



REVIEW

Liquid biopsies for liquid tumors: emerging potential of circulating free nucleic acid evaluation for the management of hematologic malignancies

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ABSTRACT

Circulating free nucleic acids; cell free DNA and circulating micro-RNA, are found in the plasma of patients with hematologic and solid malignancies at levels higher than that of healthy individuals. In patients with hematologic malignancy cell free DNA reflects the underlying tumor mutational profile, whilst micro-RNAs reflect genetic interference mechanisms within a tumor and potentially the surrounding microenvironment and immune effector cells. These circulating nucleic acids offer a potentially simple, non-invasive, repeatable analysis that can aid in diagnosis, prognosis and therapeutic decisions in cancer treatment.

KEYWORDS

Circulating nucleic acids; DNA; miRNA; hematologic malignancy; biopsy

Introduction

The presence of circulating cell free nucleic acids (CFNA), more specifically circulating free DNA (cfDNA), was first recognized in 1948¹ but it was not until 1977 that it was identified that patients with malignancy had greater amounts of cfDNA than healthy individuals². The potential of this medium was first demonstrated in 1994 following the identification of tumor associated RAS gene mutations in the blood of patients with MDS/AML³ thus confirming the capacity to identify cancer specific mutations in the peripheral blood of cancer patients. Another key discovery came in 2008 when it was shown for the first time that tumor associated microRNAs (miRNA) were detectable in the peripheral blood of lymphoma patients⁴. These discoveries have led to an increasing number of studies investigating the utility of CFNA for the characterization, monitoring and therapeutic targeting of both hematologic and solid malignancies⁵.

Current methods employed for the evaluation of cancer genomes requires tissue biopsy; either bone marrow (BM) biopsy or biopsy of affected nodal/soft tissue. These

procedures are invasive, not without complications and are limited by being representative of only a single site of the disease under evaluation. The latter is of increasing importance with the recognition that cancers may exhibit spatial genomic heterogeneity, with genetically different cancer sub-clones being located at different sites of disease within an affected individual⁶. Thus 'single-site' tissue biopsies may not truly reflect the entire mutational profile of an individual's disease and from a logistical perspective may not be suited to repeat biopsy over short periods of time. With this in mind, the potential for 'liquid' biopsies that are non-invasive may not only provide better representation of cancer genetic makeup but provides a strategy that is ideally suited to repeated sampling.

Thus, cfDNA offers the potential of a truly representative cancer genome 'biopsy' that may aid in the diagnosis, prognostication and treatment of cancer. Similarly, the study of circulating miRNA may provide insights into not only diagnosis and prognosis but also previously unrecognized inter-cellular communications aiding in our understanding of cancer biology. This review focuses on the currently available literature focusing on CFNA in hematologic malignancies.

Circulating free DNA (cfDNA)

The first identified cancer-related mutations in the peripheral

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blood were mutant RAS genes^{3,7}, identified in 1994, with their discovery prompting further investigation in the field with numerous publications by the turn of the century⁸. While cfDNA is universally found in the plasma of healthy people as well as those with benign diseases⁹, it has been observed that patients with malignant disease (both solid tumors and hematologic) have higher levels of cfDNA in their plasma². The source of cfDNA is predominantly thought to be due to cells undergoing either spontaneous or chemotherapy induced apoptosis^{10, 11}. As this process takes place nuclear material is packaged into apoptotic bodies and engulfed by macrophages, however, proportional amounts of cfDNA are also released into the circulation¹². Additionally it has been postulated the presence of cfDNA may be due to active cellular release^{13,14}.

As is the case with solid tumors cfDNA may also be more representative of the entire tumor genome in hematological cancers than the information derived from single BM or nodal biopsies, as emerging evidence supports the notion that a range of hematologic malignancies including multiple myeloma (MM)¹⁵, chronic lymphocytic leukaemia (CLL)¹⁶ and non-Hodgkin's lymphomas (NHL)¹⁷, are likely to harbour significant sub-clonal and spatial genetic heterogeneity. Thus, as novel platforms and techniques in the field continue to evolve^{18,19} the potential of the medium rises in parallel (see **Table 1**). Moreover, the interrogation of cfDNA can provide information not only in relation to point mutations but also chromosomal translocations, epigenetic modifications, including hypermethylation, and loss of

heterozygosity (LOH).

Acute myeloid leukaemia (AML)

The first study evaluating cfDNA mutational profiles in hematologic malignancies was in patients with AML and MDS³. In this seminal publication, 3 patients had paired cfDNA and BM samples, whereas 7 had paired cfDNA and DNA derived from peripheral blood (PB) blast cells. Five cases consistently demonstrated detectable mutations in cfDNA that were not necessarily found in the paired PB or BM DNA. The authors concluded that plasma could prove to be an easily accessible and useful material for the detection and monitoring of myeloid disorders. Importantly, their findings were recapitulated a decade later when it was shown that the peripheral blood of patients with AML/MDS is enriched for tumor DNA²⁰.

Circulating nucleosomal fragments represent another source of cfDNA²¹ and it has been shown these are increased in the peripheral blood of patients newly diagnosed with AML²². In a small study of 25 AML patients undergoing induction chemotherapy, irrespective of the remission status eventually achieved, all patients demonstrated an eventual drop in their circulating nucleosomal DNA fragments. But, in those patients who went on to achieve a CR, an initial rise on days 2 to 4 following the commencement of induction chemotherapy was seen, possibly reflecting increased apoptosis in response to treatment. Cytogenetics and specific molecular profiles (NPM1, c-Kit, FLT3, CEBPA) in AML are

Table 1 Techniques and targets of measurement of circulating nucleic acids, with relative advantages and disadvantages.

cfNA	Target	Technique	Comment
DNA	Mutation specific (eg. KRAS/NRAS)	PCR	Tumor specific and sensitive; potential use in longitudinal monitoring; may not be practical for broad screening
	Loss of heterozygosity (LOH)	PCR	Issues of reproducibility; circulating measurements inconsistent with primary tumor findings
	Epigenetic (eg. methylation)	Bisulfite sequencing, Methylation specific PCR	Specific and sensitive tool; requires knowledge of candidate genes for specific testing; complex process
	Viral DNA	PCR	Potential in malignancies associated with specific viruses (DLBCL, Burkitts); benign viral infection may complicate results
	Nucleosomes	ELISA	Useful for tumor with high turnover/rates of apoptosis; may also be elevated in non-malignant conditions
MicroRNA	-	Microarray	Broad application for miRNA identification; cannot quantify miRNA; issues of sensitivity and reproducibility; inter-array variability and inconsistency
	-	RT-PCR	Useful for monitoring single miRNA; issues with standardization
	-	NGS	Potential for novel miRNA discovery; costly; highly specialized techniques

used to define prognosis and guide treatment decisions following induction²³. It has been shown in a recently published study that NPM mutations can be detected and quantified in the PB²⁴ at a rate commensurate with the rate of detection in historical BM samples²⁵. However, this study did not report parallel assessment of NPM mutations in paired cfDNA with BM samples and consequently conclusions about the sensitivity of cfDNA detection could not be drawn. While these studies provide proof of concept of the potential of interrogating cfDNA in AML, they are preliminary and many questions surrounding the ultimate utility of cfDNA analysis remain.

Myelodysplastic syndromes (MDS)

Epigenetic modification is a hallmark of the pathogenesis of MDS, particularly methylation²⁶. Therefore, the potential to study cfDNA methylation utilizing highly sensitive and novel technologies such as DREAMing (Discrimination of Rare EpiAlleles by Melt) presents an exciting opportunity. In a proof of concept study of 10 MDS and myeloproliferative neoplasm (MPN) patients specifically attempting to address the issue of intra-tumoral epigenetic heterogeneity, analysis of CpG methylation at BRCA1 in the PB was undertaken with bisulfite conversion followed by methylation specific PCR²⁷. The degree of epiallelic heterogeneity was then derived and confirmed by pyrosequencing. In a second study, methylation analysis with bisulfite pyrosequencing at specific CpG sites of the LINE1 promoter was serially analyzed in patients treated with azacytidine²⁸. Following treatment, an initial decline in DNA methylation was observed followed by a progressive rise until the next cycle of therapy. Similarly, TET2 mutations were measured in paired cfDNA and BM samples and showed concordance. These data, although preliminary, set the scene for further studies of cfDNA methylation.

Lymphoid neoplasms

Patients with NHL have higher levels of total cfDNA than healthy controls^{29,30}. However, whether cfDNA levels at diagnosis correlate with baseline disease characteristics is still unclear. To evaluate lymphoma-specific cfDNA, several groups have analyzed circulating clonal IgH gene rearrangements utilizing PCR³¹, high-throughput sequencing (HTS)^{32,33} or next-generation sequencing (NGS)^{34,35}. Irrespective of the technique used the majority of patients had measurable rearrangements in the plasma and the detection of plasma cfDNA IgH gene rearrangements was

shown to have greater sensitivity than HTS of circulating tumor cells³³. Importantly, higher disease burden correlated with higher levels of IgH gene rearrangements³²⁻³⁴ and clearance of IgH gene rearrangements was seen in patients who achieved a CR^{32,34}. At progression or relapse detectable clonal IgH genes re-appeared within cfDNA in parallel with radiological relapse. More specifically, in the largest study to date, Roschewski³⁴ found 3 patterns of IgH gene rearrangements in the context of progressive disease-1-failure of clearance, 2-transient clearance followed by rapid reappearance or 3-clearance followed by clinical progression, then reappearance. Whilst the finding was not statistically significant, those who failed to clear cfDNA derived clonal IgH genes experienced the shortest survival, an observation that if confirmed by others may have significant utility as a MRD strategy to inform risk-adapted changes in therapy. The experience with alternative lymphoma-specific cfDNA targets is limited with preliminary published data describing the use of NGS for single nucleotide variants in CD79A/B, EZH2, CARD11 or MYD88³⁶ (present in 11 of 12 patients), p61 tumor suppressor gene methylation³⁷ (present in 73%) and TP53 mutations³⁸ (present in 30%).

Similarly, in pediatric patients with ALL two groups have shown the presence of higher levels of cfDNA at diagnosis compared with healthy controls, and the feasibility of cfDNA as a marker for MRD^{39,40}. Whilst in multivariate analysis cfDNA PCR MRD demonstrated a prognostic significance for EFS/RFS, there was no correlation with bone marrow based FACS MRD, and in several cases FACS MRD was positive whilst cfDNA PCR MRD was negative⁴⁰. Thus whilst showing promise as an adjunct in disease assessment, lower sensitivity than currently employed methods may pose an issue.

EBV cfDNA has been detected in patients with various subtypes of NHL and NK/T-cell lymphomas⁴¹⁻⁴⁴. Findings are consistent across all groups, with EBV associated lymphoma patients almost universally having detectable EBV cfDNA whereas paired healthy donor samples do not⁴³. Moreover, in almost all cases, where patients achieved a CR, EBV cDNA became undetectable but patients who achieved a radiologic CR but failed to clear EBV DNA, subsequently relapsed and died⁴³. Not surprisingly, high EBV cfDNA at presentation was predictive of poorer outcomes, with patients experiencing shorter overall survival (OS), even in those with early stage disease⁴³. For patients who achieved only a partial remission (PR) or were refractory to therapy, persistent or rising EBV cfDNA levels were seen⁴¹⁻⁴³. These studies show clear potential for cfDNA biomarkers in the diagnosis and monitoring of patients with NHL. Further

evaluation is warranted to see if increasing intensity of therapy or stem cell transplant can abrogate the negative impact of persistence of cfDNA.

In proof of concept studies in CLL Sonnenberg et al.^{19,45} evaluated the detection of clonal IgH gene rearrangements in cfDNA using a novel method of DNA isolation-dielectrophoretic isolation (DEP)-that allows isolation of DNA from unprocessed blood for subsequent PCR. This was compared with alternative conventional methods of DNA isolation from paired leukemic cell samples. DEP was found to be comparable for both DNA isolation¹⁹ and clonal IgH gene detection. This technique may therefore provide a simpler, rapid and more cost effective approach of isolating and analyzing cfDNA in CLL.

Hodgkin's lymphoma is associated with EBV in up to 50% of cases⁴⁶. Several groups have shown that EBV cfDNA can be detected circulating in the plasma of HL patients⁴⁷⁻⁵⁰. However, to date, due to inadequate sensitivity it cannot be used as a surrogate marker for EBER-ISH on nodal tissue⁴⁷. This fact notwithstanding, higher levels are associated with more advanced disease and patients that are EBV+ prior to therapy experience inferior failure free survival (FFS)⁴⁹ and progression free survival (PFS)⁴⁷. Furthermore, response to therapy is associated with undetectable viral loads in the plasma, whilst a single patient who remained positive for EBV viral cfDNA relapsed five months later⁴⁸. These data raise the prospect of EBV cfDNA as both a marker of prognosis and for monitoring response to therapy in HL.

As in NHL clonal rearrangements of the IgH gene are present in MM and can be detected utilizing allele specific oligonucleotide (ASO) PCR with probes designed for individual patients. A study describing the use of ASO PCR to analyze IgH VDJ rearrangements in matched bone marrow mononuclear cells (BMMCs), PB mononuclear cells (PBMCs) and cfDNA from MM patients has been reported⁵¹. A statistically significant correlation was observed between the BMMC and PBMC compartments with both levels declining in response to therapy. Using the same ASO primers cfDNA was amplified, confirming the source of the DNA as the malignant MM cells in the BM. However, the circulating DNA level failed to drop following treatment, suggesting the persistence of disease at perhaps spatially disparate sites not demonstrated in single bone marrow biopsy samples.

miRNA

MicroRNAs (miRNA) are small, up to 25 nucleotides long, single stranded non-coding RNAs that bind to specific

mRNA. In doing so, miRNAs regulate gene expression by either promoting mRNA hydrolysis and degradation or inhibiting its translation⁵². MiRNAs are found in virtually all fluid compartments of the body^{53,54} including blood where they circulate bound to proteins⁵⁵, high-density lipoproteins⁵⁶, within exosomes or apoptotic bodies⁵⁷⁻⁶⁰. This binding or encapsulation renders them resistant to RNases and thus highly stable⁶¹.

In addition to physiologic circulating miRNA, increased levels of circulating miRNA have been observed in malignancy, immune and inflammatory diseases⁶². In malignancy the origin of the deregulated levels of miRNA is questioned, whether they originate from tumor cells and/or non-malignant cells including the supporting micro-environment is an area of ongoing research⁶³. Additionally, whether these are passively released into the circulation during apoptosis or specifically secreted, within exosomes is not clear. If malignant tumor cells actively secrete miRNA, it may represent a potential intercellular communication network whereby tumors could influence the genetic machinery of target cells, potentially influencing and enhancing supporting stroma or possibly inhibiting immune responses. Indeed if this were the case, then it is easy to imagine how disease-related miRNA signatures might correlate with prognosis.

There are currently three methods typically employed to measure circulating miRNA: (i) micro-arrays (eg. Taq Low Density array, Agilent Microarray), (ii) RT-PCR, and (iii) NGS⁶⁴. Typically, microarrays are employed first to screen and identify miRNAs of interest, with subsequent confirmation and validation using RT-PCR, whereas NGS assays are more time consuming and expensive and are not routinely used (**Table 1**). Unfortunately, none of these methodologies are as yet validated or standardized, complicating generalizability and inter-study comparisons. Many groups report using 'internal' controls by comparing the levels of miRNA of interest to other species of miRNA that appear to remain stable or unaffected over periods of time, however, there is no universal agreement on which miRNA might be best suited to this purpose. The potentially problematic nature of this strategy is exemplified by the frequent use of miR-16⁴ for such purposes, as miR-16 has itself been observed to change expression over time in response to therapy. An alternative and perhaps more robust approach is absolute quantitation of target miRNA per unit serum/plasma⁶⁵ as is often done with cfDNA studies.

AML

A small number of groups have identified miRNA signatures

that demonstrate elevated or decreased levels of specific miRNA species at diagnosis in AML when compared with healthy controls. However, despite using similar techniques and investigational protocols there is surprisingly little, if any, overlap between the different miRNAs identified by different groups of investigators. Two differing miRNA sets-let-7d, miR-150, miR-339, and miR-342⁶⁶, and 199b-5p, miR-301b, miR-326, miR-361-5p, miR-625, miR-655⁶⁷ have been shown to be elevated at diagnosis in two independent studies. Additionally, in one of these studies let7b and miR-523 have been shown to be reduced⁶⁶. Where agreement was seen, was that in patients achieving a CR the initially elevated miRNA levels normalized to that observed in healthy controls. In other studies miR-92a has been identified by two groups to be significantly reduced in the plasma of patients with newly diagnosed AML and in patients with ALL⁶⁸. In both instances miR-92a increased following therapy, however, this partial normalization was not statistically different between those who did, or did not, achieve a CR⁶⁹.

Zhi et al.⁷⁰ employed a genome-wide approach using Solexa sequencing, to define a set of miRNA that might differentiate AML from healthy controls. Utilizing training and verification sets from newly diagnosed AML ($n=140$) and normal volunteers ($n=135$), 6 miRNA were found to be differentially expressed at statistically significant levels-miR-10a-5p, miR-93-5p, miR-129- 5p, miR-155-5p, miR-181b-5p, miR-320d and validated with RT-PCR. Using cluster analysis of these miRNA to differentiate AML from healthy controls in the validation set (both $n=95$), 11% of controls were misclassified as AML and only 4% of AML patients misclassified as 'healthy'. The same investigators also examined the prognostic significance of miRNAs in AML and showed that those patients with lower levels of miR-181b-5b at diagnosis had longer OS compared to those with higher levels. As the morphologic diagnosis of AML is rarely complicated, except in specific circumstances e.g. hypoplastic marrows or borderline blast counts, it may be in these settings that miRNA may have diagnostic utility in AML but these questions remain to be answered, as does the prognostic implications of altered levels of miRNA at the time of diagnosis.

MDS

Several miRNA have been shown to be elevated in the peripheral blood of patients with MDS-miR-17-3p, miR-17-5p, miR-18a, miR-15, miR-21, miR-142-3p⁷¹, or reduced let-7a, miR-16^{71,72} and miR-21⁷³, when compared with healthy controls. Additionally, elevated levels of miR-181a and miR-

222 in the BM but not the PB are associated with post MDS AML⁷¹. High levels of miR-16 correlated with low risk IPSS with the converse also holding true. With respect to correlation with survival, patients with higher levels (closer to healthy controls) of let-7a and miR16 experienced longer PFS and OS⁷², whereas lower levels of miR-21 correlated with better responses to hypo-methylating agents and longer PFS, but not OS⁷³. In all of these studies patient numbers were small and the populations studied were heterogeneous, including a mixture of MDS sub-types such as RCMD, RAEB1 and 2, CMML and the 5q- syndrome. Larger studies enriched for specific MDS sub-types are needed before this kind of data may be extrapolated to clinical practice.

Lymphoid malignancies

In diffuse large B-cell lymphoma (DLBCL) circulating miR-21 has been shown to be elevated compared with healthy controls. However, counter intuitively, those with higher levels of miR-21 had earlier stage disease (stages I and II vs. III and IV) and experienced longer relapse free survival (RFS) but not OS⁴. Additionally, higher miR-21 was associated with an activated B-cell subtype⁷⁴ (ABC) rather than a germinal center B-cell subtype (GCB), which again would appear contradictory to existing evidence of the adverse prognosis associated with an ABC subtype. Moreover, a possible explanation for the observations, that the study population was enriched for early stage ABC was proven to be not the case. A further panel of four miRNA have been shown to be significantly elevated in DLBCL compared to healthy controls⁷⁵, however, no clinical correlations with any of the individual miRNA were identified. In both DLBCL and follicular lymphoma (FL) circulating miR-92a has been shown to be reduced⁷⁶. Following treatment these levels trended towards normal, however, did not reach that of healthy individuals. In those achieving a CR, who did not undergo hemopoietic stem cell transplant, those with a lower miR-92a at six months were more likely to relapse than those with higher levels.

Elevated levels of miR-155 have been shown to differentiate between healthy controls and individuals with monoclonal B-cell lymphocytosis (MBL) or CLL⁷⁷. Importantly, patients with lower levels of miR-155 treated with FCR (Fludarabine, Cyclophosphamide, Rituximab) were more likely to achieve CR and experience longer OS⁷⁷, moreover, even when patients with high levels of miR-155 attained a CR their OS was shorter than those with lower miR-155. Similarly, elevated miR-150 can differentiate between healthy controls and CLL⁷⁸ and higher plasma miR-

150 levels have been shown to correlate with a higher tumor burden and advanced disease stage, expression of the poor prognostic marker CD38⁷⁸, ZAP70 positivity⁷⁹ and patients are more likely to require treatment⁷⁸. Patients with lower miR-150 expression experienced both longer treatment free survival (TFS) and OS⁷⁸. A panel of 14 miRNA has been reported to be able to discriminate between CLL, hairy cell leukemia (HCL) and MM⁷⁹, however, this requires confirmation by others.

It is perhaps not surprising that two groups have independently demonstrated a correlation between miR-150 and the diagnosis and subsequent prognosis of CLL given its established role in both normal and malignant hemopoiesis⁸⁰. Furthermore, increased circulating miR-150 has also been observed in AML⁶⁶ suggesting that it may be a non-specific marker of malignant hemopoiesis but this will only be clarified by further studies. It should also be noted that in two of the CLL studies^{77,78} miR-16 was used for the normalization of their RT-PCR experiments. MiR-16 is found on chromosome 13q, deleted in up to 50% of cases of CLL⁸¹, whilst both groups state its expression was consistent in the patients evaluated, it again raises the question as to what may constitute the best method of standardization for the reporting or miRNA studies.

A single study has evaluated circulating miRNA in HL⁶⁵. MiRNA of interest were identified via comparative microarray of HL involved lymph nodes (not micro-dissected HRS cells) and non-malignant lymph nodes, with validation by RT-qPCR. Five of the 7 identified nodal miRNAs (miR-494, miR2861, miR-21, miR155 and miR-16) were also significantly elevated in the plasma of HL patients when compared with healthy controls and positively correlated with Hasenclever scores. Additionally, changes in miR-494, miR-1973 and miR-21 correlated with disease response, with the plasma levels of all three miRNA at six months post CR (as per CT/PET) comparable to those seen in healthy controls. Moreover, interim evaluation of miRNA did not show a statistically significant difference between interim PR and post-therapy CR. Again highlighting the issue of normalization for circulating miRNA estimation, in this study it was found that RNA-U6 was elevated in the plasma of HL patients and that miR16 fluctuated with disease response. Both these RNAs are frequently used as controls in RT-PCR quantitation but were not used in this study that perhaps more appropriately utilized absolute quantitation of miRNA per volume unit of plasma.

The largest body of miRNA research in relation to hematological malignancies is in MM where several different

miRNA signatures have been identified to help to differentiate the continuum of plasma cell dyscrasias ranging from normal plasma cells through monoclonal gammopathy of undetermined significance (MGUS), MM, extra-medullary MM and to plasma cell leukaemia (**Table 2**)⁸²⁻⁹⁰. Despite most groups employing similar strategies, screening disease groups (MGUS versus MM etc.) with micro-array platforms followed by RT-PCR validation, different miRNA panels have been identified with very little overlap. Of particular concern is the conflicting data regarding miR20a, with two papers showing a decreased level in MM whilst another showed an increase. Additionally, highlighting the caution required in over-interpreting significance of specific miRNA, the study of Besse et al.⁸² on initial screening found both miR-130a and miR-34a to be elevated in patients with extra-medullary MM (EM-MM). However, on a second set of patients, marker validation showed the opposite, patients with EM-MM was shown to have reduced levels of miR-34a, thus raising very pertinent questions about the reproducibility of the methodologies employed.

Limited data supports an association between the expression of specific miRNA and underlying cytogenetic abnormalities, for example, several groups have found that specific miRNA correlate with del13q. In the largest cohort of MM patients studied ($n=288$), Rocci et al.⁹¹ found low miR-16 and miR-19b were associated with del13q, whereas others have shown low let7e⁸⁷ and low miR-221⁸⁶ to be associated with del13q. Whilst one group found low miR-19a⁸⁹ expression in the context of the co-existence of 1q amplification and del13q. Only one group found any other cytogenetic correlation, with high miR-99b correlating with the presence of t(4;14)⁸⁶. It should be noted these were only correlations without proven pathologic relationship and that patients may have any of these miRNA phenotypes without an associated cytogenetic abnormality.

Various miRNA have shown correlation with survival outcomes in MM. Elevated miR-16, miR-30a and miR-25 are all independent markers for longer OS, with miR-25 retaining significance in a multivariate model⁹¹. High levels of miR-19b and miR-331 were associated with longer PFS after a CR is achieved with autologous stem cell transplant⁸³. Conversely, high miR-148 and miR-20a⁸⁶, and low let7e and miR-744⁸⁷ are correlated with shorter RFS and OS. In a similar vein a low level of miR-19a was associated with shorter PFS and OS, however, this adverse prognostic association was overcome with bortezomib-based but not thalidomide-based treatment⁸⁹.

Table 2 Papers evaluating miRNA changes to differentiate healthy donors (HD) from MGUS and MM. Those in bold have been replicated in different studies.

Author	MiRNA increased	MiRNA decreased	Comment
Besse ⁸²	MiR-130a		Elevated in patients with extra-medullary disease over MM
Navarro ⁸³	-	MiR-16, miR-17, miR-19b, miR-20a, miR-660	MiRNA levels increased in patients achieving CR but did not reach healthy normal
Wang ⁸⁴	-	Let7a, let7b, let7i, miR-15a, miR-15b, miR-16, miR-20a, miR-21, miR-106b, miR-223, miR-361	4 of 11 reduced in MGUS, 8 of 11 reduced in SMM, 11 of 11 reduced in MM - suggests progressive 'accumulation' of miRNA dysregulation with progressive disease
Sevcikova ⁸⁵	MiR-142-5p, miR-660, miR-29a		
Huang ⁸⁶	MiR-148a, miR-181a, miR20a, miR221, miR-99b		MiR20a finding conflicts with other groups
Kubiczkova ⁸⁷	MiR-34a	Let7d, let7e, miR-130a, miR-744	Combination of let7e and miR-744 provided best ROC characteristics to differentiate HD and either MGUS or MM
Jones ⁸⁸	MiR-720, miR-1246	MiR-1308	Different combinations of the three required to differentiate HD from MGUS or MM
Hao ⁸⁹	MiR-4254	MiR-19a	Combination could distinguish HD or MM
Lionetti ⁹⁰	42 upregulated	41 downregulated	Differentiate MM & PCL

Conclusions and future directions

The analysis of CFNA clearly offers a potential for diagnostics, prognostics, management and pathologic insights into various hematologic malignancies. Despite longstanding knowledge of the existence of CFNA^{1,2}, it is only in the past two decades that research has focused initially on cfDNA³ and more recently miRNA⁴. The science is nascent but rapidly evolving.

The blood of patients with hematologic malignancy contains higher levels of cfDNA² that harbors mutations, chromosomal translocations and epigenetic changes present in the tumor population and as such presents an opportunity for simple, non-invasive, repeatable analysis of these genetic profiles. Furthermore, with increasingly sensitive methods³²⁻³⁵ of interrogation this source of genetic material may be more sensitive than single biopsies of primary sites of disease or that obtained from circulating tumor cells³³ and this theoretically may be of particular importance in the case of spatially and genetically heterogeneous malignancies¹⁵ whereby CFNA analysis may provide a more 'holistic' description of a tumor's mutational profile. Clearly larger studies are needed to compare mutational profiles identified in CFNA with those found in tissue biopsies from primary sites of disease and identify any correlation with patient outcomes. Current prognostic algorithms²³ have been

established for mutations detected in primary tumors, but to date their significance when detected in CFNA is unclear. Simple but important questions such as the significance of differing mutational burdens in the peripheral and the issue of how the identification of a novel mutation in the blood that may otherwise not have been identified in primary tissues should be addressed remain unanswered.

The prospective role of circulating miRNA is less evolved and less clear than that of cfDNA. The source of circulating miRNA remains contentious, with studies frequently finding no correlation between that which is found in the peripheral blood and primary tumor cells^{63,92}. Current micro-array platforms, whilst very sensitive, identify vastly different miRNA signatures with little or no overlap between independent studies of the same diseases (see section regarding miRNA in AML and MM). This raises questions of mere association as opposed to a true signal and a potentially significant role in disease pathogenesis. Further efforts investigating circulating miRNA may best be focused on specific miRNA known to play roles in normal and malignant hemopoiesis^{80,93}, as well as those already recurrently identified in more than one disease. For instance miR-150 is elevated in both AML⁶⁶ and CLL⁷⁸, whilst miR-92a is reduced in both AML^{68,69} and NHL⁷⁶.

Many questions have emerged in relation to the possible pathologic effects exerted by dysregulated circulating

miRNA. Does the prognostic significance associated with an elevated specific miRNA result from its ability to interfere with tumor suppressor genes, the theoretical possibility that it may act as an inter-cellular messenger potentially inhibiting activity of immune effector cells or is it simply an association? How might reduced levels of specific miRNAs exert a prognostic effect? Is it due to increased cellular uptake influencing tumor and/or the microenvironment or is it representative an associated underlying genetic lesion? To properly understand the significance of dysregulated miRNA, functional studies are required. These would include evaluating mechanisms of cellular release and uptake; 'genetic interference' in tumor cells, the supporting micro-environment and immune effector cells; followed by correlation with clinical outcomes. Albeit complex these would provide insight into the true significance of dysregulated miRNA.

Prospects for clinical application are wide ranging, as novel testing platforms become more sensitive, quicker and more affordable, validation and standardization will be urgently required not only for 'inter-study' comparison but for potential integration into standard pathology practice⁶⁴. Whilst controls for cfDNA analyses have largely been established, little or no agreement exists for controls and standardization of miRNA PCR and is urgently needed. The field of CFNA is rapidly evolving, with the prospects of liquid biopsy either complementing or even replacing BM and/or tissue biopsy a real possibility in the future. However, until platforms and practice are standardized, greater concordance in the data achieved, paired with an understanding of the mechanisms behind the observations, hematologists shouldn't throw out the marrow needle just yet.

Conflict of interest statement

No potential conflicts of interest are disclosed.

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