Investigating the biology of microRNA links to ALDH1A1 reveals candidates for preclinical testing in acute myeloid leukemia

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Abstract. Aldehyde dehydrogenase 1 family member A1 (ALDH1A1) is a member of the aldehyde dehydrogenase gene subfamily that encode enzymes with the ability to oxidize retinaldehyde. It was recently shown that high ALDH1A1 RNA abundance correlates with a poor prognosis in acute myeloid leukemia (AML). AML is a hematopoietic malignancy associated with high morbidity and mortality rates. Although there are a number of agents that inhibit ALDH activity, it would be crucial to develop methodologies for adjustable genetic interference, which would permit interventions on several oncogenic pathways in parallel. Intervention in multiple oncogenic pathways is theoretically possible with microRNAs (miRNAs or miRs), a class of small non-coding RNAs that have emerged as key regulators of gene expression in AML. A number of miRNAs have shown the ability to interfere with ALDH1A1 gene expression directly in solid tumor cells, and these miRNAs can be evaluated in AML model systems. There are indications that a few of these miRNAs actually do have an association with AML disease course, rendering them a promising target for genetic intervention in AML cells.

Contents

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1. Introduction

Aldehyde dehydrogenases (ALDHs) comprise a family of 19 NAD(P+)-dependent enzymes that metabolize endogenously and exogenously produced aldehydes, by irreversibly catalyzing their oxidation to their respective carboxylic acids (1,2). ALDHs have a broad spectrum of biological activities, including but not limited to biosynthesis of retinoic acid and alcohol metabolism.

ALDHs are expressed in stem cells in general; ALDHs to some extent are also expressed in cancer cells that resemble normal stem cells in terms of cell cycle dormancy and metabolic adjustments for decreased generation of reactive oxygen species (3). These cancer cells with slower proliferation most often have tumor-initiating properties and tend to be resistant to chemotherapy and cytotoxic agents. Their capacity to withstand oxidative stress is limited, but it is noteworthy that they may give rise to aggressive cancer cell clones with a high pace of proliferation and growth (4-5). Some of these effects are often attributed to polyploid cancer cells (6), or to cancer cells that have been fused to macrophages or other non-tumor cells (7,8); however the cancer 'stem-like' cells are not exclusively polyploid. Furthermore, the degree of metabolic dormancy and the precise phase of cell cycle arrest vary, as well as the readiness to re-enter the cell cycle (9). The shared aspect among these cell phenotypes is that they all exhibit an obligatory state of arrested tumor growth, which confers cancer resistance to adverse conditions.

Quiescent cells are in a metabolic state that generates a lower level of oxidative stress, thus resulting in decreased expression of enzymes that protect from reactive oxygen species. However, the cancer stem-like cells have alterations in chromatin in key genes that encode enzymes operating as components of essential antioxidant systems. These chromatin alterations allow them to express those genes rapidly

and therefore adapt and survive acute exposure to oxidant stress (5,10). During chemotherapy or inflammation, in the critical phase of cytotoxic exposure that causes cell stress and growth suppression, ALDH enzymes may protect cancer stem cells (CSCs), before favorable conditions and appropriate stimuli permit the generation of daughter clones with different phenotypes. A key member of the ALDH family that possesses properties that are well-suited to its central role in the initial cellular recovery, occurring prior to the acute expression of key rapid-response genes, is aldehyde dehydrogenase 1 family member A1 (ALDH1A1) (11).

ALDH1A1 is a member of the aldehyde dehydrogenase gene subfamily that encodes enzymes with the ability to oxidize retinaldehyde, owing to a larger substrate entry channel (1,12). The protein ALDH1A1, which localizes to the cytosol and the nucleus, is overexpressed in a number of diverse cancer types; however it is not consistently associated with a negative disease prognosis: ALDH1A1 plays the role of a tumor suppressor under certain conditions that can be attributed to the maintenance of an optimal intracellular milieu. Its precise funtion in normal stem cells, such as hematopoietic stem cells (HSCs), is indicated by research findings that suggest a degree of redundancy between certain similar ALDH enzymes (13,14). Nevertheless, ALDH1A1 is an enzyme with critical functions in CSCs (2). In contrast to normal HSCs, in some leukemia cells ALDH1A1 may posses non-redundant functions.

Acute myeloid leukemia (AML), is a hematopoietic malignancy associated with high morbidity and mortality rates (15). Understanding the molecular mechanisms underlying AML is crucial for developing effective therapies. The expression of ALDH1A1 specifically protects leukemia-initiating cells (LSCs) from a number of antineoplastic agents; i) protection from cyclophosphamide by ALDH1A1 gene transfer in cultured cells (16), and ii) conversely, ALDH1A1 gene knockout sensitizes LSCs to cyclophosphamide (17), while the enzymatic activity of ALDH in AML blast cells, has been proven to be essential for the establishment of human AML xenografts in mice (18-20). ALDH(+) cells from samples of patients with AML with ≥1.9% ALDH(+) cells were quiescent, refractory to cytarabine treatment, and capable of leukemic engraftment in a xenogenic mouse transplantation model (21).

Conversely, AML cells null for ALDH1A1 RNA expression were obtained from patients with a favorable prognosis, and were sensitive to chemotherapeutic agents (22).

It is important to emphasize that even after generation of a multi-omic profile of samples of patients with AML, the integrated classification continues to categorize ALDH1A1-overexpressing samples to the worst AML prognosis group: This analysis indicates the significant impact of ALDH1A1-expressing AML cells in an unfavorable disease course (23).

2. Biological links between AML, miRNAs and ALDH1A1

It was recently shown that ALDH1A1 RNA abundance is correlated with the outcome of AML; especially when compared to the other members of the ALDH family, ALDH1A1 had the greatest statistical capacity to differentiate between patients with AML with a favorable and an unfavorable prognosis (24,25). A number of agents are known to inhibit ALDH activity, with at least one, DIMATE, demonstrating the ability to selectively kill LSCs while leaving normal HSCs intact (26). However, there is always room for improvement, especially in respect to the development of methodologies for genetic interference.

The need to develop alternatives arises from the plasticity of leukemia cell populations, that allows the emergence of altered phenotypes. This is due to the capacity of leukemia stem-like cells to undergo phenotype changes in response to the metabolite content of their microenvironment, and most notably, in response to changes in the oxidative state (4,27).

Over a decade ago, miRNAs, a class of noncoding RNAs, emerged as key regulators of gene expression in AML, making them one of several potential avenues for genetic intervention in AML cells (28). Especially relevant in AML biology, are the mutual interactions between miRNAs, including miR-146 for example, with NF-κB, a transcription factor that regulates a substantial proportion of inflammatory genes and miRNAs involved in malignant progression (29-31). In addition, recent data suggest a strong association of miRNA expression with macrophage polarization, which regulates immune responses against AML (32). The list of miRNAs and their mRNA targets that are relevant in AML disease progression continues to grow, rendering therapeutic manipulation of miRNAs an increasingly relevant aim, especially in light of interesting preclinical data that emerge from a recent study (33).

As examined in the present review, the interactions between miRNAs and ALDH1A1 can be complex and not ubiquitous between different cell phenotypes. In other words, the mutual effects between a given miRNA and ALDH1A1 can be enhancing or suppressing, but in different cells this may change. Furthermore, it cannot be excluded that ALDH1A1 induces the expression of a given miRNA, which then acts as a negative feedback trigger and leads to repression of ALDH1A1. For this reason, in the present review, the miRNAs that have exhibited potential to act directly on ALDH1A1 expression are focused on, since this type of interaction can be expected to have the least variability.

3. miRNAs that may be included in the list of miRNAs with the potential to target ALDH1A1

There is a substantial number of miRNAs that may target the gene ALDH1A1. A few of them have already been recognized as tumor suppressors in AML, making their preclinical assay the next step forward in elucidating their application potential. As is reviewed next, experiments on cultured cells provide direct evidence, while bioinformatic analysis also suggests that there are numerous miRNAs that interfere with ALDH1A1 expression.

A number of the prospective ALDH1A1-interacting miRNAs have been identified via high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP), by photoactivatable ribonucleoside-enhanced CLIP [PAR-CLIP], and similar methods, aimed to determine the Argonaut: miRNA binding sites in the transcriptome, as a means for localizing the RNA bound by each relevant species, since Argonaute proteins use small RNA guides to identify complementary sites in transcripts targeted for silencing or repression (34).

As will be discussed further, both activating as well as repressing miRNAs have been identified. For some miRNAs, experimental evidence has directly demonstrated their capacity to interfere with ALDH1A1 expression negatively, making them thereby strong candidates for further research.

In vitro assays of miRNAs interfering with ALDH1A1 expression. In leukemia study models, there are no published studies that examine direct interference of miRNAs with ALDH1A1 gene expression. However, there are a few studies on solid tumor model systems that describe ALDH1A1 RNA-interacting miRNAs.

The human papillomavirus HPV16 caused an increase both in ALDH1A1 mRNA as well as ALDH1 enzymatic activity in oropharyngeal squamous cell carcinoma cells, which was mediated by repressing miR-181a/d, two miRNAs, that otherwise suppressed anchorage independent growth and CSC phenotype (35). However, in AML research, miR-181a has shown both favorable as well as unfavorable prognostic associations and molecular mechanistic effects, rendering this miRNA a challenging candidate for developing ALDH1A1 inhibitors for AML (36-40). One potential use for miR-181a, based on both favorable and unfavorable associations, is the trigger of cell proliferation to render AML cells sensitive to both pharmacological, as well as immunological intervention. Preclinical studies have shown encouraging results, making miR-181a, a candidate for context-dependent development of interventions (40).

In gastric cancer cells, miR-625 reversed multidrug resistance by repressing ALDH1A1; miR-625 silencing increased the IC_{50} values of four chemotherapeutic agents (ADR, VCR, 5FU and CDDP). Depletion of ALDH1A1 by siRNA reversed those effects (41). In AML, miR-625 has shown the potential to suppress metastatic and proliferative functions (42), cell viability (43,44) and invasiveness (45). miR-625 is therefore a noteworthy candidate for repression of ALDH1A1 in AML.

In breast cancer, it was revealed that miR-140 was significantly downregulated in stem-like cells from ductal carcinoma *in situ* tumor cells in comparison to normal mammary stem cells. miR-140 directly targeted the 3' untranslated region of ALDH1A1, to inhibit protein expression (46). miR-140 has shown the ability to function as a tumor suppressor in AML study models (47,48), and a previous study demonstrated the same effect specifically for miR-140-3p (49). miR-140 is therefore a plausible candidate for inhibition of ALDH1A1 in AML model systems, where it can be examined to verify whether it functions via the same mechanism as that in breast cancer cells. To underscore the importance of the evidence provided for miR-140 regulation of ALDH1A1, the widely recognized curated miRNA platform, miRTarBase, only selected miR-140 as a candidate regulator for ALDH1A1 (50,51). In addition, the database, mirtargetlink 2.0, confirmed this assessment (miR-140-5p, as supported by the experimental evidence), with the additional listing of miR-181a-5p as weakly supported, due to the lack of experimental evidence (52) (Fig. 1).

In cervical CSCs derived from tumorspheres of the cell lines, Hela and CaSki, miR-23b reduced ALDH1A1 protein levels, by specifically binding to the 3'UTR of ALDH1A1 mRNA. Overexpression of miR-23b substantially reduced the size and number of tumorspheres, and rendered cells sensitive to cisplatin (53). miR-23b appears to decreased in AML (54). Re-expression in leukemia cells can increase oxidative stress, by repressing peroxiredoxin III (55). However, miR-23b has been correlated with the Warburg effect and with a poor prognosis, making its utility in AML uncertain (56).

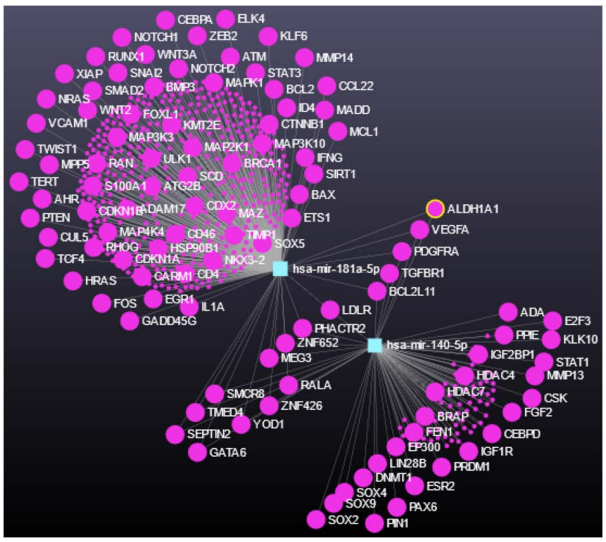

Figure 1. Prospective gene target network for miR-140-5p and miR-181a-5p in human bone marrow with data obtained from miRTarBase v.9 (https://dianalab.e-ce.uth.gr/tarbasev9), constructed using miRNet 2.0 (https://www.mirnet.ca/).

It can therefore be concluded that from the miRNAs that regulate ALDH1A1 in solid tumor study systems, miR-181, miR-625, miR-140, and even miR-23b can be further investigated to determine their effects on ALDH1A1 in AML. These investigations however, must employ a rigorous approach in respect to the precise time course, dose response, and dynamic distribution in model systems that resemble human tissue as close as possible, to address the key issue of context-dependent effects that is pervasive in miRNA biology, and which is also evident especially in the case of miR-23b as aforementioned.

miRNAs predicted to regulate ALDH1A1 expression by bioinfomatic analysis platforms. There are a number of miRNAs predicted to target ALDH1A1 as revealed using the miRNA database, TarBase (57), accessed through miRNet2.0 (58). These are summarized in Table I. A similar result was obtained by directly using the database Tarbase (Table II).

miR-16 has been revealed to be typically downregulated in leukemia, an event which contributes to the uncontrolled growth and survival of leukemic cells (59,60). It has been shown to be increased in patients with AML in remission (61). In murine myeloid cells expressing internal tandem duplications of the juxtamembrane region of the gene FLT3 (FLT3/ITD) that constitutes a marker for poor prognosis for AML, miR-16 was significantly down-regulated; and conversely, it was upregulated upon FLT3 inhibition (62). Its reduced expression was revealed to be associated with the dysregulation of several target genes involved in cell cycle control and apoptosis (63). miR-16 was demonstrated to target multiple oncogenes and regulators of apoptosis, such as BCL2 (an anti-apoptotic protein) and cyclins (involved in cell cycle progression) (60).

Table I. List of microRNAs predicted to interfere with ALDH1A1 gene expression.

miRNet	miR_id	miR_acc	Experiment	PMID or database
mirnet-hsa-29920	hsa-mir-181a-5p	MIMAT0000256	qRT-PCR	26693182
mirnet-hsa-44218	hsa-mir-140-5p	MIMAT0000431	Luc/Wblot	23752191
mirnet-hsa-647281	hsa-mir-185-5p	MIMAT0000455	HITS-CLIP	TarBase
mirnet-hsa-647282	hsa-mir-200c-3p	MIMAT0000617	PAR-CLIP	TarBase
mirnet-hsa-647283	hsa-mir-21-5p	MIMAT0000076	PAR-CLIP	TarBase
mirnet-hsa-647284	hsa-mir-221-3p	MIMAT0000278	HITS-CLIP	TarBase
mirnet-hsa-647285	hsa-mir-221-5p	MIMAT0004568	HITS-CLIP	TarBase
mirnet-hsa-647286	hsa-mir-222-3p	MIMAT0000279	HITS-CLIP	TarBase
mirnet-hsa-647287	hsa-mir-222-5p	MIMAT0004569	HITS-CLIP	TarBase
mirnet-hsa-647288	hsa-mir-22-5p	MIMAT0004495	HITS-CLIP	TarBase
mirnet-hsa-647289	hsa-mir-362-3p	MIMAT0004683	HITS-CLIP	TarBase
mirnet-hsa-647290	hsa-mir-374a-3p	MIMAT0004688	HITS-CLIP	TarBase
mirnet-hsa-647291	hsa-mir-4517	MIMAT0019054	PAR-CLIP	TarBase
mirnet-hsa-647292	hsa-let-7b-5p	MIMAT0000063	Microarrays	TarBase
mirnet-hsa-647293	hsa-mir-103a-3p	MIMAT0000101	Microarrays	TarBase
mirnet-hsa-647294	hsa-mir-107	MIMAT0000104	Microarrays	TarBase
mirnet-hsa-647295	hsa-mir-16-5p	MIMAT0000069	Microarrays	TarBase
mirnet-hsa-647296	hsa-mir-191-5p	MIMAT0000440	Microarrays	TarBase
mirnet-hsa-647297	hsa-mir-195-5p	MIMAT0000461	Microarrays	TarBase
mirnet-hsa-647298	hsa-mir-21-3p	MIMAT0004494	Microarrays	TarBase
mirnet-hsa-647299	hsa-mir-210-3p	MIMAT0000267	Microarrays	TarBase
mirnet-hsa-647300	hsa-mir-26a-5p	MIMAT0000082	Microarrays	TarBase
mirnet-hsa-647301	hsa-mir-27a-5p	MIMAT0004501	Microarrays	TarBase
mirnet-hsa-647302	hsa-mir-30d-5p	MIMAT0000245	Microarrays	TarBase
mirnet-hsa-647303	hsa-mir-34b-5p	MIMAT0000685	Microarrays	TarBase
mirnet-hsa-647304	hsa-mir-34c-5p	MIMAT0000686	Microarrays	TarBase
mirnet-hsa-647305	hsa-mir-941	MIMAT0004984	Microarrays	TarBase

Accessed using miRNET (https://www.mirnet.ca/) April 6, 2024. Luc, luciferase; Wblot, western blotting; 3p, 3 prime; 5p, 5 prime.

By targeting these genes, miR-16 inhibited cell proliferation and promoted programmed cell death. Thus, miR-16 may be a prospective candidate for ALDH1A1 inhibition in AML model systems, due to the established anti-leukemic effects of this miRNA.

Another miRNA, miR-34a has been revealed to be associated with prognosis in AML (64), and experiments on epithelial cancer cells indicate that miR-34a has the potential to repress ALDH1A1, without findings revealing whether repression is direct or indirect (65-67). Research is required to elucidate the mechanism of miR-34a interference with ALDH1A1 gene expression, and specifically whether or not miR-34a can directly target the 3' untranslated region of ALDH1A1 in AML cells.

For the miR-30 family, members 30a, 30b and 30c, were repressed in AML bone marrow samples, while miR-30d was found underexpressed in serum samples of patients with chronic lymphocytic leukemia, but an association with AML has yet to be shown (68,69). However, in oral squamous cell carcinoma specimens, miR-30a was shown to promote expression of ALDH1 member ALDH1A2 (70), making miR-30a an unlikely candidate for development as an ALDH1A1 inhibitor.

Lastly, miR-200c has exhibited the potential to regulate ALDH1A1 expression (71,72) even if this effect can be indirectly linked to miR-200c. This miRNA, has shown relevance in blocking oncogenic signaling in AML; in particular, miR-200c repression was identified as a key molecular mechanism of oncogene MUC1 induction of PD-L1 expression, which has a critical function in the progression of AML (73). miR-200c, therefore is a noteworthy candidate to assess its potential as an ALDH1A1 inhibitor in AML model systems.

miRNAs with a potential to regulate ALDH1A1 expression. Integrating miRNA and mRNA expression profiling in AML revealed that miR-155 has a critical association with immunity (74). miR-155 was revealed to suppress ALDH1A1 in a solid tumor model. In a metastatic cell line model that allows investigation of extravasation and colonization of circulating cancer cells to lungs of mice, miR-155 overexpression in tumors suppressed ALDH1A1, PIR and PDCD4 (75). However, in AML, miR-155 has an association with poor disease outcome; in cytogenetically normal patients, overexpression of miR-155 was associated with a shorter disease-free and overall survival (76). miR-155 was also revealed to be associated

Table II. List of microRNA species that interfere with ALDH1A1 expression.

miRNA_name	miRNA_id	Experiments	Publications	Cell_lines	micro_tscore
hsa-miR-103a-3p	MIMAT0000101	2	2	2	-0.27
hsa-miR-15a-5p	MIMAT0000068	2	2	1	0.37
hsa-miR-16-5p	MIMAT0000069	2	2	2	0.46
hsa-miR-210-3p	MIMAT0000267	2	1	2	0.42
hsa-miR-26b-5p	MIMAT0000083	2	2	2	0.62
hsa-miR-34a-5p	MIMAT0000255	2	2	2	-1
hsa-let-7g-3p	MIMAT0004584	1	1	1	-1
hsa-miR-101-3p	MIMAT0000099	1	1	1	-1
hsa-miR-107	MIMAT0000104	1	1	1	-1
hsa-miR-1271-3p	MIMAT0022712	1	1	1	-1
hsa-miR-130a-3p	MIMAT0000425	1	1	1	-1
hsa-miR-130b-3p	MIMAT0000691	1	1	1	-1
hsa-miR-195-5p	MIMAT0000461	1	1	1	-1
hsa-miR-199a-3p	MIMAT0000232	1	1	1	-1
hsa-miR-199b-3p	MIMAT0004563	1	1	1	-1
hsa-miR-21-5p	MIMAT0000076	1	1	1	0.74
hsa-miR-221-5p	MIMAT0004568	1	1	1	0.41
hsa-miR-29b-3p	MIMAT0000100	1	1	1	-1
hsa-miR-301a-3p	MIMAT0000688	1	1	1	-1
hsa-miR-301b-3p	MIMAT0004958	1	1	1	-1
hsa-miR-30d-5p	MIMAT0000245	1	1	1	-1
hsa-miR-454-3p	MIMAT0003885	1	1	1	-1
hsa-miR-542-5p	MIMAT0003340	1	1	1	0.38
hsa-miR-941	MIMAT0004984	1	1	1	-1

Data obtained from platform TarBase v.9 (https://dianalab.e-ce.uth.gr/tarbasev9) (accessed April 6, 2024).

with a 'core enriched' stem cell gene expression score; other miRNAs that were associated with this score were miRNAs known to be highly expressed and functionally relevant in embryonic (miR-20a) (77) or HSCs (miR-99, miR-125a/b and miR-126) (78). For some miRs in that signature (the 'core enriched' stem cell gene expression score), there are functional studies showing that their overexpression causes leukemia in model systems [miR-92a (79) and miR-125b (80)]. Furthermore, primary AML blast cells harboring the FLT3-ITD mutation had high expression of miR-155; 8-chloro-adenosine killed LSCs and supressed miR-155 without killing normal stem cells (81). Other miRNAs that regulate the maintenance of stemness of primitive hematopoietic progenitor cells, include miR-22 and miR-29 (82).

A notable observation was made with another miRNA, namely miR-143; overexpression of a miR-143-3p mimic repressed viability and proliferation of AML cells and overexpression of lysine acetyltransferase 6A (KAT6A) partially reversed the inhibitory effects of the miR-143-3p mimic on viability and proliferation of AML cells. A miR-143-3p mimic decreased the expression levels of IL-1β, TNF-α and IL-6, and increased the expression levels of TGF-β and IL-10 (83). The induction of TGF-β and IL-10 may be potentially detrimental in AML, if these two cytokines are secreted by AML cells in the microenviroment, since they can have a negative effect on the antitumor immune response by inhibiting the function of T cells (84). Nevertheless their effects require extensive characterization in more clinically-relevant research models (85,86).

It is extremely important to emphasize that the role of individual miRNAs is highly context-dependent; overexpression of miR-125b in osteoblasts, for example, leads to increased bone mass and strength, while preserving bone formation and quality (87). Thus, it is crucial to determine the characteristics of any given miRNA before selecting it for intervention. Furthermore, any such intervention can be expected to have complex pathological consequences, which necessitates a precise understanding of the effects of any given miRNA.

Impact of the ALDH1A1-targeting miRNAs on the cellular phenotype. As aforementioned, the interaction between miRNAs and ALDH1A1 may not have ubiquitous effects for all cell types, due to the complexity of their interacting pathways. In this context, it is not yet known whether the interactions between the aforementioned miRNAs and ALDH1A1 occur in all cell types, and especially in AML cells. However, there are also indications that miRNA-mediated control of ALDH1A1 levels in cells may function as part of a general adaptation mechanism and should be further investigated. For example, the expression of most of the miRNAs aforementioned has been revealed to be regulated by TGF-β, and it was shown that

they are involved in the process of epithelial-mesenchymal transition (EMT); in particular, miR-140 suppressed the TGF-β pathway in mouse embryonic fibroblasts and conversely, TGF-β suppressed the accumulation of miR-140 forming a double negative feedback loop (88). EMT is a phenotype adaptation that is triggered in cells to adjust to new conditions, which is not limited for epithelial cells as the name suggests, but it is also used by various types of acute leukemia cells (89). In this sense, after being stimulated by various factors in the local microenvironment, including TGF-β, transcriptional reprogramming is activated to transform cells into a mesenchymal phenotype (90). As regards CSCs, numerous studies have reported that cells undergoing EMT exhibit CSC or CSC-like properties (91,92) under the influence of TGF-β (93). On the other hand, core pathways operating in CSCs may also induce EMT. For example, ALDH1A1 and ALDH1A3 may induce TGF-β expression by activating retinoic acid receptor, RARA, and androgen receptor in prostate cancer (94). In concordance, retinoic acid was shown to increase TGF-β2 expression and secretion of active and latent forms of TGF-β2 in pancreatic cancer cells (95).

Prospects of targeting miRNAs that regulate ALDH1A1 expression. Although the field of RNA therapeutics has made substantial progress over the last decade, there are currently only a few miRNAs that are clinically targeted in intervention studies (96), due to the observation of off-target effects and toxicity (97). This is to be expected given the complex manner of miRNA function.

Of the miRNAs reviewed herein, only two are currently targets of intervention in clinical trials, namely miR-29 and miR-155.

Cobomarsen (MRG-106) is a miR-155 inhibitor developed by Viridian Therapeutics, and has demonstrated efficacy in the treatment of cutaneous T-cell lymphoma (98).

In addition, Remlarsen and MRG-229 also developed by the same manufacturer, are miR-29 mimics. Remlarsen repressed collagen expression and the development of fibroplasia in incisional skin wounds (99). MRG-229, developed for idiopathic pulmonary fibrosis, reduces experimentally induced fibrotic activity in both *in vitro* and *ex vivo* (lung slices derived from donors without a history of lung disease) human disease models in non-human primates, and was reportedly well tolerated at clinically relevant doses with no adverse findings observed (100).

By contrast, numerous studies focus on developing miRNA-based biomarker applications, such as the study NCT05809050, 'Study of miRNA-155 in Acute Leukemia'.

4. miRNAs that can be studied further in model systems for AML preclinical drug development, based on database output

In conclusion, in AML research, ALDH1A1 repression by miRNAs is a rather under-studied topic. From the miRNAs identified through bioinformatic analysis, it is suggested that miR-16 and possibly also miR-200, are potential candidates for further analyses. To underscore this assessment, miR-16-5p was implicated by miRNet in ALDH1A1 regulation in chronic myeloid leukemia (101). Another potential incentive for considering miR-16 development, is the rather acceptable safety profile observed in a phase 1 clinical trial for patients with recurrent malignant pleural mesothelioma. The approach undertaken was to use 'bacterial minicells', which were anucleate nanoparticles produced by inactivating the genes that control normal bacterial cell division, allowing efficient packaging of cytotoxic drugs and internalization into cancer cells (102). In acute lymphoblastic leukemia (ALL), a distinct type of leukemia from AML, miR-16-5p was proposed to enhance sensitivity to the p53-Mdm2 inhibitor, RG7388, which was evaluated in a clinical trial (NCT04029688) (103), making an application of miR-16-5p in ALL at least theoretically conceivable. To support this additional prospective application research, when examining RNA samples from pediatric patients with either AML or ALL using the program 'Therapeutically Applicable Research to Generate Effective Treatments (TARGET)' (https://www.cancer.gov/ccg/research/genome-sequencing/target/about) (104,105), it becomes apparent that high ALDH1A1 RNA expression is associated with a decreased patient survival in both types of acute leukemia (Fig. 2). In general however, caution should be exercised when translating the data for the miR-16-5p regulation of ALDH1A1, for the development of prospective preclinical treatment schemes, either for AML or for ALL.

Nevertheless, the miRNAs identified as direct regulators of ALDH1A1 in solid tumor cell studies, namely miR-181a/b, miR-625, miR-140 and miR-23b, can be studied in preclinical AML model systems with an anticipated beneficial outcome. This assumption is based on the implication of their repression in mechanisms of leukemia progression, which suggests that their exogenous re-introduction could inhibit at least a portion of the leukemic clones, prompting the question of whether these clones comprise cells expressing high levels of ALDH1A1 RNA.

The available miRNA database information and the existing experimental evidence render it possible to implement a strategy for the development of candidate inhibitors of ALDH1A1 expression (Fig. 3), taking into account the impact of the miRNA candidates on the metabolic status of the cells, where the inhibition is aimed to take place.

Although several miRNAs, especially miR-181, have been identified as prospective candidates for the development of AML therapy, in clinical trials, miRNAs are mostly evaluated as prospective biomarkers (40).

Prospective delivery methods and study systems for targeting miRNAs that regulate ALDH1A1 expression. miRNAs can be delivered to the bone marrow through a number of methodological developments that include exosomal delivery, activated hydrogel, cell-specific ligand-decorated nanocarriers, and encapsulating co-polymers (60,106-109). The advances that have been made during the last 10 years in RNA therapeutic applications, and in particular in small interfering RNAs, can help accelerate progress of research in miRNA delivery (96). Strategies explored in miRNA delivery research include lipid-based nanoparticles, polymeric vectors, dendrimer-based vectors, cell-derived membrane vesicles, three-dimensional scaffolds, as well as biodegradable and biocompatible nanoparticles derived from polymers and metals (110). Antagonists of miRNAs may be clinically

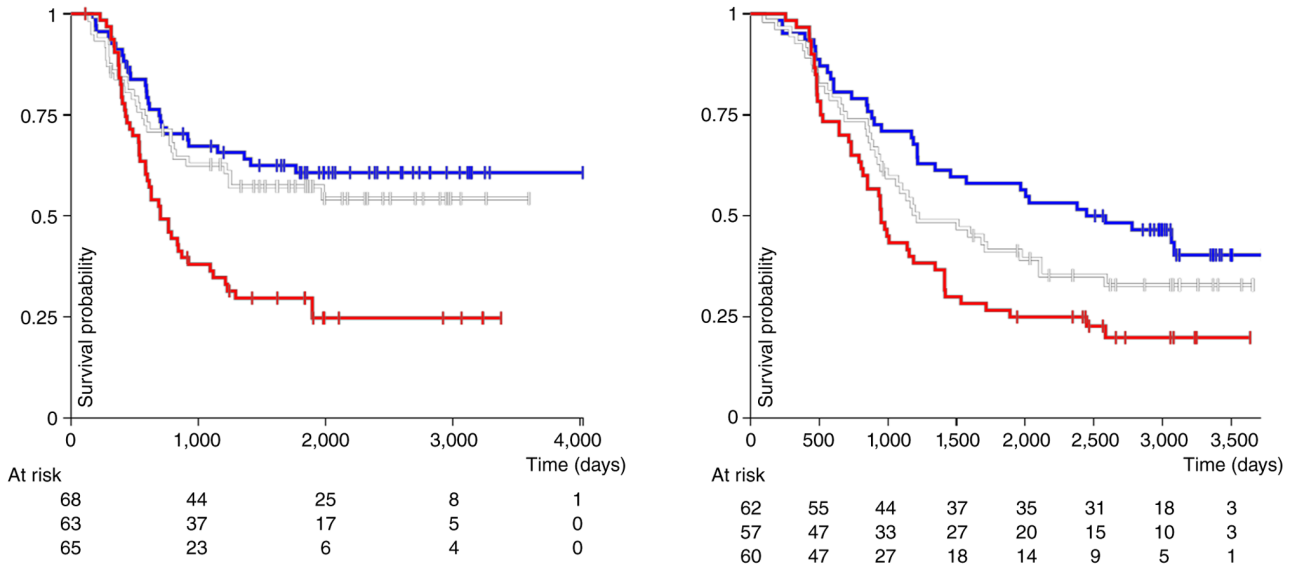

Figure 2. Analysis of the association of RNA expression from the gene ALDH1A1 with overall survival of pediatric patients with either acute myeloid leukemia (left panel, P=0.00014; log-rank test=17.68) or acute lymphoblastic leukemia (right panel, P=0.005; log-rank test=10.25), performed using the online software platform UCSC Xena (https://xenabrowser.net/, accessed on March 10, 2024) (123). The x-axis corresponds to the time passed in days, and the y-axis corresponds to patient overall survival. Units used are $\log_2$ (normalized counts +1). The red line corresponds to samples with the highest expression (>8.12, n=65 for AML; and >8.7, n=60 for ALL). The white line shows samples with intermediate ALDH1A1 mRNA expression (>4.61 and <8.12, n=63 for AML; >6.0 and <8.7, n=57 for ALL). Shown by the blue line are samples with the lowest ALDH1A1 RNA expression (<4.61, n=68 for AML; and <6.0, n=62 for ALL). The results published in this figure are in whole based upon data generated by the study 'Therapeutically Applicable Research to Generate Effective Treatments' (https://www.cancer.gov/ccg/research/genome-sequencing/target) initiative, phs000218. The data used for this analysis are available at the Genomic Data Commons (https://portal.gdc.cancer.gov).

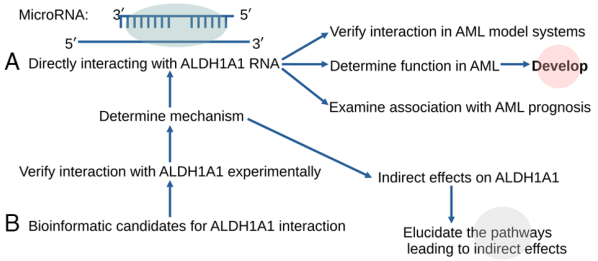

Figure 3. Flow diagram of the strategy for developing applications based on miRNAs that have been identified as potential regulators of ALDH1A1 expression.

evaluated using antisence oligonucleotides, an approach that currently appears most feasible (111,112).

Recently, a novel approach that was based on programmable editing of primary miRNA, switched stem cell differentiation and improved tissue regeneration, promoting *in vitro* cartilage formation and calvarial bone healing in rats (113). The bone, and especially the bone marrow, are targets for potential anti-osteoporosis treatments in experimental research (114). Furthermore, treatments for bone metastasis for solid tumors may affect not only tumor cells but also the balance between osteoclasts and osteoblasts, and thereby modulate the properties of the bone as a niche (115-117). While the development of such applications is not directly related to AML, it is a field that may provide effective methods for delivery of miRNAs into the bone marrow for treatment of AML, also including *ex vivo* manipulation of selected marrow cell types that can be used as vehicles with anti-leukemia activity. Another significant development to anticipate are bone marrow organoids, which can help bridge *in vitro* research and clinical applications, while limiting the use of animal models (118). The organoids can help with the accurate selection of the cell types that are targeted with the experimental miRNA-based intervention, enabling improved assessment of the outcomes in a cell-specific manner.

Although miRNAs are intensively studied, the complexity of their regulation has limited their clinical application mostly to a biomarker-oriented field. However, there are a few studies that continue to explore interventions based on direct regulation of miRNA function (96-99). In this sense, it is urgent to overcome two fundamental problems that may be encountered in miRNA-based therapy. The first is the development of a treatment strategy that targets only specific types of cells and tissues. Since miRNA target all cells in a systemic application using miRNA mimics without a specific tropism, side effects are inevitable. Therefore, the design of target-selective constructs (such as a modified viral vector) that will express a specific miRNA based on genetic engineering appears to be a more relevant approach (119). In such a case, using a promoter of a gene that has limited expression only in target cells (or tissues) and placing the miRNA in the construct under the control of this promoter may provide possible success in terms of ensuring expression only in the intended target cells. The second issue that may be encountered in miRNA therapy is the off-target effects caused by miRNAs generally targeting more than one mRNA. In fact, overcoming the off-target effects is challenging in the native miRNA-involving applications when compared with the synthetic modified versions of miRNAs. Although there have been attempts to increase the selectivity and specificity of experimental interventions, significant

progress is still required in order to develop approaches that permit a rigorous selection of target genes for the artificial miRNA constructs (120-122).

5. Conclusions

Despite extensive research on miRNAs, the intricacy of their regulation has limited their clinical application mostly to a biomarker-focused field. However, there are a few studies that continue to explore interventions based on miRNA regulation. Due to the certainty of off-target effects, it is imperative to accurately ascertain the clusters of candidate target genes in relevant model systems. In the case of ALDH1A1, while there are miRNAs, such as miR-155 with varying effects in different systems, there are other miRNAs that may qualify for preclinical development of interventions, such as miR-181.

It is enticing to consider including miRNA-targeted interventions in standard or experimental AML treatments. To combine two novel approaches is extremely risky from the point of view of drug development, but may be fruitful as an experimental approach for the aim of enriching our understanding of AML biology. The next more immediate step in drug development would be to consider combining miRNA-targeted interventions with standard AML treatments. Although combination of miRNA-based approaches with approved anti-neoplastic agents is an appealing aim, at this stage the main challenge that needs to be overcome before moving forward, is to determine the methodological approach that will permit a greater investment of resources in the field of preclinical development of miRNA-based interventions. The reason for recommending this caution is due to the inherent complexity of miRNA-interacting pathways, which inevitably exert numerous effects. The primary concern is therefore to determine and manage the substantial biomedical impact of a given miRNA, before the drugs that are pharmacologically compatible with that miRNA can be included into a testing protocol.

ALDH1A1 has critical roles in LSC biology and thereby in therapy resistance. miRNAs are directly involved in the regulation of ALDH1A1 in cells. Although miRNAs directly targeting ALDH1A1 have been mostly demonstrated in solid tissues, there is a strong possibility that they also target ALDH1A1 in LSCs. Given this perspective, it is understandable that these studies are somewhat overlooked, despite the critical roles of ALDH1A1 in LSCs and its impact on therapy resistance. It is crucial to comprehensively identify miRNAs that target ALDH1A1 in both HSCs and LSCs. Once the miRNA networks targeting ALDH1A1 in HSCs and LSCs are revealed, any differences between the two should be identified and the molecular mechanisms that cause these differences can then be rigorously investigated.

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Authors' contributions

All authors (SAV, LV, PZ, DAS and VZ) contributed to the conceptualization of the project, to the interpretation and analysis of the data to be included in the review, and wrote and prepared the draft of the manuscript. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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