002	0.0858	0.4007	0.0187	0.8548
In the tumor area	–0.0387	0.7050	–0.0464	0.6503	0.0817	0.4237

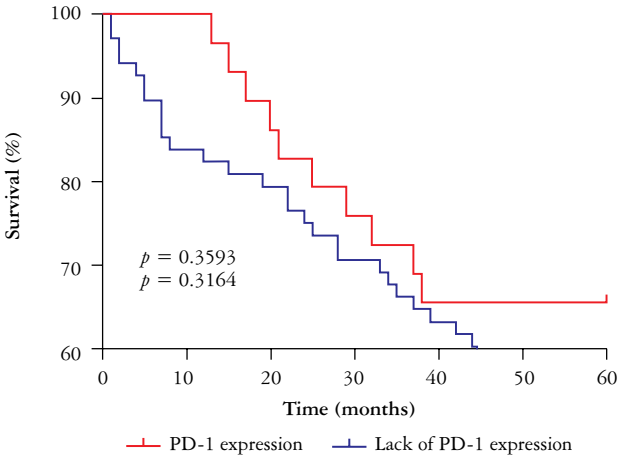
protein expression in lymphocytes was observed more frequently in patients who did not have lymph node metastases. In his study of PD-L1 protein expression, Masugi observed in colorectal cancers both a membranous and cytoplasmic reaction in the histochemical reaction [41], which is confirmed by other researchers [42–47]. The authors emphasized that evaluation of PD-L1 expression in tissue was challenging because there is no universally accepted immunohistochemical method to demonstrate this expression, and PD-L1 expression in the cell membrane is likely to be associated with the possibility of binding to the PD-1 receptor. Membrane expression may be masked in cells with strong cytoplasmic expression, so both cytoplasmic and membrane expression levels were used for calculations, and were evaluated independently by two specialists [41]. Also in the present study, a two-level evaluation was used – slides were evaluated by an experienced pathomorphologist, and then calculations were made using image analysis software to determine PD-L1 protein expression in cell membranes.

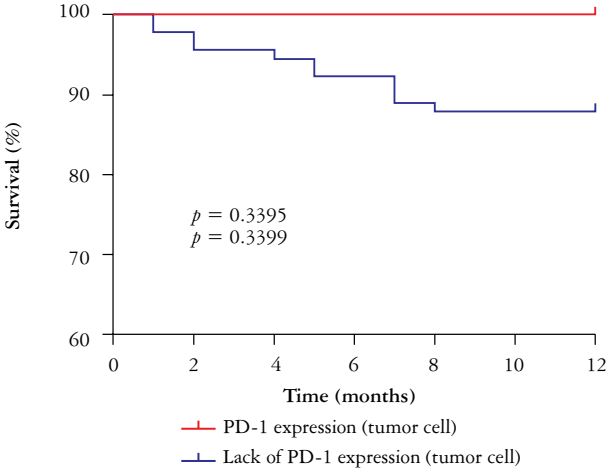
As reported previously, good therapeutic results with the drugs nivolumab and pembrolizumab, as well as atezolizumab, have been obtained in the treatment of NSCLC. Mansour *et al*. studied by immunohistochemistry both formalin-fixed and paraffin-embedded histologic and cytologic samples of lung cancer. In both variants, they detected PD-L1 protein expression in 55% of cases, but high PD-L1 expression in the tumor in histological specimens correlated with better response to treatment. Squamous cell carcinomas showed higher PD-L1 expression than adenocarcinomas.

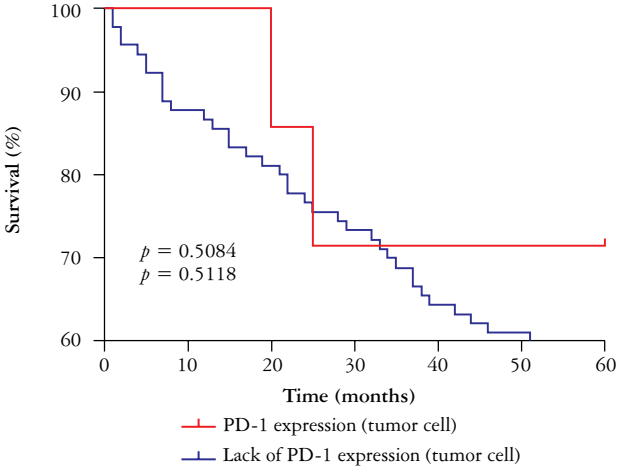
There are reports on the association of *KRAS* and epidermal growth factor receptor (EGFR) gene status with PD-L1 expression. The highest PD-L1 expression occurred in cases with *KRAS* mutations, and the lowest for cases with *EGFR* mutations and in cases without mutations in both *KRAS* and *EGFR* [48]. In the case of colorectal cancer, *KRAS* gene status was one of the first biomarkers to be studied, and is now the primary criterion that qualifies colorectal cancer patients for treatment with the monoclonal antibodies cetuximab and panitumumab. These drugs target the EGFR receptor [49–52]. The efficacy of therapy directed against EGFR in patients without mutations in the *KRAS* gene is 8–11%. Mutations in the *KRAS* gene are detected in about 40% of patients with colorectal cancer [53–55]. It is therefore necessary to find new predictive biomarkers for patients who, despite having the wild-type *KRAS* gene, are resistant to treatment directed against the EGFR. In the present study, 28 patients with defined *KRAS* gene status were examined. The *KRAS* mutation was detected in half of the cases. However, none of the 28 patients, either with or without mutations in *KRAS*, had PD-L1 protein expression in colorectal cancer cells. The association of PD-L1 protein expression in lymphocytes with *KRAS* gene status was tested, but no statistical significance was found. A significant correlation was obtained by analyzing the association of *KRAS* gene status with PD-1 protein expression in lymphocytes – none of the cases with *KRAS* mutation showed PD-1 protein expression. However, the low abundance of samples tested for *KRAS* gene status should be kept in mind here.

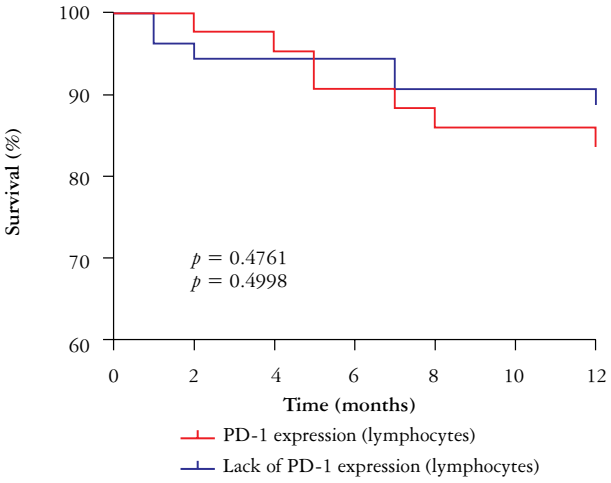
Studies have reported that the clinical response rate in various types of cancer treated with PD-1/PD-L1 inhibitors is 30–50% [56]. Therefore, it seems that identifying predictive biomarkers to select patients and improve treatment efficacy without additional and unwarranted side effects is necessary to create targeted therapies for the individual patient. Immunotherapy, based on the use of immune checkpoint inhibitors, has revolutionized the treatment of various types of cancer in recent years. However, when it comes to colorectal cancer, currently only patients with either a stable DNA repair system or a high degree of microsatellite instability can benefit from such therapy, and they represent only 5% of patients with advanced colorectal cancer. Clinical trials are underway to evaluate treatment effi-











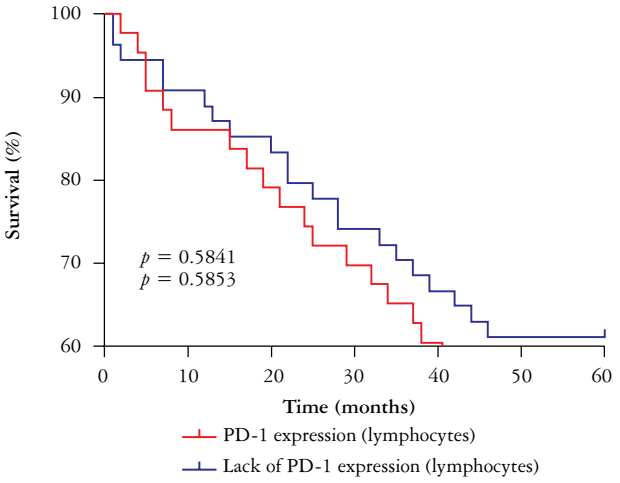


Fig. 4. Survival of patients 12 months after diagnosis in relation to the expression of PD-1 and PD-L1 proteins in cancer cells and lymphocytes

Fig. 5. Survival of patients 60 months after diagnosis in relation to the expression of PD-1 and PD-L1 proteins in cancer cells and lymphocytes

cacy when PD-1/PD-L1 inhibitors are combined with other immune checkpoint inhibitors, as well as with chemotherapy, radiotherapy and molecularly targeted therapies [57–59]. The role of PD-L1 expression in colorectal cancer is not well defined, and published studies show inconsistent results regarding the association of PD-L1 expression with prognosis [60, 61]. In the present study, the low percentage of patients showing PD-L1 protein expression in cancer cells shows that the study should have been conducted on a larger number of patients, which would have provided more reliable results.

Conclusions

Despite a slight improvement in the survival of colorectal cancer patients, approximately half still require new therapies. Unlike other malignancies, such as kidney cancer, lung cancer, or melanoma, colorectal cancer shows a very low response rate to PD-1 or PD-L1 protein blockade [62–64], but studies conducted on the PD-1/PD-L1 pathway indicate that it may be an important mechanism for cancer cells to escape from immune surveillance [65]. This leads us to believe that in the future, the therapeutic use of this pathway will become increasingly applicable in the treatment of colorectal cancer as well. However, the study of the association of PD-1 and PD-L1 protein expression with clinical, morphological, genetic and molecular parameters or prognosis of colorectal cancer requires further research.

Disclosures

1. Institutional review board statement: Not applicable.
2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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Address for correspondence

Sylwia Jankowska
Department of Pathology
Pomeranian Medical University
Unii Lubelskiej 1
71-252 Szczecin, Poland
Phone: +48663369866
e-mail: sylwia_jankowska@interia.eu