Journal for ImmunoTherapy of Cancer

Immune modulatory microRNAs in tumors, their clinical relevance in diagnosis and therapy

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To cite: Vaxevanis C, Bachmann M, Seliger B. Immune modulatory microRNAs in tumors, their clinical relevance in diagnosis and therapy. *Journal for ImmunoTherapy of Cancer* 2024;**12**:e009774. doi:10.1136/jitc-2024-009774

Accepted 23 July 2024

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ABSTRACT

The importance of the immune system in regulating tumor growth by inducing immune cell-mediated cytotoxicity associated with patients' outcomes has been highlighted in the past years by an increasing life expectancy in patients with cancer on treatment with different immunotherapeutics. However, tumors often escape immune surveillance, which is accomplished by different mechanisms. Recent studies demonstrated an essential role of small non-coding RNAs, such as microRNAs (miRNAs), in the post-transcriptional control of immune modulatory molecules. Multiple methods have been used to identify miRNAs targeting genes involved in escaping immune recognition including miRNAs targeting CTLA-4, PD-L1, HLA-G, components of the major histocompatibility class I antigen processing machinery (APM) as well as other immune response-relevant genes in tumors. Due to their function, these immune modulatory miRNAs can be used as (1) diagnostic and prognostic biomarkers allowing to discriminate between tumor stages and to predict the patients' outcome as well as response and resistance to (immuno) therapies and as (2) therapeutic targets for the treatment of tumor patients. This review summarizes the role of miRNAs in tumor-mediated immune escape, discuss their potential as diagnostic, prognostic and predictive tools as well as their use as therapeutics including alternative application methods, such as chimeric antigen receptor T cells.

INTRODUCTION: IMMUNE ESCAPE STRATEGIES OF TUMORS

Tumor development is a multifactorial process mediated by independent genetic and epigenetic events as well as different regulatory processes, which are influenced by alterations in the tumor microenvironment (TME) and can accumulate during tumor progression. The complexity of cancer phenotypes and genotypes resulted in the establishment of the hallmarks of cancer, which was extended over the years and included next genetic and epigenetic alterations changes associated with neoplastic transformation and evasion from immune cell recognition.[1] A critical role of the immune system in the immune surveillance, tumor initiation and progression is based on the cancer immunoediting concept,[2 3] which proceeds through three phases termed elimination, equilibrium and escape. This results in editing of tumor immunogenicity and acquisition of immune suppressive mechanisms that enable metastasis formation and resistance to T cell-based immunotherapies.[4]

Tumor antigens (TAs) presented by major histocompatibility class I (MHC-I) on the cell surface of tumor cells could be recognized and eliminated by $CD8^+$ cytotoxic T lymphocytes (CTLs)[5] while NK cells exert their cytotoxic activity in an antigen-independent manner.[6] The importance of both effector cells in controlling tumor growth has been strengthened by the link between a high density of $CD8^+$ T and NK cells with a good prognosis in the majority of tumor patients.[7 8] However, tumors have developed different strategies to escape immune response, which could occur at distinct levels as summarized in figure 1. These include loss or downregulation of MHC-I surface expression often mediated by an impaired antigen processing via the APM and interferon (IFN) signal transduction, an upregulation of the non-classical human leukocyte antigens (HLA) as well as immune checkpoint (ICP) molecules, secretion of immune suppressive cytokines and metabolites and metabolic reprogramming[9–13] thereby affecting the frequency and function of immune cell subpopulations.[14 15] Thus, cancer cells are able to fool the immune system by intrinsic factors, but also by remodeling their microenvironment in order to proliferate and escape immune recognition,[16] which is a result of an evolutionary pressure due to the complex interaction of the immune system with tumor cells and established by genetic abnormalities or by deregulatory mechanisms of immune response relevant factors.[2 17]

FEATURES OF MIRNAS

MicroRNAs (miRNAs) are small non-coding RNAs (18-24nt) that function as

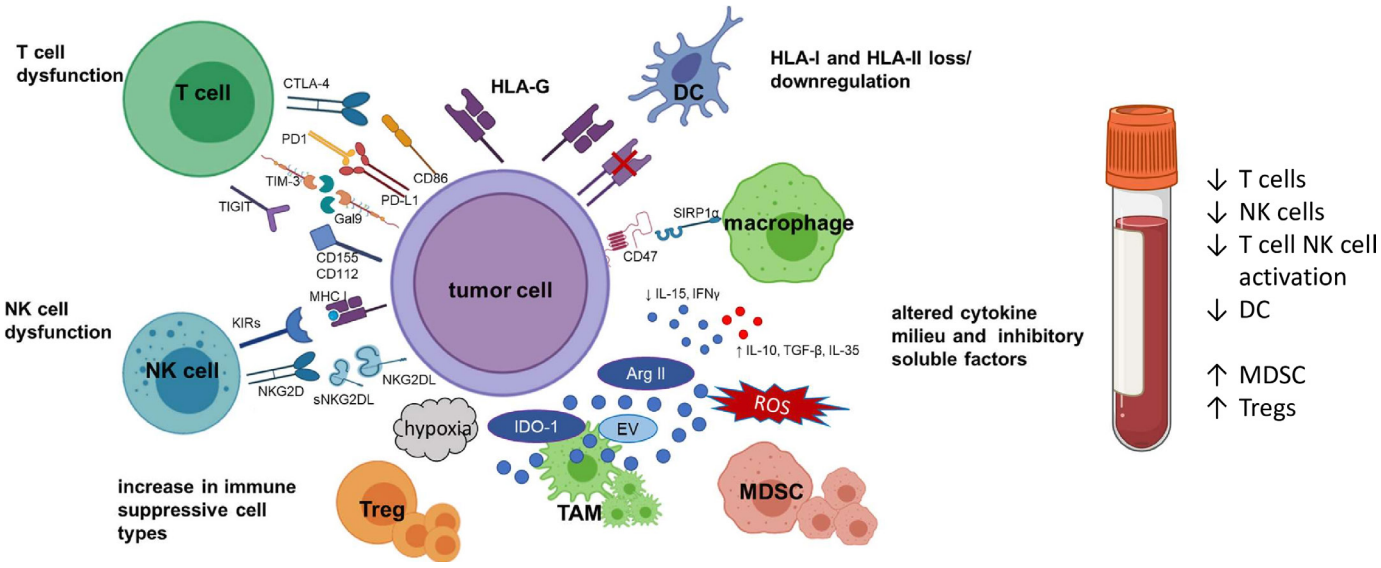

Figure 1 Schematic with various immune escape mechanisms used by tumor cells. Among these, the interaction with cytotoxic, antigen presenting and immune suppressive subpopulations are shown. The important molecules, such as receptors and cytokines, are pictured on the scheme along with the observed resulting effect from these interactions. DC; dendritic cell, TAM; tumor associated macrophages, MDSC; myeloid derived suppressor cells.

post-transcriptional regulators.[18] They mainly bind to the 3' untranslated region (UTR), but also to the coding sequence or to the 5'-UTR of their respective mRNA targets to either induce mRNA degradation, impair their stability or inhibit their translation.[19–23] Due to the small size of their seed regions, multiple miRNAs are able to bind to more than one target mRNA while one single mRNA can have a large number of binding sites for miRNAs. Despite miRNAs mainly acting as inhibitory molecules, recent evidence demonstrated a miRNA-mediated upregulation of targets by increasing their mRNA stability or targeting AU-rich elements on genomic DNA.[24 25]

miRNAs are involved in many physiological and pathophysiological cellular processes.[19 26] In cancer, miRNAs could affect the expression of targets in tumor cells, in cellular components of the TME as well as in the peripheral blood.[27] In addition, miRNAs are present in exosomes thereby increasing their plethora of activities.[28] Despite the detection of a large number of miRNAs, their expression and activity are highly dependent on the (tumor) cell type, the experimental set-up and tools used for their identification suggesting that further insights into their pluripotent functions and mechanism of actions in individual pathways and cancer types are required.[29]

Different methods/tools for the identification of miRNAs

For the identification of miRNAs, distinct unbiased and biased approaches have been applied. These include in silico analyses using different prediction tools, unbiased RNA sequencing strategies as well as target-specific biased technologies, such as Nanostring analyses, miRNA cross-linking immunoprecipitation (CLIP), miRNA Enrichment Technique via RNA affinity Purification Protocol (miTRAP) have advantages and disadvantages as described in table 1. Based on the central miRNA database (miR Base,[30]), algorithm-driven in silico prediction tools were used for the identification of miRNA-specific targets by cross-referencing the seed regions of miRNAs from the primary miRNAs[31] and various mRNA sequence databases and calculating the putative binding site and free energy on the target (figure 2A). A list of selected prediction tools and their features is presented in table 2.[32] Next to the in silico analysis, high-throughput RNA sequencing (RNA-seq) followed by bioinformatics analyses was employed for the identification of coding and non-coding RNAs to identify differentially expressed miRNAs[33] while small RNA-seq was abundantly used to investigate differences in the miRNA expression pattern[33] (figure 2B). The identified (differentially expressed) miRNAs can give important insights into the biology of tumors and therapy resistance mechanisms and might be used as diagnostic, prognostic or predictive markers.[34–36]

An alternative to (small) RNA-seq is hybridization-based approaches, such as nCounter (Nanostring),[37] which offers quantitative analysis of miRNAs with a sensitivity down to five copies using a relatively small amount of starting material.[38] Furthermore, target-specific methods, like the CLIP, were employed by mainly coprecipitating one RISC component (usually an Argonaut protein) together with the bound miRNA-mRNA complex[39–41] while a variation of the CLIP protocol used biotin-labeled miRNA of interest as bait to identify the whole reactome of the miRNA in question (miR-CLIP,[42]). The miTRAP method (figure 2C)[43 44] allows to identify miRNAs bound to a specific target gene of interest, which was used by our laboratory and others to identify immune modulatory miRNAs targeting, for example, selected ICPs, HLA-I and APM components followed by their functional validation.[45–50] In addition, genome-wide high throughput

Table 1 Advantages and disadvantages of various miRNA identification methods

	In silico prediction tools	High throughput miRNA analysis		miTRAP
		Unbiased (RNAseq, small RNAseq	Targeted (Nanostring)	
Advantages	Variety of tools with multiple algorithms	Total identification of miRNAs	Identification of selected miRNAs	Sequence-based miRNA identification
	Specific focus of prediction based on tool	Discovery based on biologically relevant material (blood, tissue, body fluids)		Simple protocol
	Data availability for all discovered miRNAs	Putative identification of miRNAs as diagnostic/prognostic/predictive biomarkers and therapeutic targets		Rapid identification of multiple target specific miRNAs
	Not species limited	low hands-on time due to automation		Simultaneous identification of miRNAs and RBPs
	No costs			Low cost compared with high throughput techniques
				Cell lysate origin allows for tissue-specific identification
				Low number of false positives
Disadvantages	Large number of false positives due to computational approach	High cost		Time-consuming
	Large number of putative candidates to validate	Thorough statistical analysis necessary		Results are sample specific (based on lysate used)
		Large number of false positives		Large number of consumables needed
		Unknown origin of miRNAs depending on sample type		Possible false negatives due to overlapping binding sites

List of the advantages and disadvantages of the three types of miRNA identification methods discussed in the manuscript, namely in silico, high throughput (RNAseq, hybridization based) and specific (miTRAP).

flow cytometry-based miRNA screening has been used to identify miRNAs targeting specific molecules by transfection of miRNA mimic libraries into cells followed by their monitoring via by flow cytometry.[51]

IMMUNE-RELEVANT MIRNAS IN TUMORS

So far, a large number of miRNAs differentially expressed in tumors have been identified that are involved in regulating pathways of malignant transformation, immune surveillance and the composition of the TME[52] thereby classifying miRNAs into tumor suppressive, oncogenic and immune modulatory miRNAs (im-miRNAs).[50 53] There exists increasing evidence that miRNAs are involved in immune escape by affecting the expression of a plethora of immune response-relevant molecules accompanied by an altered susceptibility of tumor cells to $CD8^+$ T cell-mediated cytolysis.[48 49 54 55] This review will focus on the miRNAs identified in tumor cells to be involved in the regulation of immune surveillance and immune escape and their clinical relevance.

MiRNAs targeting immune checkpoint molecules on tumor cells

ICP molecules are overexpressed in multiple cancer types, but also in the infiltrating immune cells and non-immune cells surrounding the tumor.[56] Consequently, ICP inhibitors (ICPi) have been developed over the last two decades, which have revolutionized the treatment of tumor patients, but an improved long-term outcome has been only described for a limited number of patients.[57 58] In this context, it is noteworthy that the post-transcriptional regulation of ICP molecules is frequently mediated by either miRNA families (miR-17-92), miRNAs produced from the same pre-miRNA stem loop (miR-125-5p, miR-125-3p) or even single miRNAs targeting multiple immune pathways.

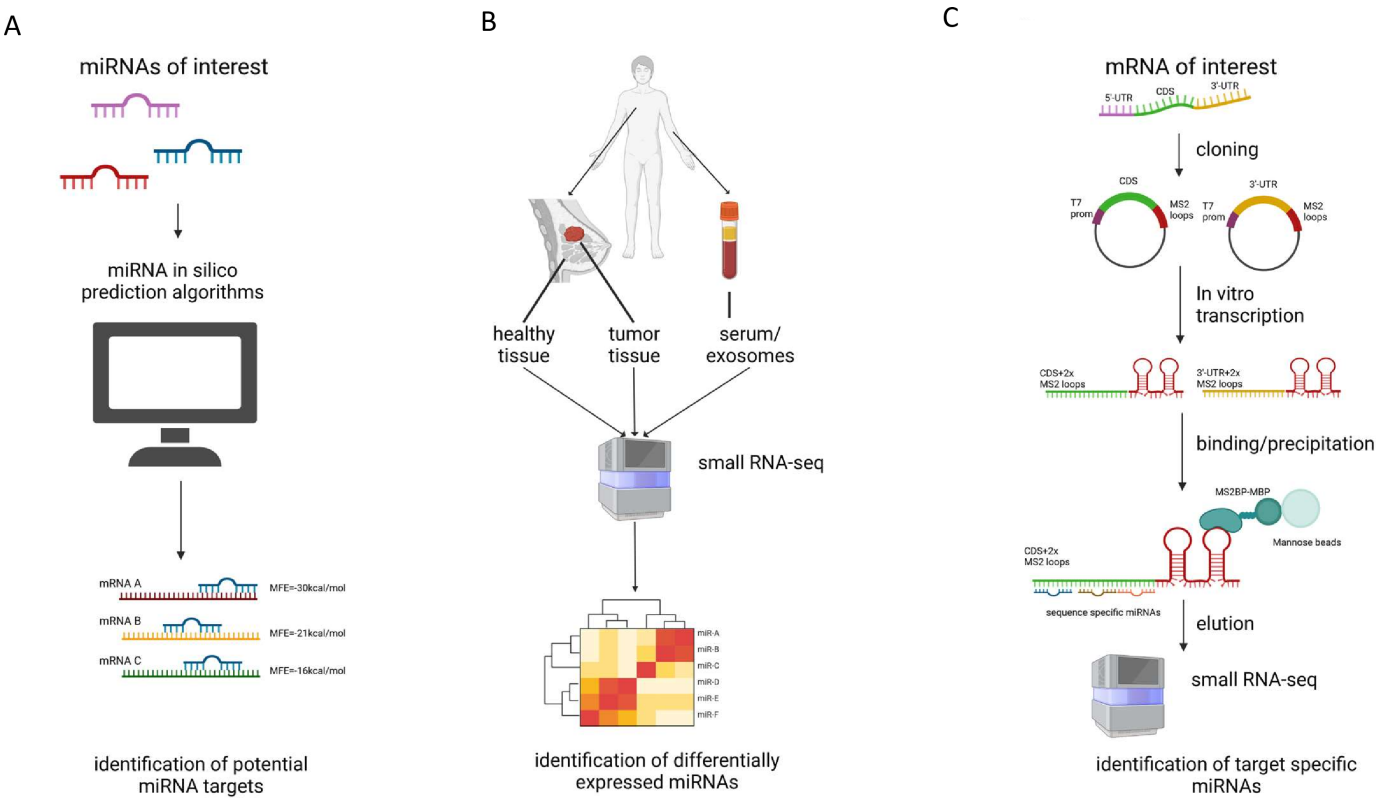

Figure 2 Schematic representation of the most commonly used methods for microRNA (miRNA) identification. (A) In silico analysis tools can be used to identify potential miRNA targets as well as their predicted binding regions in the mRNA of interest. (B) Analysis of total miRNA expression derived from tumor, healthy tissue as well as patient serum allow the identification of disease-specific/related miRNAs. (C) The miTRAP method, briefly shown, can be used for the identification of target specific miRNAs by coprecipitating them along with the mRNA sequence used as bait. Small RNA seq can be then used to identify the most prominent candidates. This figure was created with Biorender. miTRAP, miRNA trapping by RNA in vitro affinity purification.

CD274, the prototype of ICPs, also known as programmed death ligand 1 (PD-L1) was upregulated in different tumors due to distinct mechanisms including a post-transcriptional control mediated by factors stabilizing the produced mRNA[59] and by disruption or mutations of miRNA binding sites in the 3'UTR of CD274.[60] Despite some groups having identified CD274/PD-L1-specific miRNAs as summarized in table 3, the number of miRNAs targeting CD274 described is low considering the large size of the PD-L1 3'UTR. Two members of the miR-16 family, which regulate PD-L1 in neuroblastoma and lung adenocarcinoma (LUAD)[61 62] and miR-125a-3p, were identified as target of CD274 in lung cancer and esophageal adenocarcinoma.[63] Interestingly, miR-16 was also detected in cancer-derived exosomes and downregulated PD-L1 when transferred to cancer cells in vitro. Using the miTRAP method, our group identified six miRNAs that were able to downregulate PD-L1 on transfection into melanoma cells,[64] which was accompanied by an increased T cell response. The heterogenic PD-L1-specific miRNA expression in different cancer types could be a result of the combination of physiological miRNA and basal PD-L1 expression in individual tumor subtypes.[65–69]

Furthermore, miRNAs targeting other ICP have been identified in tumors[70–73] or in antigen-presenting cells, such as the CD86 ligand of the cytotoxic T lymphocyte-associated protein-4 (CTLA-4).[74] Inverse correlations were found within tissue sections regarding the expression of ICPs and certain miRNAs as it was, for example, described for CTLA-4 and miR-20b-5p in renal cell carcinoma (RCC), for miR-424-3p in prostate cancer[75 76] as well as for PD-1 and miR-33a in LUAD.[77] In addition, the miRNA cargo of cancer-derived exosomes influenced the expression of ICP in a head and neck squamous cell carcinoma (HNSCC) model with an enrichment of miRNAs targeting and affecting the expression of CTLA-4, lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) and PD-L1. Thus, several miRNAs have been shown to alter the ICP expression levels thereby directly enhancing the potency of immune responses. Most importantly, some miRNAs could affect multiple ICPs and thus might enhance antitumoral immune responses. Next to tumor cells, an miRNA-mediated post-transcriptional regulation of ICP expression was also found in immune cell subpopulations,[78] which was recently been extensively summarized.[79]

miRNAs targeting classical MHC-I antigens and APM components

There is an increasing evidence that downregulation or loss of MHC-I surface antigens accompanied by impaired

Table 2 Major in silico prediction tools and their features

Tool	Species	Custom sequences	Binding energy	Additional structural information	Evolutionary conservation	Experimental validation	non-conventional binding	Nline	Ref.
miRanda	n.r.	–	+	–	+	–	+	–	152
RNAhybrid	n.r	+	+	+	–	–	+	+	153
miRDB	r.	+	+	+	+	–	–	+	154
Targetscan	r.	–	+	+	+	–	+	+	155
miRWalk	r.	–	+	+	–	–	–	+	156
miR-Tar-Base	n.r.	–	–	–	–	+	–	+	157

Overview of the features of six selected in silico prediction tools, miRanda, RNAhybrid, miRD, Targetscan, miRWalk and miR-Tar-Base. The features in the list include, whether there is a restriction in the program used regarding the species of origin of the miRNA and target, the ability to analyze custom nucleotide sequences, additional structural information about the complex (eg, seed match, 3' compensatory pairing, site accessibility), information about the evolutionary conservation, experimental validation, consideration of non-conventional binding sites and finally its availability online. The plus symbol (+) indicates the existence while the absence of the feature is indicated with a minus (–). In the species column, in silico prediction tools with species restriction are marked with r while tools, where no restriction is applied with n.r.

expression of APM components can be mediated by miRNAs of tumor cells. MiRNAs targeting the transporter associated with antigen processing (TAP)1 and TAP2, responsible for the transport of intracellular peptides from the cytosol to the endoplasmic reticulum, have been identified. These include miR-200a and miR-21-3p, which bind to the TAP1 3'-UTR thereby inhibiting TAP1 expression in melanoma and breast cancer, respectively,[80 81] while miR-125a-5p target TAP2 expression in esophageal adenocarcinoma.[63] An inverse expression of miRNAs and TAP1 was confirmed in melanoma specimen and by in silico analysis of The Cancer Genome Atlas (TCGA) datasets.[80] An indirect effect of miR-148-3p, a member of the miR-148/152 family targeting MHC-I, has been reported by downregulating the chaperone calnexin[82] while MHC-I downregulation in esophageal adenocarcinoma cell lines was due to binding of miRNA-148-3p to their 3'-UTR and coding sequence.[63] Furthermore, the two miRNAs miR-9 and miR-19 downregulate with the expression of MHC-I molecules as well as IFN-regulated genes leading to an even stronger effect.[83 84] These synergistic activities should be taken into account by determining the best miRNA candidates for therapy.

Next to MHC-I antigens, MHC-II antigen expression could also be decreased by miRNAs as shown for miR-212,[85] but HLA-II-specific miRNAs have mainly been investigated in a non-cancer context on antigen-presenting cells.[51 86] However, a flow cytometry-based high throughput RNA screening for miRNAs was recently employed leading to the identification of a number of miRNAs upregulating or downregulating HLA-DR expression in melanoma cells.[51]

miRNAs targeting non-classical HLA-I antigens of tumor cells

The expression of non-classical MHC-I molecules, mainly HLA-G and -E, on tumor cells, results in the evasion of T cell-mediate and/or NK cell-mediated cytotoxicity. The high sequence overlaps between classical and non-classical MHC-I molecules combined with the sequence-specific mechanism of miRNA action suggest that a simultaneous miRNA-mediated regulation of both classes of MHC-I antigens should be taken into account. Indeed, miR-19, a member of the miR-17-92 cluster, was shown to target HLA-B, but also HLA-G, HLA-E and HLA-F.[84] The miR-152 family was proven to directly bind to the HLA-G 3'-UTR in HNSCC[87] and in RCC[88] while it indirectly affected HLA-G expression in a TGF-β-dependent manner in gastric cancer.[89] In addition, miR-138-1-3p shown to target HLA-G[90] has been often downregulated in papillary thyroid carcinoma (PTC). Using the miTRAP method, the HLA-G-regulating miRNAs miR-16 and miR-744 were identified, which also modulate the expression levels of HLA-ABC.[91] In contrast to the conventional miRNA-mediated inhibition of gene expression, miR-16-5p upregulates the HLA-G and HLA-I mRNA and protein expression.[91] Finally, a correlation between soluble HLA-G levels and the expression of four miRNAs was found in B cell acute lymphoblastic leukemia

Table 3 MiRNAs identified targeting immune modulatory molecules in cell lines, tumors and related diseases

miRNAs targeting immune checkpoint molecules				
miRNA	**Target**	**Disease**	**Material tested**	**Reference**
let-7a/b	CD274 (TCF-4)	HNSCC	Patient samples/cell lines	158
let-7i-5p	CTLA-4, PD-L1	HNSCC	Cancer exosomes	159
miR-15a	**PD-1, LAG-3 TIM-3 (mTOR)**	**Glioma**	**$CD8^+$ cells**	110
miR-15a/5	**CD274**	**NB**	**cell lines**	61
miR-16-5p	**CD274**	**LUAD**	**cell lines**	62
miR-16-5p	**PD-1, LAG-3 TIM-3 (mTOR)**	**Glioma**	**CD8+cells**	110
miR-17–5 p	CD274	Melanoma	Cell lines	64 160
miR-20b-5p	CTLA-4	RCC	Patient samples	75
miR-21-5p	**CTLA-4, LAG-3**	**HNSCC**	**Cancer exosomes**	159
miR-23a	TIGIT (MEG3)	Autoimmune aplastic anemia	CD4+	161
miR-26a	TIGIT (EZH2)	T1D	Tregs	162
miR-29a-3p	CD274	melanoma	Cell lines	64
miR-30e-3p	**CTLA-4, LAG-3, TIM-3**	**HNSCC**	**Cancer exosomes**	159
miR-33a	PD-1	LUAD	Patient samples	77
miR-34a-5p	CD274	TNBC	Cell lines	68
miR-103b	CD274	Melanoma	Cell lines	64
mir-125-3p	CD274 (NRG1)	NSCLC	Serum exosomes	112
miR-138	PD-1, CTLA-4	Glioma	Cell lines, mice Tregs	126
miR-142-5p	**IDO (ARID2)**	**CSCC**	**Cancer exosomes**	71
miR-142-5p	**CD274**	**HPV^+ cervical cancer**	**Cell lines**	65
miR-146a	PD-1, CTLA-4. TIM-3, LAG-3	HIV	$CD4^+$ HIV^-1^+ cells	69
miR-148	**HLA-G**	**HNSCC**	**Patient samples**	87
miR-148a-3p	**CD274**	**CRC**	**Patient samples**	151
miR-149-3p	PD-1, TIM-3, BTLA	Bca	$CD8^+$ T cells	111
miR-152	HLA-G	HNSCC	Patient samples	87
miR-155	**CTLA-4**	**Atopic dermatitis**	**$CD4^+$ T** cells	163
miR-155	**TIM-3**	**HCV**	**NK cells**	164
miR-155-5p	**CD274**	**Melanoma, LUAD**	**Cell lines**	64 66
miR-181b-5p	CD274	melanoma	Cell lines	64
miR-186-5p	CD274	melanoma	Cell lines	64
miR-199a-3p	CD86	Heart transpl.	Mice	74
miR-199a-5p	CD274	FTC	Cell lines	67
miR-214-3p	B7-H3	HNSCC	Cell lines	70
miR-224-5p	CTLA-4	Tuberculosis	Patient samples/cell lines (macrophages)	165
miR-324-5p	CTLA-4	Tuberculosis	Patient samples/cell lines (macrophages)	93
miR-330-5p	TIM-3	Myocardial ischemia	Cell lines myocardial cells	73
miR-424	**CD274**	**Ovarian cancer**	**Patient samples**	166
miR-424-3p	**CTLA-4**	**Prostate cancer**	**Patient samples**	76
miR-488-5p	CTLA-4	Tuberculosis	Patient samples/cell lines (macrophages)	165
miR-498	TIM-3	AML	Cell lines	72
miR-619-5p	CTLA-4, LAG-3	HNSCC	Cancer exosomes HN cells	159
miR-744	HLA-G	RCC	Cell lines/patient samples	91
miR-3960	TIM-3	HNSCC	Cancer exosomes	159
miR-7704	CTLA-4, LAG-3	HNSCC	Cancer exosomes	159
miRNAs targeting classical and non-classical MHC molecules				
mirna	**target**	**Disease**	**Material tested**	**Reference**

Continued

Table 3 Continued

miRNAs targeting immune checkpoint molecules				
miRNA	**Target**	**Disease**	**Material tested**	**Reference**
let-7f-2-3p	MHC-II	n.a.	Cell line	51
miR-9	MHC-I	NPC	Cell lines	83
miR-16-5p	**HLA-G**	**RCC**	**Cell lines/patient samples**	91
miR-19a/b	MHC-I	NPC	Cell lines	84
miR-21-3p	MHC I (TAP1)	BCa	Cell lines	81
miR-125a-5p	MHC I (TAP2)	Eso Ca	Cell lines	63
miR-142-5p	**MHC II**	**n.a.**	**HUVECs**	86
miR-148-3p	MHC-I	Eso Ca	Cell lines	63
miR-148-3p	MHC-I (CANX)	CoCa	Cell lines	82
miR-151a/b-5p	MHC-II	n.a.	Cell line	51
miR-200a	MHC I (TAP1)	Melanoma	Cell lines/patient samples	80
miR-205-3p	MHC-II	n.a.	Cell line	51
miR-214-3p	MHC-II	n.a.	Cell line	51
miR-456-5p	sHLA-G	B-ALL	Patient samples	92
miR-513a-3p	MHC-II	n.a.	Cell line	51
miR-567	MHC-II	n.a.	Cell line	51
miR-1202	MHC-II	n.a.	Cell line	51
miR-3115-3p	MHC-II	n.a.	Cell line	51
miR-3972	MHC-II	n.a.	Cell line	51
miR-4487	MHC-II	n.a.	Cell line	51
miR-4488	sHLA-G	B-ALL	Patient samples	92
miR-4516	sHLA-G	B-ALL	Patient samples	92
miR-4753-5p	MHC-II	n.a.	Cell line	51
miR-5003-3p	MHC-II	n.a.	Cell line	51
miR-5096	sHLA-G	B-ALL	Patient samples	92
miR-5581-5p	MHC-II	n.a.	Cell line	51
miR-5693	MHC-II	n.a.	Cell line	51

List of identified miRNAs, with proven binding and effect on immune molecules such as immune checkpoints and APM components. Along the miRNAs found, the cancer model and the biological system (patient samples, cell lines, etc) (when applicable) used for validation are provided. The miRNAs validated to bind and downregulate multiple ICPs and/or APM components are marked in bold.
AML, acute myeloid leukemia; B-ALL, B cell acute lymphatic leukemia; Bca, breast carcinoma; CRC, colorectal carcinoma; CSCC, cutaneous squamous cell carcinoma; Eso Ca, esophageal adenocarcinoma; FTC, follicular thyroid cancer; HNSCC, head and neck squamous cell carcinoma; LUAD, lung adenocarcinoma; miRNAs, microRNAs; n.a, not available; NSCLC, non-small cell lung carcinoma.

(B-ALL).[92] Concerning HLA-E, little information is available on its regulation by miRNAs and so far, only the edited miR-376a has been identified to downregulate HLA-E as a response to cytomegalovirus infection.[93]

miRNAs involved in the regulation of NK recognition receptors

Recently, multiple ligands/receptors have been investigated to regulate innate immune responses directed against pathogens and in the context of cancer, in particular with a focus on their post-transcriptional regulation by miRNAs.[94] A number of NK cell-specific receptors and ligands often aberrantly expressed in different human cancers[95] could be targeted by miRNAs, which was associated by impaired NK cell functions as recently summarized.[94] The expression of NKG2D, a receptor for NK cell activation and its ligands MICA, MICB and ULBP1-6, could be regulated by various means.[96] For example, NKG2D can be upregulated by miR-30c transfection due to targeting the inhibitory transcription factor HMBOX1 thereby increasing the efficacy of anti-cancer responses.[97] In addition, a number of miRNAs have been shown to regulate the MICA/B and ULBP2 mRNA expression[98] by their direct binding to the respective 3'-UTR thereby downregulating MICA surface expression and inhibiting the NKG2D-mediated MICA immune recognition[99–101] or indirectly through targeting of STAT3 as recently summarized.[94] These include miR-10a, miR-93, miR-106b, miR-146b, miR-302d, miR-372, miR-373 and miR-520bd.[94 102–105] Overexpression of miR-17–5p, miR-20a, miR-93, miR-373 and miR-520bd have been shown to downregulate MICA accompanied by a decreased NK

cell susceptibility. While most of the MICA regulating miRNAs bind to its 3'-UTR region, miR-520d also targets the 5'-UTR of MICA.[106] Attempts suppressing the expression of the NKG2D ligand-targeting miRNAs, like miR-93 in glioma cells, were able to increase the NK cell-mediated cytotoxicity, supporting the contribution of miRNAs from the innate immune system in immune escape.[100]

CLINICAL RELEVANCE OF IMMUNE MODULATORY MIRNAS

Immune modulation miRNAs as diagnostic and prognostic markers for tumors

Based on the differential expression pattern in tumors, the use of im-miRNAs as diagnostic and/or prognostic tools for various cancer types to predict patients' outcome has been investigated.[107] In addition, the clinical relevance of im-miRNAs was demonstrated based on the targeted pathway and their relevance in the respective cancer type. Regarding, for example, HLA-G targeting miRNAs, a prognostic value was described for miRNA-148a expression, which was lower in primary esophageal squamous cell carcinoma and RCC when compared with adjacent normal tissue.[88 108] The reduced expression of the HLA-G targeting miR-138-1-3p has also prognostic value in papillary thyroid cancer (PTC) and was associated with tumorigenesis.[90] The disruption of the 3'-UTR of PD-L1 has been used as genetic marker for cancers capable of immune evasion.[60] The tumor suppressive miR-138-5p inhibits PD-L1 expression, which is linked to a poor prognosis and worse clinical outcomes in patients.[109] However, despite the differential expression of PD-L1-specific miRNAs had a significant effect on T cell cytotoxicity, their clinical benefit was not apparent in melanoma patients unless the T cell infiltration was taken into account. Thus, the prognostic value of miRNA signatures might be limited, unless additional immune response-relevant information is available.[64]

Immune modulatory miRNAs regulated by cancer therapeutics and its role in therapy resistance

Multiple miRNAs have been reported to predict possible patients' response to therapy, but to a variable extent. This could be a direct result of miRNAs targeting mRNAs involved in the mechanism of the therapeutic regimen or indicative of different disease stages as well as cytogenetic aberrations thereby affecting the patients' response rate. Based on their pivotal role in immune responses, different groups have focused on the regulation of ICPs on T cells via miRNAs. Targeting of the mTOR pathway by the miR-16 family resulted in an upregulation of programmed death receptor (PD)-1, LAG3 and TIM-3, which was reversed in miR-15/16 deficient mice leading to a stronger immune response against glioma.[110] In contrast, miR-149-3p overexpression reversed $CD8^+$ T cell exhaustion in BC.[111] Manipulation of $CD8^+$ T cells in mice using miRNAs allows to test their use as therapeutics but also helps to shed light on the pathways regulated by miRNAs in T cells.

The plethora of tumor-related miRNA targets suggests their use as therapeutics as well as a tool for studying tumorigenesis, disease progression and therapy response. For example, miRNA expression levels were correlated to response to anti-PD-L1 therapy proving further the clinical significance of these non-coding RNA molecules.[62 112]

The identification of miRNAs that could target ICPs increased the therapeutic tool arsenal targeting the molecules and the understanding of the underlying mechanisms of their deregulated expression in tumors and their role in therapy resistance.[46] Targeting these deregulated miRNAs is an effective tool to overcome therapy resistance. Some miRNAs lead to an upregulation, others to a downregulation of ICP expression,[59] which have associated with therapy resistance.

Despite improving the patients' outcomes, multiple established standard-of-care therapies have still only a limited efficacy for all patients, which is due to intrinsic and acquired resistance mechanisms to the respective therapeutics. Recently, miRNAs as crucial post-transcriptional regulators have been suggested to contribute or predict to chemotherapy or radiation therapy resistance.[113–116]

In sum, these results provide novel insights into the miRNA biology that need to be taken into account during therapy or could be even harnessed to drive immune responses. Despite the efficacy of therapeutics on the tumor, these could be affected by alterations of the TME, which through the exosomal release of miRNAs can further alter the immunogenicity or resistance of malignant cells to therapy leading to detrimental results for the patients' progression-free and overall survival.

Distinct methods targeting miRNAs

Introduction of intact small RNAs of interest into cells is a big challenge. Despite the therapeutic modulation of miRNA expression being a promising approach for tumor prevention and treatment,[117] the difficulties in utilization of miRNAs as therapeutics involve the molecule used along with their modifications, their stability in the cell as well as the delivery method.[118] Over the last years, a number of strategies have been developed to target miRNAs, such as drugs affecting miRNA transcription and processing as well as inhibitors that block miRNA function. Another approach is to transfect miRNAs for the treatment of cancer with reduced miRNA expression. In general, synthetically produced miRNAs, which can be either mimics restoring miRNA levels thereby compensating their decreased expression or miRNA antagonists inhibiting miRNA expression, are generated with locked nucleic acid (LNA) bases, either encapsulated or conjugated to another molecule increasing their resistance to RNases and their cellular uptake.[119]

Currently, various small RNA-based drugs have proceeded into clinical trials with completely different approaches regarding nanoparticle origin, such as lipids, polymeric or inorganic nanoparticles.[120 121] The synthetic RNA is loaded into the nanoparticles, which can be added to cultured cells of the patients for autologous cell

transplant or directly intravenously applied to the patient and is then transferred into the cells via endocytosis.[122] An alternative to nanoparticles is the delivery of miRNAs via an expression cassette on a virus that could infect the target cells thereby introducing the miRNA into the patient. Regardless of the miRNA delivery systems, each method has severe drawbacks, such as the immunogenicity of the nanoparticles. Virus-based introduction cannot be modified to the extent of a synthetic miRNA thereby limiting additional options for increased miRNA stability while infection of non-desirable cells might lead to detrimental effects. A promising alternative to synthetic nanoparticles is in vitro-generated extracellular vesicles, which are difficult to generate on a large scale.[123] A more extensive analysis of the preferred methods will be discussed in the 'Currently available clinical trials using miRNAs for tumor treatment' section.

Immune modulatory miRNAs and cancer therapeutics

The large number of interactions of miRNA with components of the immune system suggested their therapeutic implementation alone or in combination with immunotherapies to optimize treatment efficacy. MiRNAs can either directly interact with modulators of the immune system or affect the outcome of the immune responses after ICPi-based immunotherapy.[124 125] However, miRNA-based therapies in cancer are still in early stages but may represent promising novel approaches in cancer immunotherapies. In mice, therapy with miR-138 targeting ICP molecules was effective for glioma treatment by reducing the PD-1 and CTLA-4 expression accompanied by an increased overall survival.[126] Concerning the human application, exosomes containing miR-125a-3p negatively affect the response of NSCLC patients to a PD-L1 therapy due to the miRNA-mediated PD-L1 upregulation via binding of miR-125-3p to neuregulin 1 (NRG1), revealing this miRNA as a stronger predictive marker for ICPi response than the expression of PD-L1 itself.[112] Furthermore, miRNAs targeting PD-1 have been described in various tumor entities, but in particular in melanoma and non-small lung carcinoma.[127] Higher levels of miR-100-5p and miR-125-5p allowed for better responses to anti-PD-1 therapy. The direct immune-enhancing role of miRNAs, such as miR-155, being able to target CTLA-4, might have adverse effects when not investigated in the right context. Despite a link between miR-155, CTLA-4 and Tregs associated with an immune-suppressed TME, metastatic melanoma patients non-responding to anti-PD-1 therapy showed lower levels of CTLA-4 in their blood. In this case, the benefits of immunotherapy outweigh the potentially detrimental miR-155-mediated CTLA-4 regulation. Such an interplay has to be taken into account, in particular since the available immunotherapeutic arsenal is increasing.

However, there exists evidence that (1) the response to chemotherapy and radiotherapy is not only dependent on the cytotoxic effect of the treatment applied, (2) but also due to the ability of these therapies to promote tumor antigenicity thereby enhancing an immune response and (3) miRNAs contribute to these mechanisms of action. In addition, miRNAs are able to change the levels of cytokine secretion and activation in immune cells and consequently miRNAs affecting chemotherapeutic activity can alter the immune responses by directly interacting with immune cells. Treatment with metformin, a type 2 diabetic medication with expected anticancer activity resulted in an overexpression of miR-150 and miR-155 in NK cells and an increase in NKp46+FasL+IFN-γ+ NK cells with a strongly improved cytotoxic potential and enhanced antitumor responses.[128] Furthermore, proinflammatory signals are crucial for the recruitment of innate and adaptive immune cells at the tumor site. The radiation-mediated upregulation of miR-223-3p was able to inhibit pyroptosis through direct targeting of the inflammasome component NLRP3.[102] Since therapy can alter the expression of multiple mRNAs associated with the immune modulatory activity of miRNAs targeting T cell activation and maturation, cytokine secretion and signal transduction, the multivalent miRNAs have to be monitored to increase the chances of a second line treatment.

Currently available clinical trials using miRNAs for tumor treatment

So far, two clinical trials used lipid nanoparticle (LNP)-encapsulated miR-193-3p and miR-34a for the treatment of various advanced solid tumors (NCT05499013, NCT01829971). While the former is still recruiting, the drug MRX34 was terminated due to strong immune-related adverse effects.[129] Thus, the uptake of LNPs without specificity can be detrimental and the implementation of exogenous miRNA mimics requires further development to avoid or at least reduce cytotoxicity. An alternative to the LNP-miRs is the implementation of TargomiRs, which are non-viable minicells of bacterial origin loaded with synthetic miRs, such as miR-16, and coated with, for example, an anti-EGFR antibody to specifically target EGF-R-expressing tumor cells (NCT02369198[130]). This treatment was better tolerated and demonstrated some moderate tumor suppression.

The use of antisense oligonucleotides is the most advanced technology to target miRNAs. LNP-encapsulated miR-155 antagomiRs (MRG-106) was developed and tested in cutaneous T cell lymphoma (CTCL), chronic lymphatic leukemia (CLL) and acute T cell leukemia lymphoma (ATCL) patients (NCT02580552) by either intratumoral or subcutaneous administration. Based on the success of this phase I clinical trial, a phase II clinical trial was developed (NCT03713320) in CTCL and diffuse large B cell lymphoma (DLBCL), which was terminated due to financial reasons. Another phase I clinical trial (NCT04675996) using LNP-formulated miR-193a-3p mimic is currently under investigation in several solid cancers. Similar holds for a miR-106 inhibitor conjugated with advanced dextran-coated iron oxide nanoparticles (NCT01849952). Next to TTX-MC138, another miR-106 inhibitor, RGLS5579, was developed for the treatment

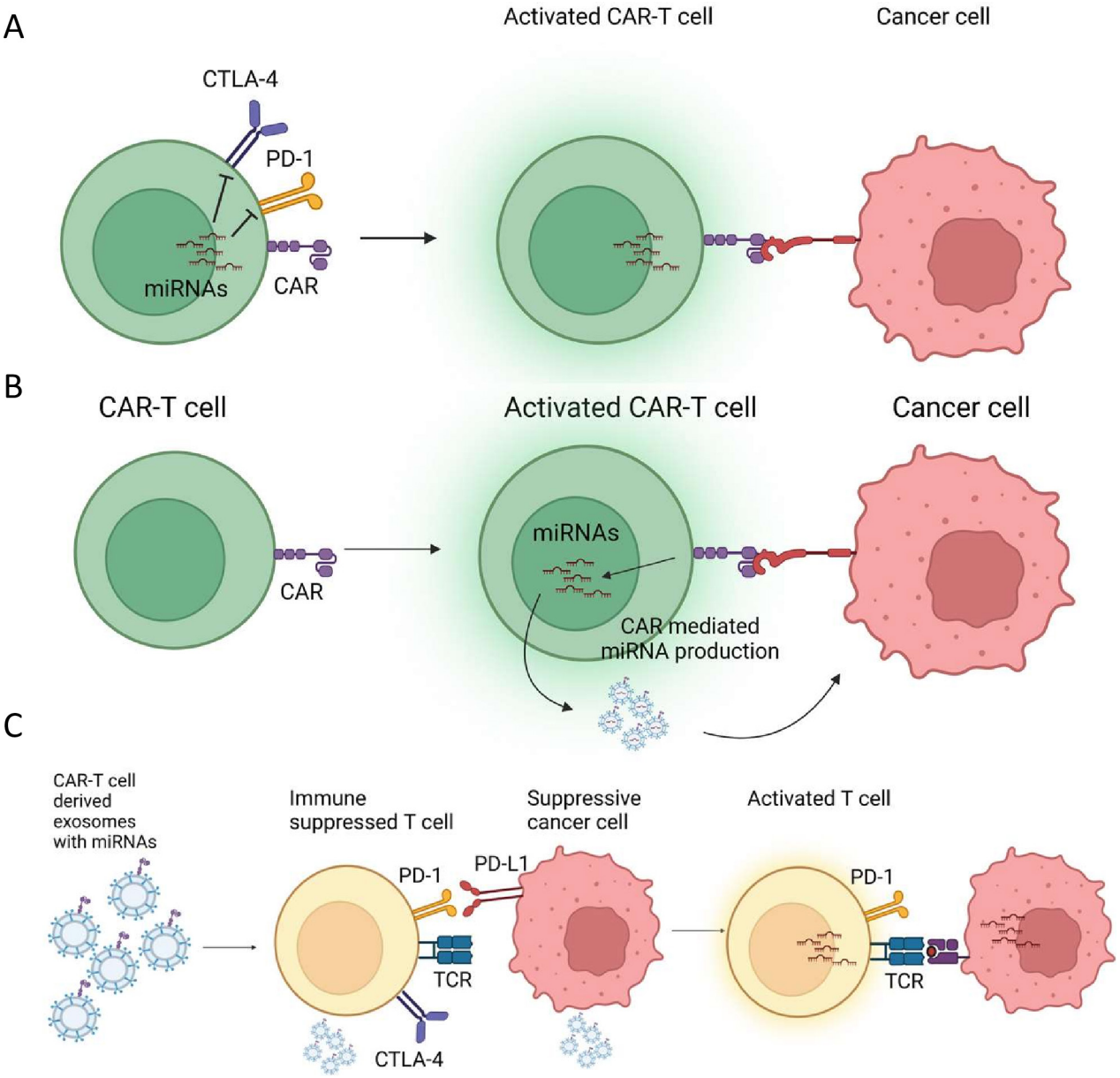

Figure 3 Possible approaches in combination of chimeric antigen receptor (CAR) T cells and microRNAs (miRNAs). (A) Careful selection of a miRNA has to be used in order to simultaneously activate the CAR T cells and inhibit the expression of immune checkpoint molecules. (B) MiRNA-loaded exosomes can be produced directly by CAR T cells on engagement of their CAR on the tumor site on injection to the patient. The miRNA payload could affect the expression of immune-relevant molecules on the surrounding tumor cells. (C) Ex vivo generated CAR T cell derived exosomes in genetically engineered miRNA expressing CAR T cells. The cytotoxic capabilities of these exosomes alone could help to eliminate tumor cells while the miRNA payload could affect the expression of immune relative molecules in tumor and immune cells. This figure was created with Biorender.

of glioblastoma. All these methods aim to increase the successful miRNA/siRNA delivery with higher specificity of the target cells. The majority of these current studies are in phase I and mainly focused on advanced tumors. Furthermore, the benefit of these therapies might be progressively lost due to changes in the TME of the patients. Despite their pleiotropic effects, miRNA therapy has still many challenges including toxicity, low efficacy and adverse effects.[131]

Future perspectives of miRNA therapies utilizing the chimeric antigen receptor T cell system

During the last decade, a number of in particular preclinical, but also clinical trials have been developed using miRNA approaches with advanced delivery technologies. While the various ongoing trials intend to alter gene expression via LNA-LNPs or viral vector-based miRNA approaches, another option for miRNA transfer is chimeric antigen receptor (CAR) T cells, which are engineered T cells with a CAR, currently used for the treatment of hematopoietic malignancies.[132 133] The development of sophisticated CARs, from the fourth generation of CARs

secreting cytokines to increase immune response[134] to the modular UniCAR model allows for the selective "turning on" of CARs based on the presence of the target module,[135] stably miRNA overexpressing CAR T cells are a promising strategy. Selection of overexpressed miRNAs should improve the cytotoxic activity and antitumoral responses of the CARs (figure 3A). Modifying the efficacy of T cells by miRNAs has been already applied in the context of oral squamous cell carcinoma (OSCC) by taking advantage of γδ T cell-derived exosomes overexpressing miR-138.[136] In addition, an anti-CD19 CAR system has been applied with a simultaneous coexpression of miR-155 leading to CAR T cells with increased TNF-α and IFN-γ production and increased cytotoxicity in vivo.[73] Furthermore, multiple miRNAs involved in T cell metabolism and mitochondrial reprogramming were suggested as prominent candidates to increase the persistence of CARs and patients' clinical outcome.[137]

Since changes in the miRNA expression could influence the cytokine levels necessary for T cell activation, such as IL-2,[138] or activating cytokines produced by T cells themselves,[139] this approach could increase the efficacy of the generated CAR T cells. Furthermore, a protein family, acting as cytokine suppressors, the SOCS proteins, known to be involved in the JAK/STAT-mediated cytokine secretion and regulation of multiple cytokines could be targeted by miRNAs,[140–143] potentially altering the TME composition and implicating a role for CAR T cells beyond their cytotoxic effect. MiRNAs overexpressed in CAR T cells could have the additional benefit of potential delivery to the cancer site altering the TME. As a differential efficacy of CARs has been demonstrated based on the miRNA expression of cancer cells,[144] alterations of the basal miRNA expression of tumors via exosomes are suitable and currently tested in the iExosomes trial using mesenchymal stromal cell exosomes. T cell-derived exosomes have been shown to contain miRNAs, which alter not only the translational profile of tumor cells and tumor mesenchymal cells,[145 146] but also directly affect and reprogram immune cells.[136 147 148] Ideally, carefully selected overexpressed miRNAs should be able to affect T cell activation and through exosomal release, should have a cytotoxic effect on the tumor[149] and manipulate tumor immunogenicity as well as the immune infiltrate at the tumor site (figure 3B). One could speculate that a further equipment of CAR T cells with an orthogonal cytokine receptor[150] coupled with an exosome release signal could allow this miRNA-mediated reprograming only on the tumor site, based on the cytokine signal selected. Alternatively, the use of exosomes derived from UniCAR T cells (figure 3C) could allow for easier dosage optimization and antigen selection through the target module with similar benefits.

CONCLUSIONS

One of the major obstacles of miRNA-based therapy is the selection of the ideal miRNA with the capacity to act on both immune and tumor cells. Despite the relatively small number of im-miRNAs so far identified and summarized in this review, many of them showed relevance for both immune and tumor cells due to their deregulation in the context of cancer. Some miRNAs were able to influence more than one ICP (miR-16, miR-155, miR-34a, miR-146a) suggesting their use as possible candidates for a CAR T cell system (Supplemental file 1). On the other hand, a careful selection of miRNA is necessary since miRNAs could simultaneously target both immune stimulatory and immune inhibitory molecules.[63 82 87 151] Undoubtedly, further experiments are necessary to clearly distinguish their possible benefits in a respective clinical context. In addition, a deeper knowledge of the potential unknown oncogenic effects of these miRNAs should be investigated, while the identification of novel targets is further required to increase the number of possible therapeutic miRNAs but also to relinquish the attributed bias due to the long-lasting investigation of this small group of targets.

Acknowledgements We would like to thank M. Heise and S. Wolf for excellent secretarial help.

Contributors CV and BS designed and wrote the review, MB discussed the review and corrected the review. BS and CV accepted full responsibility for the work and/or the conduct of the study, had access to the data and controlled the decision to publish.

Funding This review was supported by grants from the DFG (SE581/33-1 and SE581/34-1) and the Deutsche Krebshilfe (DKH Nr. 70113861).

Competing interests None declared

Patient consent for publication Not applicable.

Provenance and peer review Commissioned; externally peer reviewed.

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