miREIA – an immunoassay method for the assessment of microRNA levels in tumor tissue – a pilot study. The impact of miR-93-5p, miR-142-5p, and IFNγ on PD-L1 level in colorectal cancer

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Colorectal cancer (CRC) is the second and third most common cancer in females and males, respectively. The PD-L1/PD-1 immune checkpoint is an important source of immunosuppression in the tumor microenvironment and is associated with IFNγ. Recent studies revealed that a significant number of tumor-suppressive miRNAs can regulate the expression of PD-L1. The objective quantification of selected microRNAs using the miREIA method in CRC tissue was performed. We investigated the role of miR-93-5p and miR-142-5p expression and the levels of IFNγ in regulating the expression of PD-L1 in tumor and margin tissues of CRC in relation to the histological grade, TNM classification, and tumor localization. 37 samples of tumor and margin tissues from CRC patients were evaluated. MiR-93-5p and miR-142-5p levels were measured using a novel method for the quantitative measurement of human microRNA (miREIA). The concentrations of PD-L1 and IFNγ were determined using ELISA kits. We found higher concentrations of miR-93-5p, PD-L1, and IFNγ in tumor samples compared to the tumor margin samples. A significant correlation was found between PD-L1 and IFNγ levels in tumor and margin specimens; between miR-142-5p and miR-93-5p levels in tumor and margin specimens. A higher level of miR-93-5p was found in tumor margin tissues on the left side of the colon. Patients with distant metastases were characterized by higher miR-93-5p concentration compared to patients without metastases. CRC is an important source of PD-L1, IFNγ, and miR-93-5p expression. Understanding the mechanisms underlying intratumoral PD-L1 expression may create new opportunities for targeted immunotherapy of colorectal cancer.

Keywords: miREIA, miR-93-5p, miR-142-5p, PD-L1, IFNγ, colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease that affects the colon and rectum, which are parts of the gastrointestinal system. It is one of the most serious illnesses irrespectively of gender. It has been recognized as the second and third most common cancer in females and males, respectively. According to Global Cancer Statistics, the prevalence of CRC in 2018 was about 6.1% of new cancer cases (Rawla *et al.*, 2019). It is the second leading cause of cancer-related death worldwide (Keum and Giovannucci, 2019). A 60% increase in CRC cases is expected worldwide by 2030 (Al-Zalabani, 2020).

The discovery of the PD-1/PD-L1 immune checkpoint had an immense impact on novel cancer therapies. Overexpression of PD-L1 has already been confirmed in many types of cancer, including lung, hematopoietic, esophageal, gastric, and colorectal cancer. PD-L1 expressed on tumor cells interacts with PD-1 on tumor-infiltrating lymphocytes, such as T cells, B cells, and myeloid cells. This interaction can activate the down-stream signaling of PD-1 receptors in T cells, thus inhibiting proliferation, cytokine generation and cytotoxity of T cells. This down-regulation is used by tumors to protect themselves from immune attack (Wu *et al.*, 2019).

Recent studies revealed that interferon-gamma (IFNγ), which is a proinflammatory cytokine, and a significant number of tumor-suppressive microRNAs play an important role in cancer development and can regulate the expression of PD-L1, which is a component of the immune checkpoint (Wang *et al.*, 2017b). Inflammatory cells, especially activated T lymphocytes, are usually the major source of IFNγ. Probably, PD-L1 in tumor cells is up-regulated in response to inflammation and suppresses the excessive immune response. This mechanism allows tumors to evade immunity (Wang *et al.*, 2017b).

There are many studies on the correlation between dysregulated microRNAs and the abnormal regulation of signaling pathways involved in the initiation and progression of CRC.

Additionally, it was confirmed that microRNAs can target PD-L1 (To *et al.*, 2018).

MicroRNAs are small single-stranded non-coding RNAs of about 18-22 nucleotides in length, that regu-

late up to 30% of human genes (Petrocca *et al.*, 2008; Tsang *et al.*, 2015). They have an impact on gene expression at the transcriptional and post-transcriptional levels by completely or incompletely binding to the 3'-UTR of their target gene messenger RNA and by repressing the translation or promoting the degradation of the target gene to exert biological functions (Wang *et al.*, 2017d). They are involved in several biological processes, including proliferation, metabolism, apoptosis, and inflammation. Apart from their crucial role in physiological processes, microRNAs are frequently dysregulated in human cancers (Tsang *et al.*, 2015; To *et al.*, 2018).

The gene encoding miR-142-5p is located on human chromosome 17. Recent studies reported that miR-142-5p is aberrantly expressed in different pathological conditions, including cancers (Islam *et al.*, 2018). Yao and others (Yao *et al.*, 2019) showed that miR-142-5p regulated pancreatic cancer cell proliferation and apoptosis by regulating RAP1A.

MiR-93-5p is a member of the miR-106b-25 cluster, its sequence is 23 nucleotides in length and its gene is located on chromosome 7. MiR-93-5p was reported to be up-regulated in several cancer types such as hepatocellular, lung, gastric, and particularly colorectal cancer. Wang and others (Wang *et al.*, 2017c) reported that miR-93-5p increases multidrug resistance in CRC cells by down-regulating cyclin-dependent kinase inhibitor 1A gene expression.

It was suggested that miR-93-5p and miR-142-5p can regulate PD-L1 expression by biding to its 3'UTR. However, the role of this pathomechanism in CRC remains largely unknown, and hence this study was undertaken (Cioffi *et al.*, 2017; Jia *et al.*, 2017; Wang *et al.*, 2017b; Chen *et al.*, 2020).

It was reported that microRNAs may regulate PD-L1 expression in a direct and indirect manner. Some microRNAs (miR-93-5p,miR-142-5p, miR-15a, miR-15b, miR 106b, miR 142-5p, miR 193a-3p, miR-200, miR 324-5p, miR 338-5p, miR-424, miR-513) directly target the 3'-UTR of PD-L1 mRNA whereas other microRNAs (including miR-181a, miR-378, miR-27a, miR-23b, miR-10a) might regulate the PDL1 level by affecting pathways of PD-L1-related genes such as FN-γ, IFNGR, IRF1, PTEN, JAK, STAT, PI3K, AKT, MEK and ERK (Wang *et al.*, 2010; Ma *et al.*, 2011; Yu *et al.*, 2015; Kao *et al.*, 2017; Wang *et al.*, 2017b).

We used miREIA – an immunoassay method for quantification of miR-142-5p and miR-93-5p in the samples of colorectal tumor tissue and surgical margin tissue. The method involves the hybridization of microRNA to a complementary biotinylated DNA oligonucleotide probe. In this method, the DNA-RNA hybrids are then captured by a microtiter plate-immobilized monoclonal antibody specific to DNA/microRNA hybrids. In the next step standard ELISA techniques are used (Kappel *et al.*, 2015). To assess the usefulness of the miREIA method for the absolute quantification of the miRNA in tumor tissue samples we chose miR-93-5p and miR-142-5p. The reason was that as of November 1, 2019, of the available miREIA kits, only these two microRNAs had binding sites on the 3'-UTR side of the PD-L1 mRNA provided by TARGETSCAN.

Our study aimed to investigate the roles of miR-93-5p and miR-142-5p expression and the levels of IFNγ in regulating the expression of PD-L1 in tumor

and margin tissues of CRC in relation to the histological grade, TNM classification and tumor localization.

MATERIALS AND METHODS

Study sample

The samples from 37 patients obtained during surgery due to CRC were included in the study. Patients were treated in the 1st Specialistic Hospital in Bytom, Poland (approval of the Research Ethics Committee No. KNW/0022/KB1/42/III/14/16/18). The collected specimens included colorectal tumor tissues and surgical margin tissues. Patients were enrolled in the study after meeting the following criteria: age>18 years, signed written consent, histological confirmation of colorectal adenocarcinoma, and surgical "tumor-free" margin tissue. The exclusion criteria were as follows: no consent to participate in the study, tumors other than adenocarcinoma, tumors with involved margins, age<18 years. To classify the cancer stage, the TNM staging system and grading were used. Research sample characteristics are presented in Table 1.

Sample preparation for the evaluation of microRNA level

Upon collection, the fragments of the tumor tissue and surgical margin tissue were stored in RNAlater Stabilization Solution (Ambion, Inc., USA) at –80°C. The samples were prepared for RNA isolation by prior tissue disruption. The samples were homogenized

Table 1. Patient characteristics

	Female	Male	
	16	21	37 (100%)
Age	62.56±11.5	61.91±9.14	62.19±10.08
T parameter			
T1	0 (0%)	0 (0%)	0 (0%)
T2	7 (43.75%)	3 (14.29%)	10 (27.03%)
T3	7 (43.75%)	12 (57.14%)	19 (51.35%)
T4	2 (12.5%)	6 (28.57%)	8 (21.62%)
N parameter			
N0	8 (50%)	9 (45%)	17 (45.95%)
N1	6 (37.50%)	6 (30%)	12 (32.43%)
N2	2 (12.50%)	5 (25%)	7 (18.92%)
M parameter			
M0	15 (93.75%)	14 (70.00%)	19 (51.35%)
M1	1 (6.25%)	6 (30.00%)	7 (18.92%)
TNM stage			
I	6 (37.50%)	2 (9.52%)	8 (21.62%)
II	2 (12.50%)	6 (28.57%)	8 (21.62%)
III	7 (43.75%)	7 (33.33%)	14 (37.84%)
IV	1 (6.25%)	6 (28.57%)	7 (18.92%)
Grading			
G1	1 (6.25%)	0 (0.00%)	1 (2.70%)
G2	15 (93.75%)	21 (100%)	36 (97.30%)
G3	0 (0%)	0 (0%)	0 (0%)

using FastPrep®-24 Classic Instrument (MP Biomedicals, Solon, CA, USA) at 11,000 rpm in Lysis Buffer (BioVendor, Czech Republic) in Lysing Matrix D Column (MP Biomedicals, Solon, CA, USA). MiRIA RNA Isolation Kit (BioVendor, Czech Republic) was used to isolate RNA. The total RNA concentration was determined using NanoPhotometer® P-Class (Implen GmbH, Germany).

Evaluation of hsa-miR-142-5p and hsa-miR-93-5p levels

MicroRNA levels in the isolates were assessed by an enzyme immunoassay for the quantitative measurement of human microRNA. It is a novel method that involves the hybridization of microRNA isolated from the patient sample to a complementary biotinylated DNA probe (miREIA). We conducted the assay (miREIA) according to the manufacturer's instructions. The level of hsa-miR-142-5p was assayed using the human hsa-miR-142-5p miREIA kit (BioVendor, Czech Republic) with a sensitivity of 0.13 amol/μl. The level of hsa-miR-93-5p was determined using the hsa-miR-93-5p miREIA kit (BioVendor, Czech Republic) with a sensitivity of 0.13 amol/μl. Hybridization was conducted using Eppendorf 5333 MasterCycler Thermal Cycler (Eppendorf AG, Germany). The absorbance of the samples was determined using a Universal Microplate Spectrophotometer (μQUANT, Biotek Inc., Winooski, VT, USA) at a wavelength of 450 nm. The obtained results were recalculated to the corresponding total RNA level and presented as ng/μl of RNA.

Preparation of samples for the evaluation of PD-L1 and INFγ levels

Fragments of the tumor tissue and surgical margin tissue were weighted and homogenized using a PRO 200 homogenizer (PRO Scientific Inc., USA) at 10 000 rpm in nine volumes of phosphate-buffered saline (BIOMED, Poland). The suspensions were sonicated with an ultrasonic cell disrupter (UP 100, Hilscher, Germany). Subsequently, the homogenates were centrifuged at 12 000 rpm for 5 min at 4°C. The total protein level was determined using a Universal Microplate Spectrophotometer (μQUANT, Biotek Inc., Winooski, VT, USA).

Evaluation of PD-L1 and INF-γ levels

To assess the levels of the investigated proteins, an enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturer's instructions. PD-L1 level was evaluated using a human PD-L1 ELISA kit (Cloud Clone, China) with a sensitivity of 0.057 ng/ml. INFγ level was determined using the human INFγ level ELISA kit (KHC4022, Germany) with a sensitivity of 4 pg/ml. The absorbance of the samples was determined using a Universal Microplate Spectrophotometer (μQUANT, Biotek Inc., Winooski, VT, USA). The measurement was conducted at a wavelength of 450 nm. The obtained results were recalculated to the corresponding total protein level and presented as g/g of protein.

Statistical analysis

Data distribution was assessed using the Shapiro–Wilk test. The log transformation of the levels of the examined molecules provided a better fit to the Gaussian distribution. Data are presented as mean ± S.D. for the variables with normal distribution and as median with interquartile range for the variables with non-normal distribution. To compare the tumor and margin levels, the paired Student's t-test was used. Independent variables were compared using the Student's t-test. Pearson's coefficient was used to assess the relationships between the examined variables with normal distribution. The Spearman's correlation coefficient was used for variables with non-normal distribution. P values<.05 were considered statistically significant. The statistical analysis was performed using STATISTICA 13 software (TIBCO Software Inc., Palo Alto, CA, USA).

RESULTS

We found significantly higher concentrations of miR-93-5p, PD-L1 and IFNγ in the tumor samples compared to surgical margin tissues (Table 2). The difference in miR-142-5p concentrations between tumor and margin tissues was not significant (Table 2).

Table 2. Levels of miR-142-5p, miR-93-5p, PD-L1, and IFNγ molecules in tumor and margin tissues; miR levels are presented as log-transformed values (ng/μl) and protein levels as g/g.
We observed significantly higher concentrations of miR-93-5p, PD-L1 and IFNγ in the tumor samples compared to surgical margin tissues.

	Tumor		Margin		P
	Mean	S.D.	Mean	S.D.	
log miR-142-5p	–5.49	0.42	–5.37	0.38	0.191
log miR-93-5p	–5.27	0.29	–5.44	0.36	0.029
log PD-L1	–4.31	0.30	–4.47	0.23	0.002
log IFNγ	–8.01	0.31	–8.15	0.35	0.003

We found a significant correlation between miR-142-5p and miR-93-5p concentrations in tumor and margin tissues (Table 3). A significant correlation was also found between PD-L1 and IFNγ levels in tumor and margin specimens (Table 3). Additionally, IFNγ levels in the tumor were correlated with PD-L1 levels in margin tissues (Table 3).

Patients with distant metastases were characterized by higher miR-93-5p concentration compared to patients without metastases (Fig. 1). We found a higher level of

Table 3. Correlation between the levels of the examined molecules.
We observed a significant correlation between miR-142-5p and miR-93-5p levels in tumor and margin tissues, as well as between PD-L1 and IFNγ levels in tumor and margin tissue. Furthermore, IFNγ concentrations in the tumor were associated with PD-L1 concentrations in margin tissues.

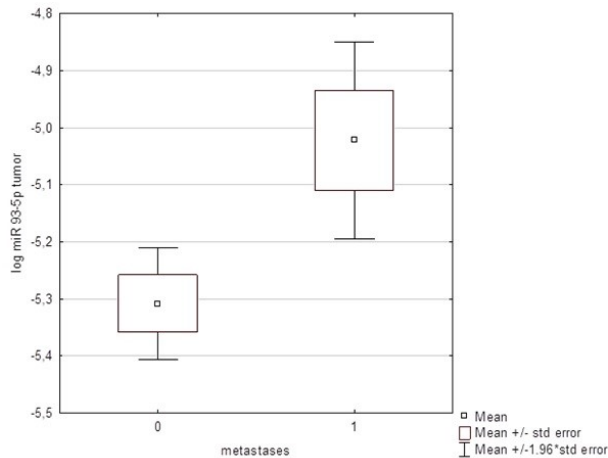| Pair of variables	R	P
Tumor log miR-93-5p and tumor log miR-142-5p	0.52	0.002
Margin log miR-93-5p and margin log miR-142-5p	0.40	0.022
Margin log PDL 1 and margin log IFNγ	0.72	<0.0001
Tumor log IFNγ and margin log IFNγ	0.69	<0.0001
Tumor log PDL 1 and tumor log IFNγ	0.57	0.001
Margin log PDL 1 and tumor log IFNγ	0.43	0.012

Figure 1. Comparison of miR-93-5p tumor levels in patients with (1)/without (0) metastases ($P=0.015$).
Patients with distant metastases were characterized by higher miR-93-5p concentration compared to patients without metastases.

miR-93-5p in the margin tissue of tumors located on the left side of the colon (Fig. 2).

DISCUSSION

Recently, the development of immune checkpoint therapy has been the most exciting event in the anti-cancer fight. The PD-1/PD-L1 immune checkpoint is the main target of this therapy. The overexpression of PD-L1 in CRC has been widely studied and assessed (Kim et al., 2016; Lee et al., 2017; Berntsson et al., 2018; Korehisa et al., 2018; Valentini et al., 2018). As a result, several trials that targeted PD-L1 were initiated. Atezolizumab, durvalumab, avelumab are anti-PD-L1 antibodies that are still under investigation for the therapy of CRC patients (Yaghoubi et al., 2019). Unfortunately, no response was seen in CRC patients when atezolizumab was administered (Brahmer et al., 2012). Moreover, no partial response was obtained in patients treated with durvalumab or avelumab (Callahan et al., 2014; Heery et al., 2017). Therefore, a novel approach to down-regulate the PD-L1 proteins and tumor immune evasion is sought.

Under physiological conditions, PD-L1 mRNA is commonly found in many tissue types. However, the PD-L1 protein was detected only in a few tissue types (e.g. tonsil tissue, the fraction of macrophage-like cells in the liver, lung and placenta). This finding indicates the existence of post-transcriptional PD-L1 regulation (Dong et al., 2002; Hirano et al., 2005). This regulation may occur via microRNAs, which is in line with previous studies (Cioffi et al., 2017; Jia et al., 2017; Wang et al., 2017b). MicroRNAs could be a novel therapeutic target for immune checkpoint modulation in anticancer therapy. In previous studies, the impact of miR-142-5p on PD-L1 levels was assessed in the mouse pancreatic ductal adenocarcinoma cell line Panc02 (Jia et al., 2017) and NSCLC cell lines (Wan et al., 2018). The influence of miR-93-5p on the investigated protein level was determined in mesenchymal stem cells (Cioffi et al., 2017). and in colorectal cancer tissues (Chen et al., 2020). In our study, we investigated the correlation between miR-93-5p and the PD-L1 protein level as well as miR-142-
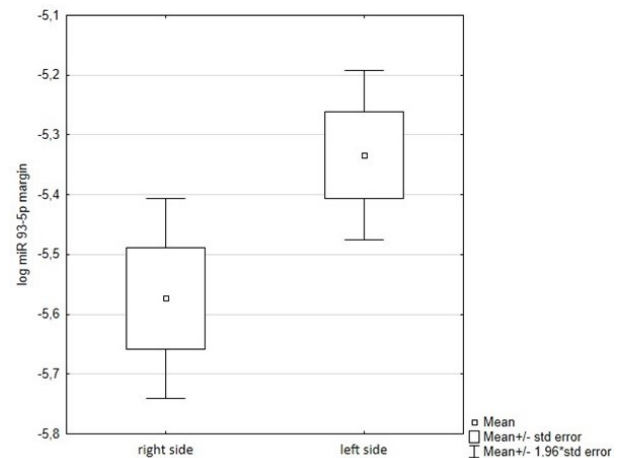

Figure 2. miR-93-5p levels in the margin tissues in patients with the tumor location in the colon ($P=0.042$).
We observed a higher level of miR-93-5p in the margin tissue of tumors located on the left side of the colon.

5p and the PD-L1 protein level to identify a new potential therapeutic target.

RT-qPCR vs miREIA

To assess the levels of miR-93-5p and miR-142-5p we used a new method of microRNA quantification, i.e. microRNA enzyme immunoassay (miREIA). RT-qPCR is the gold standard in studies on microRNAs. In turn, we obtained the absolute amount of microRNA using the miREIA method (attomol/µl) whereas in RT-qPCR the quantification is relative (Stejskal et al., 2019). Nevertheless, the Pearson correlation coefficient for results obtained using miREIA method and RT-qPCR is higher than 0.9 (Stejskal et al., 2019). What is more, the efficiency of amplification in PCR technique can depend on the content of GC-rich sequences that do not denature efficiently during PCR procedure and may cause inaccurate results (Kalle et al., 2014). The microRNA enzyme immunoassay method does not require an amplification step, so the obtained results are not disturbed by this phenomenon. Moreover, comparative studies of both methods showed that the coefficients of variations (CVs) of immunoassays are better than in RT-qPCR (Krepelkova et al., 2019; Stejskal et al., 2019). The RT step causes most of the variability produced in RT-qPCR (Ståhlberg et al., 2004), and based on the results of comparative studies, RT-qPCR is the least appropriate for microRNA quantification. However, it is still the most sensitive method and it definitely has a place in microRNA measurements (Krepelkova et al., 2019).

The correlation between miR-93-5p and PD-L1

In our study, we found a significant difference in miR-93-5p levels between cancer tissues and margin tissues ($P>0.05$). However, we did not find any correlation between miR-93-5p levels and PD-L1 levels, which also differed significantly between cancer tissues and margin tissues ($P>0.05$). Coefii and others (Cioffi et al., 2017) provided evidence that the immune modulator (PD-L1) is a target of miR-93/106b in bone marrow (stromal) cells, monocytic myeloid-derived suppressor cells (M-MDSC) and primary cancer cells. However, their sam-

ple size was small (n=4-5), the tests were conducted on mice and tumor cells obtained from murine pancreatic cancer models, which may partially explain the difference in the results. Chen and others (Chen *et al.*, 2020) also found a negative correlation between miR-93-5p levels and PD-L1 in CRC tissues and the adjacent normal tissues (n=125). The miR-93-5p levels were assessed by qRT-PCR, whereas the PD-L1 levels by immunohistochemistry (IHC). Their results showed that the miR-93-5p was significantly down-regulated in CRC tissues compared to normal tissues. They found that miR-93-5p concentration was higher in patients negative for PD-L1 expression in IHC (Chen *et al.*, 2020). However, our data revealed higher miR-93-5p levels in the tumor tissue. Moreover, we did not find a correlation between miR-93-5p and PD-L1 levels. We did not, however, divide our group into patients with negative or positive PD-L1 staining. Therefore, it may be necessary to separate a subgroup of PD-L1-positive and -negative patients based on IHC staining to clarify the relationship between miR-93-5p and PD-L1.

The correlation between miR-142-5p and PD-L1 and IFNγ

Some researchers reported that miR-142-5p played an important role in regulating the immune response. A relationship between miR-142-5p and PD-L1 expression was also confirmed experimentally. Jia *et al.* found that miR-142-5p could regulate PD-L1 expression by binding to its 3'UTR. They discovered that miR-142-5p overexpression in tumor cells inhibited the expression of PD-L1 on tumor cells, which resulted in an increase in lymphocyte and IFNγ levels. Those authors even reported that miR-142-5p overexpression could enhance anti-tumor immunity by blocking PD-L1/PD-1 pathway (Jia *et al.*, 2017). Wikberg M.L. *et al.* in their study on the relationships between plasma levels of microRNA and clinicopathological factors proved that patients with a higher T-stage had significantly lower levels of 142-5p (Wikberg *et al.*, 2018). On the other hand, a study in 125 CRC patients found that the expression of miR-142-5p in cancer tissue samples was significantly higher compared to non-cancer samples. High miR-142-5p expression was associated with the biological aggressiveness of cancer cells (Islam *et al.*, 2018). The above studies show inconclusive results related to the roles and levels of miR-142-5p in CRC.

In our study, no significant correlation was found between miR-142-5p expression and the concentrations of PD-L1 and IFNγ. Additionally, no difference was observed between tumor and margin tissues in terms of miR-142-5p concentrations. First of all, such results could be due to a small number of samples. Secondly, some studies which showed a positive relationship between miR-142-5p, PD-L1 and IFNγ were conducted in exogenous pancreatic cancer cell lines. The conditions of our experiment were different due to another cancer type, tissues taken from patients during the surgery and other methods used for the quantitative measurement of microRNA expression, which probably had an impact on obtaining different results compared to the previous findings.

The correlation between miR-93-5p and miR-142-5p

There are two general mechanisms of regulating PD-L1 expression by microRNA in cancers. Some microRNAs have been found to directly target the 3'UTR of PD-L1 mRNA, whereas indirect regulation is based on

signaling molecules such as PTEN or IFR-1. Studies demonstrated that miR-142-5p and miR-93-5p regulated PD-L1 expression directly by binding to its 3'-UTR (Wang *et al.*, 2017b). Our study demonstrated a significant correlation between miR-142-5p and miR-93-5p concentrations in tumor and margin tissues. This result may suggest that miR-93-5p and miR-142-5p are strongly involved in tumor progression. The presented correlation may be also related to a similar mechanism of regulating PD-L1 expression by miR-142-5p and miR-93-5p and may suggest their common involvement in this process. However, carefully designed experiments are needed to explain the cause of this correlation and the exact pleiotropic role of these microRNAs.

The correlation between PD-L1 and INFγ levels

For many years, IFNγ has been considered a crucial antitumor cytokine that is produced mainly by CD8-positive T Cells, natural killer cells, natural killer T cells, and macrophages (Mandai *et al.*, 2016; Ayers *et al.*, 2017). It inhibits tumor growth by inducing macrophage polarization, activation of cytotoxic T lymphocytes, modulation of dendritic cells, and up-regulation of MHC I expression (Cornetta *et al.*, 1994; Mandai *et al.*, 2016). Due to its antitumor activity, IFNγ has been used clinically to treat a variety of tumors, including bladder cancer, melanoma and CRC (Mandai *et al.*, 2016; Mojic *et al.*, 2017). In clinical trials, interferon therapy provided conflicting outcomes and even aggravated the course of the disease (Mojic *et al.*, 2017). Recent studies reported that the role of IFNγ in tumor development has a dual aspect (Mandai *et al.*, 2016). IFNγ can promote tumor progression by up-regulating PD-L1 in different malignancies, including ovarian, breast, lung, gastric cancers, CRC, melanoma, and glioma.

It was reported that the PD-L1 gene promoter region has a response element to IRF-1, which is a mediator of the IFNγ signaling pathway (Garcia-Diaz *et al.*, 2017). These findings may explain the differences in response to IFNγ treatment in clinical trials and the correlation between the levels of PD-L1 and IFNγ in different types of cancer. The correlation between IFNγ and PD-L1 gene expressions measured by qPCR was observed in surgically resected CRC ($P=0.03$, $r=0.33$) (Droeser *et al.*, 2013). The level of PD-L1 correlates positively with the level of IFNγ in patients with metastatic melanoma, gastric and ovarian cancers. Furthermore, it was reported that this correlation changes during anti-PD-1 therapy, whereas IFN therapy increases the level of PD-L1 (Chen *et al.*, 2018; Mimura *et al.*, 2018). Our results demonstrated the positive correlation between IFNγ and PD-L1 levels not only in the tumor but also in the surrounding margins. It is consistent with results obtained from studies of various solid tumors as well as CRC (Droeser *et al.*, 2013; Chen *et al.*, 2018; Mimura *et al.*, 2018). This finding shows the significance of normal tissue that does not contain cancer cells but is involved in immune processes in the tumor microenvironment and may play an essential role in tumor expansion.

The correlation with clinicopathological features

Some studies showed a correlation between the levels of PD-L1 and IFNγ in the tumor or serum and clinicopathological parameters such as the TNM stage, histological grade and poor prognosis. However, the results are inconclusive. Droeser *et al.* demonstrated that in CRC the increased level of PD-L1 is associated with the presence of CD8+ T cell infiltration, an early T stage,

the lack of lymph node metastases, lower grading, the lack of vascular invasion, and a longer median overall survival (Droeser et al., 2013). On the contrary, other researchers indicated that PD-L1 overexpression in CRC was associated with advanced TNM stage, lymph node metastases, distant metastases, and decreased overall survival (Guo et al., 2018). Also in cervical squamous cell carcinoma, an increased level of PD-L1 correlated with advanced TNM stage, a decrease in the number of lymphocytes infiltrating the tumor and poor prognosis (Feng et al., 2018). Lack of association between the level of PD-L1 and clinicopathological parameters in our study may be due to a small, homogeneous study group. However, previous studies concerning the correlation between PD-L1 and clinicopathological features were contradictory and ambiguous. Therefore, perhaps PD-L1, despite its role in cancer-related immune response regulation, is not a suitable biomarker for clinicopathological features. This assumption seems to be also confirmed by a recent meta-analysis (Alexander et al., 2021).

Additionally, the association between miR-93-5p, miR-142-5p and the clinical parameters in different types of cancers are not clear. The results of our study did not show any correlation between miR-142-5p and clinical parameters. However, it was reported that in CRC the level of miR-142-5p in the tumor was elevated and correlated with proximal CRC (Islam et al., 2018). In intrahepatic cholangiocarcinoma, the serum level of miR-142-5p was increased in patients with a more advanced TNM stage and higher histological grade (Wei et al., 2019). However, in breast cancer, a lower serum level of miR-142-5p correlated with more advanced grading and larger tumor size (Ozawa et al., 2020).

Our data showed a significantly elevated margin level of miR-93-5p in patients with left-sided tumor location compared to the right-sided location. This association was not investigated in other studies, but miR-326 (another microRNA) was reported to be correlated with right-sided CRC (Moreno et al., 2019). Moreover, we found a positive correlation between miR-93-5p and metastases. The literature describes only the correlation of miR-93-5p with the phenomenon of multi-drug resistance, but the experiment was carried out on colorectal cancer cell lines (Wang et al., 2017c). To confirm the possible role of miR-93-5p as a biomarker of metastases in CRC and possibly other clinical parameters it is necessary to conduct a larger study. However, among other solid tumors, the dependence of clinical parameters on miR-93-5p was studied more extensively. In gastric and lung cancers, as well as head and neck squamous cell carcinoma, an increased level of miR-93-5p in the tumor was reported compared to the adjacent normal mucosa (Li et al., 2015; Lu et al., 2016; Ma et al., 2017). In non-small lung cancer, the level of miR-93-5p was associated with lymph node involvement and staging, whereas the overexpression of miR-93-5p in head and neck cancers correlated with the T parameter, lymph node involvement and advanced stage (Li et al., 2015; Lu et al., 2016). In gastric cancer, clear cell renal cell carcinoma and cervical cancer, miR-93-5p was also overexpressed in the tumor tissue and correlated with the stage of disease or poor patient prognosis (Guan et al., 2017; Wang et al., 2017a; Zhang et al., 2018).

STUDY LIMITATIONS

This study has potential limitations. First of all, it is limited by a small number of samples. Nevertheless, in our study, we obtained significant differences in the levels of the investigated molecules. Additionally, the mRNA expression and IHC staining were not evaluated for PD-L1. Therefore, further studies should focus on the analysis of mRNA expression of PD-L1 as well as determining the localization of PD-L1. It is crucial to establish the localization of PD-L1 by IHC to confirm that PD-L1 levels obtained in the ELISA test came from cancer cells and not from the tumor-infiltrating immune cells (myeloid cells). In future studies, we plan to analyze the mRNA expression of PD-L1 and perform microRNA quantification by RT-qPCR to compare microRNA levels with miREIA results. Additionally, we plan to evaluate proinflammatory cytokines and separate the subgroup of patients positive and negative for PD-L1, using IHC staining.

CONCLUSION

The miREIA method can be used to obtain absolute levels of microRNA in the colon tissue. PD-L1 plays an important role in tumor immune escape and it is up-regulated in CRC tissues. MiR-93-5p levels were significantly higher in surgical margin tissues, but they did not correlate with PD-L1 levels. However, miR-93-5p level correlated with metastases parameter thus could be considered as a potential biomarker of metastasis in CRC. Currently, little is known about the exact impact of microRNAs on the expression of PD-L1 in tumor cells. However, there are many directions in which research is conducted on the mechanisms of regulating the expression of many key proteins and signaling pathways involved in cancer development through microRNA. At the same time, therapeutic methods using microRNAs are being developed, many of them are in the pre-clinical stage. Moreover, the results of many preclinical studies allow for the creation of hypotheses and rationale for combining miRNA-based therapy with Immune Checkpoint Inhibitors (ICI) for increasing ICI efficacy or overcoming immunity to immune checkpoint blockade (Huemer et al., 2021). Both levels of miR-93-5p and miR-142-5p in the tumor and surgical margins are associated with and strongly involved in tumor progression. Further research and expansion of knowledge about these molecules may be potentially useful in cancer therapies. However, it is necessary to accurately determine all gene targets for a specific microRNA, as one microRNA can regulate the expression of many genes. MicroRNAs have received a lot of attention in the last decade and are widely studied. With the development of new molecular biology techniques like miREIA, qualitative and quantitative analysis of microRNAs becomes more adequate, easier, cheaper, and requires less specialized equipment.

Conflict of interest

The authors declare that they have no conflict of interest.

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