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Review

miR signatures and the role of miRs in acute myeloid leukaemia

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ABSTRACT

Acute myeloid leukaemia (AML) is a hematopoietic stem cell disorder in which neoplastic myeloblasts are arrested in an immature stage of differentiation. Recent publications have underlined the involvement of non-coding RNAs in cancer and particularly in AML development, with several studies regarding their possible contribution to the evolution of the disease. MicroRNAs (miRs) are a class of single-stranded non-coding RNAs that bind to the 3′-untranslated region of target mRNAs and thus negatively regulate gene expression, by translation repression or mRNA degradation. Abnormal expression of miRs in AML has been described and we here review the current data from miR expression profiles. Additionally, we review the current knowledge on the biological function of individual miRs in the development of AML.

1. Introduction

Acute myeloid leukaemia (AML) is a hematopoietic stem cell disorder that can be characterised by rapid growth of a clonal population of neoplastic cells that accumulate in the bone marrow as a result of a blockage in hematopoiesis. The neoplastic myeloblasts are 'frozen' in an immature stage of differentiation, with a loss in the normal hematopoietic function due to alterations in the mechanisms of self-renewal, proliferation and differentiation.[1]

AML is a very heterogeneous disease with many specific cytogenetic abnormalities being found in about 55% of adult AML patients. Chromosomal abnormalities often have prognostic significance: t(8;21), t(15;17) or inv(16) is usually correlated with good prognosis and –5, –7, del(5q) with adverse prognosis.[2] These chromosomal translocations encode for fusion proteins whose altered properties may cause differentiation arrest. Besides cytogenetic alterations, there are many patients without any visible chromosomal alterations – cytogenetically normal (CN) – but who have certain mutations or deregulation in the expression of specific genes. About 40–50% of AML patients are CN and they represent the largest subset of AML.[3] This huge variety of AML subsets illustrates the heterogeneity of AML, particularly in patients exhibiting normal karyotype.[4,5] Nowadays, it is possible to detect genetic alterations in over 90% of all AML patients by the combination

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of the chromosomal banding technique, fluorescence *in situ* hybridisation (FISH) and molecular analysis, as chromosomal aberrations are detectable in 55% of patients and molecular mutations in 85% of normal karyotype cases.[6]

2. miR signatures in AML

MicroRNAs (miRs) are a class of single-stranded non-coding RNAs that bind to the 3'-untranslated region of target mRNAs and thus negatively regulate gene expression, by translation repression or mRNA degradation (Fig. 1). Different studies have recently been published concerning miRNA profiling in different cancer types[7,8] amongst which AML profiling has also been documented.[9–13]

The first miR expression profile in AML was conducted by Mi et al.[14] The authors evaluated if miR signatures would discriminate between AML and acute lymphoblastic leukaemia (ALL). Using samples from 11 ALL patients, 47 AML patients (both with similar chromosomal translocations), as well as from 7 cell lines of each malignancy, they found 27 miRs differently expressed between ALL and AML. Of those, miR-128a, miR-128b, let-7b and miR-223 had significantly altered expression between ALL and AML. Using any two of these four miR signatures was sufficient to accurately (97–99%) discriminate ALL from AML.[14]

Comparing 50 AML samples with 7 normal bone marrow samples as well as 5 $CD34^+$ blood samples, Isken and colleagues[9] analysed 154 miRs and found 4 differently expressed in AML. From those, miR-23b was downregulated and miR-34a, miR-221 and miR-222 were upregulated in AML samples when compared to controls. Three other miRs (miR-26a, miR-26b and miR-29b) had differential expression between the groups, with an intermediate expression level in AML, highly expressed in normal bone marrow samples and a low expression in $CD34^+$ cells, appearing to be related with differentiation. Indeed, these miRs were upregulated upon ATRA induced differentiation in HL-60 cells.[9]

Another study comparing 122 newly diagnosed AML patients and $CD34^+$ cells from 10 normal donors identified 26 downregulated miRs in AML.[10] These results were validated by quantitative RT-PCR for 7 of the downregulated miRs (miR-93, miR-106b, miR-125a, miR-126, miR-130a, miR-135 and miR-146) and the results were confirmed, in six new AML samples and four new $CD34^+$ blood samples, for all but miR-135. Of these miRs, miR-93, miR-125a, miR-126, miR-130a and miR-146 were not only downregulated in AML samples, when compared with $CD34^+$ cells, but also downregulated in healthy precursors and mature peripheral blood myeloid cells. These results indicate that the downregulation of these miRs is probably not directly related to disease but rather to differentiation.[10]

In a study performed on 100 AML patients (analysing 157 miRs), representative of the range of karyotypes known in AML,[11] researchers found 17 upregulated and 16 downregulated miRs in AML when compared to bone marrow controls. Some of the upregulated miRs had previously been described as being hematopoietic tissue-specific: miR-142-5p, miR-155 and miR-181.[15–17] Additional miRs upregulated in AML were miR-221 and miR-222, which was in agreement with other publications.[9] Amongst the downregulated miRs were some previously described as having anti-oncogenic potential in other cancers – miR-26a, miR-34c and miR-199a.[18,19]

Some of the studies reporting miR profiles took into account the diverse cytogenetic alterations present in AML patients. In fact, Jongen-Lavrencic and colleagues[13] conducted an experiment with 215 genetically defined *de novo* AML patients and four samples of $CD34^+$ cells from healthy donors and, by analysing 260 miRs and using an unsupervised clustering approach they were able to cluster together AMLs with t(8;21), t(15;17), inv(16), *NPM1* or *CEBPA* mutations. Using a supervised analysis they identified miR signatures that could characterise cytogenetic abnormal AMLs – t(15;17), t(8;21) and inv(16) – as well as signatures that distinguished *NPM1* mutations and *CEBPA* mutations or *FLT3-ITD*. Although miRs were able to characterise different genetic AMLs, mRNA expression profiling (over the same 215 samples) was more accurate in predicting the same genetically different AMLs as it required a smaller number of probes. Nonetheless, the small number of miRs studied (260 compared with 54675 mRNAs) shows the high potential of profiling miRs for the molecular diagnosis of AML.

Regarding AMLs harbouring the translocation t(8;21) or inv(16), two studies presented different results. In one of the studies, upregulation of miR-126 and miR-126* was exclusive of these cytogenetic alterations (Table 1).[12] In the other study, downregulation of miR-133a and upregulation of miR-146a characterised t(8;21) AMLs[11] whilst upregulation of miR-99a, miR-100 and miR-224 defined inv(16) AMLs (Table 1).[11]

Patients bearing the translocation t(15;17) were characterised by different miR expression alterations, with some miR alterations being common to different reports. Indeed, upregulation of miR-127, miR-323, miR-368 and miR-382 was described in two of three different studies (Table 1).[11–13] Curiously, miR-134, miR-154, miR-154*, miR-299, miR-370 and miR-376a, as well as the above-mentioned miRs, are located in chromosome 14q and were all described as being upregulated in translocation t(15;17) AMLs (Table 1).[11,13]

MLL-rearrangement AMLs were differently characterised by miR profiling. The absence of expression of miR-10a, miR-331 and miR-340 was reported to define this cytogenetic alteration in AML (Table 1)[11] whilst another study found an altered expression of 24 miRs in *MLL* rearrangements, seven of which belonging to the polycistronic miRNA cluster, mir-17-92 (Table 1).[12] Garzon and collaborators[10] have also identified a miR signature for MLL-rearrangement AMLs with 8 upregulated and 14 downregulated miRs. Amongst the downregulated miRs were many tumour suppressor miRs already known to target critical oncogenes: miR-34b (targets *CDK4* and *CCNE2*,[20]), miR-15a (targets *BCL-2*,[21]), let-7 family (targets *RAS*[22]), miR-29 family (targets *MCL-1*[23]) and miR-196 (targets *HOX-A7, HOX-A8, HOX-D8 and HOX-B8*[24]) (Table 1).

Some research groups have been attempting to determine miR signatures for specific types of AMLs bearing characteristic mutations. Amongst those, Garzon and colleagues[25] studied AMLs carrying *NPM1* mutations in 85 *de novo* AML samples (55 with *NPM1* alteration and 30 with wild-type *NPM1*). A distinctive pattern of miRs had altered expression between the two groups, with 36 upregulated and 21 downregulated miRs in NPM1 mutated samples. Amongst the upregulated miRs were miR-10a and miR-10b which were able to differentiate

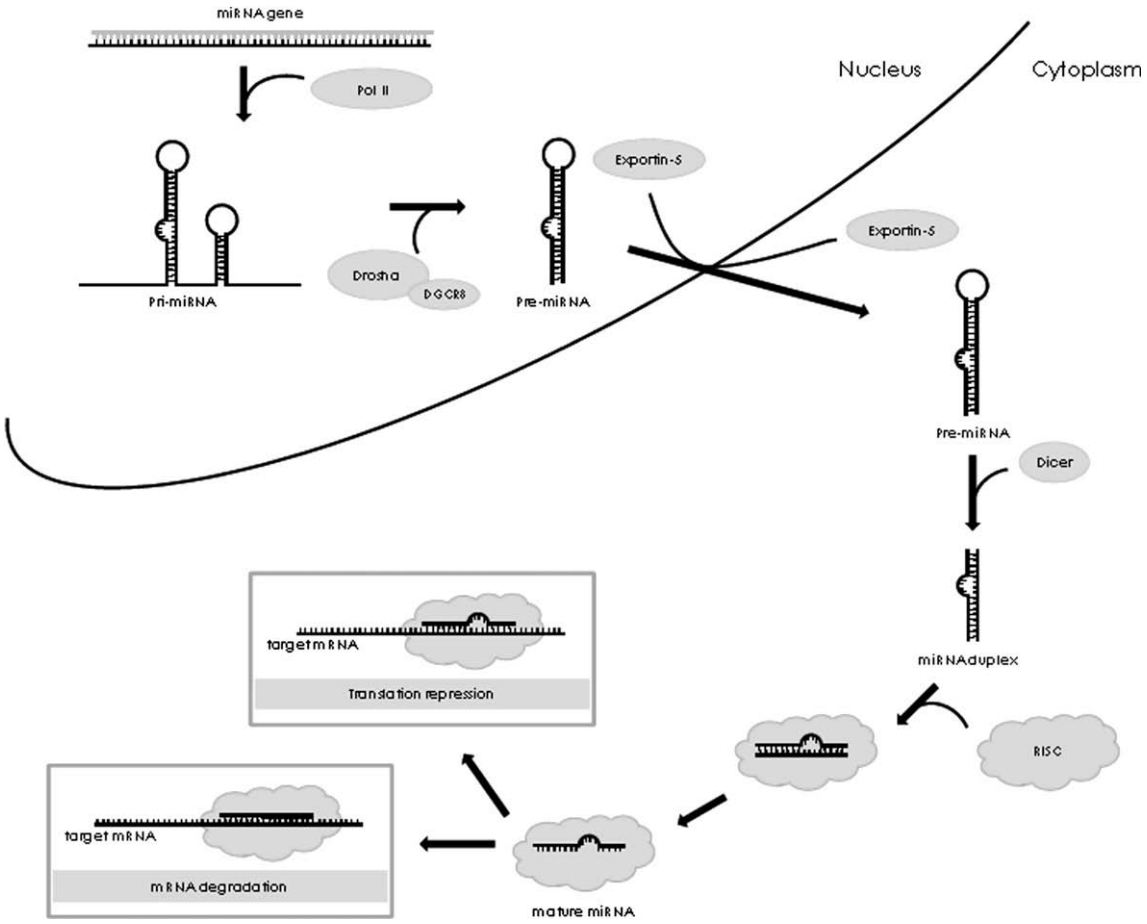

Fig. 1 – miRNA biogenesis and mode of action. Biogenesis of miR starts in the nucleus with the synthesis of a relatively long dsRNA molecule, known as pri-miR, by RNA polymerase II. Whilst still in the nucleus, pri-miR is processed to pre-miR by an RNase III enzyme, Drosha, and its interacting partner DGCR8.[65,66] This pre-miR is then exported to the cytoplasm by exportin-5 and is subsequently converted to the mature miR form by another RNase III enzyme – Dicer. The mature miR is then incorporated into the RNA-induced silencing complex (RISC), the double stranded miR separated by an RNA helicase and finally the mature miR strand is used to drive RISC to its target mRNA. The strand which becomes the mature miR is the one whose 5′ end is more unstable, thus more easily unwound by the helicase.[69,70] miRs act through a post-transcriptional suppression of the target mRNA expression, by binding to the 3′-UTR region of that mRNA. Depending on the degree of complementarity between the miR and its target mRNA, downregulation of the expression of the protein coded by that mRNA will follow, by one of two possible mechanisms: (i) translation repression when the miR is only partially complementary to the target mRNA or (ii) degradation of the target mRNA when the miR has full complementarity to that mRNA.[71–73] Partial complementarity of the miRNA is thought to target this mRNA to mRNA-processing bodies (P-bodies) which are sites for RNA decay.[74,75] The 3′-UTR of mRNAs may include binding sites for different miRs or several sites for the same miR,[76,77] which is suggestive of an intricate regulation system.[78,79]

the two groups, as also shown by others (Table 1).[13,26] A study focusing on BAALC (Brain and Acute Leukaemia, Cytoplasmic) reported that from over 50 patients – 24 with high and 26 with low expression of BAALC – there were no differences in miR profiles. However, miRs predicted (by *in silico* analysis) to target BAALC mRNA were analysed and miR-148a showed a strong inverse correlation with BAALC expression.[27] Additionally, AMLs harbouring *CEBPA* mutations showed a characteristic miR signature with 2 downregulated and 15 upregulated miRs.[28] Of the latter, 8 belonged to the miR-181 family, known to be involved in erythroid and lymphoid differentiation[15,29,28] (Table 1). A more recent study profiled a miR signature for meningioma 1 (MN1) high-expression AMLs and found five upregulated miRs (miR-126, miR-126*, miR-129-5p, miR-130b and miR-424) and five downregulated miRs (miR-16, miR-19a, miR-20a, miR-100 and miR-196a) (Table 1).[30]

Additionally, when analysing AMLs bearing FLT3-ITD, three miRs – miR10a, miR-10b and miR-155 – were found to be upregulated in FLT3-ITD when compared with FLT3-wt patients (Table 1).[10] Marcucci and collaborators[31] studied 64 CN AMLs with adverse molecular characteristics – *FLT3-ITD*, wild-type *NPM1* or both, and found 8 miRs associated with event-free survival, none of them overlapping with Garzon et al. results.[10] Overexpression of miR-124, miR-128-1, miR-194, miR-219-5p, miR-220a and miR-320 was positively associated with the risk of an event (failure to achieve

Table 1 – Highlighted upregulated and downregulated miRs in AML according to 'genetic alteration'.

Genetic alteration	Highlighted upregulated miRs	Highlighted downregulated miRs	References
t(8;21)	miR-126, miR-126*		12
	miR-146a	miR-133a	11
inv(16)	miR-126, miR-126*		12
	miR-99a, miR-100 and miR-224		11
t(15;17)	miR-127, miR-134, miR-323, miR-376a and miR-382		13
	miR-127, miR-154, miR-154*, miR-299, miR-323, miR-368, miR-370		11
	miR-368, miR-382		12
MLL rearrangements		miR-10a, miR-331, miR-340	11
	miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92		12
		let-7, miR-15a, miR-29a, miR-29b, miR-29c, miR-34b and miR-196a	10
NPM1 mutation	miR-10a, miR-10b		25
	miR-10a, miR-10b		13
	miR-10a, miR-10b		26
CEBPA mutation	miR-181a, miR-181a*, miR-181b, miR-181c and miR-181d		28
High MN1	miR-126, miR-126*, miR-129-5p, miR-130b and miR-424	miR-16, miR-19a, miR-20a, miR-100 and miR-196a	30
FLT3-ITD	miR-10a, miR-10b and miR-155		10

complete remission, relapse or death) whilst miR-181a and miR-181b overexpression was negatively associated with that risk. These authors also investigated gene-expression signatures to identify genes that could be regulated by the depicted miRs. Some predicted targets of the miR-181 family had an inverse correlation with these miRs' expression (*TLR4*, *CARD8*, *CASP1* and *IL1B*), suggesting that miR-181 family downregulation can be linked to more aggressive AMLs. A more recent study was able to directly correlate miR-181a expression with a better prognosis in CN AML, independently of other variables including *CEBPA* mutations.[32]

Overexpression of miR-191 and miR-199 was recently correlated with shorter overall survival and shorter event-free survival in a group of intermediate and poor prognosis karyotypes.[10] Cytogenetic prognostic risk could also been determined by the expression of two other miRs: miR-9 and let-7b whose expression was found to be low in good prognosis groups and high in intermediate or adverse AMLs.[11]

3. The role of miRs in AML

In addition to the study of miR expression profiles, the function of particular AML related miRs has also been investigated and is described below.

3.1. *miRs in hematopoietic differentiation*

As mentioned above, AML is a malignance associated with lack of differentiation of hematopoietic cells. This cellular process is therefore a candidate target for deregulated miRs interfering with AML progression.

Indeed, some miRs have been shown to play a role in hematopoietic differentiation and have been linked with AML progression in specific genetic backgrounds. AML1/ETO is a fusion protein characteristic of translocation t(8;21) in AMLs. AML1 normal expression was shown to downregulate miR-24 transcription but patients bearing translocation t(8;21) overexpressed miR-24, which inhibited a mitogen-activated protein kinase (MAPK) phosphatase (MKP-7), thus leading to the activation of downstream partners.[33] miR-24 was also shown to block myeloid differentiation and to accelerate cell proliferation.[33]

Another miR that has been correlated with AML t(8;21) carriers is miR-223.[34] Patients' primary leukaemia blasts showed a low level of miR-223, a previously known regulator of myelopoiesis.[35] This miR expression is decreased by the interaction of the AML1/ETO fusion protein with the miR promoter region. AML1/ETO has the capacity to oligomerise with histone deacetylase (HDAC) and DNA methyltransferases (DNMTs) bringing them to the AML1-binding site on the pre-miR-223 gene, hence silencing miR-223. Demethylating treatment, RNAi against AML1/ETO or ectopic expression of miR-223 restored cell differentiation, showing the importance of this miR in the normal myelopoiesis. Interestingly, another study focusing on miR-223 and AML showed no association between the expression of this miR and hypermethylation of its promoter region; furthermore, this study showed that miR-223 suppression in AML was rather due to impaired miR-223 upstream factors mediated by the conserved CEBP/PU.1 responsive element in front of the pri-miR-223 transcript start site.[36] The known role of miR-223 in myelopoiesis has been supported by a recent study that correlates CEBPA and E2F1 transcription factors with miR-223 expression.[37] CEBPA was shown to upregulate miR-223 expression, which in turn directly targets E2F1 3′-UTR, inhibiting its translation. Further studies elucidated that E2F1 is also a repressor of miR-223 by binding to its promoter region, producing a negative feedback loop. It was also shown that overexpression of miR-223 blocked cell proliferation allowing cells to differentiate, thus confirming its role in myelopoiesis.[37]

miR-125 has been associated with a specific cytogenetic translocation, t(2;11).[38] Indeed, AML patients with

t(2;11)(p21;q23) translocation showed a big increase in miR-125b expression; additionally, *in vitro* experiments showed that miR-125b was able to both hamper primary human $CD34^+$ cell differentiation and inhibit myelo-monocytic differentiation in HL-60 and NB4 leukaemic cell lines. Accordingly, upregulation of miR-125b may represent a new mechanism of myeloid cell transformation in patients carrying the t(2;11) translocation.

Another miR recently shown to be specifically overexpressed in AML patients with MLL rearrangement is miR-196b.[39] The overexpression of miR-196b in bone marrow progenitor cells seems to increase proliferation and survival capacity, as well as partially block differentiation, indicating that miR-196b plays a role in the development of MLL leukaemias.[39]

In a study performed in HL-60 cells, it was shown that transferrin receptor 1 (TfR-1), a molecule involved in increased proliferation and decreased differentiation of leukaemic cells,[40] is a target of miR-320.[41] This miR emerges as a potential therapeutic target for TfR-1 overexpressing malignancies such as breast carcinoma, colorectal cancer or AML.[41–44]

Functional loss of CCAAT/enhancer binding protein alpha (CEBPA), a key transcription factor involved in the regulation of cell proliferation and differentiation in the hematopoietic system, can result in a differentiation block in granulopoiesis and contribute to leukaemic transformation.[45] DNA methylation of *CEBPA* upstream promoter region has been correlated with t(15;17) and inv(16) AMLs.[46] The use of demethylating agents, *in vitro*, led to an increase of *CEBPA* mRNA but, surprisingly, to a decrease of CEBPA protein expression. In addition, it was shown that miR-124a targets *CEBPA* mRNA, both by luciferase reporter assay (using a vector containing the 3′-UTR of *CEBPA* mRNA) and by analysis of CEBPA protein levels following transfection of K562 cells with miR-124a. Indeed this miR, often silenced by epigenetic mechanisms in AML, becomes activated after demethylation treatment and targets the 3′-UTR of *CEBPA* mRNA.[46] This might explain why some patients respond well to epigenetic therapy whilst others do not.[46]

3.2. *miRs and epigenetic regulation*

DNA methylation is a recognised regulatory molecular mechanism that leads to gene transcriptional silencing[47] and plays a role in AML development.[48] The role of miRs in the epigenetic field is being unveiled[49] and miR-29b has been shown to regulate DNA methyltransferases (DNMTs) in the context of leukaemia.[50] This miR targets DNMT3A and DNMT3B directly and DNMT1 indirectly by interfering with Sp1, a DNMT1 transcription factor. Overexpression of miR-29b, in leukaemic cell lines, led to global DNA hypomethylation and re-expression of tumour suppressor genes *ESR1* and cyclin-dependent kinase inhibitor 2B ($p15^{INK4b}$).[50]

3.3. *miRs and apoptosis*

It has also been shown that miR-29b expression reduced cell growth and induced apoptosis in cell lines and primary AML samples; furthermore reduced tumour growth was found in a leukaemia xenograft model in mice, following injection of miR-29b into the tumours.[51] Moreover, Mcl-1 was shown to be a miR-29b target and the analysis of 45 primary AML samples revealed an inverse correlation between miR-29b and Mcl-1 expression.[51]

Cyclic AMP-responsive element binding protein (CREB) is occasionally overexpressed in AML.[52,53] Myeloid cell lines, known to overexpress CREB, presented significantly lower levels of miR-34b. Furthermore, the exogenous expression of miR-34b directly targeted *CREB* mRNA, diminishing CREB protein levels and altering CREB target gene expression including several proteins known to regulate apoptosis: BCL-2, cyclin A1, cyclin B1, cyclin D, NF-KB, JAK1 and STAT3.[54] Curiously, the downregulation of miR-34b in these cell lines seems to be due to promoter methylation. Moreover, an inverse correlation between miR-34b and CREB expression was observed in 78 paediatric AML patients, supporting the *in vitro* experimental results.[54]

3.4. *miRs and multidrug resistance*

In a study using the HL-60 AML cell line, Zhao and colleagues reported that miR-138 could be related with multidrug resistance of leukaemic cells. This miR was found in lower amounts in a HL-60 resistant cell line when compared with parental cells. Additionally, transfection of miR-138 to the HL-60 resistant cells was able to reverse P-glycoprotein-related resistance (by involvement in regulation of MDR1 transcription) and to induce apoptosis via regulation of expression of Bcl-2 and Bax proteins.[55]

4. Discussion

From the publications mentioned above, it becomes clear that miR expression profile studies have not been providing concordant results. This observation could be explained by the different AML samples used, with different cytogenetic and molecular characteristics, and the different platforms used to generate miR expression profiles. Normalisation of miR expression to other small RNAs (snRNA or tRNAs) other than miRs themselves may provide more reliable results. One of the above-mentioned studies has made a normalisation of the results for miR-223 expression,[17] whilst the same miR was shown in other studies to be differently expressed amongst AML samples.[34,37] The method of RNA extraction and RNA quality could also interfere with the specific miR quantities detected.[59,60] Despite these drawbacks, miR profiling can add extra knowledge towards better treatment of AML. Along with copy number alteration studies,[61] miR expression profiles and functional studies of individual miRs aberrantly expressed in AMLs can introduce new insights in the perception of leukaemogenesis, possibly improving diagnosis and finding better prognostic markers as well as contributing to more tailored patient treatment. Additionally, miRs themselves may also represent a future therapeutic approach for AML patients, either alone or in combination with currently used therapies. Loss of expression of tumour suppressor miRs could be re-established by synthetic miRs and inhibition of oncogenic miRs could be achieved by using 'antagomirs'. In fact, some promising results have already been achieved using 'antagomirs' in animal models.[62–64] Although still far from being used in the clinic due to lack of studies concerning off-target effects and difficulties in

target delivery, miR-based drugs could hold much promise for the future of cancer therapy.

Additionally, miRs have been proposed to be promising serum biomarkers for the detection of cancer.[56,57] A pioneering study has revealed that miR-92a is reduced in the plasma of acute leukaemia patients. In addition, the ratio of miR-92a/miR-638 (a miR shown to be equally expressed in all the samples, regardless of age, sex or leukaemia classification) in plasma was valuable to distinguish healthy donors from acute leukaemia patients.[58]

Conflict of interest statement

None declared.

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