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Circulating MicroRNAs: functional biomarkers for melanoma prognosis and treatment

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Abstract
MicroRNAs (miRNAs) hold significant promise as circulating cancer biomarkers and unlike many other molecular markers, they can provide valuable insights that extend beyond tumour biology. The expression of circulating miRNAs may parallel the cellular composition and dynamic activity within the tumour microenvironment and reveal systemic immune responses. The functional complexity of miRNAs—where a single miRNA can regulate multiple messenger RNAs (mRNAs) to fine tune fundamental processes, and a single mRNA can be targeted by multiple miRNAs—underscores their broad significance and impact. However, this complexity poses significant challenges for translating miRNA research into clinical practice. In melanoma, specific miRNA signatures have shown notable diagnostic, prognostic and predictive value, with lineage-specific and immune-related miRNAs frequently identified as valuable markers. In this review, we explore the role of circulating miRNAs as potential biomarkers in melanoma, and highlight the current status and advances required to translate miRNA research into therapeutic opportunities.

Keywords MicroRNA, Melanoma, Treatment prediction, Biomarkers, Immune checkpoint inhibitors, Targeted therapies

Introduction

Melanoma is an aggressive malignancy that arises from melanocytes, specialised pigment-producing cells derived from the neural crest. While melanocytes are primarily found in the skin, they are also present in the eyes, ears and mucosal membranes, and melanoma can develop in any of these locations [1]. Cutaneous melanoma accounts for over 90% of all melanoma diagnoses, while mucosal and uveal melanomas are rare, and comprise less than 5% of cases. The global incidence of cutaneous melanoma continues to rise, particularly in fair-skinned populations [2, 3] – worldwide melanoma incidence in 2019 was six times higher than 40 years prior [4], with an estimated 325,000 cases globally in 2020 [2]. Since 2011, significant advancements in treatment, including US Food and Drug Administration (FDA)-approved molecular targeted therapies (e.g. V-Raf Murine Sarcoma Viral Oncogene Homolog (BRAF) and mitogen-activated protein kinase kinase (MEK) inhibitors), as well as immune checkpoint inhibitors (e.g. programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) inhibitors), have contributed to a 17.9% reduction in melanoma mortality rates between 2013 and 2016 [5, 6]. The clinical benefits of these therapies are limited, however, by the development of innate and acquired resistance and the incidence of severe treatment-related adverse events. Approximately 70–90% of patients with advanced melanoma experience disease progression despite treatment with these therapies [7, 8], and nearly 60% of melanoma

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patients receiving combination anti-CTLA-4 and anti-PD-1 therapy experience grade 3 or 4 adverse events [8].

Further improvements in melanoma outcomes are expected through earlier screening and correct diagnosis [9, 10], alternative combination therapies such as relatlimab (Lymphocyte-activation gene 3 (LAG3) inhibitor) with nivolumab (PD-1 inhibitor) rather than nivolumab alone [11], and optimised treatment timing such as perioperative and response-driven adjuvant therapy for patients with high-risk disease [12, 13]. However, these advancements underscore the urgent need to identify biomarkers to monitor treatment response, detect resistance, and identify post-surgical recurrence. miRNAs are key regulators of cancer biology, playing crucial roles in tumour initiation, progression, and metastasis [14]. The expression of miRNAs is dysregulated in many cancers, including melanoma [15] and miRNAs are emerging as promising biomarkers for disease diagnosis and prognosis. In this review, we examine the role of circulating miRNAs in melanoma, focusing on their diagnostic, prognostic, and predictive potential. Additionally, we evaluate the therapeutic potential of miRNAs, exploring their role in enhancing treatment strategies for melanoma.

MicroRNAs: biosynthesis and functional roles

miRNAs are single-stranded ribonucleic acids (RNAs), typically 21–25 nucleotides in length [16, 17]. Most miRNAs are derived from longer non-coding or intronic primary transcripts, which are capped and polyadenylated. A smaller number of miRNAs are derived from exonic or intergenic deoxyribonucleic acid (DNA) regions [18, 19]. miRNAs are transcribed in the nucleus and exported to the cytoplasm, where they are cleaved into mature miRNAs by the Dicer endonuclease enzyme in complex with RNA binding proteins (Fig. 1) [20]. Comprehensive details on miRNA biogenesis are provided in several recent reviews [21, 22].

The 2024 Nobel Prize in Medicine was awarded to US scientists Victor Ambros and Gary Ruvkun for the

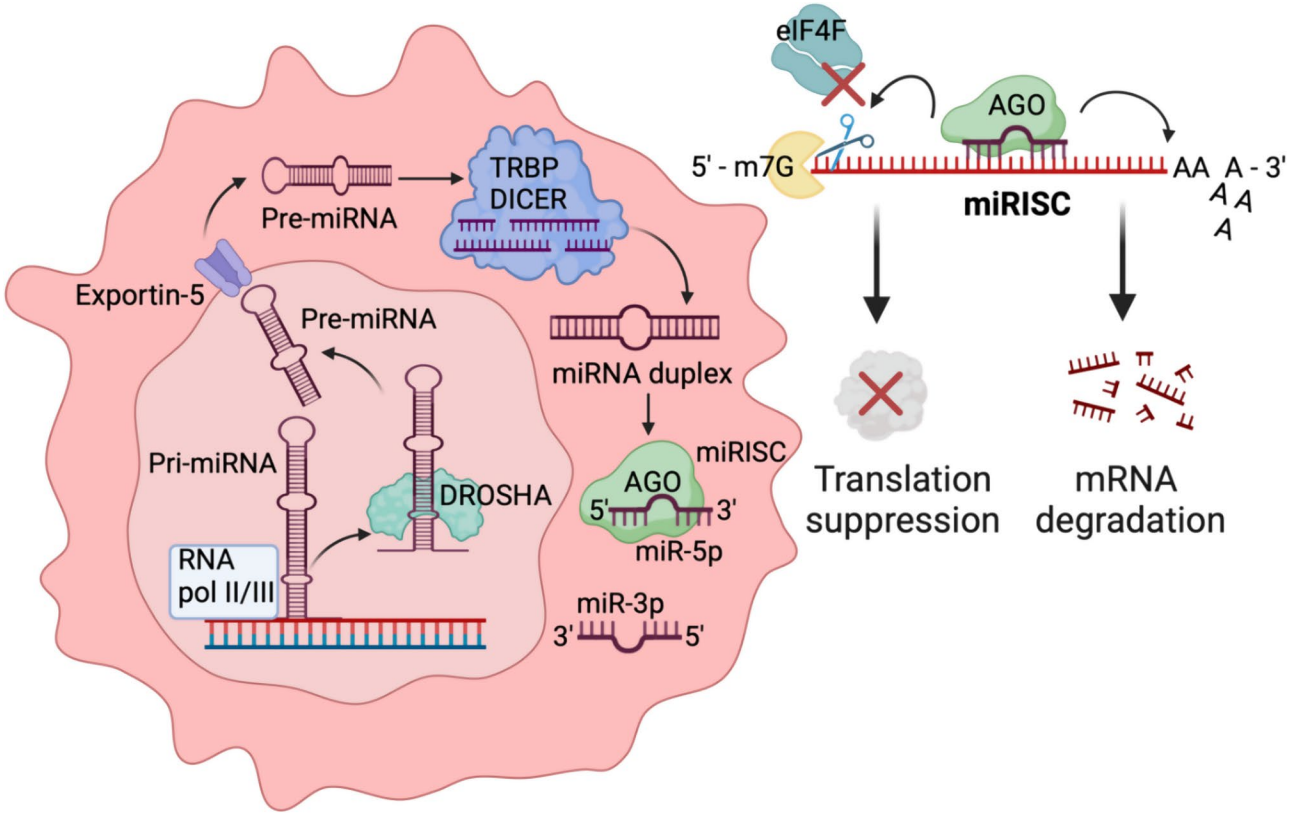

Fig. 1 Canonical miRNA biosynthesis and miRNA function. Following transcription by RNA polymerase II/III (RNA pol II/III), primary miRNAs (pri-miRNAs) are cleaved at the base of a hairpin structure by the nuclear DROSHA RNase III to yield ~ 60–70 nucleotide precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm via an Exportin-5 receptor-dependent process where they undergo further cleavage by the Dicer RNase III bound to RNA-binding proteins, such as TRBP. The resulting miRNA duplex associates with an AGO protein, where one miRNA strand (the guide strand) is selected to form the functional miRISC. The miRNA may originate from the 5′ end of the pre-miRNA or the 3′ end of the pre-miRNA giving rise to the -5p or -3p miRNAs, respectively. The unbound strand of the miRNA duplex is ejected from the AGO protein and degraded. Once formed, miRISC binds to target mRNAs and inhibits translation by competing with eIF4F recognition of the 7-methyl-G (m7G) cap, inhibiting cap-dependent translation initiation and destabilizing the target mRNA through the recruitment of de-adenylation complexes, which remove the stabilizing 3′ poly(A) tail

discovery of miRNAs and their pivotal role in post-transcriptional regulation of gene expression. miRNAs bind to complementary sequences in the 3' untranslated region (3' UTR) or the 5' UTR of target mRNAs to regulate the expression of approximately 60% of human genes [23]. This miRNA-mRNA interaction promotes the degradation or direct translational suppression of the target mRNAs [24–26] (Fig. 1). According to the miRNA repository (miRBase registry V22.1), there are currently 2656 mature human miRNA species [27]. A single miRNA can regulate multiple mRNA targets, with its binding specificity determined by the complementarity between the miRNA seed region (bases 2–8 of the miRNA [28]) and the 3' UTR of target mRNAs [29, 30]. For example, miR-34 interacts with and downregulates the expression of several cell cycle regulatory genes, including cyclin-dependent kinase 4 (CDK4) [31], CDK6, cyclin D1 [32], and E2F transcription factor 3 [33]. Conversely, a single mRNA can be targeted by multiple miRNAs, leading to complex regulation of gene expression. For instance, over 15 miRNAs have been predicted to bind with high probability to the 3' UTR of microphthalmia-associated transcription factor (*MITF*), a key transcriptional regulator of melanocyte development and function (Fig. 2).

miRNAs regulate many biological processes, including cell development, growth, differentiation, metabolism, and homeostasis, both under normal physiological conditions and in various diseases [34, 35]. Generally, oncogenic miRNAs or oncomiRs promote cancer development and are overexpressed in cancer, whereas tumour suppressor miRNAs may promote apoptosis or cell cycle arrest and are under expressed in cancer [36]. Importantly, this classification lacks consistency and may not be informative, as miRNAs can act as either tumour-promoting or tumour-suppressive (e.g. miR-125 [37]), a distinction most likely dictated by the transcriptional milieu of the cancer.

miRNAs are also released into extracellular fluids, and circulating miRNAs have been detected in plasma, serum [38, 39], cerebrospinal fluid [40], and urine [41]. In cancer, both viable and dying tumour cells actively secrete miRNA to produce circulating miRNAs [39, 42]. Most circulating miRNAs are stabilised due to their association with proteins, such as the miRNA-inducing silencing complex (miRISC) effector protein Argonaute 2 (AGO2), nucleophosmin, high-density lipoproteins [43, 44] or extracellular vesicles such as exosomes, microvesicles and apoptotic bodies [45]. The lineage, differentiation and tissue-specificity of miRNAs make them valuable disease biomarkers, while their stability enhances their utility as reliable liquid biopsy biomarkers.

Circulating MiRNAs as diagnostic biomarkers in melanoma

The current standard for melanoma diagnosis involves the histopathological assessment of an excision biopsy, interpreted in conjunction with clinical information such as patient age, lesion site, and lesion history [46]. Despite the standardisation of diagnostic criteria, the diagnosis of melanomas can be challenging. A histopathological review of over 3300 melanocytic lesions demonstrated

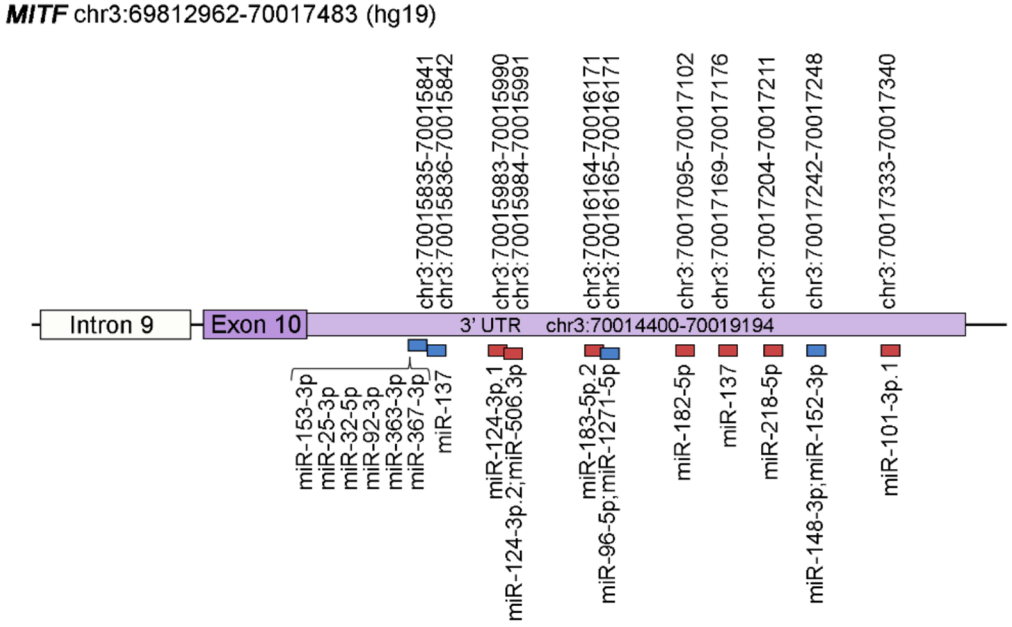

Fig. 2 miRNA regulators of the *MITF* gene. Multiple miRNAs can bind to identical and overlapping regions within the 3' UTR of the target human *MITF* mRNA. miRNAs with 8mer or 7mer seed sites are highlighted in red and blue, respectively. Data are derived from TargetScanHuman (release 7.2) [181]. The hg19 chromosome coordinates of the *MITF* mRNA and the miRNA binding sites are shown

substantial diagnostic variation, with 24% of cases receiving equivocal diagnostic results between dermatopathologists [47]. Diagnostic ambiguity was associated with variations in treatment [47], and heightened diagnostic scrutiny has led to an increased rate of misdiagnosing benign melanocytic lesions as melanoma [4]. Consequently, clinicians are evaluating and implementing adjunct diagnostic tools, including immunohistochemistry stains (e.g. PReferentially expressed Antigen in MElanoma or PRAME [48]), gene expression signatures [49], genomic analysis [50] and digital pathology workflows [51] to support the diagnosis of challenging cases.

A recent systematic review [52] identified nine independent studies [53–61] investigating the diagnostic utility of circulating miRNAs in melanoma. All studies used a melanoma-healthy control design and employed reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to measure the expression of individual or panels of circulating miRNAs. Across these studies, 82 distinct miRNAs were detected in blood, plasma or serum, with individual diagnostic sensitivity ranging from 0.71 to 0.97 and specificity from 0.62 to 0.98. Notably, despite minimal overlap in miRNAs investigated across the nine studies, a pooled analysis of these 82 miRNAs demonstrated a diagnostic sensitivity of 0.89, specificity of 0.85, and an area under the curve (AUC) of 0.93 [52]. Importantly, several miRNAs that distinguished melanoma from healthy controls also discriminated melanoma from other cancers. For instance, loss of serum miR-29c distinguished melanoma from metastatic colon and renal cancer [62]. miRNA cancer specificity also likely reflects lineage specific differences as some miRNAs, such as miR-211-5p, are located within an intron of *TPRM1*, which is a target gene of MITF [63].

The circulating MEL38 miRNA signature emerges as a notable diagnostic panel for melanoma with significant validation data. MEL38 miRNAs were identified as differentially expressed between individuals with or without cutaneous melanoma by whole miRNA profiling [53]. This signature consists of skin-cell derived miRNAs and 22/38 miRNAs have been previously identified in melanoma [53]. The MEL38 miRNA signature is enriched for pathways related to melanogenesis, T cell activation and mitogen-activated protein kinase (MAPK) activation [53]. The diagnostic utility of MEL38 has been independently validated in peripheral blood [53], in formalin-fixed paraffin-embedded tissue biopsies [64], and recently, in 582 plasma samples derived from melanoma patients or control individuals with non-melanoma skin conditions [54]. In the latter study, using a threshold of 5.5, MEL38 achieved a 93% true-positive rate (sensitivity) and a 98% true-negative rate (specificity) for the presence of invasive melanoma [54]. MEL38 was associated with melanoma, irrespective of tumour thickness and melanoma type (superficial, nodal or amelanotic) [53]. Importantly, despite being designed as a diagnostic signature, MEL38 also showed prognostic value as a continuous predictor of melanoma-specific survival (MSS) [54]. Although circulating miRNAs hold potential as an adjunct to pathological assessment in diagnosing challenging lesions, their clinical implementation remains limited. In fact, we identified only one miRNA-based diagnostic tool currently in use in oncology. The ThyGeNEXT oncogene panel, combined with the ThyraMIR v2 miRNA panel, is applied to improve the diagnostic accuracy of cytologically indeterminate thyroid nodules, with a reported sensitivity of 96% and specificity of 99% [65].

Overall, miRNAs have yet to demonstrate consistent and reliable diagnostic utility in broader clinical settings, and their application remains largely exploratory. Advancing the diagnostic potential of miRNA signatures will require large-scale studies involving representative cohorts, comprehensive clinical annotation and standardised experimental workflows. These studies should also integrate multi-omics data, including genetic, clinicopathological, and imaging information, to enhance the robustness and clinical utility of miRNA-based diagnostic markers.

Circulating MiRNAs as prognostic biomarkers in melanoma

Immune and targeted therapies have revolutionised the treatment of patients with advanced (stage IV) melanoma and these therapies are being used in the earlier-stage setting, including in patients with stage II (node-negative) [66, 67] and stage III melanoma (disease has spread to regional lymph nodes [13, 68, 69]). The outcomes of these patients are heterogenous, with substantial risks of recurrence. For instance, 27.2% of patients with stage II melanoma will recur within 5 years [70] and the 5-year MSS rates for Stage IIB (87%), IIC (82%) and IIIB (83%) are comparable [71]. There is, therefore, significant interest in developing reliable prognostic biomarkers, across all stages of melanoma, to guide treatment-decision making. Several commercially available gene expression profiles have been developed to stratify melanoma patients according to risk of recurrence. These include the DecisionDx-melanoma 31-gene expression profiling (GEP) assay (Castle Biosciences, USA) [72, 73], the Merlin 8-GEP test (Skyline Dx, Netherlands) [74] and the MelaGenix 11-GEP test (NeraCare, GmbH) [75].

Numerous studies have also investigated the association of circulating miRNAs with melanoma patient outcomes, including overall survival (OS), disease free survival (DFS), MSS and the development of brain or distant metastases (Table 1). These investigations typically focus on a narrow set of miRNAs, and we have identified over 50 reported prognostic circulating miRNAs in

Table 1 Prognostic Circulating MiRNA biomarkers in cutaneous melanoma

miRNA	Detection method	Sample Type	Cohort	Clinical associations
miR-10b	RT-qPCR	Serum	85 melanoma patients, 30 healthy controls	High serum miRNA is an independent predictor of poor OS and DFS [57]
miR-15b, **miR-30d**, **miR-150**, miR-425	RT-qPCR	Serum	210 stage I-III melanoma patients (discovery), 82 stage I-III melanoma patients (validation)	Serum miRNA signature is associated with risk of recurrence. In combination with stage, these miRNAs improved prediction of recurrence with AUC of 0.760 (discovery) and 0.790 (validation) [76]
miR-15b, miR-33a, **miR-150**, miR-424, miR-199a-5p	RT-qPCR	Serum	80 stage I-III melanoma patients (discovery cohort), 60 stage I-III melanoma patients (validation cohort)	Serum miRNA signature is associated with disease stage and predicted recurrence with sensitivity of 95%, specificity of 41% in validation cohort [77]
miR-16	RT-qPCR, miRNA microarray	Serum	20 melanoma patients, 20 healthy controls (discovery cohort), 120 melanoma patients, 120 healthy controls (validation cohort)	Low serum miRNA is associated with melanoma stage and discriminates melanoma patients from healthy controls with AUC of 0.779, sensitivity of 80%, specificity of 71.7%. Low serum miRNA is an independent predictor of OS [78]
miR-21	RT-qPCR	Plasma	16 primary, 10 stage III, 4 stage IV melanoma patients, 11 healthy controls	High plasma miRNA is associated with increased tumour burden [79]
miR-23a	RT-qPCR	Serum	192 melanoma patients, 51 healthy controls	Low serum miRNA is associated with metastasis and is an independent predictor of poor OS. miR-23a is shown to regulate autophagy by targeting ATG12 [80]
miR-99a, **miR-221**, miR-320, **miR-494**, miR-1908, miR-4487	RT-qPCR	Plasma	22 Stage I and II melanoma patients	Plasma miRNA signature is associated with shorter DFS and worse OS [81]
miR-107	RT-qPCR	Plasma	46 stage III melanoma patients	Plasma miRNA is a biomarker for DFS [82]
miR-150-5p, **miR-142-3p**	RT-qPCR	Serum	52 stage III and 40 stage IV melanoma patients (discovery cohort), 31 melanoma patients, 43 healthy controls (validation cohort)	Low serum miRNA in stage IV melanoma patients is associated with shorter OS [83]
miR-206	RT-qPCR	Serum	60 melanoma patients, 30 healthy controls	Low serum miRNA is an independent predictor of poor OS and DFS [84]
miR-210	RT-qPCR	Plasma	60 stage III and 70 stage IV melanoma patients (cohort A), 88 stage III melanoma patients (cohort B)	High plasma miRNA is associated with poor DFS and MSS [85]
miR-221	RT-qPCR	Serum	72 melanoma patients, 54 healthy controls	High serum miRNA is an independent prognostic factor for poor 5-year OS and DFS [86]
miR-221	RT-qPCR	Serum	94 melanoma patients, 20 healthy controls	High serum miRNA is correlated with increased tumour thickness [87]
MELmiR-7 signature[1]	RT-qPCR	Serum	86 stage I/II, 50 stage III, 119 stage IV melanoma patients, 130 healthy controls	Serum MELmiR-7 signature discriminates melanoma patients from healthy controls and predicted OS [56]
MEL12 signature[2]	miRNA sequencing	Plasma	232 melanoma patients	Plasma MEL12 signature is associated with disease outcome, sentinel lymph node biopsy status, and clinical stage [54, 88]
6[3] and 29[4] panel miRNA signatures	miRNA sequencing	Plasma	36 pre-operative melanoma brain metastasis patients, and 48 healthy controls (discovery), 24 melanoma brain metastasis patients	Plasma 6 miRNA signature distinguishes melanoma brain metastases from other brain metastases and glioblastoma [89]. Plasma 29 + 6 miRNA signature is associated with melanoma brain progression [89]

miRNAs highlighted in bold have been reported to be involved in immune regulation

[1]MELmiR-7 signature includes miR-16, miR-211-5p, miR-4487, miR-4706, miR-4731, miR-509-3p, miR-509-5p

[2]MEL12 signature includes miR-122-5p, miR-107, miR-125a-5p, miR-151a-3p, miR-30d-5p, miR-598-3p, miR-22-3p, miR-204-5p, miR-378i, miR-21-5p, miR-4516, miR-630

[3]6 miRNA signature includes miR-5694, miR-6796-3p, miR-6741-3p, miR-4664-3p, miR-4665-5p, miR-671-5p

[4]29 miRNA signature includes miR-3937, miR-1299, miR-1273e, miR-670-3p, miR-1225-3p, miR-574-5p, miR-6780b-5p, miR-3674, miR-7111-5p, miR-1273a, miR-6877-5p, miR-6775-5p, miR-4279, miR-5585-3p, miR-548d-5p, miR-7114-3p, miR-1207-5p, miR-3135a, miR-6789-3p, miR-1273c, miR-1228-3p, miR-1273g-5p, miR-143-5p, miR-6795-3p, miR-6162, miR-920, miR-378f, miR-3663-5p, miR-3184-3p

melanoma from various stages; most have not been validated and only a few have been identified across multiple studies (Table 1).

An intriguing finding from melanoma prognostic studies is the link between immune-modulatory miRNAs (im-miRNAs) and patient outcomes. For instance, miR-150-5p is predominantly derived from tumour infiltrating lymphocytes [90] and has been shown to be released into the circulation upon T cell activation [91]. Predictably, low miR-150-5p expression in primary melanoma tissue correlated with low intratumoural $CD45^+$ immune cells [90]. Low tissue expression of miR-150 was also associated with poor survival outcomes in stage III and IV melanoma patients [83]. In the circulation, serum miR-150 expression shows an inconsistent association with melanoma prognosis, although two out of three studies report lower serum miR-150 levels associated with poorer outcomes in early-stage and advanced melanoma [76, 83] (Table 2). miR-15b (targets programmed cell death ligand 1 (PD-L1) [92]), and miR-23a (targets transcription factor B lymphocyte-induced maturation protein-1 [93]), activate cytotoxic $CD8^+$ T cells and lower serum levels of these immune regulators are linked to poor outcomes in melanoma patients [77, 80]. Finally, miR-221, a commonly identified prognostic melanoma marker, inhibits Janus kinase/Signal transducers and activators of transcription (JAK/STAT) signalling by targeting protein coding genes such as *SOCS3* and *IRF2* [94], and modulates T helper 17 cell responses [95]; overexpression of this miRNA in serum is consistently associated with worse OS in melanoma patients [81, 86, 87].

These findings underscore the value and potential limitations of miRNAs. Their pleiotropic roles result in markers that provides a dynamic fingerprint that may reflect tumour-specific features, local immune responses and systemic immunity alongside patient outcomes. However, these extensive effects can also complicate the derivation of specific miRNA signatures with high prognostic specificity.

Circulating MiRNAs in melanoma treatment response and resistance

The development of therapies targeting BRAF and MEK and immune checkpoint inhibitors have dramatically improved the outcomes of patients with advanced and high-risk, stage III melanoma. Targeted therapies are an option for patients with $BRAF^{V600}$-mutant melanoma, which represents about 40% of metastatic melanoma [96]. Approximately 70% of these patients will respond to combination BRAF/MEK inhibitors but acquired treatment resistance is common, and few patients will achieve durable responses. The 5-year progression-free survival (PFS) and OS rates for patients with advanced BRAF-mutant melanoma receiving combination BRAF/MEK inhibitors range from 14–25% and 31–35%, respectively [97–99]. Conversely, immune checkpoint inhibitors, including ipilimumab, an antibody inhibitor of CTLA-4, and PD-1 inhibitors, nivolumab and pembrolizumab, demonstrate long-term efficacy. In the phase 3 CheckMate 067 clinical trial, 5-year PFS and OS rates were 36% and 52%, respectively, in patients with advanced melanoma receiving combination nivolumab and ipilimumab [100]. The combination of nivolumab with relatlimab also shows durable responses with 5-year PFS and OS rates of 28% and 49%, respectively [11].

Despite the effectiveness of current molecular and immune-based therapies, more than 50% of melanoma patients will exhibit innate resistance or develop acquired resistance to therapy [7, 101, 102]. Many promising new treatments are being tested, including tumour-infiltrating lymphocytes (2024 FDA-approved Lifileucel [103]), mRNA and multi-peptide vaccines (e.g. phase 2 KEYNOTE-942 and Mel44 clinical trials [104, 105]), and bispecific fusion proteins (e.g. PD-1/interleukin-2 bispecific antibody fusion protein IBI363 [106]). Ongoing efforts are focussed on identifying reliable biomarkers that can accurately predict and monitor treatment response.

The miRNAome undergoes large changes in response to BRAF inhibitor treatment and during the acquisition of resistance in melanoma cell lines. Although, the majority of miRNA changes are cell line dependent, it is evident that common pathways, including the MAPK cascade, survival networks and melanoma differentiation are deregulated during BRAF inhibitor resistance [107, 108], and miRNAs that regulate these pathways may function as potential biomarkers of treatment response and resistance. For example, miR-4443 (targets multiple survival pathways [109]), miR-199b-5p (targets vascular endothelial growth factor A (*VEGFA*) and hypoxia inducible factor 1 subunit alpha (*HIF1α*) [107]) and miR-4488 (targets differentiation marker nestin [110]) in plasma samples of melanoma patients discriminated BRAF/MEK inhibitor responders from non-responders with an AUC of 0.894 [107]. Melanoma dedifferentiation, characterized by *MITF* downregulation [111], leads to a slow-cycling cell state with increased resistance to BRAF/MEK and immune checkpoint inhibitors [112–114]. miRNAs targeting *MITF* (Fig. 2) are upregulated in BRAF inhibitor resistant melanoma cell models [108], but the relationship between MITF-targeting miRNAs in the circulation and BRAF inhibitor resistance remains to be investigated.

As reviewed elsewhere [115], im-miRNAs can regulate immune checkpoint expression, antigen processing/presentation and interferon gamma (IFNγ) signalling, and these miRNAs can additionally function as predictive biomarkers of immune checkpoint inhibitor therapy. For instance, a circulating miRNA signature (miR-146a, miR-155, miR-125b, miR-100, let-7e, miR-125a, miR-146b,

Table 2 miRNA-based diagnostics and therapeutics in cancer clinical trials

Clinical trial ID and status	miRNA target/s	Cancer type/s	Study description	Study outcomes
NCT00862914 Completed	Dicer	Cutaneous malignant melanoma, benign and dysplastic melanocytic naevi	Clinical study evaluating the immunohistochemical distribution of Dicer in cutaneous melanoma, benign and dysplastic melanocytic naevi compared to healthy controls	Dicer expression is elevated in melanoma cells compared to benign melanocytes and correlated with Breslow tumour thickness [150].
NCT01444560 Completed	Dicer, Drosha, Exp5, DGCR8, PACT, AGO1/2, TARBP1/2, MTDH and SND1	Cutaneous melanoma, cutaneous melanoma metastases, benign melanocytic naevi	Clinical study evaluating the mRNA expression of miRNA machinery components in cutaneous melanoma, benign and dysplastic melanocytic naevi compared to healthy controls	*AGO1*, *TARBP2* and *SND1* expression are lower in biopsies from patients with primary cutaneous malignant melanoma ($n=7$) compared to those with benign melanocytic naevi ($n=7$) [151].
NCT01482260 Completed	1205 miRNA targets	Primary cutaneous malignant melanoma, cutaneous melanoma metastases, benign melanocytic naevi	Clinical study evaluating miRNA expression profiles using microarrays in patients with primary cutaneous malignant melanoma, cutaneous malignant melanoma metastases and benign melanocytic naevi	22 upregulated and 28 downregulated miRNAs identified in patients with malignant melanoma ($n=13$) compared to those with benign melanocytic naevi ($n=8$) [152].
NCT05179174 Unknown	miR-506-514 cluster, miR-592 and miR-199a-5p	Uveal melanoma	Clinical study evaluating the diagnostic and prognostic role of genetic and epigenetic biomarkers	Not reported
NCT01829971 Terminated	miR-34a	Solid tumours or haematological malignancies (hepatocellular carcinoma, non-small and small cell lung cancer, melanoma, lymphoma, multiple myeloma, renal cell carcinoma)	Phase I study evaluating the safety, pharmacodynamics/kinetics of miR-34 mimic (MRX34) delivered intravenously via liposomal nanoparticles	Of 85 patients, 56% had severe adverse events (e.g. sepsis, hypoxia, cytokine release syndrome, hepatic failure), of which 38% experienced treatment-related severe adverse events. Of 66 patients evaluable for RECIST response, three had partial response, 16 had stable disease and 31 had progressive disease [145].
NCT02862145 Withdrawn	miR-34a	Melanoma	Clinical study evaluating biomarkers, pharmacodynamics/kinetics of miR-34 mimic (MRX34) delivered intravenously via liposomal nanoparticles	Study withdrawn
NCT02369198 Completed	miR-16	Malignant plural mesothelioma and non-small cell lung cancer	Phase I study evaluating miR-16 mimic (TargomiRs) as second- or third-line treatment, delivered by EGFR-targeting nanoparticles (EnGeneIC delivery vectors)	Of 26 patients, at the maximum tolerated dose, 9 (35%) experienced grade 3/4 adverse events. Of 22 patients evaluable for RECIST response, one had partial response, 15 had stable disease and 6 had progressive disease [146].
NCT02580552 Completed	miR-155	Cutaneous T-cell lymphoma, mycosis fungoides subtype, chronic lymphocytic leukemia, diffuse large B-cell lymphoma, adult T-cell leukemia/lymphoma	Phase I study evaluating the safety, pharmacokinetics and efficacy of anti-miR-155 (Cobomarsen, MRG-106), a LNA -based oligonucleotide inhibitor for treatment of blood cancers	Of six patients with mycosis fungoides, no severe adverse events were noted. Five patients showed reduction in the baseline Composite Assessment of Index Lesion Severity score; one had progressive disease and discontinued treatment [147].
NCT03713320 Terminated	miR-155	Cutaneous T-cell lymphoma, mycosis fungoides subtype	Clinical trial evaluating the safety and efficacy of anti-miR-155 (Cobomarsen, MRG-106), a LNA -based oligonucleotide inhibitor compared to vorinostat.	Not reported
NCT03837457 Terminated	miR-155	Cutaneous T-cell lymphoma, mycosis fungoides subtype	Clinical trial evaluating the safety and efficacy of anti-miR-155 (Cobomarsen, MRG-106), a LNA -based oligonucleotide inhibitor in patients with disease progression following treatment with vorinostat.	Not reported

Table 2 (continued)

Clinical trial ID and status	miRNA target/s	Cancer type/s	Study description	Study outcomes
NCT01849952 Recruiting	miR-10b	Brain tumours (gliomas)	Clinical trial evaluating the expression levels of miR-10b as diagnostic and prognostic biomarkers and in vitro testing of anti-miR-10b in primary tumours as a therapeutic agent	Study ongoing
NCT05908773 Completed	miR-10b	Advanced solid tumours	Phase 0 study evaluating the delivery of anti-miR-10b, a LNA -based antagomiR (TTX-MC138) in patients with advanced solid tumours and radiographically confirmed metastases.	First patient showed good tolerance to drug dose with no adverse events [148].
NCT06260774 Recruiting	miR-10b	Advanced solid tumours	Phase I/II study evaluating the dose and safety of anti-miR-10b, a LNA -based antagomiR (TTX-MC138) in patients with advanced solid tumours and radiographically confirmed metastases	Study ongoing
NCT04675996 Terminated	miR-193a-3p	Advanced solid tumours	First-in-human study evaluating the safety, pharmacokinetics/dynamics and efficacy of miR-193a-3p mimic (INT-1B3) encapsulated in lipid nanoparticles for treatment of solid tumours	Not reported
NCT04811898 Completed	miR-221	Refractory multiple myeloma and advanced solid tumours	Phase I study evaluating the safety and maximum tolerated dose of an LNA-based miR-221 inhibitor	Of 17 patients, no grade 3/4 adverse events were reported. Of 16 patients evaluable for RECIST response, one had partial response, eight had stable disease and seven had progressive disease [138]

Source ClinicalTrials.gov. Clinical trials with reported outcomes are referenced within the table

and miR-99b) identified in extracellular vesicles released by melanoma tumours was associated with increased myeloid-derived suppressor cells and resistance to ipilimumab and nivolumab therapy [116].

Another study reported that patients with high plasma levels of miR-155-5p, miR-320a and miR-424-5p prior to first-line anti-PD-1 therapy had longer PFS and OS. This study also confirmed the immune regulatory role of miR-155-5p, with plasma miR-155-5p levels correlating with $PD1^+$ $CD4^+$ T cells [117]. Additionally, miR-28 (targets PD-1, T-cell immunoglobulin and mucin domain 3 and B- and T-lymphocyte attenuator [118]) and miR-17-5p (targets PD-L1 [119]), suppress expression of immune checkpoint molecules, thus modulating anti-tumour immune functions, and serum levels of miR-17-5p inversely correlated with tumour PD-L1 expression in patients with metastatic melanoma [119]. In a cohort of 33 patients, serum levels of miR-16-5p, miR-17-5p, miR-451a and miR-20a-5p were higher in melanoma anti-PD-1 responders ($n = 10$) compared to non-responders ($n = 23$), and elevated serum levels of miR-1972 and miR-4502 were associated with non-response to anti-PD-1 [120]. Multivariate logistic regression analysis indicated miR-16-5p, miR-17-5p and miR-20a-5p to be independent predictors of response to PD-1 inhibitor therapy [120]. These data suggest that miRNAs predictive of PD-1 inhibitor response may contribute to patient outcomes by modulating the immunosuppressive PD-1/PD-L1 axis, and more than 100 miRNAs have been implicated in the regulation of immune checkpoints, including PD-1, PD-L1 and CTLA-4 (reviewed in [121]). Collectively, these data indicate that miRNAs can act as predictive biomarkers, but also actively modulate treatment response and resistance. As a result, miRNAs are emerging as promising therapeutic targets.

Therapeutic potential of MiRNAs in melanoma

Given the critical roles that miRNAs play in melanoma progression and therapy response, targeting miRNAs has emerged as a compelling therapeutic strategy. Several miRNAs have been reported to regulate the MAPK pathway in melanoma (Fig. 3), and manipulating the expression of these miRNAs can alter melanoma sensitivity to BRAF/MEK inhibitors. For instance, miR-7 suppresses epidermal growth factor receptor (EGFR), insulin like growth factor 1 receptor (IGF-1R) and RAF proto-oncogene serine/threonine-protein kinase (CRAF) expression, consequently inhibiting MAPK and phosphatidylinositol 3-kinase/protein kinase B signalling, and transfection of melanoma cells with miR-7 mimics resensitised cells to the BRAF inhibitor vemurafenib [122]. Similarly, miR-579-3p was shown to target *BRAF* and restoration of miR-579-3p expression sensitised melanoma cells to BRAF and MEK inhibitors [123], suggesting that

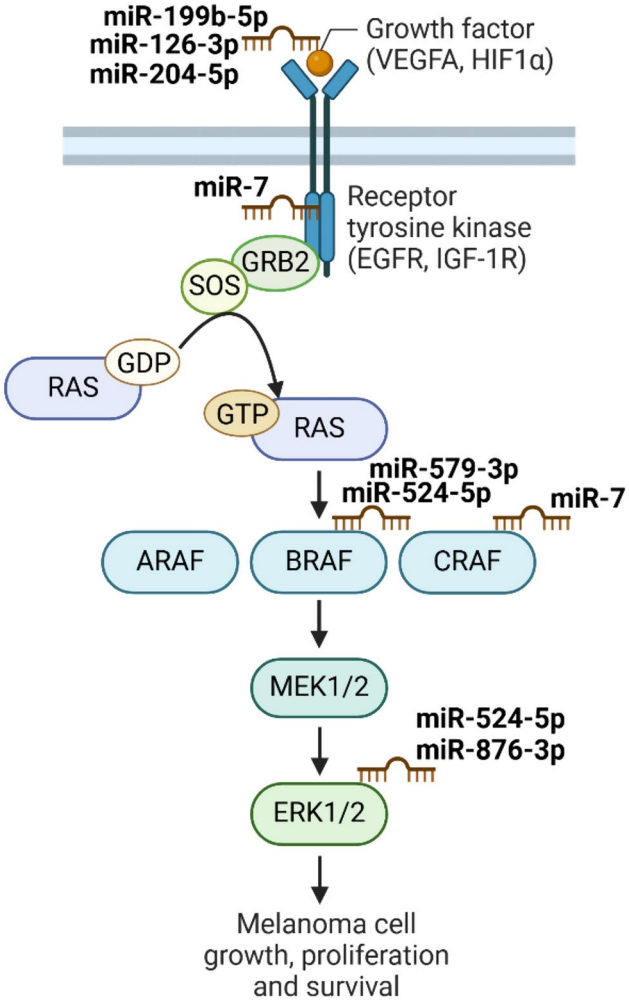

Fig. 3 miRNAs modulating components of the MAPK pathway in melanoma. Several miRNAs have been shown to regulate the MAPK pathway in melanoma by binding to the 3′ or 5′ UTR of various MAPK pathway components. These include miR-7 (targets *EGFR*, *IGF-1R* and *CRAF* [122]), miR-199b-5p (targets *VEGFA* and *HIF1α* [107]), miR-204-5p (targets *VEGFA* [182]), miR-126-3p (targets *VEGFA* and *BRAF* [182]), miR-579-3p (targets *BRAF* [123]), miR-524-5p (targets *BRAF* and *ERK* [183]), and miR-876-3p (targets *ERK2* [184])

combining miR-579-3p mimics with existing targeted treatments could be beneficial. miRNAs can also modulate anti-tumour activity during immune checkpoint inhibitor therapy. For example, miR-155 promotes the activity of effector $CD8^+$ T cells and its deficiency in T cells can dampen T cell activation by inhibiting IFNγ production; miR-155 upregulation in peripheral blood correlates with increased $CD8^+$ T cell infiltration and improved tumour control [124], whilst miR-155 deficiency enhanced tumour growth in a B16F10 melanoma mouse model [125]. This enhanced tumour growth, however, can be attenuated by the administration of immune checkpoint inhibitors (anti-PD-1, anti-PD-L1 and anti-CTLA-4 combination), suggesting that combining miR-155 overexpression with immune checkpoint blockade may augment anti-tumour responses [125]. miR-146a has also been reported to suppress Signal transducer and activator of transcription 1 (STAT1) signalling and IFNγ production in T cells, and combination of miR-146a inhibition with anti-PD-1 improved survival in a melanoma mouse model compared to anti-PD-1 alone [126].

The expression of miRNAs can be modulated, either by silencing or restoring their levels, to influence melanoma proliferation, progression, and survival. Antisense oligonucleotides, known as antagomiRs or anti-miRs, silence specific miRNAs by preventing their binding to target mRNAs (Fig. 4), and these can have broad functional effects. For example, transfection of melanoma cell lines with a miR-211 antagomiR derepressed insulin like growth factor 2 receptor (*IGF-2R*), nuclear factor of activated T cells 5 (*NFAT5*) and transforming growth factor beta receptor 2 (*TGFBR2*) expression, and increased melanoma cell invasion and migration [127]. In contrast, inhibition of the pro-metastatic miR-182 with antisense oligonucleotides reduced cell viability and suppressed migration and invasion of melanoma cell lines, likely by restoring the expression of miR-182 targets *FOXO3* and *MITF* [128]. In another study, treatment of mice with anti-miR-182 reduced the number and size of melanoma liver metastases compared to control treated mice [129], further supporting miR-182 as a therapeutic target. Inhibition of miRNAs can also be achieved using the CRISPR/Cas system, but like other gene editing technologies, this approach is limited by off-target effects and the need for selective and efficient delivery to target cells (reviewed in [130]).

BlockmiRs are modified oligonucleotides that inhibit miRNA activity by specifically binding to the 3′ UTR of the target mRNA and sterically hindering the miRNA-mRNA interaction. Indeed, miR-27a interaction with *CDH5* (encodes Cadherin 5/VE-Cadherin) is abolished by BlockmiR CD5-2, and combination treatment of CD5-2 with anti-PD-1 significantly reduced tumour burden compared to CD5-2 or anti-PD-1 alone in a liver cancer mouse model [131]. Other inhibitory methods include small molecule inhibitors that interfere with miRNA transcription, miRNA sponges that serve as decoys with multiple binding sites to prevent miRNA-mRNA interactions, and miRNA masking, which uses sequences complementary to the miRNA binding site on the mRNA to form duplexes that abrogate miRNA effects (reviewed in [132], Fig. 4). Interestingly, endogenous RNA molecules that act as miRNA sponges, sequestering miRNAs away from their targets, have been described [133]. For example, *TYRP1* mRNA binds miR-16, and prevents the suppression of miR-16 targets, including *RAB17*, which promotes melanoma proliferation. Masking of miR-16 binding sites on the *TYRP1* mRNA with small antisense

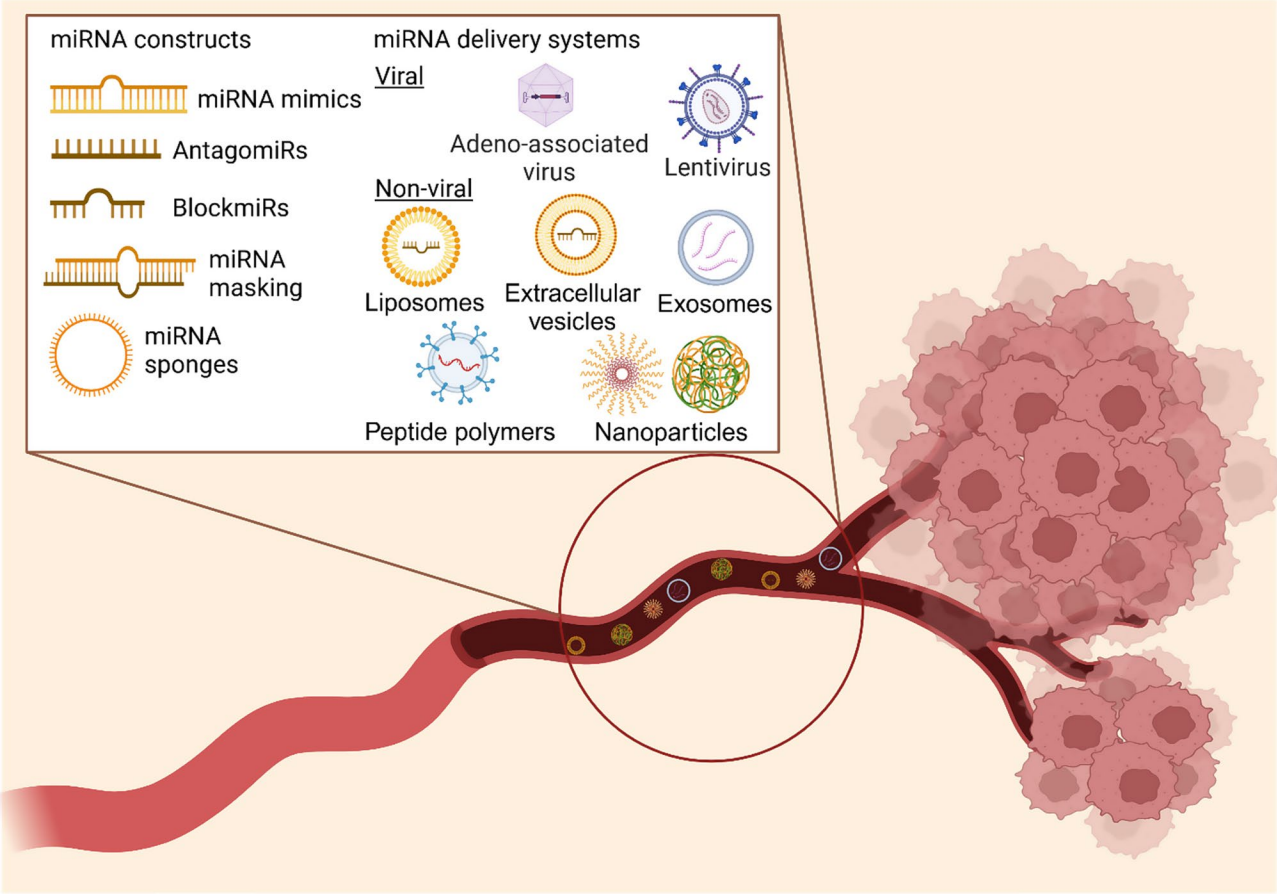

Fig. 4 Strategies and delivery systems for miRNA-based therapies. miRNA constructs can be designed to either inhibit (antagomiRs, BlockmiRs, miRNA masking, miRNA sponges) or overexpress (miRNA mimics) target miRNAs. To maximise stability of miRNAs and minimise off-target effects, miRNA constructs can be packaged within viral or non-viral delivery systems for transport to specific tumour sites

oligonucleotides increased miR-16 activity, decreased *RAB17* mRNA and reduced tumour growth in a xenograft melanoma mouse model [134].

Conversely, miRNA expression and effects on mRNA targets can be restored using double-stranded oligonucleotides referred to as miRNA mimics or mimetics (Fig. 4). For instance, transfection of melanoma cells with a miR-26a mimic decreased cell proliferation by inducing cell cycle arrest, and inhibited cell migration and invasion; transfection of the mouse B16F10 melanoma cells with the miR-26 mimic inhibited tumour growth in vivo [135]. Overall, therapeutic strategies that inhibit or restore miRNA expression have demonstrated efficacy in modulating melanoma growth and progression in preclinical models.

A major challenge in the therapeutic targeting of miRNAs is achieving delivery to precise locations, such as tumour regions and metastatic sites, whilst reducing nonspecific cellular uptake and consequent toxicity. Because miRNAs can be rapidly degraded by nucleases and have poor and nonspecific cellular uptake, several modifications and delivery strategies have been developed and tested in vivo to circumvent these challenges [136]. These approaches must be well tolerated, safe and specific, and designed to minimize off-target effects and can include chemical modifications, organic, inorganic, and polymer-based non-viral delivery methods (e.g. liposomes, nanoparticles, peptide polymers, extracellular vesicles or exosomes) and viral vectors (e.g. lentiviral, retroviral, and adenoviral, Fig. 4). For example, antisense oligonucleotides can be modified with locked nucleic acids (LNAs), RNA nucleotides with a modified ribose moiety, to offer higher thermal stability, resistance to nuclease degradation and higher specificity and affinity [137]. Administration of an LNA-anti-miR-21 reduced miR-21 expression in tumours and inhibited tumour growth in a B16F10 melanoma mouse model [137] and a LNA-modified antisense oligonucleotides (LNA-miR-221 inhibitor) demonstrated excellent safety profile and anti-tumour activity in a Phase I clinical trial [138]. Likewise, the incorporation of mesyl phosphoramidate groups in miRNA-targeting antisense oligonucleotides has been shown to increase oligonucleotide hybridisation, stability and efficacy, when compared to equivalent

phosphorothioate antisense oligonucleotides [139]. In a melanoma mouse model, mesyl phosphoramidate-modified anti-miRs targeting miR-21, miR-17 and miR-155 showed limited toxicity, including minimal dystrophy and necrosis in the liver parenchyma [140]. Moreover, combined targeting of all three miRNAs with the mesyl phosphoramidate modified antisense oligonucleotides was more effective compared to targeting single miRNA [140], demonstrating the improved efficacy and safety of targeting multiple miRNAs.

miRNAs packaged in vector systems have also been tested in melanoma. Polyethylenimine, a cationic polymer, can interact electrostatically with oligonucleotides and can protect small RNA molecules from degradation whilst increasing cellular uptake [141], and polyethylenimine-complexed anti-miR-150 and anti-miR-638 significantly inhibited tumour growth and metastasis in melanoma xenograft mouse models [142]. In addition to silencing miRNA expression with anti-miRs, vector systems can be utilised to deliver miRNAs to restore expression; delivery of lipid nanoparticles encapsulating miR-199-5p and miR-204-5p to xenograft mouse models enhanced BRAF/MEK inhibitor therapy with minimal adverse events [143]. Similarly, delivery of miR-21-3p encapsulated in gold nanoparticles increased tumour-specific cellular uptake and synergised with anti-PD-1 treatment to decrease tumour growth in a melanoma mouse model [144]. Overall, these chemical modifications and delivery systems are advancing miRNA-based therapies towards clinical application, by enhancing their stability and therapeutic efficacy while reducing toxicity and off-target effects.

Despite the improvements in miRNA targeting and delivery, to date, only a few clinical trials have investigated miRNA-based therapeutics in cancer (Table 2). In melanoma-focussed miRNA-based clinical studies, research has centred on evaluating miRNAs for their diagnostic and prognostic potential, although none have yet advanced to routine clinical application. In the therapeutic setting, some notable candidates have emerged, including miR-34 [145], miR-16 [146], miR-155 [147], and miR-10b [148], but among these, only MRX34, a miR-34 mimic encapsulated in liposomal nanoparticles, has been evaluated in a Phase I trial in patients with melanoma, liver cancer, small cell lung cancer, lymphoma, multiple myeloma, and renal cell carcinoma (NCT01829971). Results indicated that three out of 85 patients (3.5%) experienced prolonged partial responses, while 16 patients (19%) had stable disease; however, the trial was terminated due to unexpected immune-mediated adverse events resulting in deaths of four patients [145]. Other miRNA therapeutics have been evaluated in clinical trials for other cancer types and are summarised in Table 2.

Collectively, the clinical trials summarised in Table 2 underscore the low specificity and off-target effects of miRNA-based therapies, which may contribute to the occurrence of severe immune-related adverse events. Of note, many of the therapeutic trials were terminated or withdrawn, and only one (NCT06260774) has progressed to Phase II for patients with confirmed diagnoses of relapsed advanced or locally advanced solid tumours with no available standard therapy. The initial phase 0 data with a single patient receiving an injection of TTX-MC138 (nanoparticles loaded with miR-10b antagomiR) demonstrated uptake to bone, lung and liver metastases and selective retention of the drug in tumour tissue [148]. The Phase II trial is currently recruiting. This limited progress suggests minimal clinical benefit to date, raising concerns about the future viability of miRNA-based therapeutics [149]. Importantly, in the context of melanoma, the goal of miRNA-based therapies is not to replace current effective treatments but to complement them, potentially in combination with BRAF/MEK inhibitors or immune checkpoint inhibitors to enhance drug efficacy, prolong the durability of responses, or overcome resistance to these therapies.

Challenges and recommendations for circulating MiRNA research and analyses

As previously noted, the identification of circulating miRNA biomarkers across various independent studies has shown limited consistency, with conflicting findings regarding their diagnostic, prognostic, and predictive value. Numerous reports underscore the lack of standardisation in both pre-analytical and analytical steps for profiling circulating miRNAs [153–158]. As discussed in these reports, discrepancies likely arise from differences in miRNA detection methods (e.g. RT-qPCR, NanoString, microarray, whole or targeted miRNA sequencing), the quality and type of biospecimens analysed (fresh, archival, whole blood, plasma or serum), and the heterogeneity of the patient and control cohorts, including variations in subtype, stage and treatment history. Moreover, the low abundance of secreted miRNAs, and differences in extraction methods, quality control, normalisation of miRNA data and variable statistical evaluation methods contribute to the limited consistency and hinder the broader implementation of circulating miRNA biomarkers.

To address the lack of standardization, the National Cancer Institute (NCI) recently published a best practice guide for blood collection and processing, specifically for circulating miRNA research [159]. Table 3 summarises key analytical and pre-analytical considerations for miRNA research. It is important to note that this lack of standardisation extends beyond circulating miRNA

Table 3 MiRNA pre-analytical and analytical guidelines

Analytic framework	Guidelines
Processing workflows	Consider blood collection tubes, plasma/serum selection, prompt isolation (within 2 h) of biological samples, consistent and validated processing steps, small aliquot volumes, -80 °C storage and limited freeze/thaws [161, 162].
Biospecimens	Use of serum versus plasma can lead to diversity in identified miRNAs [154, 163].
Quality controls	Exclude blood fractions with haemolysis as this will confound circulating miRNA analysis [157, 164–169]. Haemolysis can be identified by visual inspection or spectrophotometric measurement of oxy-haemoglobin absorbance at 414 nm. Ratio of haemolysis dependent versus stable haemolysis independent miRNAs e.g. (miR-451a/miR-23a-3p) provides an alternative measure of haemolysis [169].
Experimental datasets	Consult miRNA expression databases and portals such as ExomiRHub and exRNA to facilitate biomarker discovery [170, 171]. These sites integrate and curate individual miRNA studies and provide a platform to analyse miRNA transcription in the context of diseases such as cancer e.g. linking to TCGA miRNA expression data.
Discovery workflows	Circulating miRNA signatures vary depending on the chosen measurement platform [55, 172–174]. Some common platforms include: • Targeted miRNA panels (NanoString hybridisation, RT-qPCR) • Whole miRNA NGS profiling (small RNASeq, EdgeSeq or Qiaseq) miRNA annotation should be based on a standardised database such as miRbase [175, 176].
Validation workflows	Validation is typically performed using single target miRNA assays. Use stable miRNAs for normalisation of single target assays and select optimal normalisation genes using algorithms such as Normfinder, geNORM or BestKeeper [177–179]. Other validation methodologies include RT-qPCR, digital droplet PCR (ddPCR) or surface-enhanced Raman scattering (SERS) [180].

research to other circulating biomarkers, including cell-free DNA and proteins [160].

Conclusions

Despite significant advancements in miRNA sequencing technologies and the inherent advantages of miRNAs—such as their stability and low detection costs—research focused on melanoma-specific miRNAs has not translated into clinical impact. To date, only one miRNA-based diagnostic signature (ThyraMIR v2 used in combination with ThyGeNEXT oncogene panel) has been approved to provide risk stratification for thyroid nodules, and only one miRNA-based therapy (MRX34) has progressed to a Phase I clinical trial in melanoma (NCT01829971), and this trial was closed early due to severe immune-mediated adverse events [145]. The intricate regulatory functions of miRNAs remain challenging to fully understand, but they also underpin the significant prognostic and mechanistic roles of miRNAs.

As miRNA research evolves, the unique technical challenges, including selection of robust internal controls and understanding the influence of biological and technical factors, will be resolved. Only then can miRNAs be integrated into multi-omics frameworks that support personalized treatment approaches. Additionally, conducting discovery and validation studies in larger, well-annotated patient cohorts, along with a focus on miRNA panels as diagnostic and prognostic biomarkers, will accelerate the miRNA field. Certainly, in melanoma, miRNAs show the greatest promise as diagnostic and prognostic tools, and continued efforts in clinical trials are essential to assess the specificity of miRNA-based biomarkers. However, miRNA-based therapies face significant hurdles, including the need to mitigate off-target effects and improve therapeutic efficacy, and future research should also explore their synergistic effects with existing treatments, such as BRAF/MEK inhibitors and immune checkpoint inhibitors. A deeper understanding of miRNAs and their interactions will be essential to realise their potential as biomarkers and therapeutic targets, ultimately enhancing the care and outcomes of cancer patients.

Abbreviations

AGO	Argonaute
AUC	Area under curve
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog
CDK	Cyclin-dependent kinases
CRAF	RAF proto-oncogene serine/threonine-protein kinase
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
eIF4F	Eukaryotic initiation factor 4 F
FDA	Food and Drug Administration
GEP	Gene expression profiling
HIF1α	Hypoxia inducible factor 1 subunit alpha
IFNγ	Interferon gamma
IGF1R	Insulin like growth factor 1 receptor
IGF2R	Insulin like growth factor 2 receptor
im-miRNA	Immune-modulatory miRNA
LAG3	Lymphocyte-Activation Gene 3
LNA	Locked Nucleic Acids
m7G	7-methyl-G
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MicroRNA	Micro ribonucleic acid
miRISC	miRNA-induced silencing complex
MITF	Microphthalmia-associated transcription factor
mRNA	Messenger RNA
MSS	Melanoma-specific survival
NFAT5	Nuclear factor of activated T-Cells 5
OS	Overall survival
PD-1	Programmed cell death-1
PD-L1	Programmed cell death ligand 1
PFS	Progression free survival
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
STAT1	Signal transducer and activator of transcription 1

TGFBR2	Transforming growth factor beta receptor 2
UTR	Untranslated region
VEGFA	Vascular endothelial growth factor A

Author contributions
SYL, SB, RJD and HR designed the review, wrote, edited and revised the manuscript. SYL and HR prepared the figures. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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