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REVIEW

Key questions about the checkpoint blockade-are microRNAs an answer?

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ABSTRACT The introduction of immune-checkpoint blockade in the cancer therapy led to a paradigm change of the management of late stage cancers. There are already multiple FDA approved checkpoint inhibitors and many other agents are undergoing phase 2 and early phase 3 clinical trials. The therapeutic indication of immune checkpoint inhibitors expanded in the last years, but still remains unclear who can benefit. MicroRNAs are small RNAs with no coding potential. By complementary pairing to the 3' untranslated region of messenger RNA, microRNAs exert posttranscriptional control of protein expression. A network of microRNAs directly and indirectly controls the expression of checkpoint receptors and several microRNAs can target multiple checkpoint molecules, mimicking the therapeutic effect of a combined immune checkpoint blockade. In this review, we will describe the microRNAs that control the expression of immune checkpoints and we will present four specific issues of the immune checkpoint therapy in cancer: (1) imprecise therapeutic indication, (2) difficult response evaluation, (3) numerous immunologic adverse-events, and (4) the absence of response to immune therapy. Finally, we propose microRNAs as possible solutions for these pitfalls. We consider that in the near future microRNAs could become important therapeutic partners of the immune checkpoint therapy.

KEYWORDS MicroRNA; PD-1; PD-L1; CTLA-4; checkpoint inhibitors

Introduction

The introduction of immune checkpoint blockade (ICB) in cancer therapy led to a paradigm change of the management of late stage cancers. This new therapy inhibits the cancer mediated suppression of the immune system. The first checkpoint inhibitor approved for cancer treatment was ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibody which was initially used only for the treatment of metastatic melanoma and more recently also as an adjuvant therapy for stage III melanoma patients[1,2]. The FDA approved other two agents, pembrolizumab and nivolumab, both of which are anti-programmed cell death protein 1 (PD-1) monoclonal antibodies. These two new agents were initially indicated for the treatment of stage IV melanoma[3,4] and for non-small cell lung cancer (NSCLC)[5,6].

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Additionally, in late 2016 and early 2017, FDA approved atezolizumab [anti-programmed death-ligand 1 (PD-L1) monoclonal antibody] for the management of advanced and metastatic urothelial carcinoma (UC)[7] and for stage IV NSCLC[8]; avelumab (anti-PD-L1 monoclonal antibody) for the management of stage IV Merkel cell carcinoma[9] and durvalumab (also an anti-PD-L1 monoclonal antibody) for the treatment of late stage UC[10].

The therapeutic indication of immune checkpoint antibodies expanded in the last few years. Based on recent clinical trials, pembrolizumab received FDA approval for any type of late stage solid tumor with microsatellite instability-high or DNA mismatch repair deficiencies[11]. Furthermore, nivolumab was also accepted for treating renal cell carcinoma, urothelial bladder cancer, metastatic epidermoid carcinoma of the head and neck and classical Hodgkin lymphoma[12].

MicroRNAs (miRNAs) are small RNAs with no coding potential, produced from long transcripts named primary miRNAs[13]. By complementary pairing to the 3' untranslated region (UTR) of messenger RNA (mRNA), miRNAs exert a

posttranscriptional control of protein expression, usually leading to a protein repression[14]. MiRNAs differ in their origin from other small non-coding RNAs [small interfering RNA (siRNA) and Piwi-interacting RNA (piRNA)]: miRNAs derive from transcripts forming stem-loops, siRNAs derive from double strand RNA precursors and piRNAs are the product of single strand fragments[15]. MiRNAs involvement in human diseases started being intensely studied after Calin et al. demonstrated their importance in chronic lymphatic leukemia development[16,17]. Afterwards, altered miRNA expression was linked with a variety of human diseases, including infectious, autoimmune, degenerative and any type of neoplastic pathology[18]. Intriguingly, most miRNAs can target multiple mRNAs and most mRNAs are targeted by several miRNAs[19]. Hence, in order to understand the underling biological phenomenon and to be able to therapeutically manipulate, it is important to study more than one miRNA that controls the expression of one protein. Using molecular networks, it is possible to characterize not only the relationship between the inhibitor and its target, but also the interaction between the different inhibitors[20,21]. A network of miRNAs directly and indirectly controls the expression of immune checkpoint receptors. The level of each of these negative regulators of the immune system is fine-tuned by several miRNAs (direct targeting) and by other proteins, which themselves are regulated by miRNAs (indirect targeting).

The roles of miRNAs as regulators of immune checkpoints were already discussed in other reviews[22,23]. In this review, we will describe the miRNA network that controls the expression of the immune checkpoints and we will present four specific issues of the ICB: (1) imprecise therapeutic indication, (2) difficult response evaluation, (3) numerous immunologic adverse-events, and (4) the absence of response to immune checkpoint therapy. Finally, we propose miRNAs as possible solutions for these pitfalls. We consider that in the near future miRNAs could become important therapeutic partners of the ICB.

MiRNAs control immune checkpoints expression

MiRNAs fine tune the expression of immune checkpoint receptors and their ligands. One miRNA can target several checkpoint molecules, mimicking the therapeutic effect of a combined ICB[22]. It is crucial to understand which are the hubs of the miRNA regulatory network (**Figure 1**) in order to design therapies that target these super-connected nodes[24]. On the other hand, the immune checkpoint molecules can control the expression of miRNAs, making the network robust and complex. A list of the miRNAs that target the immune checkpoints and of the immune checkpoints that control the expression of miRNAs can be found in **Table 1**.

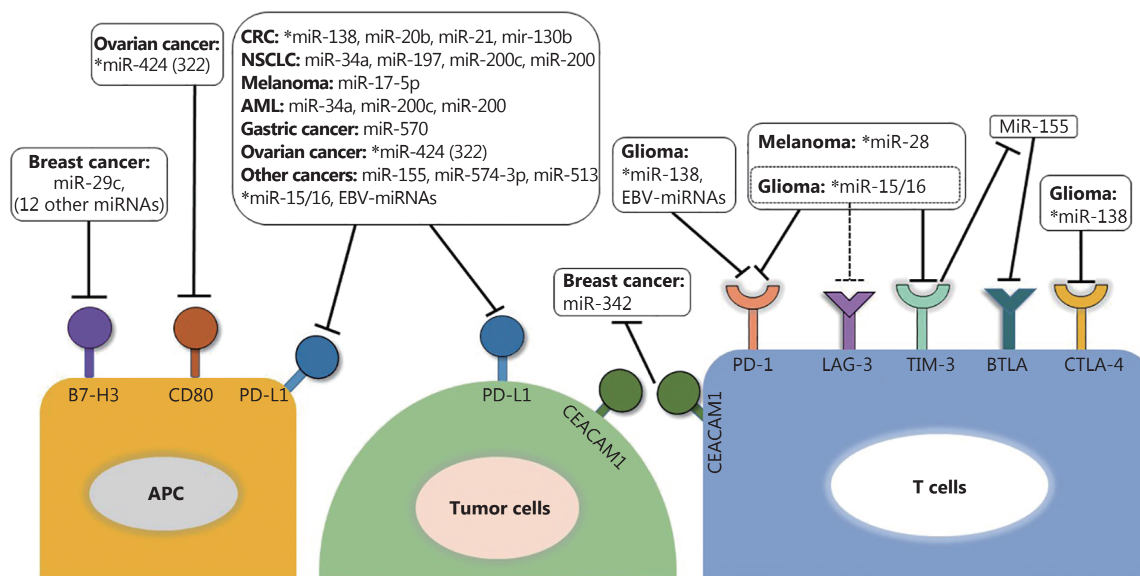

Figure 1 A network of miRNAs directly controls the expression level of the immune checkpoint molecules. Some of these miRNAs (*) target multiple immune checkpoints and are suitable therapeutic targets. Increasing the level of these hubs can lead to a multiple checkpoint blockade. Similarly, immune checkpoint can also change the expression of microRNAs.

Table 1 A panel of miRNAs controls the expression of the immune checkpoints

Item	Tissue/cell line	Relationship to immune checkpoints	Function	Ref.
MiRNAs				
MiR-424 (322)	Ovarian cancer tissue and ovarian cancer cell lines	Anticorrelates with CD80 and PD-L1	Low levels of miR-424(322) are associated with chemoresistance	26
MiR-15/16 family	Glioma mouse model	Correlates with PD-1, TIM-3, LAG-3	Low levels of miR-15a/16 prolongs mice survival	32
	MPM tissue and MPM cell lines	Anticorrelates with PD-L1	High PD-L1 is associated with low miR-15/16 levels and short overall survival	48
MiR-138	Glioma mouse model	Anticorrelates with PD-1, CTLA-4	High level of miR-138 inhibit tumor progression	33
	CRC patient samples and CRC cell lines	Anticorrelates with PD-L1	Low levels of miR-138 are associated with shorter overall survival	34
MiR-28	Exhausted T-cells from mice melanoma	Anticorrelates with PD-1, TIM3 and BTLA	Low levels of miR-28 induces T-cell exhaustion	35
MiR-155	Mouse T-cells	Anticorrelates with BTLA	Low levels of miR-155 decrease CD4+ T cell activation	37
	Dermal lymphatic endothelial cells	Anticorrelates with PD-L1	MiR-155 is part of a regulatory loop which controls the expression of PD-L1	57
MiR-29c and other 12 miRNAs	Breast cancer cell lines and tissue from breast cancer patients	Anticorrelates with B7-H3	High levels of miR-29c associate with a decreased risk of dying from breast cancer	39
MiR-570	Gastric cancer tissue	Anticorrelates with PD-L1	The inability of miR-570 to bind the PD-L1 mRNA leads to an aggressive gastric cancer phenotype	42
MiR-34a (and miR-34 family)	TCGA lung adenocarcinoma, p53 (R172HΔ)g/+K-ras (LA1/+) mouse model and various cell lines	Anticorrelates with PD-L1	P53 regulates the anti-tumor immunity by overexpressing miR-34, an inhibitor of PD-L1	43
	AML patient samples and leukemia cell lines	Anticorrelates with PD-L1	High levels of miR-34 decrease T-cell apoptosis	44
MiR-34a and MiR-200c	AML cell lines and AML mouse model	Anticorrelates with PD-L1	High levels of miR-34a and miR-200c leads to increased immune mediated killing of the tumor	45
MiR-197	NSCLC patient samples and human lung cancer cell lines	Anticorrelates with PD-L1	Low level of miR-197 predict low survival in NSCLC	46
	Oral squamous cell carcinoma	Anticorrelates with PD-L1	High levels of miR-197 predict poor overall survival	52
MiR-200	Lung adenocarcinoma databases, different mouse models and cell models	Anticorrelates with PD-L1	MiR-200 simultaneously inhibits neoplastic invasion and immunosuppression	49
MiR-20b, miR-21 and miR-130b	CRC tissue	Correlate with PD-L1	MiR-20b, miR-21 and miR-130b inhibit PTEN, which is an inhibitor of PD-L1	50
MiR-574-3p	Spinal chordoma tissue	Anticorrelates with PD-L1	Low levels of miR-574-3p are associated with worse local recurrence-free survival	51
MiR-25-93-106b cluster	Primary pancreatic cancer cells from murine models	Anticorrelates with PD-L1	The miRNA cluster controls the bone marrow metastasis	53

Continued

Continued

Item	Tissue/cell line	Relationship to immune checkpoints	Function	Ref.
EBV-miRNAs	TCGA database for various solid malignancies	Correlate with PD-L1 and PD-1	High EBV-miRNAs leads to more aggressive tumor phenotype	54
MiR-513	Human cholangiocytes	Anticorrelates with PD-L1	MiR-513 mediates the IFN-γ silencing of PD-L1	58
	Retinoblastoma cells	Anticorrelates with PD-L1	Etoposide induces a decrease of miR-513 and up-regulation of PD-L1, connecting chemoresistance to immune evasion	59
MiR-17-5p	Sera of metastatic melanoma	Anticorrelates with PD-L1	MiR-17-5p is also inversely correlated with BRAF mutation	67
Immune checkpoints				
TIM-3	Colon cancer mouse model	Downregulates miR-155	Induces M2 macrophage polarization	29
CEACAM1	MCF7 breast cancer cell line	Downregulates miR-342	High CEACAM1 induces low miR-342 and promotes luminal orientation	30
Galactin-9	Liver metastatic cell lines	Downregulates 42 different miRNAs	Tumor growth suppression	31

PD-L1: programmed death-ligand 1; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; LAG-3: Lymphocyte-activation gene 3; MPM: malignant pleural mesothelioma; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CRC: colorectal cancer; PD-1: programmed cell death protein 1; BTLA: B- and T-lymphocyte attenuator; TCGA: The Cancer Genome Atlas; AML: acute myeloid leukemia; NSCLC: non-small cell lung cancer; EBV: Epstein-Barr virus; CEACAM1: Carcinoembryonic antigen-related cell adhesion molecule 1

CTLA-4

CTLA-4 is expressed solely on T-cells and inhibits their function by binding to its ligand CD80. CTLA-4 is the first therapeutically targeted immune checkpoint molecule[25]. The function of CTLA-4-CD80 pair is controlled by miR-424 that directly binds the 3'UTR of two mRNAs involved in the immune suppressive system, CD80 and PD-L1. MiR-424 down-regulates CD80 in dendritic cell, thus increases the efficacy of chemotherapy by improving T cells immune toxicity. Further analysis revealed that higher miR-424 was correlated to the lower expression of CTLA-4 ($R=-0.1$, $P=0.0273$, $n=489$), and CD80 ($R=-0.1148$, $P=0.00111$, $n=489$)[26].

TIM-3, CEACAM1 and galactine-9

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), another immune regulator, expressed on activated T effector cells, negatively controls the responses of T effector cells by inducing T cell tolerance and exhaustion. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is expressed on activated T-cells endowing TIM-3 immunosuppressive function[27]. Galectin-9 is the most common ligand of TIM-3 that facilitates its negative immune regulatory function[28]. These checkpoints can also exert their function by changing the expression of multiple miRNAs. Over 100 miRNAs were identified dysregulated in TIM-3 knock-down macrophages suggesting that miRNAs are pivotal for TIM-3's biological function. TIM-3 negatively regulates miR-155 both *in vitro* and colon cancer mouse models. Signal transducer and activator of transcription-1 (STAT1) was confirmed as the signaling adaptor, connecting TIM-3 with miR-155 to induce M2 macrophage polarization[29]. CEACAM1 and galactine-9 can also control the expression of miRNAs. MiR-342 is a target of CEACAM1; this miRNA is down-regulated in MCF7 breast cancer cells when CEACAM1 is overexpressed. The interaction between CEACAM1 and miR-342 partially explains the mechanism by which this immune checkpoint maintains the luminal orientation in epithelial breast cells[30]. Similarly, galectin-9 can regulate 42 miRNAs in human liver metastatic cancer cell lines[31]. These data further support that the function of immune checkpoints is interconnected to the miRNA regulatory network through a dual relationship: while miRNAs controls the expression of the checkpoints, these can also change the level of miRNAs and influence their functions.

MiRNA hubs

Some miRNAs target immune checkpoints from different

cells of the tumor microenvironment and have a profound regulatory effect. In glioma, knock-out of miR-15a/16 alleviates glioma progression and prolongs mice survival by decreasing the PD-1, TIM-3 and lymphocyte-activation gene 3 (LAG-3) expression, and promotes the secretion of several cytokines from tumor-infiltrating CD8+ T cells[32].

MiR-138 was reported to inhibit glioma progression and increases the survival of tumor-bearing mice by evoking an anti-tumor immune response, by binding to the 3'UTR of PD-1 and CTLA-4. Further analysis revealed that miR-138 decreases PD-1, CTLA-4, and forkhead box protein 3 (FOXP3) in transfected CD4+ T cells. In addition, no anti-glioma effect of miR-138 treatment was found in immune-incompetent mice or in an *in vivo* T-cell depletion model, which revealed that its anti-cancer efficacy is immune system dependent[33]. In a different study, miR-138 was also reported as a direct inhibitor of PD-L1 in colorectal cancer (CRC), being able to inhibit cell growth and tumorigenesis *in vitro* and *in vivo*[34]. Similarly, miR-28 can inhibit the expression of TIM-3, B- and T-lymphocyte attenuator (BTLA), PD-1, and the secretion of cytokines IL-2 and TNF-α to modulate exhaustive differentiation of T cells[35].

BTLA

BTLA is one of the immune checkpoints that is induced during the activation of T cells. Its activation obstructs the anti-neoplastic function of CD8+ cancer-specific T cell[36]. One study showed that miR-155 targets the BTLA 3'UTR and decreases the surface BTLA expression by about 60%. As expected, knockdown of miR-155 resulted in up-regulation of surface BTLA[37]. Further studies are required to determine the function of the miR-155-BTLA interaction in neoplastic pathology.

B7-H3

B7-H3 is a B7 family member which is expressed on the surface of many cell types. The function of B7-H3 remains controversial, being unclear if its overexpression has an anti-tumor effect or an immunosuppressive function[38]. Numerous miRNAs downregulate the protein levels of B7-H3 in breast cancer cell lines. Thirteen of these miRNAs (miR-214, miR-363, miR-326, miR-940, miR-29c, miR-665, miR-34b, miR-708, miR-601, miR-124a, miR-380-5p, miR-885-3p, and miR-593) bind to the 3'UTR of B7-H3 and inhibit its translation. From these miRNAs, high expression of miR-29c was identified to show the best correlation with a substantial diminished risk of mortality from breast cancer in both discovery and validation groups[39].

PD-L1 and PD-1

The level of PD-L1 is intimately controlled by the miRNA network. PD-L1 is expressed on different types of cells, mainly on immune cells [T-cells, B-cells, monocytes, antigen-presenting cells (APCs)], but also epithelial cells. PD-L1 is overexpressed when inflammatory cytokines (e.g. IFN-γ and IL-4) stimulate the transcription factors STAT1 and IFN regulatory factor-1[40]. As an immunosuppressive mechanism, the level of PD-L1 is high in various types of neoplasia and is often linked to poor prognosis and predicts favorable responses to anti-PD-1/PD-L1 antibodies[41].

The 3'UTR of the PD-L1 mRNA harbors multiple cis-acting segments implicated in mRNA decay, as well as an adenylate-uridylate (AU)-rich element and some possible miRNA-binding sites. A single nucleotide mutation at the 3′-UTR of PD-L1 leads to the overexpression of PD-L1 by disrupting the complementarity between miR-570 and its 3'UTR binding site. This mutation is associated with high PD-L1 levels in gastric cancer and also with the aggressive phenotype[42]. The interaction between miRNAs and PD-L1 3'-UTR is dependent on the structural variations of PD-L1 3'-UTR and this type of mutation is one of the mechanism by which tumor cells can escape immune surveillance.

P53 directly controls the expression level of miR-34a, miR-34b, and miR-34c in different cell lines and tissues. The interaction between p53 and PD-L1 is mediated by miR-34, which binds to the PD-L1 3'-UTR in NSCLC models[43]. Additionally, using leukemia cell lines, Wang et al.[44] also demonstrate that miR-34a can directly bind the 3'UTR of PD-L1, downregulating its expression. Furthermore, the PD-L1 induced T cell apoptosis was decreased after transfection with miR-34a mimic. The authors also found a positive feedback mechanism among PD-L1 level and AKT activation. Another molecule that interferes with miR-34a-PD-L1 regulatory axis is mucin1 (MUC1). The inhibition of MUC1 in acute myeloid leukemia cell lines leads to a decrease of PD-L1 by overexpressing miR-34a and miR-200c, both negative regulators of PD-L1. MUC1 controls the expression of these two miRNAs by altering the level of DICER, the RNase-Ⅲ enzyme that processes precursor miRNAs into mature miRNA[45].

PD-L1 is also regulated by miR-197 through a complex regulatory mechanism involving the cyclin-dependent kinases regulatory subunit 1/signal transducer and activator of transcription 3 (CKS1B/STAT3) pathway. The expression of the immune checkpoint PD-L1 is controlled by STAT3,

which is activated by CKS1B. CKS1B is a direct target of miR-197, therefore not surprisingly, low level of miR-197 correlates with high expression of PD-L1 and predicts shorter survival in NSCLC[46]. STAT3 is also a well-known inhibitor of p53[47], therefore STAT3 is a strategic regulatory component of the network that controls the expression of PD-L1 directly and indirectly through p53-miR-34 regulatory axis.

Kao et al.[48] confirmed the role of the miR-15/16 family as an important element of the network. In malignant pleural mesothelioma (MPM) cell lines, the authors demonstrated that miR-15a, miR-15b and miR-16 directly bind to the 3'UTR of PD-L1 reducing the expression of this immune checkpoint molecule. Additionally, miR-193a-3p can directly inhibit the expression of PD-L1 in MPM.

A robust correlation between the level of epithelial-to-mesenchymal transition (EMT) involved in cancer metastasis, miR-200 and PD-L1 expression in lung adenocarcinomas have been demonstrated, where PD-L1 is directly controlled by miR-200. Additionally, miR-200 is anticorrelated with most of the EMT markers, and inhibit the phenotypical transition and reduce tumor invasion. MiR-200 forms a negative feedback-loop with the zinc finger E-box-binding homeobox 1 (ZEB1), a positive regulator of EMT[49]. Hence, we can perceive miR-200 as a node of the network that links two important hallmarks of cancer, immunosuppression and invasion.

MiR-20b, miR-21, and miR-130b are positive regulators of PD-L1 in advanced CRC. By inhibiting phosphatase and tensin homolog (PTEN), these miRNAs cause an indirect upregulation of PD-L1. These three miRNAs are one of the few that positively correlate with the expression of an immune checkpoint and would be suitable for anti-miRNA therapy[50].

For some of the miRNAs that regulate the expression of PD-L1 mechanistic insight is lacking and only statistical correlations are available. For example, in spinal chordoma, miR-574-3p was recognized to inversely correlate with PD-L1 expression: patients with high PD-L1 and low miR-574-3p chordoma were significantly associated with worse local recurrence-free survival[51]. For another miRNA only statistical inverse correlations to PD-L1 levels are available: high miR-197 anticorrelates to PD-L1 in oral squamous cell carcinoma and predicts poor overall survival[52].

The level of PD-L1 is controlled also by the miR-25-93-106b cluster. MiR-25-93-106b knockout mice have 50% higher level of PD-L1 +/CD11b + bone marrow cells compared to WT mice. Moreover, treatment with miR-93-5p and miR-106b-5p mimics or OTX015 (inhibitor of the bromodomain and extraterminal family of proteins) which is an upstream positive regulator of the same miRNA cluster, decreases the expression of PD-L1 in the peripheral blood cells of mice or primary cancer cells[53].

Very intriguing, a cluster of EBV-miRNAs is correlated with the upregulation of PD-1 and PD-L1 in solid malignancies. A large population based study from The Cancer Genome Atlas (TCGA) project showed that patients with higher level of EBV-miRNA have also high expression of PD-1, PD-L1, TGFβ1, IL-10, IFN-γ and TGFβ2, and may be candidates for immune checkpoint therapy. Additional studies are necessary to explain the mechanism behind this surprising correlation[54].

Based on the interaction between miRNAs and IFN-γ/STAT1 pathway, Baer et al.[55] found that DICER deficiency in tumor-associated macrophages (TAMs) induces an anti-tumorigenic phenotype, with tumors populated largely by M1-like TAMs. Moreover, tumors populated by DICER deficient TAMs can be completely eradicated by treatment with anti-PD-1 antibodies or CD40 agonistic antibodies. The rescue of let-7 activity in DICER-/- macrophages leads to an increased M2-like macrophage population and reduces the number of tumor-infiltrating cytotoxic T lymphocytes. These observations sustain that DICER/let-7 activity antagonizes the IFN-γ induced anti-neoplastic effect.

Recently, it was also shown that the oncogenic miR-155 is necessary to limit tumor growth and activate IFN-γ synthesis by T cells within the neoplastic microenvironment. ICB against PD-1, PD-L1 and CTLA-4 restored the antitumor immunity in conditionally deleted miR-155 in T-cells. This data suggests that the ICB and miR-155 control overlapping signaling pathways. Additionally, miR-155 deficiency leads to a decrease expression of IFN-γ genes in TAMs[56].

The interaction between IFN-γ - miR-155 and PD-L1 was proven in dermal lymphatic endothelial cells and dermal fibroblast. Treating the cells with IFN-γ or TNF-α induced an upregulation of PD-L1 and miR-155, while miR-155 inhibits the expression of PD-L1. Hence, it seems that miR-155 is controlling the expression of PD-L1 activation, building a regulatory loop[57]. On the contrary, IFN-γ stimulation of biliary epithelial cells leads to the decrease of miR-513 (in fact miR-513a-5p) and upregulation of PD-L1. MiR-513 targets PD-L1 and miR-513 transfection downregulates the IFN-γ induced PD-L1 protein expression[58]. It is not clear if these regulatory mechanisms are tissue specific or are present simultaneously when PD-L1 is activated. It has been shown by others that etoposide can increase the expression of PD-1, indicating a potential association between chemotherapy and neoplastic avoidance of immune destruction. MiR-513a-5p expression is downregulated after treatment of retinoblastoma cells with etoposide and the PD-1 expression is reduced

gradually with the increasing dose of miR-513a-5p mimics. MiR-513a-5p directly inhibits the expression of PD-1 creating a connection between the response to chemotherapy and inactivation of the immune system[59]. Additional studies are necessary to describe the interaction between miRNAs and IFN-γ pathway and the role of miRNAs on promoting specific TAM phenotypes.

MiRNAs from the network which specifically inhibit only one checkpoint are suitable for assessing if a patient will benefit from the therapy and for evaluating the response to immune checkpoint blockade after the initiation of therapy (i.e. biomarkers). The miRNAs which were reported to be dysregulated in autoimmune disease are probably suited to monitor and predict the immune related adverse events. Finally, the miRNAs which target multiple checkpoints are ideal therapeutic targets, because they mimic the blockade with multiple immune checkpoint inhibitors which proved to be superior to single antibody therapy (**Figure 2**).

Translational perspectives

The therapeutic indications of checkpoint inhibitors are imprecise and the evaluation of the therapy response is difficult

The therapeutic indication of immune checkpoint antibodies is expanding rapidly. This new therapy is approved for numerous types of cancer, but only a subset of patient can benefit from it[60]. Adding other genetic and epigenetic markers could further delimitate the indication of the ICB. For the clinician, an important challenge is to decide who can benefit. Significant research is carried out to discover new biomarkers specific for the molecular mechanism of immune checkpoint inhibition or use routinely available markers (e.g. leucocyte count, lactate dehydrogenase, C-reactive protein) that can predict the response to therapy, but none proved efficient enough[61].

Therapy with antibodies against the immune checkpoints can lead to an atypical response. In a subgroup of patients, the initial phases of treatment are accompanied by tumor growth/or the appearance of secondary lesions, but shortly after the tumor burden decreases. This unique tumor response pattern is termed "pseudoprogression"[62]. The atypical response mechanism opens new challenges for the clinician, which encounters difficulties in evaluating the treatment and also taking future therapeutic decisions. Moreover, recent studies have demonstrated that the classical tumor response criteria [WHO criteria and Response Evaluation Criteria In Solid Tumors (RECIST)] are not suitable for assessing the tumor burden in case of the ICB[63-65]. Hence, there is an unmet need for novel biomarkers which can be used to assess the response to immune checkpoint inhibitor therapy.

One of the most studied method to predict the response and outcomes of anti-PD-1 agents is by assessing the expression of PD-L1 receptors by immunohistochemistry in tumor samples. The results are controversial, but FDA approved, based on several positive studies, the treatment of

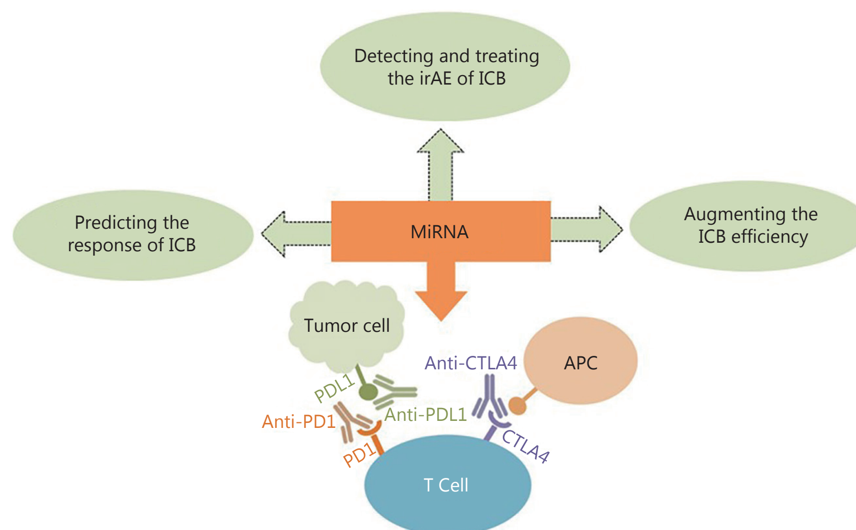

Figure 2 The ICB is a promising therapy, but clinicians encounter several difficulties. MiRNAs can be suitable partners of the ICB and be used to predict the response to therapy, detect and treat the side effects of anti-immune checkpoint antibodies and potentiate the effect of the ICB (ICB – immune checkpoint blockade; irAE – immune related adverse events).

metastatic NSCLC with pembrolizumab if the expression of PD-L1 receptors in metastatic tumors is over 50%[66]. This method has several limitations: (a) requires biopsy; (b) because of tumor heterogeneity some samples do not express PD-L1, although the overall expression is high; (c) most antibodies target the membranous and cytoplasmic PD-L1, leading to imprecise results[62].

A different strategy to determine if a patient will respond to checkpoint inhibitors could be the expression of miRNAs that control the level of immune checkpoints. Therefore, finding miRNAs that correlate with the expression of immune checkpoints is a good alternative. A possible biomarker to determine if a patient will respond to anti-PD-L1 therapy is miR-34a. Furthermore, using bone marrow samples from 44 acute myeloid leukemia and 5 healthy controls, Wang et al.[42] confirmed that the level of miR-34a is statistically inversely correlated with that of PD-L1. In a different study, using the TCGA database for 181 NSCLC it has been showed that in WT TP53 patients, the PD-L1 level is low and the level of miR-34a is high, suggesting that WT TP53 inhibits PD-L1 via miR-34a[43].

MiR-17-5p from sera of metastatic melanoma is inversely correlated with the expression of PD-L1 in tumor tissue and with the appearance of BRAF mutations. The authors propose low miR-17-5p to assess the level of PD-L1 in metastatic melanoma tumors[67].

Kao et al.[48] showed that in metastatic pleural mesothelioma tumor samples with high levels of PD-L1 correlate with a low level of miR-15b, miR-16, miR-193a-3p and miR-200c predicting a poor prognosis. Additionally, using the TCGA lung adenocarcinoma database (n = 230), Chen et al.,[49] discovered that the miR-200 family anticorrelates with the mRNA level of PD-L1 and high PD-L1 associates with a high mesenchymal score. The authors speculate that low miR-200 is a suitable biomarker for lung adenocarcinomas which responds to immune checkpoint blockade.

Two studies confirmed that the level of PD-L1 is anticorrelated with that of miR-197 in two tumor types, NSCLC and oral squamous carcinoma, respectively[46,52]. In recurrent, platinum-resistant NSCLC, miR-197 is downregulated in tumor samples compared to chemotherapy responsive tumors. Regarding the prognostic value of miR-197, the results are controversial between the studies. In NSCLC high miR-197 was linked to a good overall survival[46], while in oral squamous carcinoma high miR-197 was linked to worse overall survival[52]. These observations suggest a different mechanism for miR-197-PD-L1 regulation in the two tumor types. Additionally, Fujita et al.[46] demonstrate that knock down of miR-197 *in vitro* and *in vivo* promotes an aggressive pulmonary cancer phenotype. Taken together, the data from the NSCLC study prove the potential therapeutic role of miR-197 mimetics, at least in chemoresistant NSCLC.

We envision that miRNA could solve the problem of imprecise indication of ICB. By assessing the level of miRNAs that anticorrelate with the expression of immune checkpoint receptors, before the start of the therapy, the clinician could predict the potential response of this novel treatment. Additionally, the diagnostic approaches used now to determine the response to ICB are invasive. As shown, most of the studies measure the expression of immune checkpoint regulatory miRNAs in the tumor sample. It would be interesting to evaluate if these miRNAs also circulate in plasma and would be suitable for noninvasive methods to determine the response to ICB. If the expression of miRNAs is appropriate to predict the therapeutic response, than the same miRNAs could be tools to assess the response to treatment after the initiation of the ICB. We imagine that the miRNAs which are downregulated before the initiation of the treatment, will be restored, if the therapy is effective and could be markers of beneficial response. Further studies expanded on larger sets of samples (that are building rapidly with the advance of ICB use), are necessary to show how powerful these miRNAs are as potential noninvasive biomarkers to predict the response to ICB.

The immune checkpoint therapy is characterized by numerous immunologic adverse-events

Following the treatment with checkpoint inhibitors, one should not perceive the immune system to be in a new state of hemostasis. The immune system after being stimulated by checkpoint inhibitors reacts in an aggressive manner not only against the tumor cells, but also against self-tissues. This new state of the immunity resembles with that of an autoimmune disease. This observation is enforced by the numerous side effects related to the treatment with checkpoint inhibitors, side effects which are very different from those associated with conventional chemotherapy[68]. The checkpoint inhibitors related side effects are named immune-related adverse events (irAEs). The most frequent irAEs consist in skin reactions rush and/or pruritus, reaching an incidence of 40%–60%, depending on the type of targeted receptor[69-71]. Diarrhea and/or colitis are also side effects of the ICB: around 7% of patients treated with CTLA-4 ICB develop high grade colitis (grade 3–4) and only 1.8% of those treated with PD-1 antibodies[3]. Also common are the immune related

endocrinopathyes: hypophysitis and thyroiditis, which occur in approximately 10% of treated patients[72] and adrenalitis, a more rare, but life threatening toxic side-effect[73]. Furthermore, the incidence of irAEs increases if two immune checkpoint inhibitors are combined. The treatment of irAEs differs based on the rate of adverse reaction grade. Often, the immune stimulation with checkpoint inhibitors is interrupted and immunosuppression with corticosteroids is required[68]. Hence, the immune system is inhibited and the tumor progresses all over again. From a clinical perspective there are two important questions: (1) how can the oncologist promptly recognize irAEs? And (2) how can the side effects be managed without discontinuing the immune blockade and without turning the immune mechanism in favor of the tumor (by using steroids and other immunosuppressive agents)?

MiRNAs could be one of the elements that maintain the balance between immune tolerance and autoimmunity. By now, several studies demonstrated that miR-155 is a PD-1 suppressor[57]. Zhang and Braun[74] showed *in vivo* that autoimmune encephalomyelitis does not occur in miR-155 deficient mice, but in double knockout Pdcd1-/miR-155- mice the susceptibility to autoimmune disease is restored, accompanied by an increase pro-inflammatory cytokine production and T-cell infiltration. In a clinical study, Sonkoly et al.[75] showed that miR-155 is overexpressed in the dermal lesions of patients with atopic dermatitis and this miRNA suppresses CTLA-4 in T-cells. The role of the miR-155-CTLA-4 interaction as an element of the pathogenic chain of autoimmune disease was also proven in allergic asthma, where high miR-155 downregulates CTLA-4 expression and induces T-cell activation[76]. On the other hand, Huffaker et al.[56] underlined the function of miR-155 in immune tolerance, showing that the antitumor immunity of T cells is defective in miR-155 deficient mice, and ICB can restore the immunity in this mouse model. These data show the importance of miR-155 in regulating the interplay between T-cells and self-tissues, including neoplastic tissue, by controlling the expression of checkpoint molecules. Hence, it would be a promising approach to evaluate the expression level of miR-155 in patients treated with ICB that present irAEs and establish if high miR-155 expression level is a suitable biomarker for the onset of irAEs.

One of the limitations of the ICB therapy is the appearance of irAEs, which often leads to the interruption of the treatment. Interestingly, the miRNAs that are deregulated in autoimmune diseases show an opposite expression pattern *in vivo* studies and clinical samples of patients who present immune tolerance. This being a supplementary argument that confirms that the irAEs are a phenomenon similar to autoimmunity. We consider that a good understanding of the function of miRNAs in autoimmunity and irAEs could lead to a new therapy of the side effects of ICB. High miR-155 is frequently associated with autoimmunity, during the ICB we can hypothesize that miR-155 is overexpressed because its targets are downregulated. The role of miR-155 is not strictly depended on the immune checkpoint receptors and its overexpression will lead to an augmentation of the irAEs. Hence we believe that by manipulating the miRNAs that coordinate the side effects, the clinician will not be obligated to interrupt the therapy with immune checkpoint inhibitors in case of irAEs, he will be able to control the undesirable effects. Additionally, one can presume that in the near future by analyzing the expression of miRNAs, clinicians could be able to recognize and promptly tackle irAEs, but further preclinical studies are necessary in order to implement this strategy in the clinical arena.

Absence of response to immune checkpoint therapy

Optimistic is not the percentage of patients who can benefit from ICB, which is relatively modest, but the very low mortality rate of those who respond. Analyzing the survival curve of melanoma patients treated with ipilimumab one can observe a plateau after 3 years of follow up, but only an approximate 20% of treated patients reach this point[77]. Patients who respond appear to be cured. Therefore, finding new methods to increase the response rate of patients to ICB is highly necessary. We hypothesize that a miRNA therapy combined with ICB could increase the efficiency of the established monotherapeutic approach. The miRNA therapy is classified in miRNA mimetics (overexpression of a specific suppressor miRNA) and miRNA inhibitors (blocking the expression of a specific oncogenic miRNA)[78]. The great advantage of using a miRNA based therapy is the capacity of these short transcripts to target multiple molecules involved in the same pathway or from different pathways with synergistic global effect (e.g. immune inhibition). Therefore, choosing miRNAs that target multiple immune checkpoint molecules is the best strategy.

Xu et al.[26] demonstrate that, in ovarian cancer tumors, the expression of miR-424 is negatively associated with the level of PD-L1 and CD80 (the receptor for CTLA-4) and that a high expression of this miRNA is correlated with progression-free survival. They showed that restoration of miR-424 levels leads to a T cell activation and reverses chemoresistance. Therefore, adding miR-424 mimetics to the ICB has the

potential to increase the therapeutic efficiency of immunotherapy and chemotherapy.

Another miRNA capable to target two distinct immune checkpoints is miR-138. MiR-138 binds to the 3'UTR of PD-1 and CTLA-4 and downregulates the expression of these checkpoints *in vitro* and *in vivo*. Furthermore, miR-138 treatment activated the T-cells and consequently increased the survival of immune competent glioma mice with 43%. As expected the results were not reproducible in nude immunocompromised mice[33]. Zhao et al.[34] underlined the importance of miR-138-5p in CRC. Using CRC samples and corresponding adjacent normal tissues they observed that the expression of miR-138-5p is inversely associated with that of PD-L1, where low miR-138-5p and high PD-L1 predict shorter overall survival. Another miRNA with high therapeutic potential in the context of ICB, is miR-28. MiR-28 is an important hub of the tumor immune evasion regulatory network, being capable to inhibit multiple immune checkpoints. *In vitro* studies demonstrated that miR-28 mimics are capable to decrease the expression of PD-1 while miR-28 inhibition leads to an increases expression of PD-1, TIM3 and BTLA[35]. Future *in vivo* and clinical studies are necessary to prove the usefulness of miR-28 mimics as an additive therapy for ICB.

Because only a subset of patients respond to ICB and using combinations of immune checkpoint molecules increases also the number and gravity of adverse effects, we consider it is highly necessary to find new approaches to augment the response to ICB. One possible class of molecules which are suitable to increase the efficiency of ICB are miRNAs. The best miRNAs for this job are the ones who can target multiple immune checkpoints simultaneously, mimicking a multi-checkpoint blockade. Because physiologically miRNAs lead to a modest downregulation of their target and because high levels of exogenously administrated miRNAs can trigger immunologic side effects (citation), we consider that a miRNA monotherapy is not a good option. The best solution in the case of the non-responders to ICB would be the addition of high physiological levels of miRNA based therapy to the already approved immune checkpoint treatment. We hypothesize that such an approach could boost the immune response against the treatment and convert non-responders to responders.

Final remarks

The molecular regulatory network we describe is far from complete. There are at least two other layers of complexity which are not explored yet and need to be further researched. In this review we presented miRNAs as regulatory elements of the immune checkpoints expression. Most probably the network contains also non-coding RNAs, [i.e. long non-coding RNAs (lncRNAs), circular RNAs] which add a supplementary level of regulation to the network. To our knowledge there is only a study reporting the role of lncRNAs in tumor immune evasion. Tang et al.[79] show that the level of the lncRNA actin filament-associated protein 1 antisense RNA 1 (AFAP1-AS1) is positively correlated with that of PD-1 in nasopharyngeal cancer tissues, but the study lacks any mechanistic details regarding the interaction of the two molecules. In order to have a comprehensive understanding of regulatory network of immune checkpoints future research should also be directed towards describing the role of lncRNAs and also other types of ncRNAs in immune tolerance.

It is well known that ncRNAs[80], especially miRNAs travel via exosomes in the tumor microenvironment and change the phenotype of neighboring cells[81]. Therefore, it is crucial to evaluate if the miRNAs that control the expression of immune checkpoints are transcribed in the same cell where they perform their function or are imported from neighboring cells. Finding out that the tumor tissue secrets exosomes containing miRNAs capable to modulate the immune response would bring new insights to the mechanism of neoplastic immune tolerance. These details would help improve the design of future therapies.

In conclusion, numerous studies describe miRNAs as key regulatory elements of tumor immune evasion by changing the expression of immune checkpoints. These miRNAs build an intricate network that partially controls the immune response via immune checkpoints against the tumor cells. We propose that the miRNAs can be used to predict and evaluate the response of ICB, control irAEs and potentiate the effect of the immune checkpoint inhibitors.

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Conflict of interest statement

No potential conflicts of interest are disclosed.

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